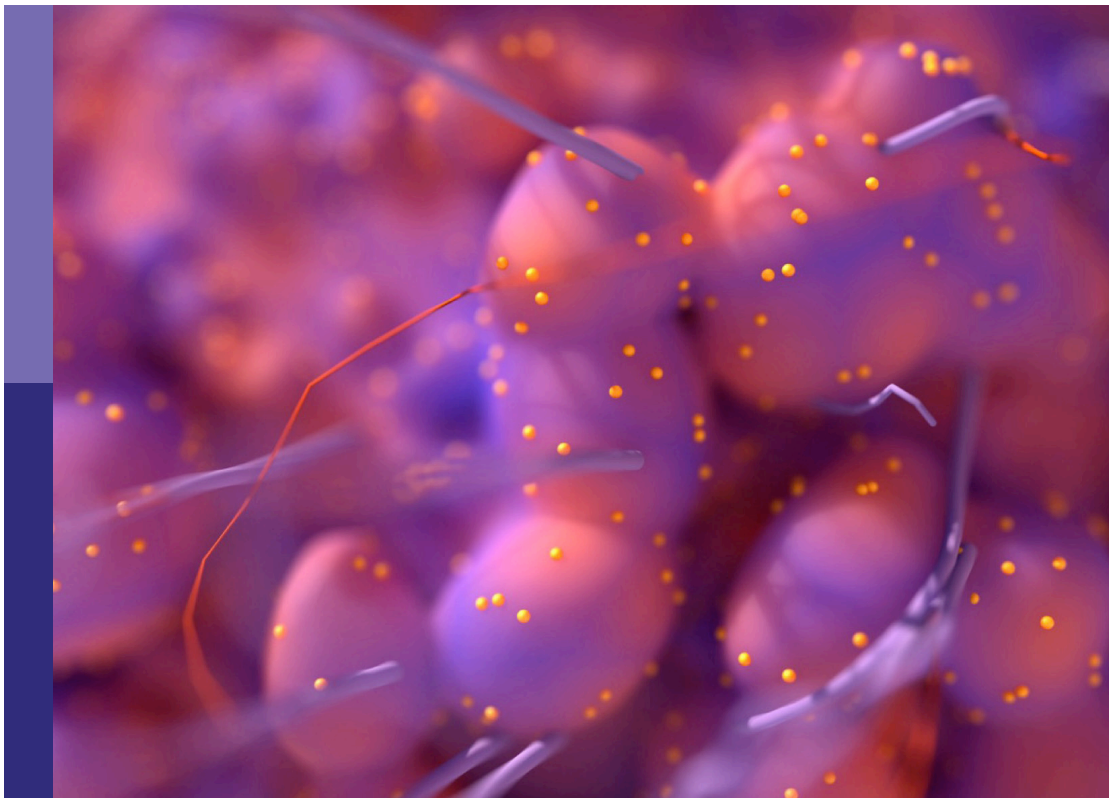
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Reviews in gastrointestinal cancers

Edited by
Fabrizio Bronte, Kanjoormana Aryan Manu
and Emilio Francesco Giunta

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Reviews in gastrointestinal cancers

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Editorial: Reviews in gastrointestinal cancers

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Editorial on the Research Topic
Reviews in gastrointestinal cancers

Gastrointestinal (GI) cancers are a group of cancers associated with the gastrointestinal tract and the most affected areas are the esophagus, stomach, colon, liver, and pancreas. GI cancers are responsible for 25% of cancer incidence and 33% of cancer-related death globally. Even though the recent advances in diagnosis and therapies have made an overall good impact, the challenges in controlling and managing GI cancers continue (1). This Research Topic with 34 articles is intended to consolidate the present status of research in the diagnosis and treatment of GI cancers.

The world is now witnessing the emergence of artificial intelligence (AI) in every field. Luo et al. assessed that AI is highly accurate in early-stage upper GI cancer detection using endoscopic images. In a systematic review, Jia et al. showed by metanalysis that AI Deep learning models have higher predictive accuracy than radiomics models in patients with rectal cancer.

Arrichiello et al. describe the emerging pathological features to predict the prognosis of patients with colorectal cancer (CRC). In a systematic review by Guan et al., the authors have performed a meta-analysis to quantify the relevance of preoperative factors for peritoneal carcinomatosis in gastric cancer when using staging laparoscopy (SL). He et al., Schlosser et al., and Sung et al. have reviewed the emerging biomarkers and their potential for clinical diagnosis of hepatocellular carcinoma (HCC). In a systematic review and meta-analysis, Yang et al. assessed the prognostic value of pan-immune-inflammation value in patients with CRC. In another systematic review, Li et al. assessed the diagnostic value of lncRNAs for gastric cancer.

In a case report, Zhang et al. described a condition of pancreatic neuroendocrine tumors and liver perivascular tumors with the involvement of multiple organs and space-occupying lesions, which is less common. Yan et al. summarized that interventional therapies such as rupture tissue ablation and TAE/TACE for those who are not tolerant to emergency surgery, reach an ideal prognosis for ruptured HCC (rHCC) cases. Xue et al. reviewed the prognostic importance of tumor budding, which is a single cell or cluster of up to four cells at the cancer invasion margin in gastric cancer. Bae et al. showed an increasing trend in the utilization of radiotherapy, adoption of advanced techniques, and overall

survival improvements in patients with HCC from a Korean tertiary hospital registry. A review by Wang Q et al. has described the pathogenesis, diagnosis, and management of an extremely rare pathological condition, primary hepatopancreatobiliary lymphoma, and offers a diagnosis and management schedule for clinicians. Chen et al. have reported a meta-analysis on neoadjuvant chemoradiotherapy for resectable gastric cancer. Li et al. have discussed the current status and future perspectives of cardia preserving radical gastrectomy, which is a promising approach with various advantages. Du et al. have reported a rare case of an ectopic enterogenous cyst in the anterior sacral and soft tissue of the buttocks and its carcinomatous transformation, an event that has never been reported before in the literature.

Comparative efficacy and toxicity of immune checkpoint inhibitors (ICIs) combined or not with chemotherapy have been analyzed in the systematic review by Ma et al.. Wu et al. updated the advancement in the research of HCC progression after radiofrequency ablation. In a systematic review, Zhong et al. assessed the efficacy and safety of ICIs combined with antiangiogenic drugs in HCC. Hu et al. described the current advances in research on the secondary resistance to imatinib against gastrointestinal stromal tumors (GISTs). A meta-analysis by Zheng et al. showed the effect of phosphoglucomutase (PGM), a key enzyme involved in the synthesis and breakdown of glycogen, on the survival prognosis of tumor patients. In this Research Topic, Jiang et al. have discussed the possibilities of targeting neutrophils against the development and progression of pancreatic cancer.

Qiu et al. reviewed the role of Intercellular Adhesion Molecule-1 (ICAM-1), a cell surface glycoprotein, focusing on expression, functions, prognosis, tumorigenesis, polymorphism, and therapeutic implications in CRCs. Gong et al. have reviewed the role of melatonin, a natural indolamine in inhibiting GI carcinogenesis and the mechanisms behind it. Xie et al. have reviewed the application of single-cell sequencing in gastrointestinal cancers. Qi et al. have described the prognostic roles of Competitive Endogenous RNAs (ceRNA) Netowork-Based Signatures in GI cancers. Wang S. et al. have reviewed the mechanisms and prospects of circular RNAs, a group of single-stranded RNAs that form a covalently closed continuous loop, and their role in GI cancer signaling networks.

Up to 80% of patients with pancreatic adenocarcinoma (PDAC) can experience PDAC-derived cachexia, a systemic disease that involves a complex interplay between the tumor and multiple organs. Yu et al. reviewed the endocrine organ-like tumor hypothesis that explains the factors involved in cachexia development. Ferroptosis is an iron-dependent form of programmed cell death and Liang et al. have reviewed the therapeutic applications of ferroptosis in CRCs. In their review, Melia et al. have explained the pro-tumorigenic role of type-2 diabetes-induced cellular senescence in CRCs and its molecular mechanism. In a systematic review, Pang et al. investigated the clinical significance of lung cancer inflammation index in patients with GI cancer in order to evaluate the postoperative complications before surgery and survival outcomes.

Taken together, the articles published in this Research Topic have discussed a broad area of research in GI cancers, from diagnosis to therapeutic resistance. Current advances in genomic techniques and AI have certainly created new and tremendous possibilities in GI diagnosis and therapy, which will hopefully improve GI cancer management in the future.

Author contributions

KM has written, FB and EG have reviewed the editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

EG received personal fees from Novartis and travel accommodation from Janssen.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Artificial Intelligence–Assisted Endoscopic Diagnosis of Early Upper Gastrointestinal Cancer: A Systematic Review and Meta-Analysis

De Luo[1†], Fei Kuang[2†], Juan Du[3†], Mengjia Zhou[4], Xiangdong Liu[5], Xinchen Luo[6], Yong Tang[7], Bo Li[1] and Song Su[1]*

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Objective: The aim of this study was to assess the diagnostic ability of artificial intelligence (AI) in the detection of early upper gastrointestinal cancer (EUGIC) using endoscopic images.

Methods: Databases were searched for studies on AI-assisted diagnosis of EUGIC using endoscopic images. The pooled area under the curve (AUC), sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR) with 95% confidence interval (CI) were calculated.

Results: Overall, 34 studies were included in our final analysis. Among the 17 image-based studies investigating early esophageal cancer (EEC) detection, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.98, 0.95 (95% CI, 0.95–0.96), 0.95 (95% CI, 0.94–0.95), 10.76 (95% CI, 7.33–15.79), 0.07 (95% CI, 0.04–0.11), and 173.93 (95% CI, 81.79–369.83), respectively. Among the seven patient-based studies investigating EEC detection, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.98, 0.94 (95% CI, 0.91–0.96), 0.90 (95% CI, 0.88–0.92), 6.14 (95% CI, 2.06–18.30), 0.07 (95% CI, 0.04–0.11), and 69.13 (95% CI, 14.73–324.45), respectively. Among the 15 image-based studies investigating early gastric cancer (EGC) detection, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.94, 0.87 (95% CI, 0.87–0.88), 0.88 (95% CI, 0.87–0.88), 7.20 (95% CI, 4.32–12.00), 0.14 (95% CI, 0.09–0.23), and 48.77 (95% CI, 24.98–95.19), respectively.

Conclusions: On the basis of our meta-analysis, AI exhibited high accuracy in diagnosis of EUGIC.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO/, identifier PROSPERO (CRD42021270443).

Keywords: artificial intelligence, upper gastrointestinal tract, early detection of cancer, endoscopy, systematic review

INTRODUCTION

Upper gastrointestinal cancer (UGIC) is among the most common malignancies and causes of cancerrelated deaths worldwide, which presents a major challenge for health-care system (1). A majority of UGIC patients are detected at a late stage and have a poor prognosis. In contrast, with early detection, the 5-year overall survival can be more than 90% (2, 3). Thus, the early detection of UGIC is essential to improve patient prognosis.

Endoscopy remains the most optimal approach of UGIC detection (4, 5). However, endoscopic features of early upper gastrointestinal cancer (EUGIC) lesions are subtle and easily missed. Moreover, diagnostic accuracy depends on the expertise of endoscopists (2). One report revealed that EUGIC misdiagnosis can be high regardless of the number of patients, developed or underdeveloped locations, or in countries performing a remarkably high volume of endoscopies (6).

Artificial intelligence (AI) is gaining much popularity in the field of medicine, including gastrointestinal endoscopy (7–11). Owing to its good pattern recognition ability, AI is a promising candidate for detection of upper gastrointestinal lesions (12, 13). However, the data on AI-assisted EUGIC diagnosis are still lacking. Hence, we conducted this study to assess the diagnostic accuracy of AI in the detection of EUGIC using endoscopic images.

METHODS

This systematic review and meta-analysis was reported in line with PRISMA guidelines and was registered with the international prospective register of systematic reviews PROSPERO (CRD42021270443).

Search Strategy and Study Selection

Two authors (FK and JD) separately searched electronic databases (PubMed, Medline, Embase, Web of Science, Cochrane library, and Google scholar) from the date of establishment until November 2021 using the following pre-specified search terms: "endoscopy", "endoscopic", "early gastric cancer", "early esophageal cancer", "early esophageal squamous cell carcinoma", "early Barrett's neoplasia", "early esophageal adenocarcinoma", "artificial intelligence", "AI", "machine learning", "deep learning", "artificial neural network", "support vector machine", "naive bayes", and "classification tree". Potentially relevant studies (based on title and abstract) were then read completely to ensure eligibility in the meta-analysis. In addition, we also reviewed the reference lists of relevant studies to search for eligible studies.

Study Eligibility Criteria

Studies meeting the following criteria were included in the meta-analysis: (1) studies that evaluated AI diagnostic performance for EUGIC using endoscopic images; (2) true positive (TP), false positive (FP), false negative (FN), and true negative (TN) values could be extracted directly or calculated from the original studies. The following studies were excluded from our meta-analysis: (1) reviews, (2) meta-analyses, and (3) comments or protocols. We followed a strict exclusion policy that any study meeting one of the abovementioned exclusion criteria was excluded.

Data Extraction

Two authors (MZJ and XDL) separately extracted data from the included studies, namely, author, publication year, study design, imaging type, AI model, sample size, TP, FP, FN, and TN. TP, FP, FN, and TN were extracted with the histology as the reference standard. Intramucosal carcinoma, T1 cancer, and Barrett's esophagus (BE) with high-grade dysplasia were considered as positive. Normal tissue, BE without high-grade dysplasia, and non-cancerous lesions were considered as negative. The authors of the studies were contacted for missing information, if necessary. Discrepancies were decided through discussion.

Methodological Quality Assessment

Two authors (XDL and XCL) evaluated the quality and potential bias risk of eligible studies based on the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) (14). Disagreements were resolved through discussion. The QUADAS-2 tool was composed of four domains: "patient selection", "index test", "reference standard", and "flow and timing". In addition, the "patient selection", "index test", and "reference standard" were further tested for "applicability". Each domain was then stratified into high, low, or unclear bias risk.

Statistical Analysis

Statistical analysis was performed using the Meta-Disc software (version 14). To assess AI performance in EUGIC diagnosis, the pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR) with 95% confidence interval (CI) were computed. In addition, we plotted a summary receiver operating characteristic (SROC) curve. The area under the curve (AUC) was computed to predict precision in diagnosis. We evaluated AI diagnostic performance based on images (image-based analysis) and patients (patient-based analysis). The forest plot was constructed. The inconsistency index (I^2) test determined presence or absence of heterogeneity among studies using sensitivity (15). A fixed-effects model was used if the I^2 value < 50%; otherwise, a random-effects model was selected. The Spearman correlation coefficient (SCC) between sensitivity and false positive rate was calculated, and a value > 0.6 indicated a threshold effect.

RESULTS

Literature Screening and Bias Evaluation

The primary screening uncovered 1,812 eligible studies. Upon removal of duplicates and other studies that were irrelevant to

this study (based on title, abstract, and full article), 34 studies (16–49) investigating AI-assisted early esophageal cancer (EEC) and early gastric cancer (EGC) detection were included in the final meta-analysis. Among 34 studies, 18 and 17 studies assessed the diagnostic ability of AI in the detection of EEC (16–33) and EGC (29, 34–49), respectively. An overview of the eligible studies screening process is illustrated in **Figure 1**. **Table 1** presents the characteristics of all eligible studies. Overall, the included studies showed high methodological quality. The quality assessment and risk of bias for each eligible study are summarized in **Figure 2**.

AI-Assisted EEC Diagnosis Using Endoscopic Images

Meta-Analysis of AI-Assisted EEC Diagnosis Using Endoscopic Images [White-Light Imaging (WLI)/ Narrow-Band Imaging (NBI) Images]

Eigtheen studies (16–33) reported the AI-assisted EEC diagnosis performance using endoscopic images. Moreover, 17 and 7 studies reported the AI-assisted EEC diagnosis performance based on per image (16–26, 28–33) and per patient (17, 18, 21, 24, 27, 31, 33), respectively. Among the 17 image-based studies, a total of 13,091 images (4,310 positive vs. 8,781 negative) were identified. Specifically, the positive group composed of the early esophageal squamous cell carcinoma (EESCC), early esophageal adenocarcinoma (EEAC), and EEC images, whereas the negative group consisted of normal, Barrett's esophagus, and non-cancerous images. In most studies, the AI algorithm type was convolutional neural network (CNN). However, single-shot multibox detector (SSD) (25) and support vector machine (SVM) (29, 31) were also employed. Among the seven patient-based studies, a total of 1,380 patients (316 positive vs. 1,064 negative) were identified. Specifically, EESCC and EEAC constituted the positive group, whereas normal, Barrett's esophagus, and non-cancerous comprised of the negative group. Most studies used the CNN algorithm. However, SVM was used in one study (31).

In the 17 image-based studies investigating AI-assisted EEC diagnosis, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.98, 0.95 (95% CI, 0.95–0.96), 0.95 (95% CI, 0.94–0.95), 10.76 (95% CI, 7.33–15.79), 0.07 (95% CI, 0.04–0.11), and 173.93 (95% CI,81.79–369.83), respectively (**Figures 3A–F**). In addition, the SCC and p-values were −0.10 and 0.70 (>0.05),

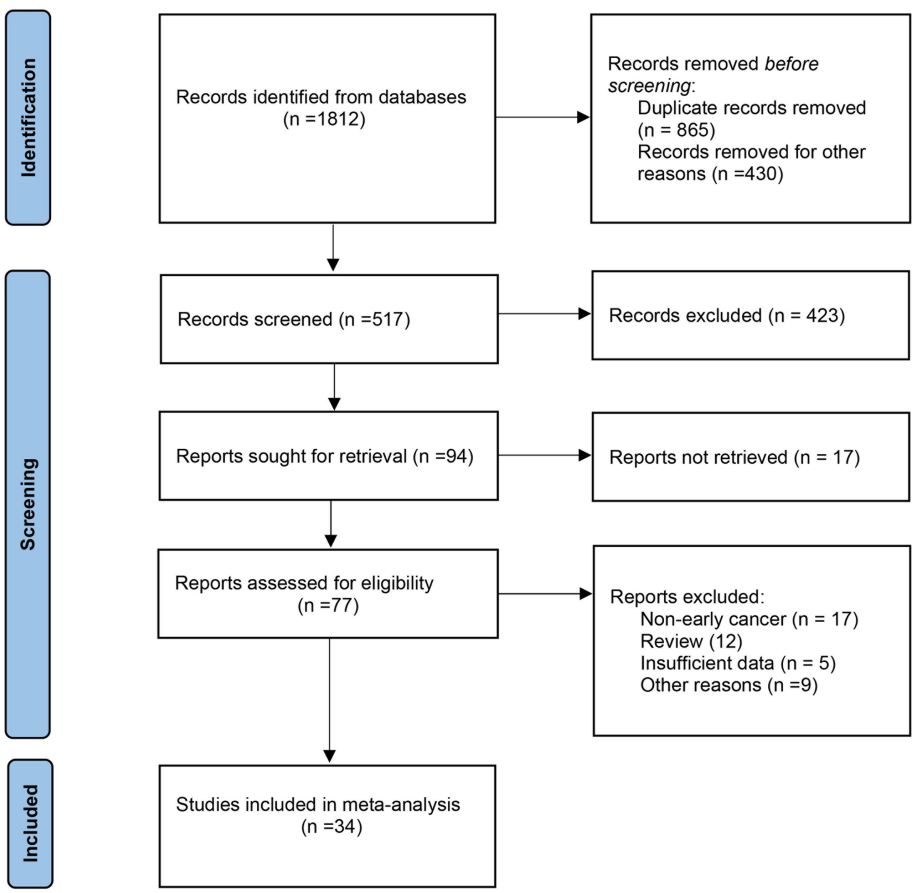


FIGURE 1 | An overview of the study screening process.

TABLE 1 | Clinical characteristics of the included studies.

Author/year	Study design	Imaging type	AI model	No. of images/patients/lesions in the test dataset		TP	FP	FN	TN	Endoscopist control
				Positive	Negative					
Cai, 2019 (16)	Retrospective	WLI	CNN	EESCC:91	Normal:96	89	14	2	82	Yes
de Groof, 2019 (17)	Prospective	WLI	CNN	EEAC:40/40*	BE:20/20*	38/38*	3/3*	2/2*	17/17*	No
de Groof, 2020 (1) (18)	Prospective	WLI	CNN	EEAC:33/10*	BE:111/10*	25/9*	15/1*	8/1*	96/9*	No
de Groof, 2020 (2) (19)	Prospective	WLI	CNN	EEAC:209	BE:248	186	31	23	217	No
Ebigbo, 2019 (1) (20)	Retrospective	WLI	CNN	EEAC:36	BE:26	30	0	6	26	No
Ebigbo, 2019 (2) (21)	Retrospective	WLI/NBI	CNN	EEAC:83①/33②/33①*/33②*	BE:91①/41②/41①*/41②*	78①/31②/32①*/31②*	5①/8②/5①*/8②*	5①/2②/1①*/2②*	86①/33②/36①*/33②*	No
Mendel, 2017 (22)	Prospective	WLI	CNN	EEAC:50/22*	BE:50/17*	47	6	3	44	No
Everson, 2019 (23)	Retrospective	NBI	CNN	EESCC:775/10*	Normal:891/7*	770	24	5	867	No
Fukuda, 2020 (24)	Retrospective	NBI	CNN	EESCC:45/45*	NC:49/99*	39/41*	5/48*	6/4*	44/51*	Yes
Ghatwary, 2019 (25)	Retrospective	WLI	SSD	EEAC:50/22*	BE:50/17*	48	4	2	46	No
Guo, 2020 (26)	Retrospective	NBI	CNN	EESCC:1,480	NC:5,191	1,451	258	29	4,933	No
Iwagami, 2021 (27)	Retrospective	WLI+NBI	CNN	EEAC:36*	NC:43*	34*	25*	2*	18*	No
Li, 2021 (28)	Retrospective	WLI/NBI	CNN	EESCC:133①/133②	Normal:183①/183②	131①/121②	31①/6②	2①/12②	152①/177②	Yes
Liu, 2016 (29)	Retrospective	WLI	SVM	EEC:150	Normal:250	140	27	10	233	No
Hashimoto, 2020 (30)	Retrospective	WLI/NBI	CNN	EEAC:146①/79②	BE:107①/126②	144①/73②	12①/1②	2①/6②	95①/125②	No
van der Sommen, 2016 (31)	Retrospective	WLI	SVM	EEAC:60/21*	BE:40/23*	50/18*	7/3*	10/3*	33/20*	Yes
Wang, 2021 (32)	Retrospective	WLI/NBI	CNN	EEAC:95①/115②	Normal:17①/37②	90①/112②	4①/12②	5①/3②	13①/25②	No
Yang, 2021 (33)	Retrospective	WLI	CNN	EESCC:474/98*	Normal:964/787*	419/94*	9/13*	55/4*	955/774*	No
Wang, 2018 (34)	Retrospective	WLI	CNN	EGC:232	NC + normal:478	206	49	26	429	Yes
Horiuchi, 2020 (35)	Retrospective	NBI	CNN	EGC:151	NC:107	144	31	7	76	No
Ikenoama, 2021 (36)	Retrospective	WLI	CNN	EGC:209	NC:2,731	122	347	87	2,384	Yes
Kanesaka, 2018 (37)	Retrospective	NBI	SVM	EGC:61	NC:20	59	1	2	19	No
Li, 2020 (38)	Retrospective	NBI	CNN	EGC:170	NC:171	155	16	15	155	No
Liu, 2016 (29)	Retrospective	WLI	SVM	EGC:130	Normal:270	118	25	12	245	No
Namikawa, 2020 (39)	Retrospective	WLI+NBI	CNN	EGC:100*	GU:120*	99*	8*	1*	112*	No
Shibata, 2020 (40)	Retrospective	WLI	CNN	EGC:533	Normal:1,208	404	127	129	1,081	No
Tang, 2020 (41)	Retrospective	WLI	CNN	EGC:4,810	NC:6,120	4,555	1,074	255	5,046	No
Ueyama, 2021 (42)	Retrospective	NBI	CNN	EGC:1,430	NC:870	1,401	0	29	870	No
Wu, 2021 (43)	Prospective	WLI	CNN	EGC:3#	NC:191#	3#	30#	0#	161#	No
Sakai, 2018 (44)	Retrospective	WLI	CNN	EGC:4,653	Normal:4,997	3,723	262	930	4,735	No
Yoon, 2019 (45)	Retrospective	WLI	CNN	EGC:330	NC:330	300	8	30	322	No
Wu, 2019 (46)	Retrospective	WLI	CNN	EGC:100	NC:100	94	9	6	91	No
Zhang, 2020 (47)	Retrospective	WLI	CNN	EGC:333	NC:311	285	189	48	122	No
Cho, 2019 (48)	Retrospective	WLI	CNN	EGC:46	NC:126	13	15	33	111	No
Cho, 2020 (49)	Retrospective	WLI	CNN	EGC:179	NC:217	111	75	68	142	No

EESCC, early esophageal squamous cell carcinoma; EEAC, early esophageal adenocarcinoma; BE, Barrett's esophagus; EEC, early esophageal cancer; GU, gastric ulcers; SVM, support vector machine; CNN, convolutional neural network; SSD, single-shot multibox detector; WLI, white-light imaging; BNI, narrow-band imaging; NC, non-cancerous; TP, true positive; FP, false positive; FN, false negative; TN, true negative; WLI/NBI indicates that one study included WLI and BNI images, and the numbers of TP, FP, FN, and TN for EEC/EGC diagnosis with WLI or NBI images were reported or could be calculated; WLI + NBI indicates that one study included WLI and BNI images, but the numbers of TP, FP, FN, and TN for EEC/EGC diagnosis with WLI or NBI images were not reported or could not be calculated.

*① indicates the number of WLI images; ② indicates the number of NBI images; *indicates the number of patients; #indicates the number of lesions.*

respectively, suggesting no significant threshold effect among these studies.

Among the seven patient-based studies investigating AI-assisted EEC diagnosis, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.98, 0.94 (95% CI, 0.91–0.96), 0.90 (95% CI, 0.88–0.92), 6.14(95% CI, 2.06–18.30), 0.07 (95% CI, 0.04–0.11), and 69.13 (95% CI, 14.73–324.45), respectively (**Figures 4A–F**). The SCC and p-values were −0.071 and 0.879 (>0.05), respectively, indicating no significant threshold effect among these studies.

AI-Assisted EGC Diagnosis Using Endoscopy Images

Meta-Analysis of AI-Assisted EGC Diagnosis Using Endoscopic Images (WLI/NBI Images)

Seventeen studies (29, 34–49) reported the AI diagnosis performance of EGC using endoscopic images. Fifteen studies (29, 34–38, 40–42, 44–49), one study (39), and one study (43) evaluated the AI diagnosis performance based on per image, per patient, and per lesion, respectively.

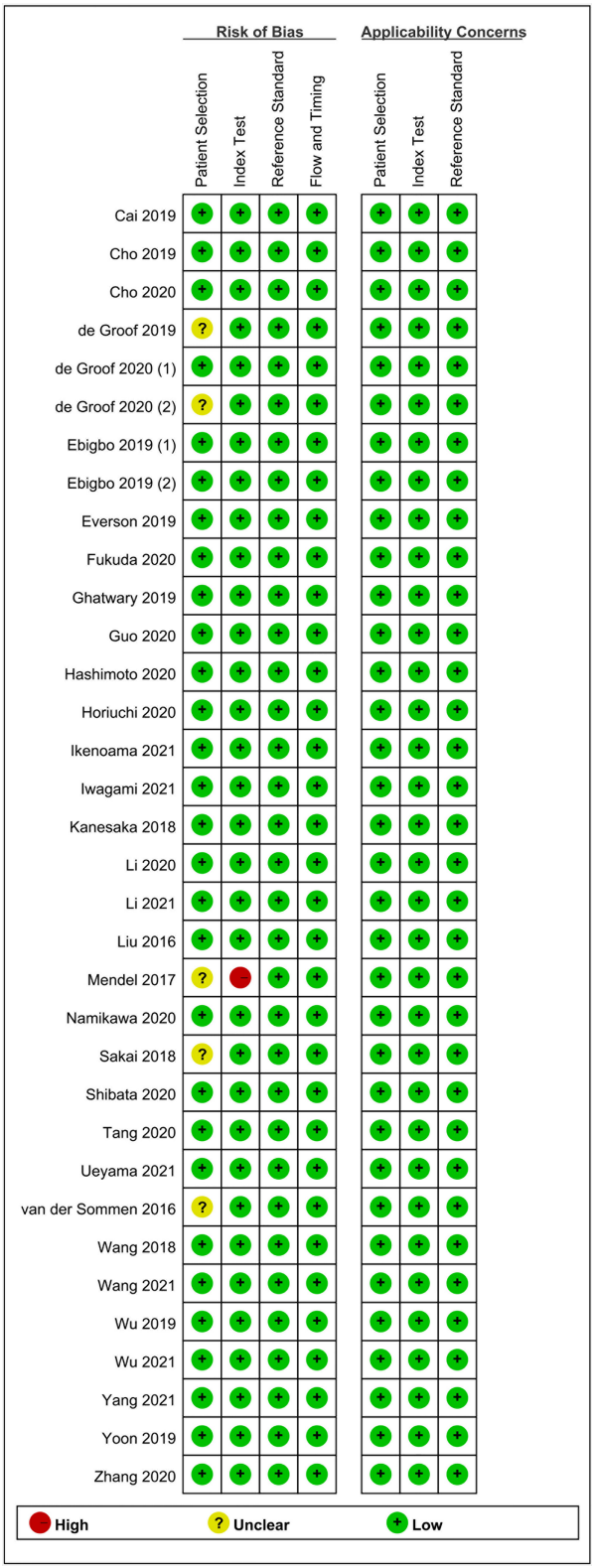


FIGURE 2 | The quality assessment and risk of bias for each eligible study.

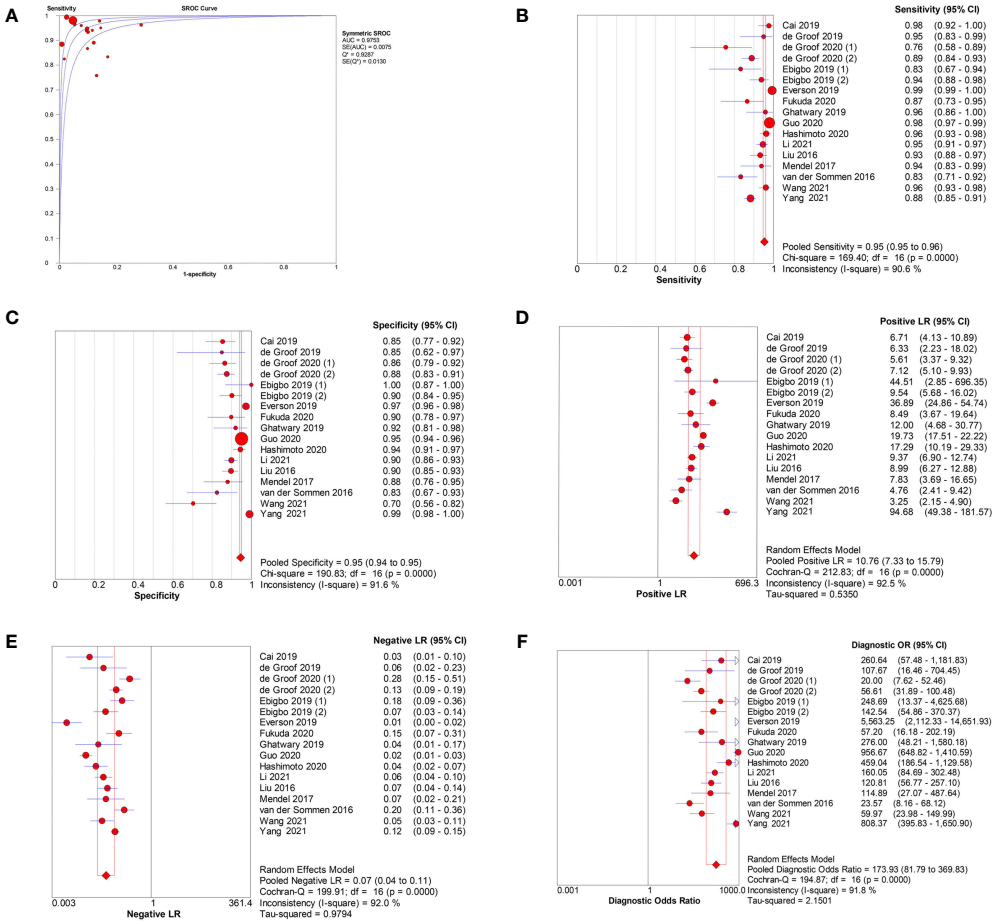


FIGURE 3 | Meta-analysis of AI-assisted EEC diagnosis (image-based analysis). **(A)** SROC curve. **(B)** Pooled sensitivity. **(C)** Pooled specificity. **(D)** Pooled PLR. **(E)** Pooled NLR. **(F)** Pooled DOR.

Among the 15 image-based studies, a total of 31,423 images (13,367 positive vs. 18,056 negative) were identified. Only the EGC images were categorized in the positive group, whereas the normal and non-cancerous images were categorized in the negative group. A majority of the studies used CNN algorithm. However, the SVM algorithm was also used (29, 37). Among the two patient/lesion-based studies, a total of 414 patients/lesions (103 positive vs. 311 negative) were identified. Only the EGC were placed in the positive group, whereas the gastric ulcers and non-cancerous were placed in the negative group. Both studies utilized CNN algorithm.

Among the 15 image-based EGC detection studies, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.94, 0.87 (95% CI, 0.87–0.88), 0.88 (95% CI, 0.87–0.88), 7.20 (95% CI, 4.32–12.00), 0.14 (95% CI, 0.09–0.23), and 48.77 (95% CI, 24.98–95.19), respectively (**Figures 5A–F**). The SCC and p-values were −0.44 and 0.10 (>0.05), respectively, suggesting no significant threshold effect among these studies.

Only two patient-based studies evaluated AI in the diagnosis of EGC, so meta-analysis was not performed. In Namikawa's study, the sensitivity and specificity were 0.99 and 0.93, respectively. In Wu's study, the sensitivity and specificity were 1.00 and 0.8429, respectively.

Subgroup Analysis Based on Imaging Type

To compare the AI diagnostic performance of EEC and EGC detection using WLI and NBI endoscopic images, we performed a subgroup analysis based on imaging type. On the basis of the results of subgroup analysis, the NBI mode showed a better diagnostic performance than the WLI mode. The results are summarized in **Table 2**.

Meta-Analysis of AI-Assisted EGC Diagnosis Using WLI Endoscopic Images

Fourteen studies (16–22, 25, 28–33) reported the performance of AI-assisted EEC detection using WLI endoscopic images. Among the 14 image-based studies, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.97, 0.92 (95% CI, 0.90–0.93), 0.93 (95% CI, 0.91–0.94), 9.11 (95% CI, 6.04-13.75), 0.09 (95% CI, 0.06–0.13), and 136.06 (95% CI, 67.20–275.49), respectively. The SCC and

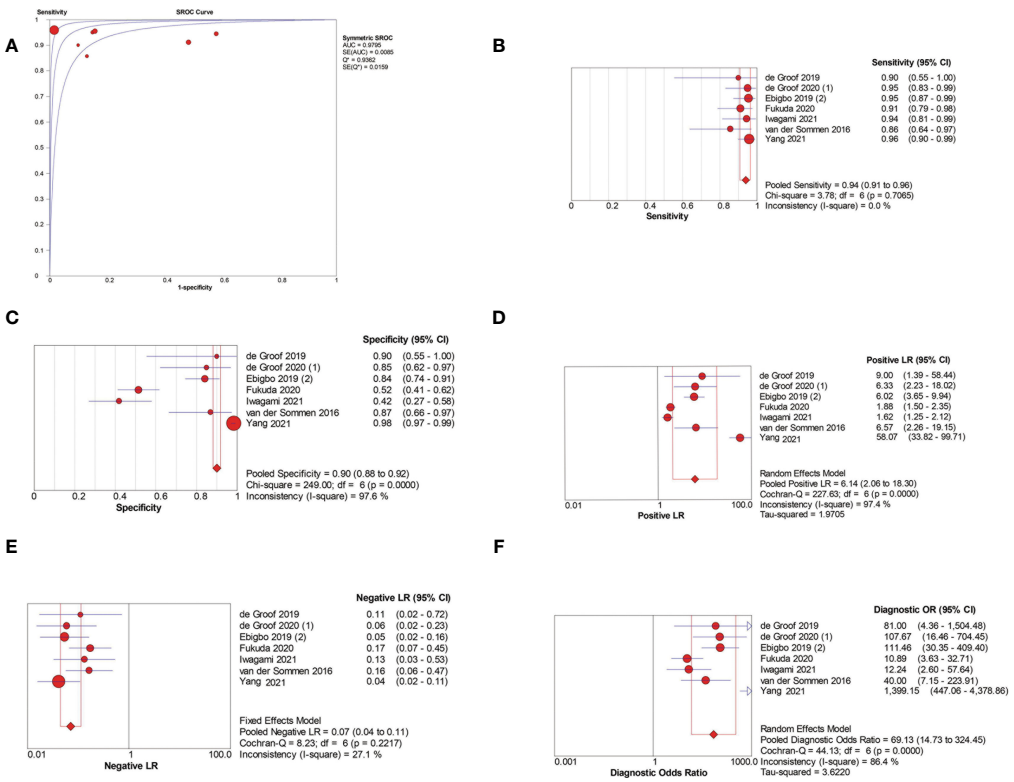


FIGURE 4 | Meta-analysis of AI-assisted EEC diagnosis (patient-based analysis). **(A)** SROC curve. **(B)** Pooled sensitivity. **(C)** Pooled specificity. **(D)** Pooled PLR. **(E)** Pooled NLR. **(F)** Pooled DOR.

p-values were 0.24 and 0.40 (>0.05), respectively, indicating no significant threshold effect among these studies.

Among the five patient-based studies (17, 18, 21, 31, 33), the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.95, 0.95 (95% CI, 0.92–0.98), 0.82 (95% CI, 0.74–0.88), 4.7 (95% CI, 3.32–6.65), 0.07 (95% CI, 0.04–0.12), and 86.48 (95% CI, 39.04–191.57), respectively. The SCC and p-values were 0.5 and 0.39 (>0.05), respectively, indicating no significant threshold effect among these studies.

Meta-Analysis of AI-Assisted EEC Diagnosis Using NBI Endoscopic Images

Seven studies (21, 23, 24, 26, 28, 30, 32) reported the AI-assisted EEC detection performance using NBI endoscopic images. Among the seven image-based studies, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.99, 0.98 (95% CI, 0.97–0.98), 0.95 (95% CI, 0.95–0.96), 14.00 (95% CI, 6.71–29.20), 0.05 (95% CI, 0.02–0.11), and 363.56 (95% CI, 108.47–1218.26), respectively. The SCC and p-values were −0.04 and 0.94 (>0.05), respectively, indicating no significant threshold effect among these studies. Only two patient-based studies evaluated AI for the diagnosis of EEC, so meta-analysis was not performed. In the study by Ebigbo et al., (21) the sensitivity and specificity were 0.94 and 0.80, respectively. In the study by Fukuda et al. (24), the sensitivity and specificity were 0.91 and 0.52, respectively.

Meta-Analysis of AI-Assisted EGC Diagnosis Using WLI Endoscopic Images

Twelve studies (29, 34, 36, 40, 41, 43–49) reported the AI diagnosis performance of EGC detection using WLI endoscopic images. Eleven studies (29, 34, 36, 40, 41, 44–49) reported the AI diagnosis performance based on per image. In addition, only Wu's study (43) reported the AI diagnosis performance based on per lesion. Among the 11 image-based EGC detection studies, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.92, 0.86 (95% CI, 0.85–0.87), 0.87 (95% CI, 0.87–0.88), 6.12 (95% CI, 3.53–10.63), 0.21 (95% CI, 0.12–0.35), and 29.92 (95% CI, 14.23–62.90). The SCC and p-values were −0.35 and 0.30 (>0.05), respectively, indicating no significant threshold effect among these studies.

Meta-Analysis of AI-Assisted EGC Diagnosis Using NBI Endoscopic Images

Four studies (35, 37, 38, 42) reported the AI diagnosis performance for EGC using endoscopic NBI images based on per image. In

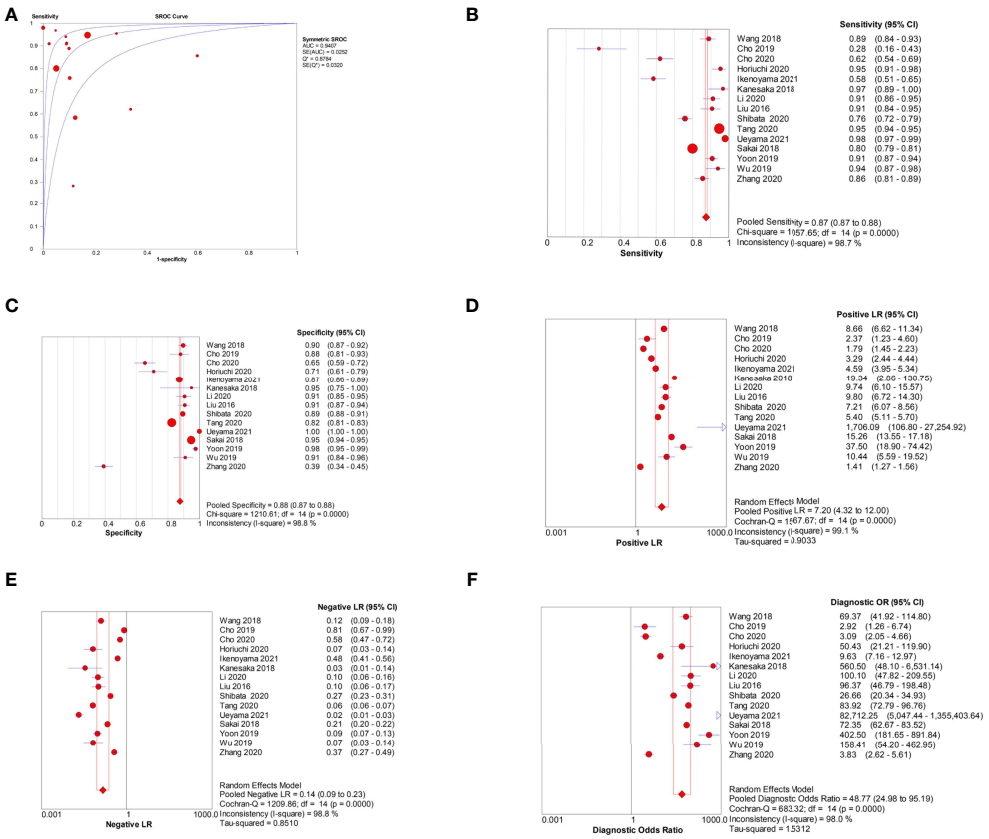


FIGURE 5 | Meta-analysis of AI-assisted EGC diagnosis (image-based analysis). **(A)** SROC curve. **(B)** Pooled sensitivity. **(C)** Pooled specificity. **(D)** Pooled PLR. **(E)** Pooled NLR. **(F)** Pooled DOR.

addition, no studies reported the AI diagnosis performance based on per lesion or per patient. Among the four image-based EGC detection studies, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.99, 0.97 (95% CI, 0.96–0.98), 0.96 (95% CI, 0.95–0.97), 25.92 (95% CI, 1.63–413.31), 0.05 (95% CI, 0.02–0.12), and 523.76 (95% CI, 37.39–7336.36), respectively. The SCC and p-values were −0.8 and 0.2 (>0.05), respectively, suggesting no significant threshold effect among these studies.

TABLE 2 | Summary of subgroup analysis based on imaging type.

Subgroup	Number of included studies	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	DOR (95% CI)	AUC
EEC							
WLI image-based analysis	14	0.92 (0.90–0.93)	0.93 (0.91–0.94)	9.11 (6.04-13.75)	0.09 (0.06–0.13)	136.06 (67.20–275.49)	0.97
patient-based analysis	5	0.95 (0.92-0.98)	0.82 (0.74–0.88)	4.70 (3.32–6.65)	0.07 (0.04–0.12)	86.48 (39.04–191.57)	0.95
BNI image-based analysis	7	0.98 (0.97–0.98)	0.95 (0.95–0.96)	14.00 (6.71–29.20)	0.05 (0.02–0.11)	363.56 (108.47–1218.26)	0.99
EGC							
WLI image-based analysis	11	0.86 (0.85–0.87)	0.87 (0.87–0.88)	6.12 (3.53–10.63)	0.21 (0.12–0.35)	29.92 (14.23–62.90)	0.92
NBI image-based analysis	4	0.97 (0.96–0.98)	0.96 (0.95–0.97)	25.92 (1.63–413.31)	0.05 (0.02–0.12)	523.76 (37.39–7336.36)	0.99

Subgroup Analysis Based on Pathologic Type in Esophagus

We also performed a subgroup analysis between early esophageal squamous cell carcinoma (EESCC) and early esophageal adenocarcinoma (EEAC). On the basis of the results of subgroup analysis, AI showed a better diagnostic performance in EESCC than EEAC. The results are summarized in **Table 3**.

Meta-Analysis of AI-Assisted EESCC Diagnosis Using Endoscopic Images (WLI/NBI Images)

Six studies (16, 23, 24, 26, 28, 33) reported the AI-assisted EESCC diagnosis performance using endoscopic images based on per image. Among the six image-based studies, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.99, 0.96 (95% CI, 0.96–0.97), 0.95 (95% CI, 0.95–0.96), 18.21 (95% CI, 10.07–32.93), 0.04 (95% CI, 0.01–0.11), and 491.74 (95% CI, 170.20–1420.71), respectively. The SCC and p-values were −0.20 and 0.70 (>0.05), respectively, indicating no significant threshold effect among these studies. Only two patient-based studies (24, 33) evaluated AI for the diagnosis of EESCC, so meta-analysis was not performed. In the study by Yang et al. (33), the sensitivity and specificity were 0.97 and 0.99, respectively. In the study by Fukuda et al. (24), the sensitivity and specificity were 0.91 and 0.52, respectively.

Meta-Analysis of AI-Assisted EEAC Diagnosis Using Endoscopic Images (WLI/NBI Images)

Ten studies (17–22, 25, 30–32) reported the AI-assisted EEAC diagnosis performance using endoscopic images based on per image. Among the 10 image-based studies, the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.96, 0.93 (95% CI, 0.91–0.94), 0.89 (95% CI, 0.87–0.91), 7.41 (95% CI, 5.09–10.77), 0.10 (95% CI, 0.06–0.15), and 87.66 (95% CI, 44.40–173.08), respectively. The SCC and p-values were −0.03 and 0.93 (>0.05), respectively, indicating no significant threshold effect among these studies.

Among the five patient-based studies (17, 18, 21, 27, 31), the pooled AUC, sensitivity, specificity, PLR, NLR, and DOR were 0.96, 0.94 (95% CI, 0.89–0.97), 0.75 (95% CI, 0.68–0.81), 4.76 (95% CI, 1.69–13.38), 0.09 (95% CI, 0.05–0.17), and 51.94 (95% CI, 20.89–129.11), respectively. The SCC and p-values were 0.6 and 0.29 (>0.05), respectively, indicating no significant threshold effect among these studies.

DISCUSSION

In this study, we conducted a comprehensive literature search and included all studies that assessed diagnostic performance of AI in EUGIC using endoscopic images. Next, we conducted a meta-analysis to explore the diagnostic performance of AI in EUGIC detection. On the basis of our results, AI demonstrated an excellent diagnostic ability, with high accuracy, sensitivity, specificity, PLR, and DOR, and with low NLR in detecting EUGIC, suggesting the feasibility of AI-assisted EUGIC diagnosis in clinical practice. To the best of our knowledge, this is the first systematic review and meta-analysis that explored the AI-assisted detection of EUGIC based on upper gastrointestinal endoscopic images.

Endoscopy is the primary tool used in the diagnosis of UGIC (50, 51). However, EUGIC lesions manifest as indistinct mucosal alterations under the classic WLI images. Therefore, EUGIC detection is often highly dependent on endoscopist's experience and expertise (52). Previous studies also revealed that WLI-based EGC diagnosis is possible, but with poor sensitivity or specificity (36, 47, 48). More recently, AI-assisted image recognition makes remarkable breakthroughs in the field of medical imaging diagnosis and is gaining popularity in clinical practice (7–11, 53, 54). Traditional AI algorithms like SVM and decision trees require experts to manually design the image features, before the algorithm extracts the feature from images (53, 55). This results in the detection of only specific lesions, and in case the features are insufficient, satisfactory identification results cannot be obtained. Simultaneously, manual design is highly dependent on the previous knowledge of designers. Thus, it is not feasible to work with large amounts of data. At present, many studies on medical image recognition adopt deep learning algorithm based on CNN. The deep learning can automatically learn the most predictive characteristics from a large image data file with no requirement of previous knowledge and classify these images. In our study, most included studies used the CNN algorithm, so we did not compare the AI diagnostic ability between the different algorithms. Many studies demonstrated excellent AI performance in detecting early esophageal and stomach cancers with the CNN algorithm. Consistent with these studies, in our study, AI exhibited an excellent diagnosis performance for EUGIC with high accuracy, sensitivity, and specificity.

Although several advanced technologies like NBI, confocal laser endomicroscopy, and blue laser imaging have shown great promise

TABLE 3 | Summary of subgroup analysis based on pathologic type.

Subgroup	Number of included studies	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	DOR (95% CI)	AUC
EESCC							
image-based analysis	6	0.96 (0.96–0.97)	0.95 (0.95–0.96)	18.21 (10.07–32.93)	0.04 (0.01–0.11)	491.74 (170.20–1420.71)	0.99
EEAC							
image-based analysis	10	0.93 (0.91–0.94)	0.89 (0.87–0.91)	7.41 (5.09–10.77)	0.10 (0.06–0.15)	87.66 (44.40–173.08)	0.96
patient-based analysis	5	0.94 (0.89-0.97)	0.75 (0.68–0.81)	4.76 (1.69–13.38)	0.09 (0.05–0.17)	51.94 (20.89–129.11)	0.96

in the endoscopic detection of EUGIC, endoscopists still need extensive specialized training and substantial experience to identify early cancer lesions accurately. NBI endoscopy is an optical image-enhanced technology that better visualizes surface structures and blood vessels than does WLI (56). Multiple studies have demonstrated NBI has a high sensitivity in detecting EUGIC (37, 57, 58). To compare the AI diagnostic performance for EUGIC detection using WLI and NBI endoscopic images, we performed a subgroup analysis based on imaging type. On the basis of our results, the NBI imaging mode has a superior diagnostic performance for both EEC and EGC detection, with higher AUC, sensitivity, specificity, PLR and DOR, and lower NLR.

There are limitations to this study. First, most studies were based on the retrospective review of selected images. At the same time, the number of positive images and negative images included in some included studies was significantly different. All retrospective studies were considered at high risk for selection bias, so those studies might overestimate the diagnostic accuracy of AI. Second, few studies compared the diagnostic efficacy between AI and endoscopists, so we could not perform meta-analysis to compared the diagnostic efficacy between AI and endoscopists.

In conclusion, on the basis of our meta-analysis, AI achieved high accuracy in diagnosis of EUGIC. Further prospective studies comparing the diagnostic performance between AI and endoscopists are warranted.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTION

DL designed the study. FK and JD screened electronic databases. MZ and XDL extracted data from the selected articles. XDL and XCL evaluated eligible study quality and potential bias risk. Statistical analyses were performed by YT and BL. DL wrote the manuscript. SS supervised the study. All authors contributed to the article and approved the submitted version.

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REVIEW
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Ruptured Hepatocellular Carcinoma: What Do Interventional Radiologists Need to Know?

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Rupture of HCC (rHCC) is a life-threatening complication of hepatocellular carcinoma (HCC), and rHCC may lead to a high rate of peritoneal dissemination and affect survival negatively. Treatment for rHCC mainly includes emergency surgery, interventional therapies, and palliative treatment. However, the management of rHCC should be carefully evaluated. For patients with severe bleeding, who are not tolerant to open surgery, quick hemostatic methods such as rupture tissue ablation and TAE/TACE can be performed. We described clinical presentation, prognosis, complication, interventional management, and current evidence of rHCC from the perspective of interventional radiologists. Overall, our review summarized that interventional therapies are necessary for most patients with rHCC to achieve hemostasis, even in some patients with Child–Pugh C. Moreover, TAE/TACE followed by staged hepatectomy is a beneficial treatment for rHCC according to current clinical evidence. TAE/TACE is the first choice for most patients with rHCC, and appropriate interventional treatment may provide staged surgery opportunities for those who are not tolerant to emergency surgery to reach an ideal prognosis.

Keywords: rupture of hepatocellular carcinoma, transarterial embolization (TAE), transarterial chemoembolization (TACE), ablation, prognosis, interventional radiology

INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most diagnosed cancer and the fourth leading cause of cancer-related death worldwide (1). Hepatitis C virus (HCV) infections and hepatitis B virus (HBV) infections account for most cases of HCC, and the rest mainly comprise people with excessive alcohol consumption, nonalcoholic fatty hepatitis, liver cirrhosis, and a family history of HCC (2, 3). Spontaneous rupture of HCC (rHCC) is a life-threatening complication with an estimated incidence of 3%–26% in patients with HCC (4, 5) and a mortality rate ranging from 25% to 75% (6–9). Furthermore, even if the hemorrhage is completely controlled, rHCC may lead to a high rate of peritoneal dissemination and affect patient survival negatively (10). Therefore, rHCC is significantly associated with high risk of recurrence and poor prognosis (11). The primary aim remains the

prevention of hypovolemic shock and the stabilization of the condition of patients (12, 13); thus, therapeutic options should be discussed according to the condition of the patient, tumor stage, liver function, and feasibility of resection. The treatment options for spontaneous rHCC mainly include emergency surgery, interventional therapy, and palliative treatment. However, the choice of emergency surgery is still controversial because some studies believed that patients with spontaneous rHCC should be managed by non-operative treatment initially (14). Moreover, emergency surgery has a high risk for elderly patients or patients with poor physical condition (15, 16). Emergency surgery may increase the risk of intraperitoneal hemorrhage, liver failure, abdominal infection, and bile leakage. According to the American Joint Committee on Cancer, all patients with spontaneous rHCC are assigned a grade of T4, even if the tumor is small, and there is no vascular invasion (17), indicating the poor prognosis of rHCC; thus, whether those with rHCC are tolerant to emergency surgery should be carefully evaluated (18, 19). Another controversial point is that emergency surgery may prolong the overall survival time for some patients, but it may increase the risk of tumor metastasis for others (18, 20). After the first 12 h of clinical management without significant improvement, supportive care could be chosen in situations of acute uncontrolled bleeding in moribund patients (21). Initial management of rHCC includes a multidisciplinary and comprehensive approach with a primary aim of patient survival, rather than HCC treatment.

Interventional therapy, including transarterial chemoembolization (TACE), transarterial embolization (TAE), transarterial radioembolization (TARE), microwave ablation (MWA), and radiofrequency ablation (RFA), remains a minimally invasive treatment for HCC, even suitable for some patients who have extrahepatic metastases or recurrence (4). TACE/TAE could embolize the blood supply of rHCC *via* the hepatic artery and other extrahepatic arteries. The application of interventional therapy is still broader than open surgery, as interventional therapy can be applied in some cases with Child–Pugh C (22). Furthermore, for patients with severe bleeding, who are not tolerant to open surgery, quick hemostatic methods such as rupture tissue ablation and TAE/TACE can be performed (23). This review will aim to clarify the clinical presentation, prognosis, complication, interventional management, and current evidence of rHCC from the perspective of interventional radiologists.

PATHOPHYSIOLOGY AND RISK FACTORS OF HCC RUPTURE

The mechanism and prognosis of rHCC have not been completely investigated. Studies have also reported that tumor location, tumor size, hypertension, hepatic parenchyma, and liver cirrhosis were associated with rHCC.

The overlying liver parenchyma plays an important role in rHCC. As tumor progresses, when the tumor invades the hepatic arteries or portal vein, the pressure inside the liver may increase and lead to rHCC (24–26). Moreover, a study found that there was almost no overlying liver parenchyma on the ruptured tumor, neither was it shown by imaging examination nor found in operation (27).

The relationship between tumor size and HCC rupture is controversial. Although the size of tumor is associated with the pressure inside the tumor and vessel, Li et al. (27) reported that tumor size is not a significant risk factor in rHCC. However, Chen et al. (25) and Zhu et al. (28) reported that HCC size of more than 5 cm is at high risk of rHCC. Undoubtedly, tumor size is an independent prognostic factor in evaluating overall survival and disease-free survival (29, 30). If the mass volume increases too fast and partial necrosis of the tumor occurs, it can lead to the collapse of the tumor surface and bleeding. Furthermore, researchers also found that an HCC as small as 2 cm has been reported to be ruptured (31). Tumor size is an important study-level factor of rHCC, but clinicians cannot predict rHCC simply by tumor size.

Previous studies have identified that vascular injury may be associated with rHCC. A widely accepted mechanism of rHCC is that hepatic arteries and veins are invaded or occluded by tumor cells, leading to high pressure within the tumor mass (32). As HCC is supplied by the tumor supply vessels, Zhu et al. (33) reported that the vascular injury was present more frequently in the patients with rHCC than that in the patients with non-ruptured HCC. Moreover, Zhu et al. (34) reported that the expression of the antigen–antibody complex including hepatitis B e1 antigen and complement C1q on vascular wall and vascular injuries was mainly presented in patients with rHCC. Their conclusion can also explain why it is more common to see patients with rHCC in Asia than in Europe, because Asia has the highest incidence rates of chronic HBV infection (especially in China) (35). In addition, due to the invasion and growth of tumor, the pressure of hepatic vein may increase, and the high pressure of hepatic vein may be associated with venous congestion, resulting in tumor ischemia, necrosis, and liquefaction. Hibi (36) reported that increased intraluminal pressure of the biliary system due to obstruction by the tumor thrombus is considered to have led to the rupture of the liver abscess into the bronchus, thus creating a bronchobiliary fistula; thus, intraluminal pressure of the biliary system may be associated with a series of risks including tumor rupture. As patients with HCC can be affected by various factors such as coagulopathy and abnormal liver function, all these factors lead to rHCC and intrableeding of tumor (12).

In addition, rHCC is associated with factors involving liver function, portal vein thrombus, location, and so on. Monroe et al. (37) reported that patients with a higher degree of liver dysfunction have an overall poor prognosis and a high possibility of rHCC. After the portal vein was embolized by tumor thrombus, dystrophic necrosis appeared in the peripheral part of the superficial tumor, and the rupture of superficial tumor may also be associated with rHCC (38); thus, the location of the tumor is a risk factor for rHCC. On the basis of the experience from our center, when the tumor is located at the superficial position of the hepatic encapsulation, it can be easily affected by

external forces. The thin tumor encapsulation and the extremely fragile cancer tissue also cause rupture and bleeding.

CLINICAL PRESENTATIONS AND DIAGNOSIS OF RUPTURED HCC

Although rHCC can present with various symptoms, acute abdominal pain can be observed in most cases (66%–100%) of rHCC (17). Shock is the second most common complication of rHCC, with an incidence range from 33% to 90% (7, 9, 20, 39–41). Liver failure occurs in 12%–42% of rHCC (12). Furthermore, in the study by Zhu et al. (28), patients with spontaneous rHCC can be asymptomatic, or experience abdominal discomfort and anemic symptoms, indicating that the presentation of rHCC varies from individuals. Even in some rare cases, patients presented with non-bleeding rHCC (42) or hemothorax (43).

Ultrasonography (US) and computed tomography (CT) are recommended for detecting rHCC, the location of ruptured tumor, and changes in hematoma density. The most direct evidence of rHCC from CT scans is hemoperitoneum (44), but hemoperitoneum cannot be observed in all cases. Moreover, highest-attenuating hematomas, active extravasation of contrast materials, contour protrusion of tumor, discontinuity of the hepatic surface, and the enucleation sign are various CT findings of usual manifestations of rHCC (25, 45–49). Furthermore, the location of highest-attenuation hematomas is close to the rHCC (50).

Patients with rHCC usually presented with acute abdominal pain, and with the utilization of medical imaging, laboratory analyses, and history of patients, a diagnosis of rHCC can usually be made. However, in some cases of rHCC, the diagnosis of rHCC is still a challenge, as some cases are noncirrhotic and there is an absence of risk factors, indicating that other potential factors may also play an important role in carcinogenesis and rHCC (51, 52).

Abdominal paracentesis can be a choice when patients are suspected with rHCC (53). If the non-coagulable blood was sucked out by a syringe, it shows that there is visceral hemorrhage in the abdominal cavity, which is the most effective and routine examination method for the diagnosis of abdominal visceral hemorrhage (54). However, the coagulation function of HCC patients should be taken into account when abdominal paracentesis is performed. Moreover, diagnostic paracentesis is in vain when performed in some rare case presented with no bleeding (42). It is worth noting that no diagnostic study has been published to investigate the sensitivity and specificity of abdominal paracentesis for the diagnosis of rHCC.

TRANSARTERIAL EMBOLIZATION/ TRANSARTERIAL CHEMOEMBOLIZATION FOR RUPTURE OF HCC

Although the prognosis of rHCC is not ideal, survival benefits for interventional radiology and hepatectomy have been reported in patients with rHCC (55–57). Actually, several studies (55, 58, 59) reported that hepatectomy provided better survival benefits than TAE/TACE, reaching an in-hospital survival of 76.5% and a 1-month survival of 71%. However, TAE is the most frequent option to achieve hemostasis and stabilize the condition of patients. The hemostatic success rate from published studies is ideal as most patients can achieve hemostasis (**Table 1**) (29, 30, 58–79). A meta-analysis (5) involving 21 studies with 974 rHCC participants (485 participants treated with TACE/TAE and 489 participants treated with emergency surgery) reported that TAE/TACE was significantly superior to emergency surgery in terms of complications [OR = 0.36, 95% confidence interval (CI): 0.22–0.57] and in-hospital mortality (OR = 0.52, 95% CI: 0.29–0.94), and emergency surgery did not provide a more favorable effect on successful hemostasis (96.2% vs. 94.6%) and 1-year survival rate (47.6% vs. 48.7%) than TAE/TACE. However, all included studies of this meta-analysis were conducted in China, which will undoubtedly lead to bias. Another meta-analysis and systematic review regarding rHCC reported that among patients treated with TACE/TAE, the in-hospital and 1-month survival ranged from 30.3% to 66.7% and from 44.4% to 87.5% (80). Furthermore, Zhou et al. (59) reported that there are no differences in successful rate of hemostasis between TAE and hepatectomy. Furthermore, due to the abnormal coagulation function, poor hepatic function reserve, hemodynamic instability, cirrhosis, and other risk factors (58, 80, 81), many patients with rHCC are not well tolerant to open surgery. Interventional therapies involving TAE/TACE for rHCC can reach an ideal effect. TACE/TAE can be performed as a temporary treatment for patients waiting for staged hepatectomy. Even some cases with poor prognostic factors such as liver function deteriorated, and aforementioned interventional options may be employed in patients with hemodynamic instability or advanced underlying patients with rHCC.

The aim of interventional therapy is not only to achieve successful hemostasis, but also to prolong the survival of patients with rHCC. Lee et al. (64) and Hsueh et al. (69) reported that those who underwent staged hepatectomy after TACE/TAE had significantly higher overall survival than those who underwent TACE alone, indicating that staged hepatectomy is encouraged for post-TAE/TACE patients who are tolerant to hepatectomy. However, whether TAE/TACE is to be followed by staged hepatectomy depends on the recovery of liver function and a thorough investigation of the tumor characteristics. Moreover, whether HCC is ruptured, staged hepatectomy is recommended (12, 20, 82). It should be noted that a meta-analysis (83) reported that long-term staged hepatectomy might not be more beneficial than emergency hepatectomy. However, in this meta-analysis, we found that the authors did not mention how they acquire survival data to estimate hazard ratio from included studies and their results contradict the studies that they included; thus, their conclusion should be interpreted with caution.

Chen et al. (38) and Kirikoshi et al. (30) reported that the main cause of death is failed hemostasis after TAE and hepatic failure. Furthermore, Cheng et al. (63) reported that

TABLE 1 | Summary of clinical studies on successful hemostasis, survival, and embolization agents of TAE/TACE for rHCC.

Study	Study type	Number of patients	Etiology	Intervention	Successful hemostasis	30-day survival	1-year survival	Embolization agents
Bakopoulos A 2018 (60)	Case report	1	HBV	TAE	1	1	1	Microspheres
Barah A 2018 (61)	Case series	2	NA	TAE	2	NA	NA	Gelatin sponge
Battula N 2009 (29)	Case series	7	NA	TAE	5	NA	NA	NA
Buczkowski AK 2005 (62)	Retrospective study	16	NA	TAE	13	NA	NA	A variety of embolic agents were used
Cheng Y 2021 (63)	Retrospective study	186	HBV: 83 HCV: 53 B+C/: 5 Non-B, non-C: 45	TAE	170	148	76	Lipiodol
Kirikoshi H 2009 (30)	Retrospective study	16	HBV: 11 HCV: 1 Alcohol: 1 Non-B, non-C: 3	TAE	15	14	4	NA
Lee HS 2019 (64)	Retrospective study	112	NA	TACE/TAE	107	NA	NA	NA
Masumuto A 1997 (65)	Case report	1	HCV	TAE	1	1	0	NA
Shinmura K 2018 (66)	Retrospective study	51	Alcohol:6 HBV: 21 HCV: 14 Non-B, non-C: 5	TAE	NA	32	NA	Gelatin sponge
Qiu Y 2021 (67)	Retrospective study	322	HBV: 94	TAE	NA	NA	152	NA
Lee K 2019 (68)	Retrospective study	118	HBV: 49 Non-HBV: 69	TAE	118	66	NA	Gel foam slurry and/or PVA
Hsueh K 2012 (69)	Retrospective study	29	NA	TAE	NA	26	18	NA
Kodama Y 2002 (70)	Retrospective study	1	NA	TAE	1	1	NA	Gelatin sponge
Miyayama S 2001 (71)	Case series	3	NA	TAE	3	NA	NA	Lidocaine
Yang P 2007 (72)	Case report	1	HBV	TAE	1	1	1	Coil
Zhou C 2018 (73)	Retrospective study	57	HBV: 47 Other: 12	TAE	57	49	21	Lipiodol or gelatin sponge
Zhou C 2020 (74)	Retrospective study	20	HBV: 20	TAE	20	NA	16	Lipiodol or gelatin sponge particles or PVA
Shin BS 2010 (75)	Retrospective study	47	HBV: 29 HCV: 4 Non-B, non-C: 17	TACE/TAE	44	35	20	Lipiodol or gelatin sponge particles or PVA
Shiozawa K 2013 (76)	Case report	1	Alcohol-related cirrhosis	TAE	1	NA	NA	Sponge particles
Yang H 2014 (58)	Retrospective study	41	NA	TAE	NA	30	4	NA
Yoshiya S 2018 (77)	Case report	1	Non-B	TAE	1	1	NA	Sponge particles
Zhang D 2015 (78)	Retrospective study	126	Alcohol:3 HBV: 114 HCV: 3 Negative: 6	TAE	126	121	99	Sponge particles
Zhong F 2016 (79)	Retrospective study	48	HBV: 41 HCV: 6 Other: 1	TAE	48	39	NA	Gel foam
Zhou C 2020 (59)	Retrospective study	59	NA	TAE	59	32	NA	Lipiodol or gelatin sponge particles or PVA

PVA, polyvinyl alcohol particles; TAE, transarterial embolization; TACE, transarterial chemoembolization; NA, not available.

Child–Pugh classification, MELD score, BCLC stage, albumin, INR, and post-TAE total bilirubin in 7 days are independent risk factors for post TAE 30-day mortality and successful hemostasis in rHCC.

Interventional radiologists should also note that extrahepatic rupture of metastatic HCC has been reported as a rare complication of TAE (84). Suoh et al. (85) reported a case of hemothorax secondary to spontaneous rHCC metastasis to the

chest wall in an 87-year-old man who was treated with TAE, and transcatheter treatment can achieve hemostasis and a favorable survival even in this setting. Nagao et al. (86) reported a case of ruptured chest wall metastasis of HCC that was controlled by TAE.

Kodama et al. (70) reported of a case with rHCC supplied by the right renal capsular artery and Child–Pugh C liver function, indicating that TAE may be chosen for poor liver function when tumor feeders are only extrahepatic collateral vessels. However, Bassi et al. (87) reported that the mortality of patients with Child–Pugh C liver function is exceedingly high in the early post-TAE period, but only three patients with Child–Pugh C liver function received TAE in this study. TAE is a palliative procedure that is used when the liver function is compromised or, in the case of multifocal-bilobar HCC, when the post-treatment mortality of poor liver function is very high regardless of treatment type. In Shin's study (75), the overall median survival time of ten patients with Child–Pugh class C who underwent TAE/TACE was 5 days.

HCC can recruit extrahepatic collateral vessels from a wide range of vessels such as gastroduodenal artery, superior mesenteric artery, and suprarenal arteries (88–90); thus, incomplete embolization of those vessels may also raise the risk of rebleeding. The estimated prevalence of extrahepatic arteries recruited by HCC ranged from 17% to 27% (91–94), indicating that some cases with extrahepatic collateral arteries cannot be successfully embolized because those vessels may originate from other organs such as stomach and duodenum. Moreover, due to the incomplete embolization of extrahepatic collateral arteries, recurrent bleeding and local tumor recurrence should be noted by interventional radiologists in clinical practice. In rHCC with celiac axis stenosis, Barah et al. (61) reported that the pancreaticoduodenal was used as a salvage alternative route for emergency TAE of hepatic arteries. However, in situations such as extensive stenosis or occlusion of the origin of the celiac axis, inaccessibility to hepatic arteries through the celiac axis is a highly challenging situation in which the technical success depends on the experience of interventional radiologists.

It is worth noting that TAE/TACE-led injuries of portal vein or hepatic vein may lead to recurrent bleeding and an unsatisfactory hemostatic effect (95, 96), as HCC can be ruptured after TAE/TACE or during TAE/TACE (97). Furthermore, some patients are at high risk of rupture because of a lack of protective surrounding parenchymal tissue, or tumor progression resulting from revascularization after embolization (98). Moreover, TACE may lead to a series of post-chemoembolization syndrome including pain and fever. Most patients who underwent chemoembolization may experience post-chemoembolization syndrome, but the data are lacking. Moreover, TACE may also be related to acute renal failure, upper gastrointestinal bleeding, and other serious complications (99–101). This may be the reason why TAE is much more frequently performed for patients with rHCC than TACE. Interestingly, Hidaka et al. reported that TACE for those with huge HCC (>10 cm) may also lead to rHCC (102), and a post-TACE huge HCC rupture can be successfully treated using interventional procedures. Tu et al. (103) reported that liver rupture can be observed in 6 of 1,120 patients who received conventional TAE/TACE therapy. According to aforementioned studies, we infer that patients with a history of TACE/TAE or a tumor diameter that is greater than 10 cm may have a higher incidence of rHCC. However, this hypothesis should be verified by further studies.

Various embolization materials can influence the patient prognosis, and the effect of different embolic agents is not completely known. Koçyiğit et al. (104) reported that the use of different embolic agents for TACE had no significant effect on survival on patients with HCC. Marelli et al. (105) reported that there is no evidence of the benefit of lipiodol, and gelatin sponge is the most used embolic agent, but PVA particles may be better. However, in clinical practice, in general, a variety of embolic agents were used, depending on tumor location, degree of bleeding, and interventional radiologist experience. Furthermore, no study has investigated the effect and safety of different embolic agents in the treatment of rHCC.

Discussing the level of embolization, Lee et al. (68) reported that selective or subsegmental TAE was performed if the tumor is solitary and/or a bleeding source was well delineated by CT and/or angiography. In our opinion, the primary aim remains the prevention of hypovolemic shock and stabilization of the condition of patients, and the secondary aim is to embolize the tumor as much as possible when feasible.

Another issue that should be noted is that aforementioned observational studies, non-randomized studies, case reports, and cluster-randomized trials have a known limitation as these studies will force us to focus more on benefits than harms (106).

Furthermore, the overall survival rate of patients with rHCC who underwent TAE/TACE is lower than those who underwent radical resection; hence, the long-term effect of TAE/TACE is still questioned (12).

ABLATION FOR RUPTURE OF HCC

Ablation is another interventional therapy for rHCC, but the efficacy and safety are only reported in a few studies (**Table 2**) (107–111). Although ablation involves radiofrequency ablation therapy (RFA), cryoablation, MWA, and irreversible electroporation (IRE), RFA is the main choice in the treatment of HCC, which can be performed using a percutaneous, laparoscopical, or laparotomic approach. However, the safety of the percutaneous approach is not clear, which is associated with the risk of intrahepatic bleeding, bile leakage, and liver injury (108); thus, Livraghi et al. (112) suggested that the open or laparoscopic method is a safer approach in the treatment of subcapsular tumors.

Gao et al. (108) reported 10 patients [size: 6.6 ± 2.2 cm (4.0–10.1 cm)] with rHCC treated with laparoscopic RFA, with a 3-year survival rate of 70%. Sasaki et al. (113) reported that ruptured peritoneal metastases of HCC can also be controlled by RFA. Sun et al. (114) reported a case of a giant HCC with a maximum diameter of 14 cm with unstable circulation (Child–Pugh C).

TABLE 2 | Summary of clinical studies on successful hemostasis and survival of ablation for rHCC.

Study	Study type	Number of patients	Etiology	Intervention	Successful hemostasis	30-day survival	1-year survival
Cheung T 2014 (107)	Case series	119*	HBV: 86	RFA	NA	93	NA
Gao J 2016 (108)	Case series	6	HBV: 6	Laparoscopic RFA	6	6	6
Warren YE 2016 (109)	Case report	1	NA	MWA	1	1	NA
Huang J 2017 (110)	Case report	1	NA	RFA	1	1	1
Bertacco A 2017 (111)	Case report	1	Alcoholic-related cirrhosis	RFA	1	NA	NA

**Only 19 patients received ablation.*
RFA, radiofrequency ablation; MWA, microwave ablation; NA, not available.

During the emergency laparotomy, the authors found that surgical excision was impossible due to the size and location of the tumor. Then, the patient received RFA as a salvage solution. Finally, patient follow-up for at least 56 months revealed a high quality of life, indicating that a giant rHCC can be successfully controlled by RFA. Chen et al. (115) reported that RFA can also be performed in a case of liver metastasis from rectal cancer and a case of liver metastasis from gastric sarcoma, reaching a beneficial survival time of 36 months and 17 months, respectively.

Among these studies, image-guided percutaneous ablation is commonly used, as it can be minimally invasive, and most cases can achieve hemostasis. Because only limited cases were reported, the benefit of ablation is not completely understood. Three studies (107, 108, 110) reported that direct puncturing of the bleeding site was performed. Similar to TAE/TACE, the primary aim of ablation remains the prevention of hypovolemic shock and the stabilization of the condition, and the secondary aim is to achieve tumor elimination when feasible.

Furthermore, another issue of ablation that should be noted is the "heat sink" effect, which is defined as a phenomenon whereby flowing blood adjacent to or within tissues being targeted for ablation results in relative tissue cooling due to heat transfer by convection (116), leading to a bad prognosis. On the other hand, rHCC usually has a large volume and rich blood supply; thus, the "heat sink" effect may have a significant impact on RFA for a ruptured giant HCC. Interestingly, several studies found that the "heat sink" effect has a significant impact on RFA but not MWA in combination with embolization, indicating that the "heat sink" effect plays a more important role in RFA than in MWA (117–119). Puza et al. (120) reported that the "heat sink" effect appears to have a more minor impact on MWA in a rabbit model and the effect of ablation can be affected when performed adjacent to major vessels. On the basis of these studies, we infer that RFA is much more sensitive in the treatment of rHCC with a smaller diameter and MWA is the optimal ablation for a huge rHCC. However, the conclusion is limited because only several well-documented cases are reported.

INTERVENTIONAL COMBINATION THERAPIES FOR RUPTURE OF HCC

The application of interventional combination therapies is used in some settings. However, interventional combination therapies for rHCC are only reported in several studies (**Table 3**) (121, 122). Baimas-George et al. (121) reported that TAE or TACE followed by laparoscopic MWA and washout may offer an advantage in the treatment of rHCC. It not only achieves hemostasis but also could have an oncologic benefit by targeting local tumor and decreasing peritoneal carcinomatosis risk, reaching a median survival of 431 days in 15 patients, but the 30-day mortality was 6/15. Takao et al. (122) reported that a case of hemobilia from HCC can be controlled by TAE followed by MWA; the patient died of liver failure with no recurrence of hemobilia. However, the prognosis of patients who received TAE/TACE followed by MWA should be evaluated by more high-quality clinical evidence. It is too early to draw final conclusions about the impact of interventional combination therapies on rHCC.

Recently, the interventional combination therapies for HCC include TACE plus brachytherapy and transarterial ethanol

TABLE 3 | Summary of clinical studies on successful hemostasis, survival, and embolization agents of interventional combination therapies for rHCC.

Study	Study type	Number of patients	Etiology	Intervention	Successful hemostasis	30-day survival	1-year survival	Embolization agents
Gao J 2016 (108)	Case series	4	HBV: 4	TAE followed by laparoscopic RFA	4	4	3	Lipiodol and gelatin sponge
Baimas-George M 2020 (121)	Retrospective study	9	NA	TACE/TAE followed by MWA	NA	NA	7	NA
Takao Y 2008 (122)	Case report	1	HCV	TAE followed by MWA	1	1	0	NA

RFA, radiofrequency ablation; MWA, microwave ablation; NA, not available.

ablation combined with TACE (123–125). Moreover, immune checkpoint inhibitors and tyrosine kinase inhibitors are adopted in clinical practice, but there is no evidence to investigate the relationship between rHCC and these drugs (126). Although such treatments are not reported in the treatment of rHCC, those studies prompted us to consider that interventional combination therapies may improve the prognosis of patients with rHCC in the future.

CONCLUSION

In conclusion, the clinical management of rHCC remains a challenge. Our review summarized that interventional therapies are necessary for most patients with rHCC to achieve hemostasis, even in some patients with Child–Pugh C. Furthermore, TAE/TACE followed by staged hepatectomy is the most beneficial treatment for rHCC according to current clinical evidence. However, the efficacy and safety of ablation are still questioned because only a few well-documented studies were reported. Interventional radiologists should also keep in mind that TAE/TACE is the first choice for most patients with rHCC, and appropriate interventional treatment may provide staged surgery opportunities for those who are not tolerant to emergency surgery to reach an ideal prognosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Applications and Achievements of Single-Cell Sequencing in Gastrointestinal Cancer

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Gastrointestinal cancer represents a public health concern that seriously endangers human health. The emerging single-cell sequencing (SCS) technologies are different from the large-scale sequencing technologies which provide inaccurate data. SCS is a powerful tool for deciphering the single-cell resolutions of cellular and molecular landscapes, revealing the features of single-cell genomes, transcriptomes, and epigenomes. Recently, SCS has been applied in the field of gastrointestinal cancer research for clarifying the origin and heterogeneity of gastrointestinal cancer, acquiring micro-environmental information, and improving diagnostic and treatment methods. This review outlines the applications of SCS in gastrointestinal cancer research and summarizes the most recent advances in the field.

Keywords: single-cell sequencing, gastrointestinal cancer, gastric cancer, esophageal cancer, colon cancer

INTRODUCTION

Single-cell sequencing (SCS) has rapidly developed in recent years. The first single-cell mRNA sequencing experiment was performed in 2009, then the first single-cell DNA sequencing experiment in human cancer cells was performed two years later, and the first single-cell exon sequencing experiment was performed in 2012. In 2013, Picelli et al. made some improvements on the smart-seq technology, an SCS protocol (1). This new technology is called smart-seq2. In 2017, 10xGenomics and the Fred Hutchinson Cancer Research Center developed a new single-cell RNA sequencing (scRNA-seq) method, and since then, thousands of immune cells had been analyzed

Abbreviations: CAF, cancer-associated fibroblasts; CNVs, copy number variations; CRC, colorectal cancer; CTC, circulating tumor cells; EC, esophagealcancer; EAC, esophageal adenocarcinoma; FIR, fractionated irradiation; GAC, gastric adenocarcinoma; GC, gastric cancer ; GI, Gastrointestinal; HDGC, hereditary diffuse gastric cancer; IL-17, interleukin-17; LCTCsmulti, large multiploid CTCs; paclitaxel-R, paclitaxel-resistant KYSE-30 cells; PC, pancreatic cancer; PD-1, Programmed cell death protein 1; PC, peritoneal carcinomatosis; scRNA-seq, single-cell RNA sequencing; SCS, single-cell sequencing; SCTCstri, small CTCs with trisomy 8; SPEM, Spasmolytic polypeptide-expressing metaplasia; TAMS, tumor-associated macrophages; Tregs, regulatory cells; WES, whole-exome sequencing.

(2, 3). Single-cell sequencing involves the isolation of the cell group in the tissue or body fluid to a single cell level, then the expansion of the extracted nucleic acid (DNA or RNA) to the lowest detection level, followed by sequencing of the genome and transcriptome, and finally correction and analysis of the data. The SCS procedure is shown in **Figure 1**.

Gastrointestinal (GI) cancers are one of the most common malignant tumors and comprise gastric cancer (GC), esophageal cancer (EC), colorectal cancer (CRC), pancreatic cancer, etc. They are characterized by high morbidity, mortality, malignancy rates, and rapid development (4). GC has a high global prevalence. It accounted for more than 1 million new cases and an estimated 769,000 deaths in 2020, ranking fifth among the causes of global cancer morbidity and fourth among those of cancer mortality (5). Due to the lack of obvious early symptoms, the mortality rate of GC was still one of the highest among malignant tumors. Intriguingly, the incidence of EC was ranked seventh (604,000 cases) for new cases and sixth in the overall mortality rate (544,000 deaths) in 2020. Moreover, CRC (including anal cancer), which accounted for over 1.9 million new cases and 935,000 deaths, ranked third in terms of incidence, but second in terms of mortality among all cancers in 2020. GI cancer is characterized by complex heterogeneity (6) and a specific tumor microenvironment and is extremely suitable for promoting tumor progression and metastasis (7).

SCS plays a significant role in cancer research. Bulk sequencing does not perform well with intratumoral heterogeneity, as it misses rare mutations. For example, in cancer cells, mutations are diluted or lost during averaging of the bulk sequencing (8). In contrast, SCS can be used for the molecular profiling of individual cells and helps in obtaining more precise information about the tumor (**Figure 2**). Therefore, SCS is a potential superior alternative to traditional sequencing methods. Moreover, although the combination of new resistant chemotherapy, molecular targeted therapy, and immunotherapy techniques has shown promising anti-tumor effects against advanced GI tumors, these techniques have several limitations. SCS can better help researchers investigate problems in tumor heterogeneity, microenvironment, diagnosis and treatment. Based on these advantages, many researchers have made important achievements in cancer research by using this technique. This review focuses on SCS and its applications and achievements in GI cancer studies.

SCS APPLICATIONS AND ACHIEVEMENTS IN GC

SCS Reveals the Origin and Heterogeneity of GC

Tumor heterogeneity exists due to cell groups with different genotypes in tumor cells during the process of growth, this different cell groups lead to phenotypic inconsistencies. Intratumoral heterogeneity which is observed in various cancers is also one of the main clinical and pathological characteristics of GC. Cellular heterogeneity is significant genotypic differences within the same phenotype and leads to the differences in the growth, invasion, metastasis and drug sensitivity of GC cells (9, 10). Many researchers have used SCS to investigate the origin and heterogeneity of GC (**Table 1**). Andor et al. characterized cell diversity in nine GC cell lines and assessed gastric cancer heterogeneity and detected scarce clones more efficiently by the high resolution of SCS (11). Peng et al. identified 201 individual cell single nucleotide variations, including 117 non-synonymous mutations, in GC

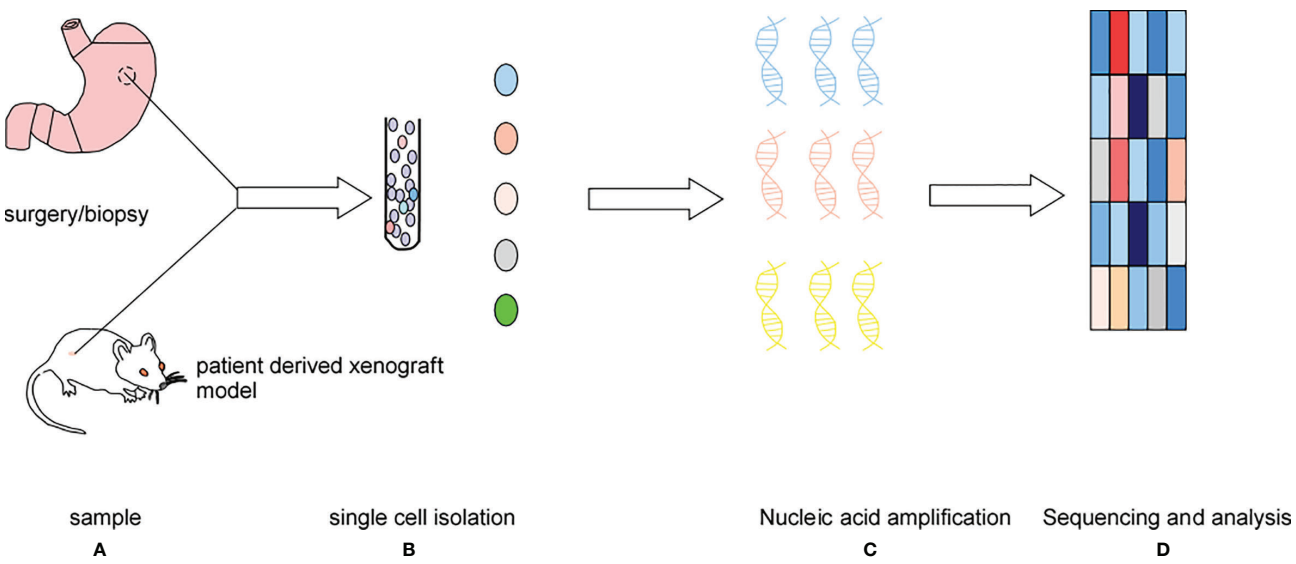


FIGURE 1 | The main processes: **(A)** sample acquisition, **(B)** cell isolation, **(C)** single-cell DNA or RNA amplification, **(D)** high-throughput sequencing and single-cell sequencing data correction and analysis.

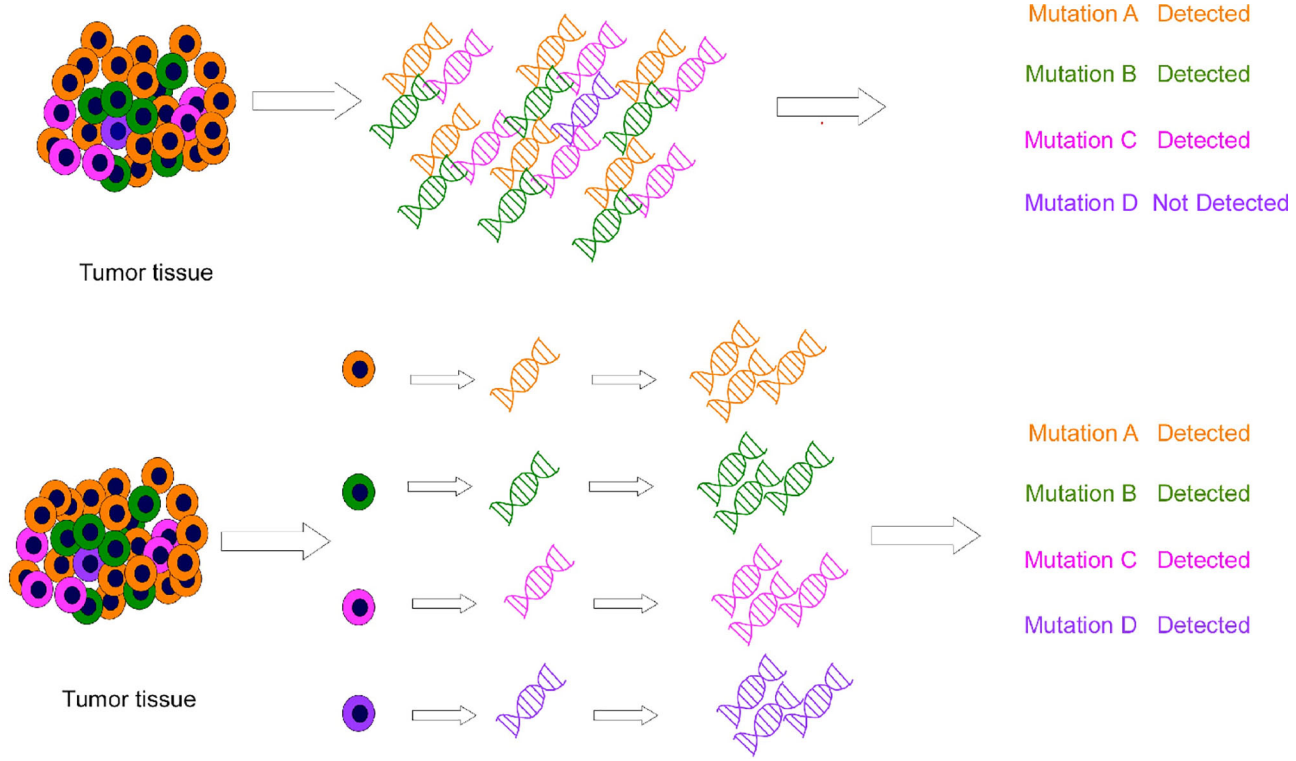


FIGURE 2 | Conventional sequencing (methods above) results in the neglect of some low-abundance information, and single-cell sequencing (methods below) combines cell heterogeneity.

TABLE 1 | SCS in gastric cancer heterogeneity.

Sample	Single Cell isolation	Molecular level	Amplification	Achievement	Reference
Gastric cancer cell lines	Flowmi cell strainer	DNA and RNA	Droplet-based reagent delivery system	Demonstrated a new scDNA-seq technology combined with scRNA-seq and enabled more accurate interrogation of intratumoral heterogeneity.	(11)
Tumor tissue of a patients	Agilent SureSelect Platform	DNA	Agilent SureSelect Platform	Revealed 24 significant mutant genes (SMGs) identified in single cell(*SORD,REXO2, REC8,PTCH2,CTAGE5,RNF20,RBBP4,MGAT4A,KIF15,XYLT1,IGF2BP3,DLC1,TG, CDC27,BAZ1A,ETV6,FLG,NEK6,NSD1,PDE4DIP,RAF1,RNF2,SMO,ZNF483*). The mutant genes CDC27 and FLG might alter the protein conformation only in single cell but not in the corresponding tumor tissue.	(12)
Tumor tissue from 3 patients	Fluorescence microscopy	RNA	Smart-seq2	Discovered some GC lymph node metastasis marker genes (*ERBB2, CLDN11* and *CDK12*), as well as potential gastric cancer evolution–driving genes (*FOS* and *JUN*). *ERBB4, NOTCH2, KIF5B* and *NOTCH2NL* were highly expressed in primary cancer, while *CDK12, CLDN11* and *ERBB2* were over expressed in metastatic cancer.	(13)
7 patients with GC and one patient with gastrointestinal metaplasia	10x Genomics	RNA DNA	10x Genomics	The transcriptional activation levels of GC1 and GC2 cells with different carcinogenic pathways were significantly different.	(14)
9 tumor and 3 non- tumor tissue.	FACS 10x Genomics	RNA	10x Genomics	Uncovered high intratumour differentiation heterogeneity in patients such as IGC1 and IGC4. Several genes specifically expressed in C4 cluster, *RNF43 , GGH, BMP4, DPEP1*.	(15)
15 patients with gastric adenocarcinoma	10x Genomics	RNA	10x Genomics	Tumor cells were divided into four clusters. Cells within C4 expressed the highest levels of entero-derived marker genes, such as *DMBT1, FCGBP, PIGR*, and *WFDC2*, whereas cells within C1 had the highest levels of marker genes expression, such as PSCA and TFF1.	(16)

cells by using SCS. They also revealed that there were 24 significant mutant genes, such as *CTAGE5, REC8, SORD, and PTCH2* genes, in single cells, wherein the change in single amino acids affected protein conformation. This study firstly showed the mutation pattern of GC cells at the intratumoral level and provided more important information for understanding individualized targeted therapy and the heterogeneity of GC cancer (12). Based on the SCS results of three primary and paired metastatic lymph node cancers in GC patients, Wang et al. identified different tumor characteristics and different patients with different microenvironment subsets; moreover, their clustering data revealed that *KIF5B, NOTCH2, NOTCH2NL* and *ERBB4* were highly expressed in primary carcinomas, whereas *ERBB2, CDK12*, and *CLDN11* were highly expressed in metastatic carcinomas (13). Similarly, in Zhang's experiment, they classified a subclass of tumor-specific epithelial cells as "GC type 1", and a subclass consisting of epithelial cells and normal tissues of GC as "GC type 2". The expression of the intestinal mucosal markers *MUC13, TFF3, SPINK4*, *FABP1*, and *REG4* were increased in the GC type 1 subclass, while, the expression of the previously identified gastric cancer marker gene *KRT7* was increased significantly in the GC type 2 subclass (14). A previous study used SCS and GC tumor cell clusters (C1-C5) to investigate that *REG4, CLDN4, TFF3*, and *CLDN7* were upregulated in the malignant epithelium as compared with that in the non-malignant epithelium. In addition, *PGC, MUC5AC, LIPF*, and *GKN1* were highly expressed in the non-malignant epithelium (15). In a recent study, Wang et al. clarified the relationship between tumor cell lineage/state composition and intratumoral heterogeneity at the transcriptional, genotypic, molecular, and phenotypic levels by using SCS. The study demonstrated the diversity of tumor cell lineage/state components in peritoneal carcinomatosis (PC) samples. The relationship was defined as the key factor responsible for intratumoral heterogeneity (16).

SCS Enables the Discovery of the Features of GC Microenvironment

Tumor cells and their microenvironments are interactive and coevolutionary. The tumor immune microenvironment comprises various tumor-infiltrating immune cells (such as B lymphocytes, T lymphocytes, mast cells, natural killer cells, and myeloid suppressor cells) (17, 18). Tumor cells are also surrounded by the stroma, which is divided into cellular and acellular parts. These compartments are composed of complex tumor microenvironments that interact with cancer cells. SCS helps to clarify the molecular level mechanism of the immune cells in the tumor microenvironment during tumor cell generation, development, metastasis, drug resistance and immune escape. It contributes to a more accurate clinical diagnosis, treatment, and prognosis of solid tumors (19, 20). In the past few years, several researchers have made significant advances in microenvironmental research by using SCS to analyze GC cells (**Table 2**). For example, Eum et al. used SCS and found that macrophages which were recovered from malignant ascites of GC patients have non-inflammatory characteristics and the anti-inflammatory properties of tumor-associated macrophages (TAMs) were controlled by tumor cells. These findings helped the researchers to continuously improve the treatment strategies for patients with GC (21). Another study by Meyer et al. demonstrated that the expression of several secretory factors, including IL4, IL5, IL9, IL13, and ARG, were

TABLE 2 | SCS in microenvironment of gastric cancer.

Sample	Single Cell isolation	Molecular level	Amplification	Achievement	Reference
7 patients with GC and one patient with gastrointestinal metaplasia	10x Genomics	RNA DNA	10x Genomics	Cyclin B1 was upregulated by the loss of *CDC27*. *CDC27* was a tumor suppressor inactivated after the mutation.	(14)
15 patients with gastric adenocarcinoma	10x Genomics	RNA	10x Genomics	In tumors with mixed gastrointestinal characteristics, the abundance fraction of B cells increased significantly, with a higher proportion of M1-like macrophages (pro-inflammatory) and a lower proportion of M2-like macrophages (anti-inflammatory).	(16)
Tumor tissue of 4 patients	C1 microfluidic system	RNA	SMART-Seq2	TAMs from GC abundantly expressed proinflammatory cytokines and the macrophages were M2 macrophages.	(21)
Carcinogen-induced mouse model	FACS	RNA	SMART-seq2	ILC2-derived factors were required for the reprogramming of the gastric mucosa after injury and ILC2s performed a central role in the coordination of gastric epithelial repair after severe damage.	(22)
Immune cells in 9 patients with gastric cancer	FACS 10×Genomics	RNA	10×Genomics	*IRF8* was associated with depleted $CD8^+$ T cells in the GC. The transcription factor RBPJ was overexpressed in the tumor-infiltrating Tregs. DC cells expressed more inhibitory receptors in GC tissues, for example, FTL and IL8.	(23)
19 patients with metastatic gastric cancer	10× Genomics	RNA	10× Genomics	Patients who showed a good response to pembrolizumab demonstrated abundant preexisting tumor-infiltrating lymphocytes , a diverse pretreatment TCR repertoire, and a high proportion of stem-like exhausted cells in dysfunctional CD8+ TILs.	(24)

involved in the function of L635-treated ILC2 (type 2 innate lymphoid) cells (22). Fu et al. found that the expression of the transcription factor IRF8 in CD8+ tumor-infiltrating lymphocytes in GC tissue was downregulated in the late stage of GC the disease by using SCS. These findings provided a further rationale for targeted immunotherapy in GC (23). Through SCS analysis, researchers confirmed that tumor-specific macrophages existed in a continuum of stimulus-dependent functional states and were regulated by a specific set of genes. They even found that the increase of the abundance of regulatory cells (Tregs) in the gastric tumor microenvironment was related to immunosuppression (14). Kwon et al. found that the dynamic tumor evolution was more related to the collapse of mutant structures in treatment response. Different T-cell receptor lineages were found to be related with a longer progression-free survival with pembrolizumab treatment by combining whole-exome sequencing (WES) with scRNA-seq. In addition, the increase in the number of PD-$1^{+}CD8^{+}$ T cells was associated with lasting clinical benefits (24).

SCS Facilitates the Diagnosis and Treatment of GC

ScRNA-seq approaches can identify optimal combination therapies that efficiently target heterogeneous cell populations. Furthermore, scRNA-seq can identify the alterations associated with treatment resistance in different cell clusters to support individualized cancer therapy (25). The optimization of existing chemotherapeutic agents and the development of targeted therapies have provided more options for the treatment of advanced gastric cancer and further prolonged the survival expectations of the patients. In addition, global efforts, including the employment of SCS, have been made to identify new specific, predictive, sensitive, and prognostic biomarkers and to establish innovative molecular classifications based on gene expression profiles (26). With the use of SCS, the researchers have made great progress in the diagnosis and treatment of gastric cancer (**Table 3**). For example, Zhang et al. used SCS to construct a single-cell network based on the cellular and molecular characteristics of gastric epithelial cells with different lesions and establish OR51E1 as a unique endocrine cell marker in early malignant lesions. They also suggested that HES6 may mark goblet precell clusters and these findings helped to identify metaplasia in the early stage. Zhang et al. also determined the specific characteristics which is clinically significant for its accurate diagnosis in early GC (27). SCS can also help to identify markers related to tumor diagnosis and personalized therapy (33, 34). Wang et al. performed SCS to classify PC samples into two subtypes that were predicted independent of clinical variables, obtained and verified the prognostic markers of 12 genes (*TPM2, FCGBP, CDK6,*

TABLE 3 | SCS in diagnosis and treatment of gastric cancer.

Sample	Single Cell isolation	Molecular level	Amplification	Achievement	Reference
15 patients with gastric adenocar-cinoma	10x Genomics	RNA	10x Genomics	*TPM2, FCGBP, CDK6, NCBP2, CLCX3, PIGR, BTF3, CKB, VPS28, TM4SF1, EIF3E, GPX4* were the prognostic markers in multiple large-scale gastric adenocarcinoma.	(16)
Tumor tissue of 4 patients	C1 microfluidic system	RNA	SMART-Seq2	Combination therapies targeting cancer cells and macrophages might have mutually synergistic effects. *CITED2* alone had prognostic value in predicting overall GC survival by representing macrophage M2 properties.	(21)
Tumor tissue of 13 patients	10x Chro-mium platform	RNA	10x Chro-mium platform	Among the genes upregulated in the endocrine cells of the EGC lesions, *OR51E1* was ranked at the top. *HES6* could be used to label cells with some goblet cell features but had not been morphologically identified as goblet cells.	(27)
Carcinogen-induced mouse model	10x Genomics	RNA	10x Genomics	Muc6 + Gif + epithelial cells were present in healthy stomachs, but did not express SPEM transcripts such as *Tff2, Cd44*, and *Cftr*.	(28)
Tumor tissue of 111 advanced GC patients	NMSCM, Cytelligen	DNA	Single Cell WGA Kit	*V7A* and *G15S* non-synonymous mutations were more frequently identified in SCTCstri, and high frequency of *MET E1214A, PIK3CA K440N FGFR1 M2761,E1214D, K1215E, L687I* and *K1215 N* mutations were detected in LCTCsmulti.	(29)
402 cells from 6 patients	From the GSE112302 dataset in the Gene Expression Omnibus	From the GSE112302 dataset in the Gene Expression Omnibus	From the GSE112302 dataset in the Gene Expression Omnibus	A prognostic risk scoring signature consisting of 8 GC differentiation-related genes was generated(*VCAN, TNFAIP2, STMN2, RNASE1, DUSP1, AQP2, ADAM8, TFF1*).	(30)
Two gastric cancer cell lines, YTN16 and YTN2 were inoculated in C57BL/6 mice	FACS 10X Genomics	RNA	10X Genomics	The combination of anti-IL-17 and anti-PD-1 mAb caused strong tumor regression and was confirmed in a murine gastric cancer model.	(31)
Carcinogen-induced mouse model	10X Genomics	RNA	10X Genomics	Expanded the definition of gastric metaplasia to include Gkn3 mRNA and GKN3-positive cells in the corpus, allowing a more accurate assessment of SPEM.	(32)
Carcinogen-induced mouse model	10X Genomics	RNA	10X Genomics	Cytokeratin 7, encoded by the differentiation-dependent gene *Krt7*, was a specific marker for early neoplastic lesions.	(20)

NCBP2, CLCX3, PIGR, BTF3, CKB, VPS28, TM4SF1, EIF3E, GPX4) in multiple large-scale gastric adenocarcinoma (GAC) cohorts (16). Another study by Bockerstett et al. established that in Spasmolytic polypeptide-expressing metaplasia (SPEM) and cervical cell proliferation hypertrophy, the expression of SPEM-related transcripts were similar, and the mechanism of drug-mediated parietal cell ablation was similar to that of SPEM induced by chronic inflammation (28). Chen performed single-cell DNA sequencing of 50 target circulating tumor cells (CTCs) and discovered that large multiploid CTCs (LCTCsmulti) and small CTCs with trisomy 8 (SCTCstri) had different gene variations. Moreover, mutations in the KRAS and Rap1 pathways were abundant in SCTCstri, while several unique mutations in the MET/PI3K/AKT pathway and *SMARCB1* genes were found in LCTCsmulti. These findings highlighted the different mechanisms of drug resistance for modulating target therapy and could help in preventing the poor prognosis of patients (29). Based on the data from scRNA-seq, some researchers analyzed GC samples to classify them into three GC differentiation-related genes molecular subtypes. They found that molecular typing based on cell differentiation could successfully predict the overall survival of the patient, immune checkpoint gene expression, clinicopathological features. This study emphasized the significance of GC cell differentiation in predicting the clinical outcomes and potential immunotherapy responses of patients (30). Furthermore, by transplanting two GC cell lines into mice and performing single-cell transcriptome sequencing of the transplanted tumors, Nagaoka confirmed that interleukin-17 (IL-17) could be a potential target for enhanced programmed cell death 1, anti-PD-1 (programmed cell death protein 1) mAb treatment for GC (31). Bockerstett et al. sequenced the transcriptome of gastric mucosal epithelial cells and found that gastrin 3 mRNA was a tumor-specific marker of the gastric epithelium of intestinal metaplasia (32). Analysis of the SCS data from mice with hereditary diffuse gastric cancer (HDGC) revealed that inactivation of Cdh1 led to metastasis along the squamous cell differentiation trajectory associated with aberrant expression of GI epithelial differentiation center genes. Cytokeratin 7 encoded by the differentiation-dependent gene Krt7, was a specific marker of early neoplastic lesions in CDH1 carriers (20). In conclusion, SCS is valuable in identifying prognostic tumor markers for predicting potential clinical outcomes and immune responses, as well as, for individualized therapy.

SCS APPLICATIONS AND ACHIEVEMENTS IN EC

SCS Reveals the Origin and Heterogeneity of EC

SCS plays an important role in studying the origin and heterogeneity of esophageal cancer. Many researchers have used this technique to achieve important results in revealing the esophageal cancer heterogeneity (**Table 4**). For example, Zhang et al. screened the ESCC cells into 38 subsets, and found that there were 24 subsets with more than 75% of the cells coming from an individual patient. These 24 subsets were defined as the cluster 1, and the remained 14 subsets were defined as the cluster 2. Cluster 1 showed increased pathway activity in cell proliferation and EMT, while cluster 2 showed activation of immune-related pathways. This study showed high heterogeneity in ESCC (35). Wu et al. found that NOTCH signaling was not activated in esophageal adenocarcinoma (EAC) and only activated in ESCC. ESCC tumors with higher NOTCH activity were associated with significantly worse survival. Furthermore, levels of *DLL1, JAG1* and *NOTCH2* were higher in ESCC and EAC tumors than in normal tissues, while products of active signature genes (*SNAI2* and *TNFSF10*) were only detected in ESCC tissues. This study revealed differences in cellular transcriptomic profiles of ESCC and EAC, and a wide range of intratumoral cellular heterogeneity. This founding had important implications for future therapeutic strategies and drug development (36). Chen et al. reclustered malignant cells from five ESCC tumor samples and identified five subsets. Each subset was corresponded to a patient. They revealed high-risk genes in different patients, and different patients showed different expression patterns for different high-level genes. Furthermore, the SCS and copy number variations (CNVs) analysis data showed relative changes in the CNV profiles of all tumor cells compared to non-malignant epithelial cells. The tumor cells in each subpopulation exhibited different CNV status.

TABLE 4 | SCS in esophageal cancer heterogeneity.

Sample	Single cell isolation	Molecular level	Amplification	Achievement	Reference
60 ESCC tumor and 4 adjacent normal tissue 60 patients	10x Genomics	RNA	10x Genomics	The quantitative data showed that in patients with the same level of intratumoral heterogeneity, patients with group 1 cluster had relatively higher levels of intertumor heterogeneity than patients without group cluster 1.	(35)
368 single cells from three ESCC and two EAC	10X Genomics	RNA	SMART-seq2	The three ESCC tumors contained an overwhelming majority of cancer cells with notable *TP63/SOX2* over-amplification, which was not apparent in EAC cancer cells.	(36)
5 ESCC patients and 5 corresponding non-malignant patients	10X Genomics	RNA	10X Genomics	*EGR1* was highly expressed in patient 2, whereas S100A8 / 9 was found to be a high-risk ESCC gene in patient 4. Compared to other sub-clus-ters, cluster 1 demonstrated an apparent CNV loss in chromosome 4 and chromosome 5.	(37)
11 patients with ESCC	10X Genomics	RNA	10X Genomics	Mac_1 expressed multiple chemokines,Mac_2 expressed Cathepsin genes,Mac_3 intriguingly expressed a number of nonclassical monocytic genes,Mac_5 was characterized by its specific expression of interferonstimulated genes.	(38)

These findings demonstrated the high heterogeneity of ESCC tumor cells in terms of gene expression and CNV status (37). Another study explained the high degree of heterogeneity in the ESCC microenvironment. Macrophages were clustered into five subsets. Among the five macrophage subsets, Mac_1, Mac_2 and Mac_3 expressed higher anti-inflammatory "M2" -related genes in Mac_C, with Mac_5 expressing M2-like genes (38).

SCS Enables the Discovery of the Features of EC Microenvironment

SCS plays an important role in determining the cellular characteristics of the EC microenvironment. To date, various studies have utilized SCS techniques to explore the problems in the microenvironment of EC (**Table 5**). For example, some researchers discovered that TAMs expressed not only genes related to immunosuppression (*TGFB1* and *COX2*) but also genes related to angiogenesis (*VEGFA, CXCL8, MMP9*, and *MMP12*), and found that *VEGFA* was upregulated in monocytes, while MMPs were mainly expressed in TAMs. These results revealed the immunosuppressive status of the esophageal squamous cell carcinoma (ESCC) tumor microenvironment and improved our understanding of ESCC (35). Similarly, several characteristics of $CD4^+$ T cells had been identified by using SCS. Three classes of CD4+ T cells were identified *via* SCS, and it was found that CD4_1 upregulates TIGIT expression in tumors, CD4_2 expresses PD-1 (encoded by PDCD1) exclusively in tumors, and CD4_3 showed tumor-specific TIGIT and CD96 expression. In addition, both CD4_1 and CD4_2 were found to express high levels of CTLA-4 in tumors (38). Wu et al. recently showed that cell cycle signaling was associated with high cancer stemness of EAC, such as *E2F3, CHEK1, CDC20, SMC3, and TFDP1*. In addition, they identified a novel cancer stem cell-associated gene, poly (ADP-ribose) polymerase 4, and they validated its association with survival in a cohort study of 121 ESCC patients (39). Another study discovered a strong correlation between FGF2 and SPRY1 expression in EC using SCS. In the fibroblasts in EC tissues, a high FGF2 expression was found to be associated with low overall survival, and the mouse tumor model confirmed that FGF2 overexpression in fibroblasts significantly upregulated SPRY1 expression in the depleted T cells, weakened the cytotoxic activity of T cells, and promoted tumor growth (40). Together, several studies have identified the features of different cells in the EC microenvironment and found some exclusively expressed genes using SCS. These researches has considerably improved our understanding of esophageal carcinogenesis.

SCS Facilitates the Diagnosis and Treatment of EC

SCS also plays an important role in the diagnosis and treatment of EC by facilitating the identification of diagnostic markers and development of new treatment. Many investigators have applied SCS to make great progress in the diagnosis and treatment of EC (**Table 6**). For example, using scRNA-seq and real-time quantitative PCR, Zhang et al. verified that *RAD51AP1, KIF2C, KIF20A,NUF2, PBK*, and *DEPDC1* are all potential biomarkers for the diagnosis and prognosis of ESCC and may be potential therapeutic targets for ESCC (41). Furthermore, researchers found that $CD4^+$ Tregs expressed the highest levels of IL-32 and had relatively low expression in proliferating natural killer cells and suggested that IL-32 may be a target for immunosuppressive therapy in EC (42). Using scRNA-seq analysis of KYSE-30 cells and paclitaxel-resistant KYSE-30 cells (paclitaxel-R), Wu et al. showed that the two subsets based on *KRT19* expression levels had different paclitaxel sensitivity, suggesting there were intrinsic paclitaxel resistance in KYSE-30 cells. They also found that the proteasome inhibitor carfilzomib could attenuate resistance to paclitaxel-R cancer cells by activating HIF-1 signaling, suggesting that the combination of carfilzomib and paclitaxel could be used as a novel cancer treatment (43). Yang et al. carried out scRNA-seq of KYSE-180 cells and found radioresistance in KYSE-180 cells after fractionated irradiation (FIR) treatment. These foundings provided an important reference for developing radiotherapy strategies (44). Another study involved the identification of 42 recurrent radioresponsive

TABLE 5 | SCS in microenvironment of esophageal cancer.

Sample	Single cell isolation	Molecular level	Amplification	Achievement	Reference
60 ESCC tumor and 4 adjacent normal tissue	10x Genomics	RNA	10x Genomics	Most of TEX cells were likely tumor-reactive T cells. Esophageal squamous carcinoma tissue was enriched in Treg and Tex cells, whereas TN, TMEM, and Teff cells were fewer, suggesting that TME was in an immunosuppressive state	(35)
Primary tumors and matched adjacent nonmalignant esophageal tissues from 11 treatment-naive ESCC patients	10x Genomics	RNA	10x Genomics	CD4_1 expressed abundant follicular-assisted T (TFH) effector genes (*CXCL13, IL2RA, TNFRSF18, TNFRSF*) CD4_2 subset was characterized by effector memory genes, including *IL7R*, and *CXCR6*.	(38)
From the SRA (https://www.ncbi.nlm.nih.gov/sra) under the accession no. SRP119465	From the SRA (https://www.ncbi.nlm.nih.gov/sra) under the accession no.SRP119465	RNA	From the SRA (https://www.ncbi.nlm.nih.gov/sra) under the accession no. SRP119465	Cell cycle signaling was associated with high cancer stemness of EAC,such as *E2F3,CHEK1, CDC20, SMC3 ,TFDP1*.	(39)
8 treatment-naïve ESCC patients	FACS	RNA	10x Genomics	*FGF2* as an important regulator of SPRY1 expression was involved in establishing the dysfunctional state of CD8+ T cells in esophageal cancer.	(40)

TABLE 6 | SCS in diagnosis and treatment of esophageal cancer.

Sample	Single cell isolation	Molecular level	Amplification	Achievement	Reference
3 ESCC patients and 208 single cells	From the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra)	RNA	From the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra)	*RAD51AP1, KIF2C, KIF20A, NUF2 , PBK*, and *DEPDC1* were associated with the diagnosis and prognosis of ESCC.	(41)
7 ESCC tumor and paired adjacent tissues of the patient	10 × Genomics	RNA	10 × Genomics	IL-32 was overexpressed in T and NK Cells in the TME. IL-32 was dominant in CD4+ Treg Cells.	(42)
Carcinogen-induced mouse model	FACS	RNA	SMART-seq2	Low HIF-1 and high proteasome expression were critical for acquired paclitaxel resistance in ESCC.	(43)
KYSE-180 cells	FACS	RNA	SMART-seq2	The *CFLAR, LAMA5, ITGA6, ITGB4*, and *SDC4* genes were verified as radioresistance genes.	(44)
Tumor tissue of 2 patients	Qiagen	DNA	REPLI-g UltraFast Mini Kit	A subset of sensitive mutations in 10 genes and resistant mutations in 18 genes defined a significantly improved prognosis and the shortest time for locoregional recurrence, respectively, indicating possible clinical utility.	(45)

genes (sensitive and resistant), including *GAS2L2*, *NOTCH1*, *OBSCN, MAML3*, *NFE2L2,TP53* and *CDKN2A*. This finding provided a reference for the diagnosis and treatment of EC (45).

SCS APPLICATIONS AND ACHIEVEMENTS IN CRC

SCS Reveals the Origin and Heterogeneity of CRC

SCS is important in revealing the origin and heterogeneity of CRC. With the help of SCS, many investigators have further revealed the origin and heterogeneity of CRC (**Table 7**). Some researchers performed single-cell analysis of colon cancer samples using high-throughput SCS based on multiple displacement amplification. They focused on a mutant gene, *SLC12A5*, and found that *SLC12A5* activation could promote cell proliferation and inhibit apoptosis, thus potentially promoting oncogenesis and demonstrating the biclonal origin of CRC cases (46). In addition, two normal or adenomatous polyps in CRC patients were studied *via* single-cell whole-exome sequencing and matched bulk WES. The results indicated that accumulation of non-random somatic gene mutations were involved in the GPCR, PI3K-Akt, and *FGFR* signaling pathways were also observed. These new driver mutations in *OR1B1* (GPCR signaling), *LAMA1* (PI3K-Akt signal in CRC

TABLE 7 | SCS in colorectal cancer Heterogeneity.

Sample	Single cell isolation	Molecular level	Amplification	Achievement	Reference
Fresh tumor and adjacent normal tissues from a patient	manual-controlled pipetting system	DNA	Multiple displacement amplification	Activation of SLC12A5 becomed a potential oncogenic driver event in colon cancer by promoting cell proliferation and inhibiting. apoptosis.	(46)
20 normal single cells, 25 polyp single cells, 20 adenomatous polyps single cells, and 50 cancer single cells .	micromanipulation system	DNA	Agilent SureSelect Platform	New mutations were found in *OR1B1* (GPCR signaling pathway) in adenoma evolution, and *LAMA1* (PI3K-Akt signaling pathway) and *ADCY3* (FGFR signaling pathway) in CRC evolution.	(47)
Cancer tissue obtained 2824 cells from CRC patient with stage III C	10x genomics	RNA	10x genomics	High degree of specificity existed for the genes clustered by five cells in the same tumor tissue.	(48)
1,900 single cells from 12 CRC patients (stage III or stage IV)	/	DNA	scTrio-seq (single-cell triple omics sequencing) technique	The feasibility of reconstructing genetic lineages with single-cell multigroup sequencing, tracking its epigenome and transcriptome dynamics was demonstrated.	(49)
9 tumor regions and 88 single cells from two rectal cancer patients	FACS	DNA	multiplexed single-cell MALBAC	Dominant subclones adapted to the surrounding microenvironment played a dominant role in a certain region of a given tumor, and their dominance changed dynamically, such as *TP53*, *ERBB2*, and *APC.*	(50)
590 cells from 11 primary CRC tumors and matched normal samples	10x genomics	RNA	10x genomics	The pathway alteration and diversity of CAFs in CRC and the treatment barriers caused by CAF heterogeneity.	(51)

evolution), and *ADCY3* (FGFR signaling) of adenoma evolution and cancer evolution, confirming that both colorectal adenomas and CRC were of monoclonal origin (47). Davel analyzed scRNA-seq data of 2824 cells from CRC cancer tissues, dividing different cells of tumor tissues into five clusters according to specific genes, further analyzing the cluster data, gene ontology terms, KEGG pathways and trajectory maps.The study found that cluster 1 was characterized by a unique set of genes, such as *IGLC7, IGLC2, IGLC3*, cluster 2 has unique *HLA-DRA, IGHM, IGHG2* other genes, and the remaining three clusters also defined themselves by unique genes. A high degree of specificity between the different clusters was found (48). These results showed that SCS was a powerful tool for studying tumor cell heterogeneity. Owing to the unique high resolution of SCS, scTRIO-SEQ (a type of single-cell triple sequencing) can simultaneously assess somatic copy number changes, DNA methylation, and transcriptomic information and facilitate single-cell heterogeneity research. The high-throughput and high-resolution characteristics of scRNA-seq are also beneficial for the detection of tumor samples (49). Some studies have shown that mutations in *ATM* and *GNAS*, as well as deletions in the tumor suppressor gene *PTEN*, likely led to tumorigenesis because these genes were potential cancer driver genes. Besides, it had been suggested that mutations in *TP53*, *ERBB2*, and *APC* may play an important role in tumorigenesis and may serve as drug targets (50). Markers for two different subtypes of cancer-associated fibroblasts (CAF) were identified using SCS studies. CAF-B cells expressed markers of myofibroblasts such as *TAGLN, ACTA2*, and *PDGFA*, and CAF-A cells expressed *DCN, MMP2*, and *COL1A2*. Only the CAF-A cells expressed the FAP (fibroblast activa tion protein α). Thus, this indicated that the heterogeneity of CAF may constitute a potential barrier to FAP-directed therapy (51).

SCS Enables the Discovery of the Features of Tumor Microenvironment in CRC

SCS facilitates the discovery of cellular features in the CRC microenvironment (**Table 8**). SCS data revealed that the proportion of somatic copy number alteration (SCNA) in cancer tissues was much higher than that in adjacent normal tissues (11.1% v.s.10.6%), and five genes (*BGN*, *RCN3*, *TAGLN*, *MYL9*, and *TPM2*) were identified as fibroblast-specific biomarkers of poor CRC prognosis. Thus CRC successfully confirmed the extensive genomic alteration in cells in CRC tumor microenvironment (52). Studies of the CRC tumor microenvironment using SCS revealed cancer type-specific T cell subsets and developmental patterns, as well as detailed molecular characterization of tumor immune-related T cell clusters. The cellular and molecular mechanisms underlying the tumor immune microenvironment composition, heterogeneity, and formation were revealed (53). Comprehensive analysis of the non-epithelial scRNA-seq data derived from precancerous lesions and CRC revealed that the proportion of $CD8^+$ T cells, natural killer cells, and γδT cells (labeled cytotoxic cells) was significantly increased in serrated polyps compared to that in adenomas (54). Another study examined TAMs in CRC and found that Bcl9 deficiency caused macrophage polarization inhibition from M0 to M2 and altered the CRC tumor microenvironment to further interfere with the inflammation of M0 and M1, the cell type balance and transcription differences in TAMs regulated by BCL9-driven Wnt signaling affected immune surveillance and inflammation in cancer (55). Together, with the support of new technologies, SCS has greatly promoted a thorough understanding of the tumor microenvironment.

SCS Facilitates the Diagnosis and Treatment of CRC

Many researchers have used SCS to make great achievements in the diagnosis and treatment of CRC (**Table 9**). Some studies demonstrated that primary tumor cells evolved for a long time and acquired many mutations such as in *KRAS*, *NRAS*, *APC*, and *TP53*, which spread to distant sites and organs by using high-throughput single-cell DNA sequencing to study the advanced transmission of model metastatic CRC. This transmission model could be extended to many human cancers with important clinical significance (56). Lei et al. conducted scRNA-seq analysis on immune and stromal populations from CRC patients and identified specific macrophage and conventional

TABLE 8 | SCS in microenvironment of colorectal cancer.

Sample	Single cell isolation	Molecular level	Amplification	Achievement	Reference
Peripheral blood from 8,982 immune cells of 12 patients with CRC	FACS	DNA	Multiplexed single-cell MALBAC	*BGN, RCN3, TAGLN, MYL9*, and *TPM2* were identified as fibroblast-specific biomarkers with a poor prognosis in colorectal cancer.	(52)
T cells from a total of 12 CRC patients in 4 MSI and 8 MSS patients	/	RNA	SMART-seq2	The CRC-specific T cell subpopulation, included Th17 (CD4_C08-IL23R), follicular T helper cells (CD4_C06-CXCR5), follicular T regulatory cells (CD4_C11-IL10), CD8_C05-CD6, and CD8_C06-CD160. The latter two clusters highly expressed CD69 and ITGAE.	(53)
Pre-cancers and CRCs, serrated polyps (SERs) consisting of hyperplastic polyps (HPs)	TruXTRAC FFPE microTUBE DNA Kit-Column Purification kit	RNA	ScRNA-seq	The proportion of CD8+ T cells, natural killer cells, and γδT cells (labeled cytotoxic cells) were significantly increased in serrated polyps compared to that in adenomas.	(54)
12 tumor samples from mice model	Magnetic-activated cell sorting (MACS)	RNA	10x genomics	The macrophages interacted with T cells through the CCL3-CCR5, CAF1R-CSF1 and ICAM1-ITGAL to change the T-cell functions in $hsBCL9_{CT}$-24 treated group. Depletion of *Bcl9* maked the CSF1R-CSF1 and CCL4-CCR5 was significantly regulated.	(55)

TABLE 9 | SCS in diagnosis and treatment of colorectal cancer.

Sample	Single cell isolation	Molecular level	Amplification	Achievement	Reference
Frozen primary colon cancer and matched metastatic CRC in liver tissues	FACS	DNA	High-throughput single-cell DNA sequencing method	APC was the first blow to trigger colon cancer before the *KRAS* and *TP53* mutations. Late transmission indicated that the primary and metastatic tumors had most of the clinically relevant gene mutations(amplification of oncogenes including *CDX2, CDK8, JAK3* and *ZNF217*).	(56)
Cells were extracted from tumors, adjacent normal tissues, and blood from 18 initially treated CRC patients	FACS	RNA	SMART-seq2	A method to dissect specifically the effects of tumor-associated immune populations was demonstrated.	(57)

dendritic cell subsets as key mediators of cellular cross-talk in the tumor microenvironment. Besides, they determined that anti-CSF1R treatment preferentially depleted macrophages with inflammatory features, and CD40 agonist antibody treatment preferentially activated the conventional dendritic cell population (57).

SUMMARY AND PROSPECTS

The incidence and mortality rate of malignant tumors in China are the highest among the world, and the overall situation of GI cancer prevention and treatment are very grim. SCS has become an important technique for studying GI cancer. Currently, with its development and integration with other technologies, further improvements and advances in SCS technologies will improve its applicability in clinical settings. However, the technique has some limitations. For example, biological noise will lead to the change of single cell sequencing data and affect the results of data (36). Another limitation is RNA leakage. It may occur during reverse transcription and then may introduce substantial bias (58). SCS also has some shortcomings. Single-cell sequencing is very sensitive to samples and therefore not suitable for analysis of preserved or poorly processed clinical samples.So it is difficult to translate the results from sequencing studies into the clinic (59). The high of SCS cost limits the ability to analyze a large number of tumors, and often only a few to dozens of samples are analyzed per study.

With the development of single-cell multiplexed technologies and the miniaturization and automation of SCS instruments, these limitations and shortcomings may be solved gradually and SCS will have more vast applications in GI cancer research. What's more, with continuous innovation and optimization of methods, the SCS technology will continue to promote the development of biomedicine and the accurate treatment of GI cancer and may likely aid in achieving high-quality long-term survival for patients with GI cancer.

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YJ and KL participated in the design, conception. ZX, JL, PH, YZ and JY wrote the review. ZX, JL, YJ and KL revised the review. All authors contributed to the article and approved the submitted version.

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Beyond N staging in colorectal cancer: Current approaches and future perspectives

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Traditionally, lymph node metastases (LNM) evaluation is essential to the staging of colon cancer patients according to the TNM (tumor–node–metastasis) system. However, in recent years evidence has accumulated regarding the role of emerging pathological features, which could significantly impact the prognosis of colorectal cancer patients. Lymph Node Ratio (LNR) and Log Odds of Positive Lymph Nodes (LODDS) have been shown to predict patients' prognosis more accurately than traditional nodal staging and it has been suggested that their implementation in existing classification could help stratify further patients with overlapping TNM stage. Tumor deposits (TD) are currently factored within the N1c category of the TNM classification in the absence of lymph node metastases. However, studies have shown that presence of TDs can affect patients' survival regardless of LNM. Moreover, evidence suggest that presence of TDs should not be evaluated as dichotomic but rather as a quantitative variable. Extranodal extension (ENE) has been shown to correlate with presence of other adverse prognostic features and to impact survival of colorectal cancer patients. In this review we will describe current staging systems and prognostic/predictive factors in colorectal cancer and elaborate on available evidence supporting the implementation of LNR/LODDS, TDs and ENE evaluation in existing classification to improve prognosis estimation and patient selection for adjuvant treatment.

KEYWORDS

colorectal cancer, tumor staging, lymph node metastases, adjuvant treatment, TNM

Introduction

Colorectal cancer (CRC) represents the third most common cancer and the second leading cause of cancer-related death in the overall population, with nearly 1,148,515 new diagnosis and 576,858 deaths in 2020. Data have shown a slight difference between the two sexes; indeed, it takes up the second place for incidence and the third for mortality in women and the third for both incidence and mortality in men (1).

Despite being considered for many years an age-related neoplasia, in recent times there appears to be a decline in CRC incidence in the population over 50-year-old, balanced by an increase of new diagnosis in individuals younger than 50 years (2).

The 5-year survival rate has considerably increased during the past decades, reaching 63% all stages combined in 2021. There are however considerable variations amongst the 5-year survival rate depending on the TNM stage of the disease at moment of the diagnosis: as a matter of fact, it amounts to 91% in the localized disease (stage I-II), 72% in the regional disease (stage III) and it dramatically drops to 14% in the advanced disease (stage IV) (3, 4).

Complete resection of the primary tumor and regional lymph nodes remains the most effective therapy for early colon cancer. Adequate surgery also allows for evaluation of the resection specimen which is considered an essential step to define prognostic factors and predict disease recurrence after surgery, thus informing clinicians on potential benefits of adjuvant treatment.

Optimal management currently relies on the tumor-node-metastasis (TNM) staging system proposed by the American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC), which assesses primary tumor (T), lymph node metastasis (N), and distant metastasis (M). This classification has now reached its eight iteration (5). Lymph node metastases, in particular, are considered a significant factor for predicting disease-free survival (DFS) and overall survival (OS) in patients with colorectal cancer without distant metastasis (6).

Beyond the above-mentioned TNM staging, other risk factors have shown an impact on the prognosis, particularly in stage II: pT4; inadequate lymphadenectomy (<12 lymph nodes); vascular invasion; lymphatic invasion; perineural invasion; high grade tumor; high preoperative CEA levels; tumor presentation with obstruction (7, 8). Moreover, MSI-H/MMRd status represents a molecular marker that has demonstrated to be related to a better prognosis in localized CRC and designates a subgroup of patients with less expected response to 5-fluorouracil-based chemotherapy (9).

However, definition of further pathological features can help improve existing classifications, to better identify patients with localized disease and a higher risk of relapse and to guide more accurately the choice of optimal adjuvant treatment.

In this review we will explore how emerging pathological characteristics, aside from existing biomarkers, can impact patient prognosis and how their factoring can improve disease management, and guide adjuvant strategies in colorectal cancer patients.

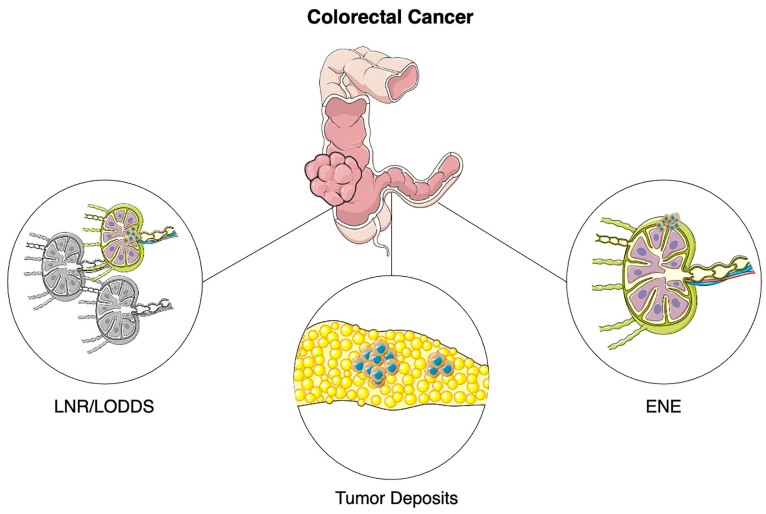


FIGURE 1
Emerging pathological features in colorectal cancer. Abbreviations: LNR, Lymph Node Ratio; LODDS, Logarithm of Positive Lymph Nodes; ENE, Extranodal extension.

We will focus on the role of lymph node ratio, tumor deposits, extracapsular node extension.

A descriptive illustration is available in Figure 1.

Methods

We reviewed the available literature on the use of lymph node ratio, tumor deposits and extracapsular node extension in CRC staging and overall management. We performed PubMed and Embase searches focused on these topics, selecting primary and review articles from peer-reviewed journals. Search terms included "lymph node ratio", "log odds of positive lymph nodes", "tumor deposits", "extracapsular node extension", "colorectal cancer". We also searched PubMed and major oncology conferences for presentations pertinent to the matter of this review.

Lymph node ratio and log odds of positive lymph nodes

Lymph node ratio

Lymph Node Ratio (LNR) is defined as the ratio of metastatic lymph nodes (LN) over total LN examined (Figure 1). LNR has been established as a prognostic indicator in several non-colorectal malignancies, such as breast cancer, esophageal and gastric cancer, medullary and papillary thyroid cancer, non-small cell lung cancer, oropharyngeal cancer (10–16).

In colorectal cancer, even though pathological nodal stage remains one of the most important predictors of patient prognosis, several studies have tried to evaluate the potential of LNR as a prognostic marker.

When examining these results, it is important to underline that since 2002, three different American Joint Committee on Cancer (AJCC) staging classifications have been issued, with differences in stage grouping especially between 6th and 7th edition (17, 18).

All the studies revised are summarized in Table 1.

Wang J et al. were among the first authors to show in 2008 the role of LNR as an independent predictor of survival in 24,477 stage III colon cancer patient from the SEER registry (19). Patients were stratified in 4 groups according to three different cutoffs (1/14, 0.25, 0.5) and LNR was deemed to be more accurate then TNM staging for stage IIIB and IIIC patients.

In 2005, Berger AC et al. published an analysis on stage II and III patients with colon cancer pooled from Intergroup trial 0089 of fluoropyrimidine-based adjuvant chemotherapy and proved LNR to be the most significant prognostic factor for both DFS and OS in patients with at least 10 LN sampled (22); interestingly, within the N1 and N2 classifications, dramatic changes were observed in recurrence rates based on the LNR value (less than 5%, 5% to 20%, 20% to 40%, or more than 40%). This work has the benefit of clarifying the prognostic relevance of LNR. Since all patients received adjuvant chemotherapy, it is unlikely that the impact of improved nodal staging is explained by more patients receiving intensified treatment. This means that a lower LNR, and consequent better prognosis, could be attributable to other variables, such as the quality of surgery performed.

It is a matter of debate whether the number of examined lymph nodes can influence the ability of LNR to stratify patients according to prognosis.

While in fact some authors suggested that discrimination provided by LNR is lost when less than 12 LN are examined (20, 23–25), work published by Rosenberg et al. showed that the LNR remained an independent predictor of outcome even when less than 12 nodes are examined and had better value than pathological nodal stage in the multivariate analysis (26).

The same observation was made by Peschaud F et al. in rectal cancer patients, where LNR predicted DFS and OS even when fewer than 12 LN were examined (27).

Ceelen W et al. eventually published in 2010 a systematic review based on 16 analyzed studies, including 33,984 patients with stage III colon or rectal cancer (21). In all the studies reviewed, LNR was an independent prognostic factor and allowed for a prognostic separation that was superior to that of the nodal stage alone in terms of OS, DFS and cancer specific survival.

Several trials have since been reported reinforcing the prognostic value of LNR in both early-stage colon and rectal cancer (28–30, 55). However, there is no consensus on the cut-off to use when applying LNR.

In 2014, a French regional study conducted by Sabbagh C et al. identified a 10% cutoff as optimum to distinguish between good and poor prognosis stage III colon cancer patients (31). This stratification allowed for significant correlation with 3-year OS and DFS.

Shinto E et al. proposed the use of different cut-offs to predict the prognosis of right or left-side primary colon cancer; by analyzing 5,463 patients with stage III colon cancer authors were able to stratify patients using values of 0.16 and 0.22 for right-sided and left-sided tumors, respectively (32).

Zhang CH et al. also designed a study to further validate the prognostic significance of LNR by evaluating 218,314 patients from the SEER database and 1,811 patients from three independent cohorts (33). Patients were divided into 5 groups according to LNR cutoffs previously investigated (0, 0.1-0.17, 0.18-0.41, 0.42-0.69, >0.7) and each group identified patients with worsening prognosis regardless of LN sampling.

Several attempts have been made to propose updates to pre-existing classifications by incorporating LNR information (34, 35).

TABLE 1 Evidence regarding LNR/LODDS implementation.

Authors	Subject	Major Findings	Reference
Wang J et al.; Chin CC et al.; Ceelen W et al.	LNR	LNR is an independent and more accurate prognostic method for stage III colon cancer patients than AJCC TNM categories	(19–21)
Berger AC et al.	LNR	After curative resection for colorectal cancer, the LNR is an important prognostic factor and should be used to stratify patients receiving adjuvant treatment	(22)
In JP et al, Shimomura M et al., Li Destri G et al.	LNR	Adequate lymph node examination is important to ensure the prognostic value of LNR in patients with stage III colorectal cancer	(23–25)
Rosenberg R et al., Peschaud F et al.	LNR	LNR remains an independent prognostic predictor in colorectal cancer even when fewer than 12 LN are examined	(26, 27)
Isik A et al.; Parnaby CN et al.; Sabbagh C et al.; Shinto E et al.; Macedo F. et al; Zhang CH et al.	LNR	LNR impacts both DFS and OS in colon cancer patients; several cut-offs have been proposed to stratify patients	(28–33)
Sugimoto K et al; Wang LP et al.; Pei JP et al.; Yang LV et al.	LNR/ LODDS	Implementation of LNR/LODDS improves prognostic accuracy of existing staging classifications	(34–37)
Madbouly KM et al.; Chen L et al.; Junginger et al.; Lykke J et al.; Karjol U et al.;	LNR	LNR can provide prognostic information in locally advanced rectal cancer and compensate for inadequate lymph node dissection in patients who did not receive preoperative therapy	(38–42)
Deng Y et al.; Alexandrescu ST et al.; Ahmad A et al.	LNR	High LNR correlates with burden of liver metastatic disease and predicts shorter RFS in patients undergoing curative resection	(43–45)
Mohan HM et al.; Jakob MO et al.	LNR	LNR is either equivalent or inferior to pathological nodal staging in patients with adequate LN harvesting	(46, 47)
Wang J et al; Fang HY et al.; Li T et al	LODDS	LODDS accurately predicts prognosis of patients with early-stage colon cancer	(19, 48, 49)
Occhionorelli S et al.	LODDS	LODDS is the only independent prognostic factor in patients with colon cancer receiving emergency surgery	(50)
Lee CW et al.; Xu T et al.	LODDS	LODDS accurately predicts prognosis of patients with locally advanced rectal cancer	(51, 52)
Baqar AR et al.; Song Y et al	LODDS/ LNR	LODDS adds no prognostic information to LNR alone, which should be preferred due to ease of application	(53, 54)

DFS, Disease Free Survival; LN, Lymph Node; LNR, Lymph Node Ratio; LODDS, Logarithm of Positive Lymph Nodes; OS, Overall Survival.

Pei JP et al. developed a revised TLNR classification by combing tumor stage and LNR based on data from 62,294 early-stage colon cancer from the SEER registry and 3,327 additional patients from an external validation cohort (36). The novel classification was found to be superior to the AJCC 8th TNM classification in predicting overall and disease-free survival.

Even though most efforts have focused on colon cancer, data has accumulated in rectal cancer patients as well (38–40).

For example, Junginger et al. demonstrated that LNR can provide prognostic information and thus compensate for inadequate lymph node dissection in patients with stage III rectal cancer who did not receive preoperative treatment (41).

Karjol U et al. recently published a systematic review and meta-analysis on this topic, encompassing 18 trials and 4,486 node-positive rectal cancer patients, confirming that a higher LNR was significantly correlated with worse OS and DFS (42).

However, not all the available evidence is in favor of LNR implementation in current staging systems. Mohan HM et al. suggested that LNR provides no additional information when compared with nodal staging, while Jakob MO et al. determined LNR to be inferior to pathological nodal staging in node-positive colon cancer patients (46, 47).

Lymph node ratio in metastatic CRC

LNR has been also evaluated as a prognostic marker in patients with colorectal cancer and liver metastases.

High LNR was significantly associated with lower 3-year relapse free survival (RFS) in patients with liver-limited disease undergoing curative resection, as observed by Deng Y et al. (43).

Alexandrescu ST et al. evaluated the role of LNR in predicting prognosis of patients with synchronous liver metastases and found that LNR was the only independent predictor of both DFS and OS (44).

LNR has been correlated with burden of liver metastases as well, as shown by Ahmad A et al. in their analysis of 53 stage IV colorectal cancer patients (45); authors found that high LNR status predicted the presence of more than 3 liver lesions and poorer OS.

Log odds of positive lymph nodes

Log Odds of Positive Lymph Nodes (LODDS) is defined as the logarithm of the ratio of metastatic lymph nodes to negative lymph node (Figure 1).

The LODDS classification system has been tested with success in both breast and gastric cancer (56, 57). When applied to colon cancer, LODDS was proven effective in discriminating between patients with overlapping LNR values as shown by Wang J in a work already reported in this review (19).

Fang HY et al. compared the prognostic assessment of pathological nodal stage, LNR and LODDS using data collected retrospectively from 192 patients with resected colorectal cancer (48). All three variables correlated significantly with survival, yet LODDS was superior to the other categories in the multivariate analysis. Li T et al. confirmed the prognostic and clinic-pathological value of LODDS in a cohort of 389 patients with colorectal cancer undergoing curative surgery (49).

An interesting work by Occhionorelli et al. proved that LODDS was the only nodal category able to independently predict prognosis in 320 patients with colon cancer receiving emergency surgery (50).

LODDS was a reliable prognosticator in locally advanced rectal cancer as well, as reported in works by Lee CW et al. and Xu T et al. (51, 52). In particular, the latter work highlighted once more the importance of different staging approaches in improving the definition of prognosis in patients with lower LN yield.

LODDS has been proposed to complement existing staging classification, too. Pei JP et al. tried combining tumor stage with LODDS to classify 45,558 patients from the SEER database and found that the novel TLODDS classification has better discriminatory ability than current TNM staging (37).

However, criticism has emerged regarding simplicity of application of LODDS.

Baqar et al. compared LNR and LODDS in a cohort of 862 patients and found no difference in the prognostic impact of the two categories, suggesting LNR use is preferrable due to its ease of calculation (53). Song YX et al. analyzed data of 1,297 patients with colorectal cancer and found the LNR classification was superior to LODDS in assessing patient prognosis (54).

Summarizing, LNR is an independent and more accurate prognostic method for early colon cancer patients than AJCC TNM categories, even though no consensus has been reached on minimum number of lymph nodes to examine and on the cut-off to implement in existing staging systems. It can also be informative in the metastatic setting, since it has shown correlation to burden of liver metastases and survival in patients undergoing curative resection. It is a matter of discussion whether LODDS adds additional information to LNR and N staging.

In conclusion, both LNR and LODDS have been thoroughly evaluated as prognostic markers and should be evaluated for incorporation in upcoming staging classifications.

Tumor deposits

Tumor deposits (TDs) are defined as discrete nodules of tumor cells in the bowel surrounding fat, lacking associated lymph node tissue and vascular or neural structures, which are found in 20-25% of colon cancer patients (Figure 1) (58).

Since its inclusion in the AJCC TNM staging system, TDs definition has changed considerably and, with every new edition, there has been an upstaging for patients with TDs between in up to 64% of cases (59). They were first defined as a separate entity in the 7th edition of the TNM classification, with the introduction of the pN1c category, categorizing the presence of TDs in the absence of LNMs, whereas, in presence of lymph node metastases, TD status is discarded.

However, presence of TDs seems to be prognostically of equal importance to N status and its evaluation should not be restricted to cases in which pathological lymph nodes are absent (60).

In fact, a retrospective analysis performed by Shen F on 19,991 patients with colorectal cancer pooled from the SEER database found that the N1c category is associated with a prognosis similar to that of the N1b category (61). Mayo et al. performed a different analysis on the same database and showed that presence of TDs is associated with lower 3-year OS in multivariable models (62). Interestingly, presence of TDs is associated with worsening hazard ratio in lower N stages. A phase III trial in colon cancer patients receiving adjuvant chemotherapy (IDEA France) also demonstrated a significantly higher risk of recurrence or death in patients with TDs, regardless of LNM substatus (63).

Moreover, factoring of TDs should not be dichotomic. TDs should rather be considered as a quantitative variable, with a higher number of TDs predicting worse survival (58).

A retrospective analysis performed by Pricolo EV et al. in stage III colon cancer patients showed how patients included in pN1c staging category with ≥ 3 TDs had a worse overall survival than those with < 3 TDs, with a prognosis resembling that of pN2 patients (64). Zheng K et al. identified a cutoff of 4 or more TDs to predict poorer disease specific survival using data pooled from SEER database (65).

A similar conclusion was produced by Wang S et al. using data from 39,155 colorectal cancer patients within the SEER database (66). Authors found that the prognostic value of one TD is equivalent to that of two metastatic LNs based on the comparison of cause-specific survival rates and proposed this approach to be superior to the N1c staging in stratifying patient prognosis.

Research produced by Mirkin KA et al. and Zheng P et al. in patients with stage III colon cancer pooled from the National Cancer Data Base and SEER registry found that the presence of both TDs and LN metastases confers additive risk. Presence of both elements was, in fact, associated with significantly worse survival than each of these risk factors alone (67, 68).

A recent *post hoc* analysis of the CALGB/SWOG 80702 phase III study by Cohen R et al. suggested that combining the number of TDs to that of pathological lymph nodes improves the prognostic accuracy of current TNM staging (69). Combining TD and the number of lymph node metastases, 104 of the 1470 patients included in the analysis were re-staged as pN2 and showed significantly worse outcomes than those patients confirmed as pN1.

Other features of TDs have been investigated beyond their number.

A retrospective review classified TDs in invasive-type TD (iTD) (vascular invasion, lymphatic invasion, perineural invasion and undefined cancer clusters) or nodular-type TD (nTD) (cancer aggregates without iTD component): DFS was significantly shorter in both node-negative and node-positive, iTD/nTD+ patients compared to TD- patients. Among node-negative patients, disease-specific survival (DSS) differed significantly between the iTD/nTD+ and TD− groups, while in node-positive patients presence of nTD had no impact on DSS (70).

A more accurate staging of these patients may also help to improve adjuvant treatment strategies. Currently, in fact, patients with TDs but no metastatic LNs are less likely to receive adjuvant chemotherapy (52% vs 74%) and have longer delay to treatment initiation, as shown by Wong-Chong N et al. These patients are also reported to be younger and to have more adverse tumor features (71).

Adequate selection for adjuvant treatment is even more relevant, considering that the number of TDs reported does not impact the benefit of adjuvant chemotherapy (72).

Finally, TDs have been evaluated as prognostic indicator in metastatic CRC as well. Lin Q et al. evaluated 146 patients with synchronous colorectal liver metastases undergoing simultaneous resection of primary tumor and metastatic lesions. Authors found that the presence of TDs was associated with significantly shorter DFS, regardless of LN status (73).

All the evidence reviewed is summarized in Table 2.

Summarizing, presence of TDs is at least of equal importance to N status and its factoring should not be restricted to cases in which lymph node metastases are absent, considering also that both features confer additive risk. Factoring of TDs should not be dichotomic as higher count of deposits predicts poorer survival. Presence of TDs is also informative in metastatic CRC, as it is associated with worse survival in patients undergoing simultaneous resection for liver colorectal metastases.

In conclusion, TDs can influence patient prognosis significantly and should be highly considered when evaluating patient prognosis and indications to adjuvant treatment.

Extranodal extension

Extranodal extension (ENE) is defined as the extension of tumor cells through the nodal capsule into the perinodal fatty tissue (Figure 1). Current AJCC TNM classification in colorectal cancer does not account for presence of ENE, even though it could theoretically identify a more aggressive disease (74).

Evidence has accumulated regarding its prognostic significance in several malignancies, including breast, head and neck, gastro-esophageal, prostate and bladder cancer (75–79).

Early evidence of its role in colorectal cancer was collected in a systematic review of literature by Wind J et al. which included 4 series of patients with lower gastrointestinal tract malignancies, where presence of ENE identified patients with significantly worse long-term prognosis (80).

More recently Veronese N et al. published a new systematic review with meta-analysis evaluating 1,336 patients with colorectal cancer from 13 different trials (81). Authors reported ENE was associated significantly with higher stage and grade of disease, increased risk of all-cause mortality (HR = 1.69, 95% CI 1.32–2.17, P < 0.0001) and increased risk of recurring disease (HR = 2.31, 95% CI 1.54–3.44, P < 0.0001).

Further evidence has since emerged. Ambe PC et al. reported data from a cohort of 147 patient with node-positive colorectal cancer, suggesting extranodal extension predicts higher risk of cancer-related death (OR= 0.44, p = 0.021) and shorter median OS (30.5 ± 42 months vs. 51.0 ± 33, p = 0.02).

Kim CW et al. reported the results of a single-institution analysis of 2,346 patients with colorectal cancer receiving curative surgery (6). Authors found that ENE was associated with younger age, more advanced tumor stage, presence of both lymphovascular invasion (LVI) and perineural invasion (PNI) in both colon and rectal cancer. Interestingly, ENE frequency was described to be increasing from the right colon to the left colon and the presence of this pathological feature was reported to independently predict DFS regardless of tumor location in patients who do not receive adjuvant chemotherapy.

A paper produced by Li T et al., already mentioned in our work in relation to LODDS, confirmed these findings in a different cohort (49). ENE frequency was once more reported to increase in distal tumors and its presence was associated with worse prognosis in both colon and rectal cancer patients.

Summarizing, presence of ENE is associated with increased risk of recurrence and worse survival. It is also frequently associated with other “high-risk” features such as higher tumor grade and stage.

All the evidence available is therefore in favor of ENE implementation in current classifications to improve patient stratification and selection for treatment intensification.

Conclusion

Current management of early colorectal cancer is based on the existing 8th edition of the TNM classification. However, emerging pathological features as described in this review, can significantly modify the prognosis of patients within a same stage group. It should be noted that most of the evidence reviewed in this paper is based on retrospective analysis, which carry intrinsic limitations. Nevertheless, until prospective evidence is available to support implementation of LNR, TDs and ENE in clinical practice, clinicians should evaluate these features in addition to traditional staging system on a patient basis in order to guide treatment and follow up in cases where risk assessment is not straight-forward.

TABLE 2 Evidence regarding TDs implementation.

Authors	Subject	Major Findings	Reference
Nagtegaal ID et al.	Staging TDs	Presence of TDs is at least of equal importance to N status and its factoring should not be restricted to cases in which LN are absent.	(59, 60)
Shen F et al.	Staging TDs	Cancer specific survival difference between N1b and N1c is not statistically significant	(61)
Mayo et al.	Presence of TDs	Presence of TDs predicts poorer survival, especially in lower N stages	(62)
Pricolo VE et al.; Brouwer NPM et al; Zheng K et al	Number of TDs	TDs number is associated with worse survival	(58, 64, 65)
Mirkin KA et al.; Zheng P et al.	Presence of TDs and LNMs	Presence of both TDs and LNM was associated with worse survival than with each factor alone	(67, 68)
Nagtegaal ID et al.; Wang S et al.; Cohen R et al.;	Number of TDs	TDs should be added to final N count. According to Wang S et al. one TD should be considered as two LNMs	(59, 60, 66, 69)
Yamano T et al.	TDs subclassification	Classifying TDs in invasive-type and nodular-type TDs may improve prognostic value	(70)
Lin et al.	TDs evaluation in metastatic disease	Presence of TDs is associated with worse survival in patients undergoing simultaneous resection for liver colorectal metastases	(73)

LNM, Lymph Node Metastasis; TD, Tumor Deposit.

In the era of precision medicine and amidst attempts to de-escalate intensity of adjuvant treatments, it appears fundamental to adequately select patients with worse prognosis who require a more aggressive management.

We believe that both pathologist and clinicians should factor LNR, LODDS, TD and ENE when assessing a patient's outcome and when selecting individuals for a more intensive treatment and follow-up strategy.

Author contributions

GA contributed to conception and design of the review. GA, MP, BF, and SF wrote the first draft of the manuscript. GA, MP, BF, and SF wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Prognostic Roles of ceRNA Network-Based Signatures in Gastrointestinal Cancers

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Gastrointestinal cancers (GICs) are high-incidence malignant tumors that seriously threaten human health around the world. Their complexity and heterogeneity make the classic staging system insufficient to guide patient management. Recently, competing endogenous RNA (ceRNA) interactions that closely link the function of protein-coding RNAs with that of non-coding RNAs, such as long non-coding RNA (lncRNA) and circular RNA (circRNA), has emerged as a novel molecular mechanism influencing miRNA-mediated gene regulation. Especially, ceRNA networks have proven to be powerful tools for deciphering cancer mechanisms and predicting therapeutic responses at the system level. Moreover, abnormal gene expression is one of the critical breaking events that disturb the stability of ceRNA network, highlighting the role of molecular biomarkers in optimizing cancer management and treatment. Therefore, developing prognostic signatures based on cancer-specific ceRNA network is of great significance for predicting clinical outcome or chemotherapy benefits of GIC patients. We herein introduce the current frontiers of ceRNA crosstalk in relation to their pathological implications and translational potentials in GICs, review the current researches on the prognostic signatures based on lncRNA or circRNA-mediated ceRNA networks in GICs, and highlight the translational implications of ceRNA signatures for GICs management. Furthermore, we summarize the computational approaches for establishing ceRNA network-based prognostic signatures, providing important clues for deciphering GIC biomarkers.

Keywords: ceRNA network, prognostic signature, gastrointestinal cancer, translational implication, lncRNA, circRNA

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Abbreviations: GIC, Gastrointestinal cancers; ncRNA, non-coding RNA; ceRNA, competing endogenous RNA; lncRNA, long non-coding RNA; circRNA, circular RNA; GC, gastric cancer; CRC, colorectal cancer; EC, esophagus cancer; TNM, tumor-node-metastasis; miRNA, microRNA; MREs, miRNA-response elements; SPONGE, Sparse Partial correlation ON Gene Expression; CLASH, crosslinking, ligation, and sequencing of hybrids; SNHG, small nucleolar RNA host gene; OS, overall survival; EMT, epithelial-mesenchymal transition; EAC, esophageal adenocarcinoma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; MSigDB, Molecular Signatures Database; ssGSEA, single-sample Gene Set Enrichment Analysis.

INTRODUCTION

Gastrointestinal cancer (GIC), mainly including gastric cancer (GC), colorectal cancer (CRC) and esophagus cancer (EC), represents a common threat to public health, with morbidity and mortality accounting for more than 15% of all cancers (1). Although significant progress in treatment strategies, e.g. surgery, chemotherapy, targeted therapy and radiotherapy, has been achieved over the past years, the outcomes of GICs are still disappointing since they mostly develop with no obvious symptoms and are frequently diagnosed at advanced stages (2). Moreover, due to complexity and heterogeneity, GIC patients with identical pathologic conditions often exhibit huge variation in treatment response and prognosis, limiting the application of traditional approaches (e.g. tumor-node-metastasis (TNM) pathological staging) to distinguish patients at high risk of metastasis or death. Therefore, it is critical to develop novel and powerful prognostic models that can provide reliable information for patient risk stratification and treatment choice.

Early researches on the molecular mechanisms of tumorigenesis were mainly focused on the function of protein-coding genes, as proteins were traditionally considered as the central function executor. In the past two decades, the technological advances in next-generation sequencing approaches have enabled the system-level understanding of biological processes, which revealed that the presence of numerous non-coding RNAs (ncRNAs) contributes to the diversity and complexity of human transcriptome (3). Importantly, due to their regulatory roles in cellular events necessary for growth and development, ncRNA abnormal expression is closely linked to cancer pathogenesis (4, 5). Therefore, the exploration of ncRNAs can provide critical clues for identifying novel diagnostic and/or therapeutic targets in multiple cancer types.

ncRNAs comprise a diverse variety of RNA species, e.g. microRNA (miRNA), long noncoding RNA (lncRNA), circular RNA (circRNA) and etc. (6). Among them, miRNAs perform post-transcriptional regulatory roles by binding to miRNA-response elements (MREs) of target mRNAs (7). Increasing studies have demonstrated that target genes carrying common MREs can compete to sponge the same miRNA. Accordingly, competing endogenous RNAs (ceRNAs) hypothesis was put forward by Salmena et al. in 2011 (8) and has received extensive attention since then. It postulates that coding and non-coding RNA molecules with common MREs can compete for miRNA binding at these sites, thus indirectly regulating the expression of each other by acting as miRNA sponge (9).

Currently, as new functional players in cancer biology, lncRNA and circRNA have emerged as the most important ceRNA types (10, 11). Especially, based on the pivotal roles of ceRNA crosstalk in modulating cancer hallmarks, systematic construction and analysis of lncRNA/circRNA-mediated ceRNA network has recently become a powerful tool for decoding the underlying molecular mechanism of cancers and identifying prognostic biomarkers in these diseases (12, 13). Besides, many pseudogenes can also crosstalk with protein-coding genes by acting as ceRNAs to sequester shared miRNAs. For example, RP11-3543B.1 has been identified as an oncogenic pseudogene that implicated in GC pathogenesis by regulating MAPK4 expression *via* a ceRNA mechanism (14). However, there is little evidence for pseudogene-related prognostic signatures in GICs. Therefore, we here introduce the functional roles of lncRNA/circRNA-mediated ceRNA crosstalks in the pathogenesis of GICs, present a review on the prognostic signatures constructed based on lncRNA/circRNA-mediated ceRNA network in GICs, and summarize the computational strategy for establishing prognostic signatures based on ceRNA network.

LNCRNA/CIRCRNA-MEDIATED CERNA CROSSTALKS IN GICS: FUNCTIONAL ROLES AND PROGNOSTIC IMPLICATIONS

As two novel classes of ncRNA regulators, lncRNAs and circRNAs play critical roles in multiple steps of cancer initiation and progression. With the innovations in biotechnology and bioinformatics, they are increasingly identified and characterized in GICs through genomic and transcriptomic studies (15, 16). Notably, given the ability to interact with miRNAs, both lncRNA and circRNA have emerged as the most important ceRNA players with prognostic significance in GICs.

Mounting evidence has demonstrated the profound impact of lncRNA/circRNA-mediated ceRNA interactions on multiple processes and events in the pathogenesis of GC, CRC and EC, such as cell proliferation, invasion, migration, apoptosis, or chemoresistance (**Figure 1**). For example, lncRNA MAGI2-AS3 can regulate the expression of epithelial-mesenchymal transition (EMT) transcription factor ZEB1 by sponging miR-141/200a to promote GC cell migration and invasion (17). By regulating the Wnt/β-catenin pathway, circFGD4 and LINC01133 serving as ceRNAs of APC, inhibit GC progression (18, 19), while circBANP and NEAT1-mediated ceRNA crosstalks contribute to CRC cell proliferation and invasion (20, 21).

Besides, lncRNA/circRNA-mediated ceRNA crosstalks are able to facilitate risk stratification and guide clinical decision-making for GIC patients (**Table 1**). For example, the small nucleolar RNA host gene (SNHG) family members (e.g. SNHG6, SNHG11 and SNHG12) are newly recognized important lncRNAs that promote tumor progression through the ceRNA mechanism (22–24). Increased SNHG6 expression was significantly correlated with poor prognosis of both GC and CRC patients (22, 25). In addition, it has been shown that ciRS-7 can act as an oncogene by inhibiting miR-7 activity *via* a ceRNA manner in GC, CRC and EC (**Figure 1**) (26–28), making it a promising prognostic biomarker and an attractive therapeutic target for GIC patients.

Furthermore, increasing ceRNA players have emerged as potential therapeutic targets for GIC patients due to their critical roles in tumor progression (**Table 1**). For example, lncRNAs (e.g. HIF1A-AS2, GCMA and HOTAIR) and circRNAs (e.g. circ-RanGAP1, TMEM87A, circLMTK2 and circTMC5) implicated in

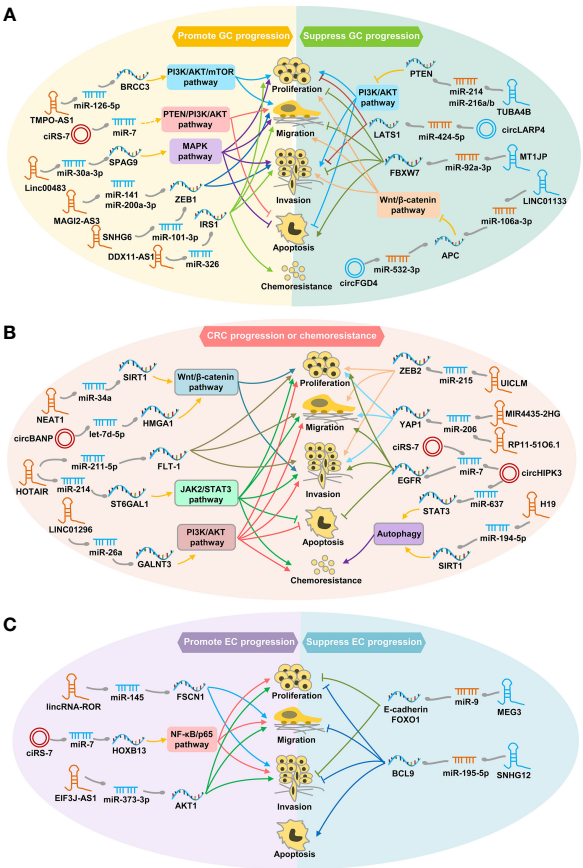


FIGURE 1 | Schematic diagram of representative ceRNA crosstalks function in GC **(A)**, CRC **(B)** and EC **(C)**. **(A)** ceRNA interaction regulates tumor cell proliferation, migration, invasion, apoptosis, or chemoresistance through PI3K/ATK, MAPK or Wnt/β-catenin signaling pathways, thereby exerting carcinogenic or tumor suppressor effects in GC. **(B)** ceRNA interaction contributes to CRC progression or chemoresistance by regulating autophagy process or pivotal pathways, such as Wnt/β-catenin, PI3K/ATK and JAK2/STAT3 signaling pathway. **(C)** ceRNA interaction promotes or inhibits EC progression by modulating cancer cell proliferation, migration, invasion, or apoptosis.

GC metastasis by acting as ceRNAs, hold promise as potential therapeutic targets for GC patients (29–35). Besides, development of chemoresistance remains a primary obstacle for GIC treatment. It has been demonstrated that DDX11-AS1 can contribute to oxaliplatin resistance in GC by sponging miR-326, implying its therapeutic role (36). circHIPK3 and H19 have been reported to promote oxaliplatin and 5-FU resistance in CRC by mediating different ceRNA interactions, respectively (**Figure 1**) (37, 38). Those findings indicate that targeting circHIPK3 and H19 are also potential therapeutic strategies to inhibit chemoresistance in CRC.

Collectively, as pivotal factors mediating cancer pathogenesis, ceRNA players have emerged as promising prognostic biomarkers and attractive therapeutic targets in the clinical management of GICs.

PROGNOSTIC SIGNATURES BASED ON LNCRNA/CIRCRNA-MEDIATED CERNA NETWORK IN GICS

As ceRNA networks connect the function of different RNA species, the characterization of cancer-specific ceRNA network may provide a valuable clue to systematically explore the potential role of RNA molecules in cancer pathogenesis. Therefore, a number of efforts have focused on construction of signatures based on lncRNA/circRNA-mediated ceRNA network in GICs (**Table 2**), illuminating new avenues to explore powerful prognostic biomarkers and therapeutic targets in the era of precision medicine.

TABLE 1 | Prognostic lncRNAs and circRNAs that function by a ceRNA mechanism in GICs.

ceRNA	Shared miRNA	Target mRNA	Clinical significance	Role in cancer	Cancer type	PMID
lncRNA-mediated ceRNA crosstalk						
XIST	miR-101	EZH2	Prognostic biomarker and therapeutic target	Promote	GC	27620004
LINC01939	miR-17-5p	EGR2	Prognostic biomarker and therapeutic target	Suppress	GC	30683847
LINC02163	miR-593-3p	FOXK1	Prognostic biomarker and therapeutic target	Promote	GC	29893595
CCDC144NL-AS1	miR-143-3p	MAP3K7	Therapeutic target	Promote	GC	32647147
TUBA4B	miR-214, miR-216a/b	PTEN	Prognostic biomarker and therapeutic target	Suppress	GC	31198405
TMPO-AS1	miR-126-5p	BRCC3	Therapeutic strategy	Promote	GC	33295056
ADPGK-AS1	miR-3196	KDM1B	Prognostic biomarker and therapeutic target	Promote	GC	30944080
FEZF1-AS1	miR-363-3p	HMGA2	Therapeutic target	Promote	GC	32638620
Lnc-ATB	miR-141-3p	TGFβ2	Prognostic predictor and therapeutic target	Promote	GC	28115163
MAGI2-AS3	miR-141/200a-3p	ZEB1	Biomarker and therapeutic target	Promote	GC	31837602
Linc00483	miR-30a-3p	SPAG9	Prognostic biomarker	Promote	GC	29761936
DLX6-AS1	miR-204-5p	OCT1	Prognostic biomarker and therapeutic target	Promote	GC	31463827
HOTAIR	miR-331-3p	HER2	Prognostic biomarker and therapeutic target	Promote	GC	24775712
BC032469	miR-1207-5p	hTERT	Prognostic biomarker	Promote	GC	26549025
XIST	miR-497	MACC1	Prognostic biomarker and therapeutic target	Promote	GC	27911852
MIR99AHG	miR577	FOXP1	Therapeutic target	Promote	GC	32874129
HIF1A-AS2	miR-429	PD-L1	Prognostic biomarker and therapeutic target	Promote	GC	33555514
LINC00184	miR-145	ANGPT2	Biomarker and therapeutic target	Promote	GC	33758610
DDX11-AS1	miR-326	IRS1	Prognostic biomarker and therapeutic target	Promote	GC	32271422
LOXL1-AS1	miR-708-5p	USF1	Prognostic biomarker	Promote	GC	31468594
GCMA	miR-124, miR-34a	Slug, Snail	Prognostic biomarker and therapeutic target	Promote	GC	32439864
PVT1	miR-30a	Snail	Therapeutic target	Promote	GC	32557622
HOTAIR	miR-1277-5p	COL5A1	Prognostic biomarker and therapeutic target	Promote	GC	32583079
SNHG6	miR-101-3p	ZEB1	Prognostic biomarker and therapeutic target	Promote	GC	28683446
LINC01133	miR-106a-3p	APC	Prognostic biomarker and therapeutic target	Suppress	GC	30134915
MT1JP	miR-92a-3p	FBXW7	Prognostic biomarker and therapeutic target	Suppress	GC	29720189
XIST	miR-185	TGF-β1	Prognostic biomarker	Promote	GC	29053187
UFC1	miR-498	Lin28b	Prognostic biomarker and therapeutic target	Promote	GC	29970131
SNHG11	miR-184	CDC25A	Prognostic biomarker	Promote	GC	33816469
LINC01503	miR-133a-5p	VIM	Prognostic biomarker	Promote	GCA	33200343
UICLM	miR-215	ZEB2	Prognostic biomarker and therapeutic target	Promote	CRC	29187907
LEF1-AS1	miR-489	DRAPH1	Prognostic biomarker and therapeutic target	Promote	CRC	32248974
MIR4435-2HG	miR-206	YAP1	Prognostic biomarker and therapeutic target	Promote	CRC	32154166
SLC30A10	miR-21c	APC	Prognostic biomarker and therapeutic target	Promote	CRC	32633358
MCF2L-AS1	miR-874-3p	CCNE1	Prognostic biomarker	Promote	CRC	33037865
HOTAIR	miR-211-5p	FLT-1	Prognostic biomarker	Promote	CRC	34470574
HOATIR	miR-214	ST6GAL1	Therapeutic target	Promote	CRC	31694696
LINC01296	miR-26a	GALNT3	Therapeutic target	Promote	CRC	30547804
NEAT1	miR-34a	SIRT1	Prognostic biomarker and therapeutic target	Promote	CRC	30312725
LUNAR1	miR-495-3p	MYCBP	Prognostic biomarker	Promote	CRC	33300052
H19	miR-194-5p	SIRT1	Biomarker of chemoresistance	Promote	CRC	30451820
SNHG6	miR-26a/b, miR-214	EZH2	Therapeutic target	Promote	CRC	30626446
CCMAlnc	miR-5001-5p	HES6	Prognostic biomarker and therapeutic target	Promote	CRC	33681178
SNHG6	miR-181a-5p	E2F5	Prognostic and therapeutic biomarker	Promote	CRC	30666158
NEAT1	miR-193a-3p	IL17RD	Potential marker	Promote	CRC	30407674
Lnc-HSD17B11-1:1	miR-338-3p	MACC1	Therapeutic target	Promote	CRC	32595704
RP11-51O6.1	miR-206	YAP1	Biomarker and therapeutic target	Promote	CRC	34038520
MALAT1	miR-106b-5p	SLAIN2	Prognostic biomarker	Promote	CRC	30797712
MEG3	miR-9	E-cadherin, FOXO1	Prognostic biomarker	Suppress	EC	28539329
EIF3J-AS1	miR-373-3p	AKT1	Prognostic biomarker and therapeutic target	Promote	EC	32811869
SNHG12	miR-195-5p	BCL9	Prognostic biomarker	Suppress	ESCC	32086782
ROR	miR-145	FSCN1	Prognostic biomarker	Promote	ESCC	29430188
circRNA-mediated ceRNA crosstalk						
circFGD4	miR-532-3p	APC	Prognostic biomarker and therapeutic target	Suppress	GC	32633323
circRHOBTB3	miR-654-3p	p21	Therapeutic target	Suppress	GC	31928527
circ-PRMT5	miR-145, miR-1304	MYC	Prognostic biomarker and therapeutic target	Promote	GC	31701767
circ-PTPDC1	miR-139-3p	ELK1	Prognostic biomarker	Promote	GC	34803498
circ_0110389	miR-127-5p, miR-136-5p	SORT1	Prognostic biomarker and therapeutic target	Promote	GC	34162830
circ-RanGAP1	miR-877-3p	VEGFA	Prognostic biomarker and therapeutic target	Promote	GC	31811909
circHECTD1	miR-137	PBX3	Prognostic biomarker	Promote	GC	34001137
circPDSS1	miR-186-5p	NEK2	Biomarker and therapeutic target	Promote	GC	30417526
ciRS-7	miR-7	NA	Prognostic biomarker and therapeutic target	Promote	GC	28608528

(Continued)

TABLE 1 | Continued

ceRNA	Shared miRNA	Target mRNA	Clinical significance	Role in cancer	Cancer type	PMID
circTMEM87A	miR-142-5p	ULK1	Prognostic biomarker and therapeutic target	Promote	GC	33155080
circLMTK2	miR-150-5p	c-Myc	Prognostic predictor and therapeutic target	Promote	GC	31722712
circ-DCAF6	miR-1231, miR-1256	NA	Prognostic biomarker	Promote	GC	31226266
circTMC5	miR-361-3p	RABL6	Prognostic predictor and therapeutic target	Promote	GC	34296378
circ0005654	miR-363	sp1	Therapeutic target	Promote	GC	34499009
circUBE2Q2	miR-370-3p	STAT3	Prognostic biomarker	Promote	GC	34611143
circLARP4	miR-424-5p	LATS1	Prognostic biomarker	Suppress	GC	28893265
circ-ATAD1	miR-140-3p	YY1	Prognostic biomarker and therapeutic target	Promote	GC	32169278
circNHSL1	miR-1306-3p	SIX1	Prognostic biomarker and therapeutic target	Promote	GC	31438963
circEGFR	miR-106a-5p	DDX5	Therapeutic target	Promote	CRC	34320120
circ3823	miR-30c-5p	TCF7	Therapeutic target	Promote	CRC	34172072
circ_0026416	miR-346	NFIB	Therapeutic target	Promote	CRC	33061846
circ_0000372	miR-495	IL6	Prognostic biomarker and therapeutic target	Promote	CRC	33534412
circBANP	let-7d-5p	HMGA1	Biomarker and therapeutic target	Promote	CRC	33981828
circMBOAT2	miR-519d-3p	TROAP	Biomarker	Promote	CRC	32796815
ciRS-7	miR-7	EGFR, RAF1	Prognostic biomarker and therapeutic target	Promote	CRC	28174233
circHIPK3	miR-7	AK, IGF1R, EGFR, YY1	Prognostic biomarker and therapeutic target	Promote	CRC	29549306
circVAPA	miR-125a	CREB5	Therapeutic target	Promote	CRC	32256212
circHIPK3	miR-637	STAT3	Prognostic biomarker	Promote	CRC	31631038
circCAMSAP1	miR-328-5p	E2F1	Prognostic biomarker and therapeutic target	Promote	CRC	31951832
ciRS-7	miR-7	HOXB13	Prognostic marker and therapeutic target	Promote	ESCC	30082829

GC, Gastric cancer; GCA, Gastric cardia adenocarcinoma; CRC, colorectal cancer; EC, Esophageal cancer; ESCC, Esophageal squamous cell cancer.

ceRNA Network-Based Prognostic Signatures in GC

GC is a serious health problem throughout the world with high morbidity and mortality. Due to the lack of early disease-specific symptoms, most GC patients are diagnosed at advanced stages with unsatisfactory prognosis. Since survival probability is a major concern for cancer patients, signatures developed based on lncRNA-mediated ceRNA network are usually used to predict overall survival (OS) of GC patients (**Table 2**). For example, based on integrative analysis of the GC-specific ceRNA network, Zhang et al. (39) established a two-lncRNA signature consisting of LINC01644 and LINC01697 as a prognostic biomarker for survival prediction of GC patients. Functionally, knockdown of LINC01644 or LINC01697 could inhibit GC cell proliferation. Similarly, Li *et al.* (40) investigated the clinical significance of genes within the circRNA-mediated ceRNA network and further build a three-gene risk model for predicting OS in GC patients. The findings not only unravel the regulatory mechanisms of circRNAs, but also guide individualized management.

Furthermore, as principal causes of cancer-related death, metastasis and recurrence have long been considered as critical events influencing prognosis and treatment effect of cancer patients. Understanding the risk of metastasis and recurrence is critical for the success of personalized cancer therapy. Therefore, prognostic signatures based on lncRNA/circRNA-mediated ceRNA network are increasingly developed to predict metastasis or recurrence of GC patients, thus helping to optimize clinical treatment and management. For example, Chen *et al.* (41) successfully established a four-lncRNA signature to predict prognosis and distinguish recurrence risk of GC patients with robust performance.

ceRNA Network-Based Prognostic Signatures in CRC

CRC remains the most common gastrointestinal tract malignancy, ranking second for cancer-related mortality globally. Emerging evidence reveals that dysregulation of ceRNA crosstalks is closely involved in the pathological biology of CRC, making ceRNA network-based prognostic signature a promising tool for guiding personalized therapy (**Table 2**). For example, based on metastasis-associated ceRNA network, Liu et al. (42) developed a three-lncRNA signature including LINC00114, LINC00261, and HOTAIR, and proved its powerful prognostic value for CRC patients. Functionally, LINC00114 can suppress CRC cell proliferation and migration by sponging miR-135a.

Notably, biological process or pathway (e.g. immune, autophagy and fatty acid metabolism)-specific ceRNA networks are widely used to establish prognostic signatures in CRC. First, given the close association between immune infiltration level and clinical outcome in cancers, unraveling cancer-specific ceRNA network tightly associated with immune regulation can facilitate the development of prognostic signatures. For example, Song et al. (43) developed a novel signature consisting of seven immune-related genes based on circRNA-mediated ceRNA network, and proved that the immune-related signature can predict OS of CRC patients with high accuracy. Second, autophagy is a conserved intracellular degradative process, which plays critical roles in maintaining cellular metabolism, homeostasis and survival. Dysregulation of the autophagy process has been shown to be closely related to the pathogenesis of various cancers. By integrating the reported autophagy-related genes and the experimentally verified miRNA-mRNA and miRNA-lncRNA interactions, Qian et al. (44) established an autophagy-related ceRNA network and

TABLE 2 | ceRNA network-based prognostic signatures in GICs.

Signature	Function	Included parameters	Performance		Cancer type	PMID
			Training dataset	Testing dataset		
Signatures based on lncRNA-mediated ceRNA network						
Gao et al.'s signature	Predicting OS	6 lncRNA	NA	NA	CC	33836755
Guo et al.'s signature	Predicting OS	2 lncRNAs, 1 miRNA, and 3 genes	AUC of 0.634 at 1 year, 0.68 at 3 years, and 0.66 at 5 years	AUC of 0.775 at 1 year, 0.836 at 3 years, and 0.804 at 5 years in validation 1 dataset; AUC of 0.586 at 1 year, 0.62 at 3 years, and 0.632 at 5 years in validation 2 dataset	CRC	34276767
Huang et al.'s signature	Predicting OS	5 lncRNAs	AUC of 0.850	NA	CC	31448228
Li et al.'s signature	Predicting OS	3 lncRNAs	NA	NA	CC	33858429
Li et al.'s signature	Predicting OS	7 genes	AUC of 0.720 at 1 year, 0.741 at 3 years, and 0.714 at 5 years	NA	CRAC	34692502
Liu et al.'s signature	Predicting OS	3 lncRNAs	AUC of 0.716 at 5 years	AUC of 0.649 at 5 years	CRC	33302562
Peng et al.'s signature	Predicting OS	8 lncRNAs	AUC of 0.738 at 1 year, 0.746 at 3 years and 0.784 at 5 years	NA	CRC	34458145
Qian et al.'s signature	Predicting OS	3 genes	NA	NA	CRC	29916526
Xu et al.'s signature	Predicting OS	1 lncRNA, 2 miRNAs, and 4 genes	AUC of 0.698 at 1 year, 0.739 at 3 years and 0.781 at 5 years	NA	CC	34692670
Yang et al.'s signature	Predicting OS	7 genes	AUC of 0.746 at 1 year, 0.744 at 3 years and 0.772 at 5 years	NA	CC	31612869
Yang et al.'s signature	Predicting OS	4 lncRNAs	AUC of 0.628	AUC of 0.649	CRC	32256018
Zhang et al.'s signature	Predicting OS and DFS	5 lncRNAs	AUC of 0.675 for OS and 0.690 for DFS at 5 years	AUC of 0.695	CRC	30714675
Zhang et al.'s signature	Predicting chemotherapy resistance and survival	8 lncRNAs	AUC of 0.87 in predicting the FOLFOX chemotherapy response in metastatic CRC patients	NA	CRC	33585448
Zhang et al.'s signature	Predicting OS	15 genes	C-index of 0.817 at 1 year, 0.838 at 3 years and 0.825 at 5 years	C-index of 0.773 at 1 year, 0.824 at 3 years and 0.801 at 5 years	CRC	31796117
Wei et al.'s signature	Predicting OS	1 lncRNA and 1 miRNA	AUC of 0.71 at 1 year, 0.79 at 3 years and 0.97 at 5 years	NA	RC	34350117
Li et al's signature	Predicting OS	3 lncRNAs	AUC of 0.639 at 3 years, AUC of 0.685 at 5 years	NA	EC	33381546
Zhang et al's signature	Predicting OS	6 lncRNA	AUC of 0.686	NA	ESCC	34603485
Chen et al's signature	Predicting recurrence	4 lncRNAs	AUC of 0.936	AUC of 0.827 in validation 1 dataset; AUC of 0.882 in validation 2 dataset	GC	33869776
Mao et al.'s signature	Predicting OS	3 lncRNAs and 3 mRNAs	AUC of 0.699 at 3 years, 0.739 at 4 years, 0.801 at 5 years, 0.766 at 6 years and 0.853 at 7 years	AUC of 0.809 at 3 years, AUC of 0.820 at 4 years	GA	33188157
Qi et al.'s signature	Predicting OS	2 lncRNAs	AUC of 0.614	NA	GC	31923354
Zhang et al.'s signature	Predicting OS	2 lncRNAs	AUC of 0.651 at 3 years	AUC of 0.615 at 3 years	GC	34603561
Signatures based on circRNA-mediated ceRNA network						
Song et al.'s signature	Predicting OS	7 genes	AUC of 0.701 at 3 years and 0.728 at 5 years	NA	CRC	32582276
Wang et al.'s signature	Predicting OS	8 genes	AUC of 0.77 at 1 year, 0.92 at 3 years and 0.78 at 5 years	NA	EAC	33376353
Han et al.'s signature	Predicting OS	11 genes	AUC of 0.741	NA	GC	33514881
Li et al.'s signature	Predicting OS	3 genes	NA	NA	GC	33969120

CRC, colorectal cancer; CC, colon cancer; CRAC, colorectal adenocarcinoma; RC, rectal cancer; EC, esophageal cancer; ESCC, esophageal squamous cell carcinoma; GC, gastric cancer; GA, gastric adenocarcinoma; EAC, esophageal adenocarcinoma; OS, overall survival; DFS, disease-free survival. NA, Not available.

further constructed multi-gene models for OS prediction in colon cancer and rectal cancer, respectively. Besides, perturbation of fatty acid metabolism has recently been recognized as a hallmark of cancer. Peng et al. (45) successfully built a prognostic signature containing eight fatty acid metabolism-related lncRNAs identified from the ceRNA network, and found that the fatty acid metabolism-related lncRNA signature can predict OS in CRC patients with high accuracy (AUC>0.7), which is superior to traditional clinical factors, such as age and stage. Therefore, process or pathway-related ceRNA network has provided a useful tool for constructing prognostic signatures in CRC.

ceRNA Network-Based Prognostic Signatures in EC

EC is also known as one of the most commonly diagnosed gastrointestinal tumors with approximately 604,100 new cases annually (1). Despite technological improvement achieved in diagnosis and treatment, the 5-year survival rate of EC patients is below 20% (46), indicating poor prognosis. Recently, increasing studies have shown that lncRNAs participate in the post-transcriptional regulation of EC carcinogenesis through the ceRNA mechanism, exhibiting prognostic potential (**Table 2**). For example, based on integrated analysis of lncRNA-mediated ceRNA network, Li et al. (47) and Zhang et al. (48) successfully developed a novel three-lncRNA and six-lncRNA panel with prognostic value for EC patients by employing multiple Cox regression analysis, respectively. Similarly, Wang et al. (49) established a novel eight-gene signature as an independent prognostic factor for predicting the OS of patients with esophageal adenocarcinoma (EAC).

COMPUTATIONAL ESTABLISHMENT OF CERNA NETWORK-BASED PROGNOSTIC SIGNATURE

Compelling functional studies have demonstrated that dysregulation of ceRNA crosstalk can contribute to tumor progression by affecting a variety of signaling pathways involved in cancer hallmarks, paving the way for the establishment of novel prognostic signatures in various cancer types. Collectively, the computational strategy for developing ceRNA network-driven signature primarily consists of a series of steps, including cancer-specific ceRNA network construction, risk model construction and validation, and functional annotation (**Figure 2**).

Cancer-Specific ceRNA Network Construction

According to the ceRNA theory, endogenous RNAs competitively bind to shared miRNAs, thereby regulating mutual expression. Therefore, the computational methods used to identify ceRNA interactions mainly rely on complementary base pairing and expression correlation between miRNA and its targets (50).

With increasing amounts of cancer data becoming available at public databases (e.g. TCGA and GEO), construction of ceRNA networks based on transcriptome analysis has been extensively employed to investigate key ceRNA crosstalks in multiple cancer types (50, 51). In this method, differential expression analysis was commonly performed to identify RNA molecules implicated in cancer-related processes, such as cancer initiation, progression or metastasis. Meanwhile, miRNA-target pairs were usually recognized by prediction algorithms (e.g. miRanda (52), TargetScan (53), RNAhybrid (54), RNA22 (55), etc.) and available databases collecting predictive or experimental information (e.g. miRCode (56), starBase v2.0 (57), miRTarBase (58), DIANA-LncBase v3 (59), CircInteractome (60), etc.). Following evaluation of miRNA regulatory similarity, expression correlation between each putative ceRNA pair was frequently evaluated by Pearson correlation coefficients. Besides, several R/Bioconductor packages, such as Sparse Partial correlation ON Gene Expression (SPONGE) and miRspongeR, are available for fast identification of ceRNA crosstalks and construction of ceRNA networks (61, 62). Then, based on the ceRNA triplets composed by differentially expressed RNAs as well as biological process or pathway information, cancer-specific ceRNA network can be constructed and visualized *via* Cytoscape software or R packages (**Figure 2A**) (63).

In addition to the above strategy, extensive efforts have been made to develop novel approaches for prediction of miRNA-mediated ceRNA crosstalks and construction of ceRNA networks. For example, Chiu et al. (64) designed an integrative framework named Cupid for context-specific prediction of both miRNA-target and ceRNA interactions simultaneously based on sequence and expression information. Helwak et al. (65) developed a crosslinking, ligation, and sequencing of hybrids (CLASH)-based method for high-throughput identification of miRNA-target interaction directly. Furthermore, considering the influence of kinetic parameters on miRNA-mediated interaction between ceRNAs, multiple computational/mathematical models have been developed to study dynamics of the ceRNA crosstalk in diverse biological settings (66). For example, Bosia et al. (67) proposed a stochastic model to explore the equilibrium and non-equilibrium characteristics of ceRNA networks based on the miRNA-target titration mechanism. Chiu et al. (68) proposed a kinetic model for ceRNA regulation that accounts for the influence of co-regulation by miRNAs with multiple targets and found that ceRNA interaction is strongly affected by the abundance of miRNA mediators and the number of miRNA targets. Therefore, increasing breakthroughs have been achieved in the development of computational approaches for ceRNA network construction.

Construction and Validation of Prognostic Signatures

Based on cancer-specific ceRNA networks, signatures can incorporate multiple types or a single type of RNAs. Among them, lncRNA was the most reported type, so the present study takes it as an example to introduce signature construction and verification methods. First, the prognostic value of lncRNAs involved in the cancer-specific ceRNA network can be evaluated by univariate Cox regression analysis of the association between lncRNA expression level and patient survival time (69). Then, lncRNA-related prognostic signature was commonly established

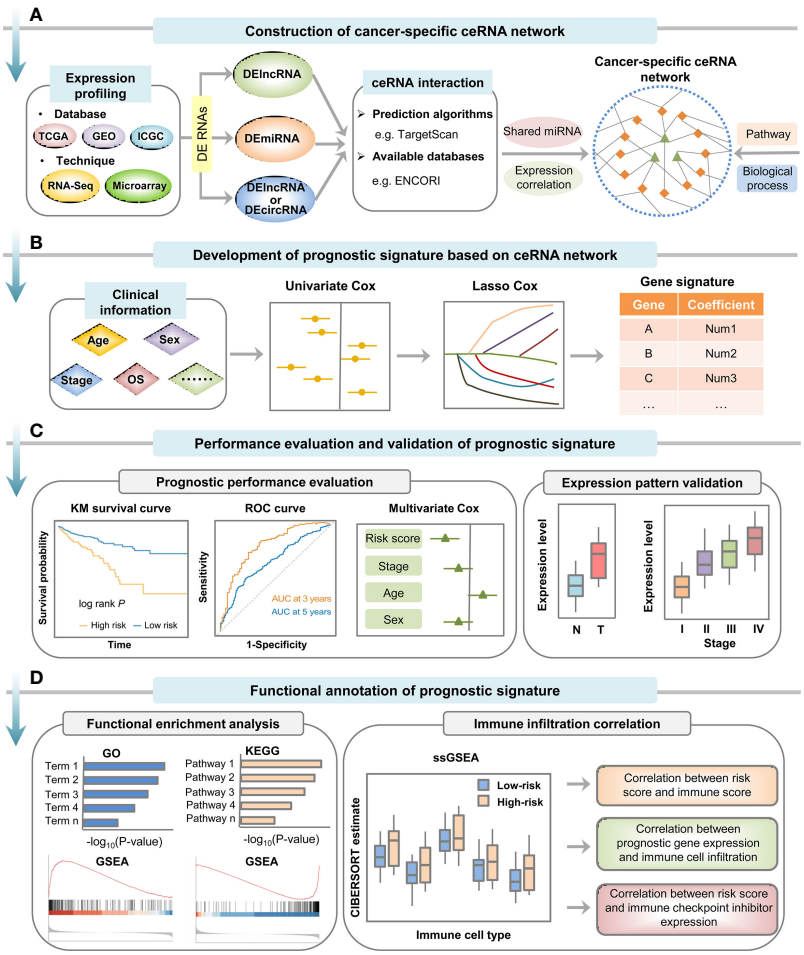


FIGURE 2 | Computational strategy for construction, validation and functional annotation of ceRNA network-based prognostic signature in cancer. **(A)** Cancer-specific ceRNA network was constructed based on expression and interaction information. **(B)** Prognostic signature was developed by employing univariate Cox regression analysis and LASSO Cox regression analysis. **(C)** Prognostic performance of the signature should be evaluated and validated by Kaplan-Meier survival curve analysis, time-dependent ROC curve analysis and multivariate Cox regression analysis, and the expression pattern of genes that make up the model can be verified in other independent datasets. **(D)** Biological role of the prognostic signature could be investigated by functional enrichment analysis and immune infiltration analysis.

by performing LASSO Cox regression analysis or multivariate Cox regression analysis (70). The risk score for each patient was calculated based on the coefficient and normalized expression value of each lncRNA included in the signature (**Figure 2B**). Furthermore, multivariate Cox regression analysis could also be employed to test whether the lncRNA-related signature was an independent predictor for patient survival (**Figure 2C**) (71). For example, based on comprehensive analysis of ceRNA network, Mao et al. (70) established a six-lncRNA signature for recurrent prognosis prediction of patients with colon adenocarcinoma by using LASSO Cox regression model. Similarly, Tao et al. (72) developed a vascular invasion-related lncRNA signature to predict the OS of hepatocellular carcinoma patients by utilizing univariate, LASSO and multivariate Cox regression analyses.

To evaluate the robustness of the constructed signature for prognosis prediction, the patients in both training and testing datasets were usually divided into high- and low-risk subgroups, followed by Kaplan-Meier survival curve analysis and time-dependent ROC curve analysis (**Figure 2C**). Indeed, the prognostic performance of most ceRNA network-based signatures has been evaluated and/or validated through Kaplan-Meier survival curve and ROC curve analyses (42, 73, 74).

Functional Annotation of Prognostic Signatures

The common functional enrichment analyses, such as Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Set Enrichment Analysis (GSEA), could be used to explore the potential functions of the established lncRNA/circRNA-related signature. Generally, given the principle that co-expressed ncRNAs and mRNAs might share biological roles, GO and KEGG enrichment analyses were frequently performed on the genes co-expressed with model ncRNAs identified by computational methods. Besides, based on

the Molecular Signatures Database (MSigDB), GSEA can also be utilized to explore the biological function of prognostic signatures (**Figure 2D**). For example, Liu et al. (42) found that the key lncRNAs that constitute the prognostic model were implicated in CRC tumorigenesis through GO and KEGG enrichment analyses on the co-expressed genes. Based on GSEA results, Chen et al. (69) found that the constructed eleven-lncRNA prognostic signature was involved in immune-related processes of hepatocellular carcinoma.

Furthermore, given the close link between immune and cancer pathogenesis, single-sample Gene Set Enrichment Analysis (ssGSEA) could be conducted to investigate the relationship between prognostic signature and immune status by calculating infiltration scores of distinct immune cell types based on the abundance of immune-related marker genes (**Figure 2D**) (75). Besides, immune infiltration correlation analyses, such as correlation between signature-based risk score and immune score, correlation between prognostic gene expression level and immune cell infiltration, and correlation between signature-based risk score and immune checkpoint inhibitor expression level, can be used to investigate the biological role of the established signature (**Figure 2D**) (43, 69, 76).

DISCUSSION AND PERSPECTIVE

In view of the complex and heterogeneous characteristic of GICs, satisfactory prognostic evaluation of patients is difficult to accomplish. With the constant effort and advances in gene expression regulation, accumulating evidence has proved that both coding and non-coding RNAs (e.g. mRNA, lncRNA, and circRNA) hold the power to communicate with each other through a miRNA-mediated ceRNA mechanism (9). Given the potential roles in cancer pathogenesis and progression, the translational significance of ceRNA molecules has recently attracted increasing attention in GICs. It should be noted that a single miRNA can bind to multiple different targets according to the mechanism of action of miRNA. The diversity of miRNA target genes determines that the ceRNA crosstalk does not work alone, but through forming a coordinated large interaction networks where significative crosstalk could take place between distant RNAs under physiological and pathological conditions. For example, Rzepiela et al. (77) discovered the hierarchical response dynamics of distinct miRNA targets to miRNA induction by combining mathematical modeling with single-cell mRNA profiling, promoting our understanding of the complexity of ceRNA networks. Miotto et al. (78) found that despite the weakness of individual ceRNA crosstalk, extended miRNA-RNA networks could facilitate the integration of a huge number of interactions, leading to significant system-level effect. Besides, Chiu et al. (68) also highlighted the impact of the number and abundance of titrated microRNA species on ceRNA regulation. Therefore, the paradigm of ceRNA biomarker discovery is gradually shifting from individual ceRNA identification and validation toward the exploration of interaction relationship in ceRNA networks under a systematic framework of gene regulation.

Currently, increasing researches towards ceRNA networks in GICs has not only enhanced our understanding of ceRNA-mediated GICs pathogenesis, but also paved the way for developing novel prognostic biomarkers and therapeutic targets for GICs patients (79). Indeed, a large number of studies have identified prognostic signatures that predict the OS, metastasis or recurrence of patients with GC, CRC or EC through an integrated analysis of cancer-related ceRNA network (41, 80, 81). However, most of those signatures have not reached the criteria of well-validated effective prognostic models that could improve risk stratification and therapeutic decision making in pre-clinical and clinical practice. On one hand, ceRNA network-based prognostic signatures were commonly established by employing expression profiling datasets collected in public databases, such as TCGA or GEO. Their prognostic value needs to be confirmed in independent large and diverse population cohorts with GIC. On the other hand, the major obstacle for clinical application of ceRNA network-based prognostic signatures are largely due to the lack of a clear understanding of their functional roles in tumorigenesis, and the specific downstream signaling pathways and targets that they regulate. Therefore, although our understanding for the functions of ceRNA crosstalks in GICs continues to deepen, there is still much to explore to bridge the gap between theoretical research and clinical translation.

As different types of GICs, such as GC, CRC and EC, possess varying clinical manifestations, course and outcomes, the reported prognostic signatures are commonly constructed based on cancer-specific ceRNA networks. Accordingly, based on the published literatures, we found no evidence that any of the reported ceRNA network-based prognostic signatures are applicable to multiple cancer types. In fact, it is challenging to create a general ceRNA signature in multiple cancer types, as ceRNA interactions mainly depend on the abundance of free RNAs, and the expression of genes required for specific functions varies widely in distinct tissues (82). However, ceRNA interactions explain that even a slight amount change in a certain transcript can affect the abundance of other transcripts in indirect ceRNA:miRNA:ceRNA interactions. Therefore, large-scale analysis is needed to explore ceRNA functions. In addition, due to the pivotal role of certain process or pathway involved in carcinogenesis, process or pathway-specific ceRNA network provides novel strategies for powerful prognostic signature building.

Single-cell RNA sequencing technologies have revolutionized the field of cancer biology as they provide unprecedented opportunities to reveal the properties of distinct cell populations at single-cell resolution (83). Considering the impact of intratumoral heterogeneity on clinical practice of GICs, construction of cellular-specific ceRNA networks will deepen the quantitative understanding of cancer pathogenesis and further promote the development of precision medicine (84). Recently, the database of cellular-specific lncRNA-mediated ceRNA networks, LnCeCell, has been constructed based on single-cell RNA sequencing datasets and published literature. It collected ceRNA interactions from a large number of cells across 25 cancer types, facilitating the decoding of ceRNA

regulations at single-cell level (85). Therefore, with the advance of single cell expression profiling approaches, cellular-specific ceRNA networks provide a new route to establish prognostic signatures in the future.

In summary, although the field of ceRNA network-based prognostic signatures is still in its infancy, we are currently witnessing their translational and clinical significance in multiple GICs and other diseases. With further convincing validations and functional explorations, those signatures will be helpful to optimize individualized management and treatment as well as to improve clinical outcomes of patients with GIC in the era of personalized medicine.

AUTHOR CONTRIBUTIONS

BS and XQ designed the review. XQ collected the related data and drafted the manuscript. XC and YZ revised the tables and figures. BS, XQ, JC, and BN revised the manuscript. All authors read and approved the final manuscript.

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REVIEW
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Tuberous Sclerosis Complex With Multiple Organ Tumors: Case Report and Literature Review

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Pancreatic neuroendocrine neoplasms (PNEN) are tumors that originate from neuroendocrine cells. Only about 1% patients are related to mutation of tuberous sclerosis complex gene. Here, we reported a rare case with involvement of multiple organs and space-occupying lesions. Initially, the patient was thought to have metastasis of a pancreatic tumor. However, the patient was diagnosed as pancreatic neuroendocrine tumors, liver perivascular epithelioid tumors, splenic hamartoma, and renal angiomyolipoma by pathological examination after surgery. We performed genetic mutation detection to identify that tuberous sclerosis complex 2 gene presented with a heterozygous variant. Tuberous sclerosis often presents with widespread tumors, but it is less common to present with pancreatic neuroendocrine tumors and liver perivascular tumors as highlighted in the case. So we analyzed the relationship between TSC gene mutations and related tumors. And we also reviewed the current molecular mechanisms and treatments for tuberous sclerosis complex.

Keywords: tuberous sclerosis complex, liver perivascular epithelioid tumors, pancreatic neuroendocrine neoplasms, splenic hamartoma, renal angiomyolipoma

INTRODUCTION

Neuroendocrine tumors are tumors that originate from neuroendocrine cells and can be distributed throughout the body. The gastrointestinal tract and the pancreas are the most common locations for these tumors (1). The incidence of pancreatic neuroendocrine neoplasms (PNEN) is approximately 5 cases per 100,000, and the rate in women tends to be moderately higher than those in men. PNEN account for 3% of primary pancreatic tumors. Patients present different clinical symptoms according to the endocrine hormones produced by tumors (2). But the variable and atypical clinical manifestations of PNEN patients means that missed and misdiagnoses are relatively common.

Tuberous sclerosis complex (TSC) is an autosomal dominant chronic disease, occurring as a consequence of mutations in the TSC1 gene on chromosome 9q34 or the TSC2 gene on

chromosome 16p13. This is a clinically rare condition, with a reported incidence of between 1/6000 and 1/10000 (3). It can cause multi-organ tumors due to mutations in the TSC gene such as the brain, skin, heart, lung, and kidneys. However, accumulation in the pancreas is relatively rare, especially PNEN.

About 10% of PNEN patients are hereditary, mainly related to autosomal dominant inheritance, such as multiple endocrine neoplasms type I (MEN1), neurofibromatosis type I (NF-1), tuberous sclerosis (TSC), etc. MEN1 is the most common type, while TSC type only accounts for about 1%. Here, we reported a patient with multiple organ occupying lesions. The patient was diagnosed with PNEN, renal angiomyolipoma, splenic hamartoma, and liver vascular peripheral epithelioid tumors with the help of surgery and pathological tests. The mutation in the TSC2 gene was found by genetic mutation detection.

CASE REPORT

A 43-year-old woman presented to the First Affiliated Hospital of China Medical University with left back pain. However, there was no jaundice, abdominal pain, weight loss, or other symptoms. After admission, relevant laboratory examinations were performed and all within the normal range, including routine blood test, liver function, renal function, coangulation test, immynoglobulin levels, insulin and glucagon, tumor indicators (CEA, AFP, CA125, CA199), etc. Enhanced CT examination of the upper abdomen revealed multiple low-density shadows in the lower right lobe of the liver, head of the pancreas, and spleen. Soft tissue-density nodules were on the surface of the renal cortex. The largest nodule was located in the right kidney and protruded out of the kidney. The arterial phase of the enhanced scan was obviously or unevenly strengthened in the nodules when compared with the surrounding tissue. Enhanced MRI of the abdomen confirmed multiple space-occupying changes in the liver, spleen, pancreas, and kidneys (**Figure 1**). PET-CT further showed that the tumor within the pancreas was malignant and exhibited obvious uptake of fludeoxyglucose (FDG). Both the kidney and spleen were considered more likely to contain benign lesions given that there was no obvious FDG uptake.

Based on these examination results, the patient underwent surgical resection. After opening the abdomen, a tumor of diameter 2.5 cm was found in the body of the pancreas (**Figure 2**). The tails of the pancreas and spleen were removed. The cut surface of the pancreatic mass was grayish-yellow and soft with hemorrhage. The cut surface of the splenic mass was grayish-red and brown. A tumor with a diameter of approximately 1 cm was observed in the caudate lobe of the liver and presented with gray-white nodules in the center. This tumor was excised in a wedge shape.

Pathological diagnosis of the spleen, liver, and pancreas was performed during and after the operation. Intraoperative cryopathology suggested that the splenic tumors were likely to be benign lesions, and the tumors in the pancreas were likely to be pancreatic pseudopapillary tumors. Microscopic examination of the paraffin-embedded histopathological section showed that the tumor cells of the pancreas (**Figure 3**) were mostly distributed in strips or diffuse sheets, with the same cell size and characteristic

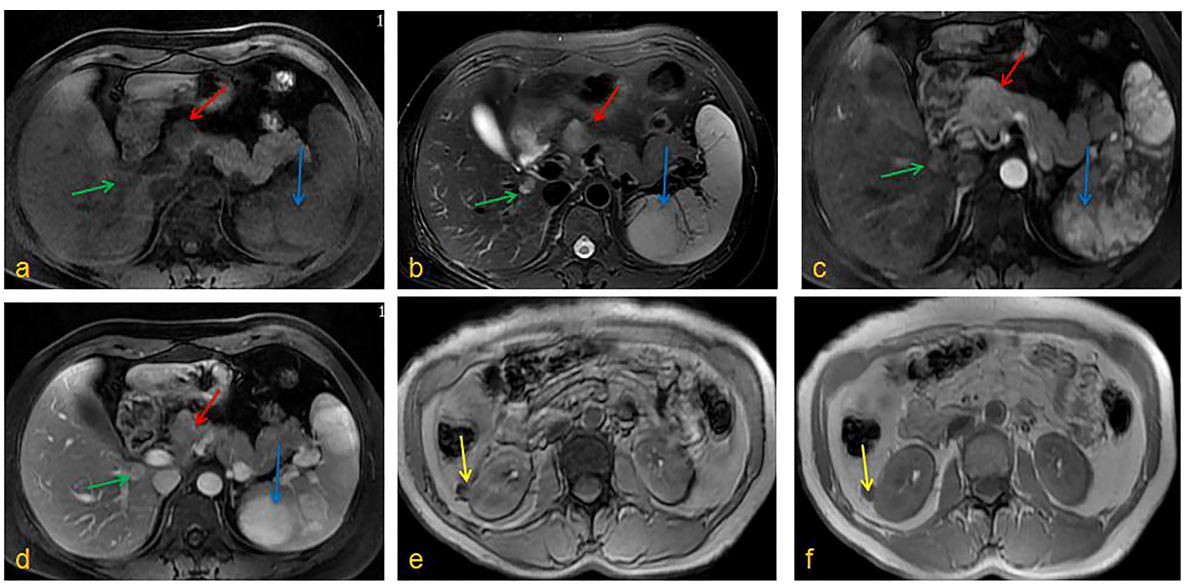


FIGURE 1 | Enhanced MRI of the abdomen. **(A)** T1WI, **(B)** T2WI, **(C)** Enhancement phase, **(D)** Delay phase: the pancreatic head could be seen a clear outline of about 2.5cm in diameter with long T1T2 signal, the lower segment of the right liver had oval shadow of about 1.4cm in diameter with long T1T2 signal, the spleen showed multiple irregular T1 signals and long T2 signals; **(E)** T1WI, **(F)** T2WI, kidneys could be seen round T1 signal and low T2 signal foci, and the enhanced arterial phase showed obvious enhancement. (red arrow: pancreatic tumor; blue arrow: splenic tumor; green arrow: liver tumor; yellow arrow: kidney tumor).

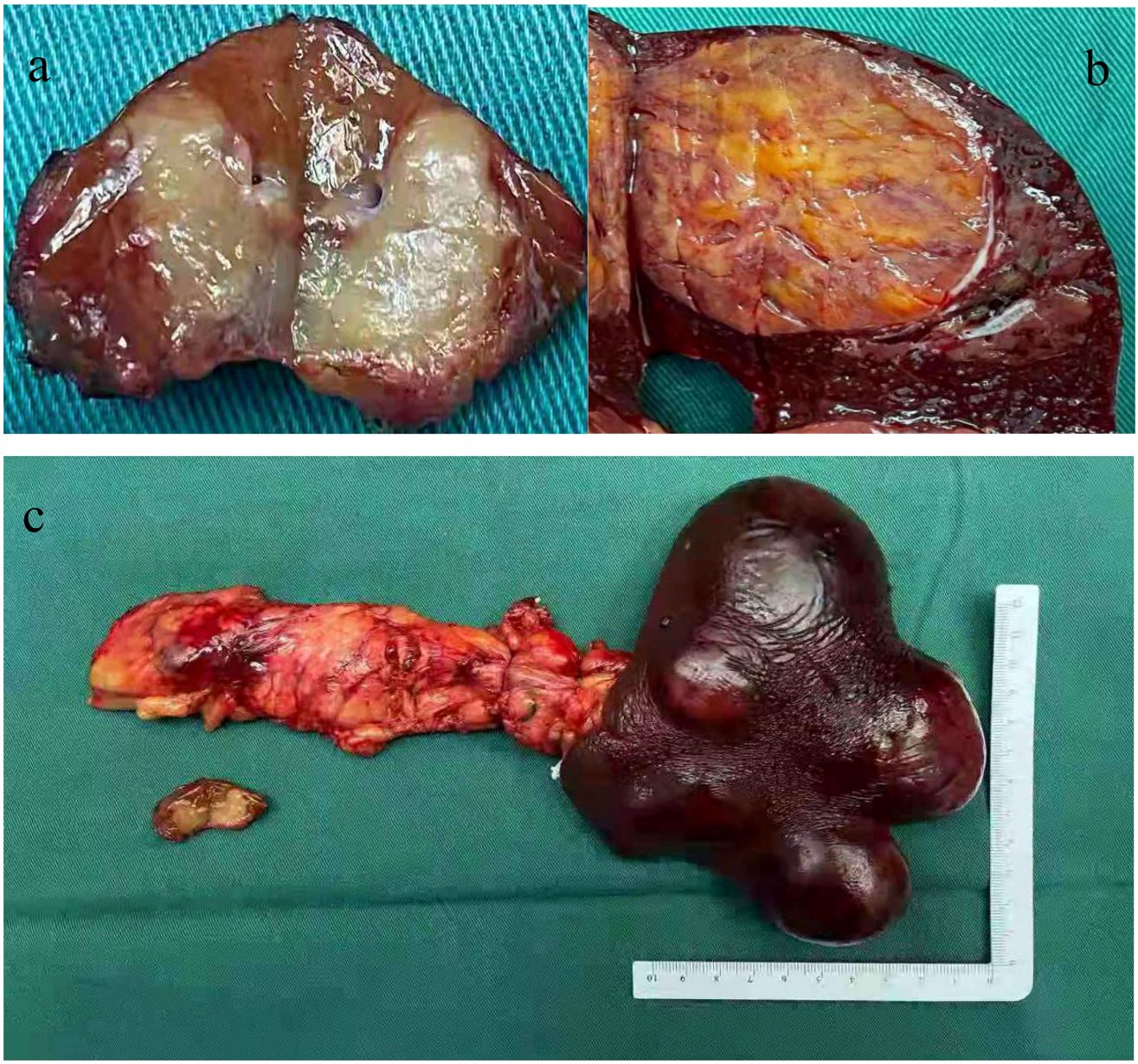


FIGURE 2 | Tumor specimens of surgical excision. **(A)** A tumor with a diameter of about 1 cm was seen in the caudate lobe of the liver with a gray-white nodule in the center; **(B)** The cut surface of the splenic tumor was grayish-red, gray-brown and medium in quality; **(C)** The pancreatic tumor was located on the body of the pancreas with a diameter of about 2.5 cm. The cut surface was grayish yellow and soft with bleeding.

salt and pepper-like nuclei. Immunohistochemistry confirmed that cytokeratin (CK) and beta-catenin were positive, which supported the diagnosis of pancreatic neuroendocrine tumors. The tumor and the surrounding tissues of the liver were unclear (**Figure 4**). The liver-associated tumor was found to contain epithelioid cells, perivascular muscle-like cells, and adipocytes. Immunohistochemistry revealed that phosphatidylinositol proteoglycan-3 (GPC-3) was negative and Melan A was positive, supporting the diagnosis of a perivascular epithelioid cell tumor (PEcoma). The splenic tumor was a round-shaped nodule (**Figure 5**) with clear boundary. The tumor comprised of uniformly sized oval-shaped tissue-like cells and spindle-shaped muscle-like cells. Immunohistochemistry showed positive staining for both smooth muscle actin (SMA) and cluster of differentiation 68 (CD68), supporting the diagnosis of splenic hamartoma (myoid hamartoma).

To ascertain the cause of the multiple tumors, we performed genetic mutation detection using the patient's genomic DNA, which was recovered from the peripheral blood. The results showed that the tumor susceptibility syndrome-related genes

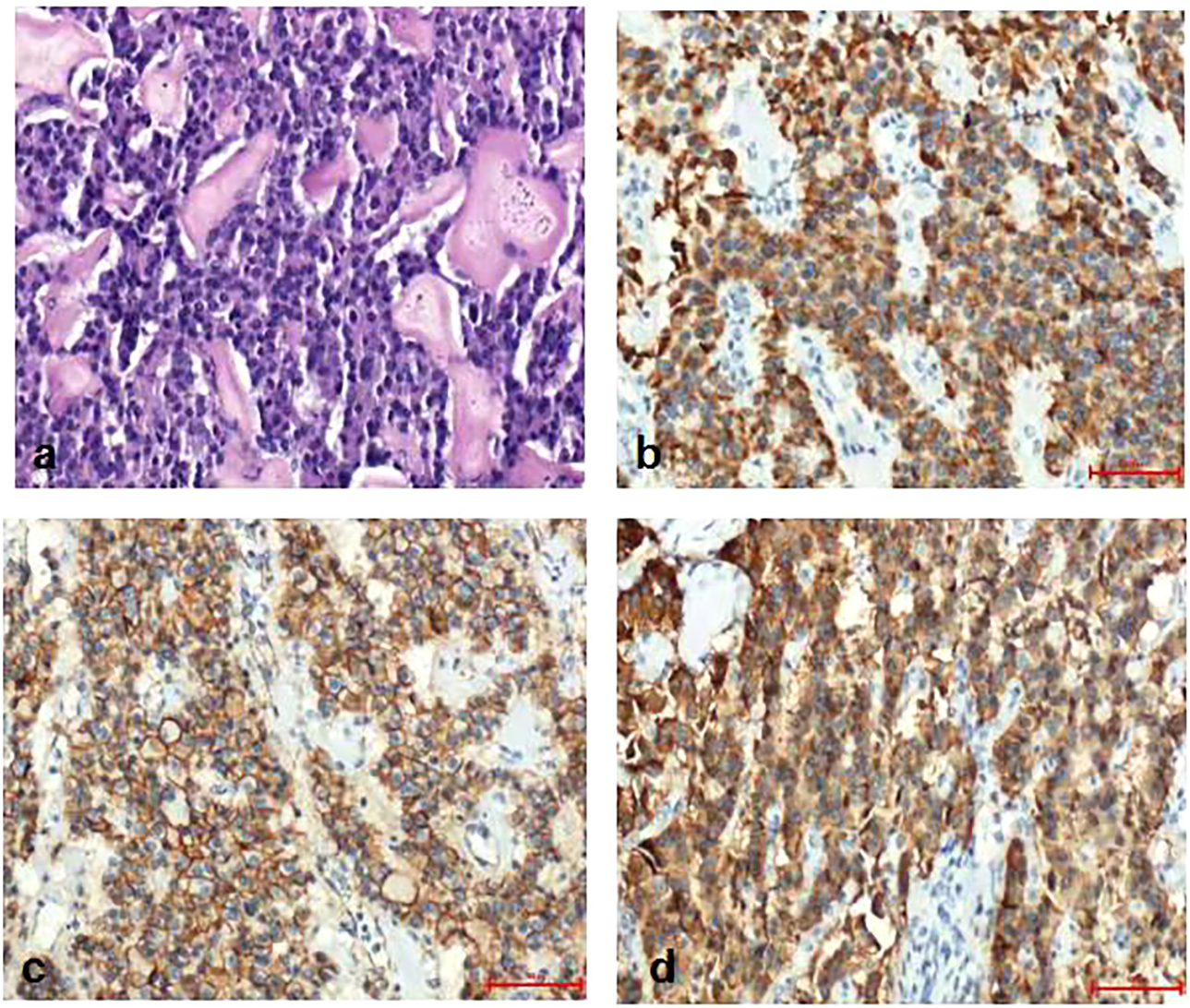


FIGURE 3 | Pathology result of the pancreas. **(A)** Pancreatic tumor cells were mostly distributed in strips or diffuse sheets, with the same cell size; **(B)** Immunohistochemistry CK(PAN)(+); **(C)** Immunohistochemistry SY(+); **(D)** Immunohistochemistry beta -Catenin (cell membrane+).

BRCA1 related protein 1 (*BAP1*) and tuberous sclerosis complex 2 gene (*TSC2)* each presented with a heterozygous variant (**Figure 6**). These findings are in line with the genetic diagnosis of tuberous sclerosis. In terms of clinical diagnosis, the liver and kidney angiomyolipomas are major indicators, and the splenic hamartoma belongs to non-renal hamartoma. Consequently (4), the most likely diagnosis was tuberous sclerosis with multiple organ involvement. After diagnosis, we completed the examination and did not find the TSC pathological features of other organs (skin and lungs). Tumor markers and ultrasound were re-examined at 3 and 6 months after surgery, and there was no indication of recurrence. The patient's diagnosis and treatment processes are shown in **Figure 7**.

DISCUSSION

Initially, the patient was thought to have metastasis of pancreatic endocrine tumor. However, after pathological findings and genetic mutation, the patient was diagnosed as tuberous sclerosis complex.

Aberrations of the TSC genes are associated with a variety of tumors. Skin damage is the most common manifestation, observed in over 95% of cases. Patients may present depigmented spots on the extremities or red hard papules on the face, which then manifest as depigmented spots, facial fibers, and perithyroid fibroma (5). Brain tissue lesions are the main cause of morbidity in children with TSC (6). Specific lesions include cortical nodules, subependymal nodules, and

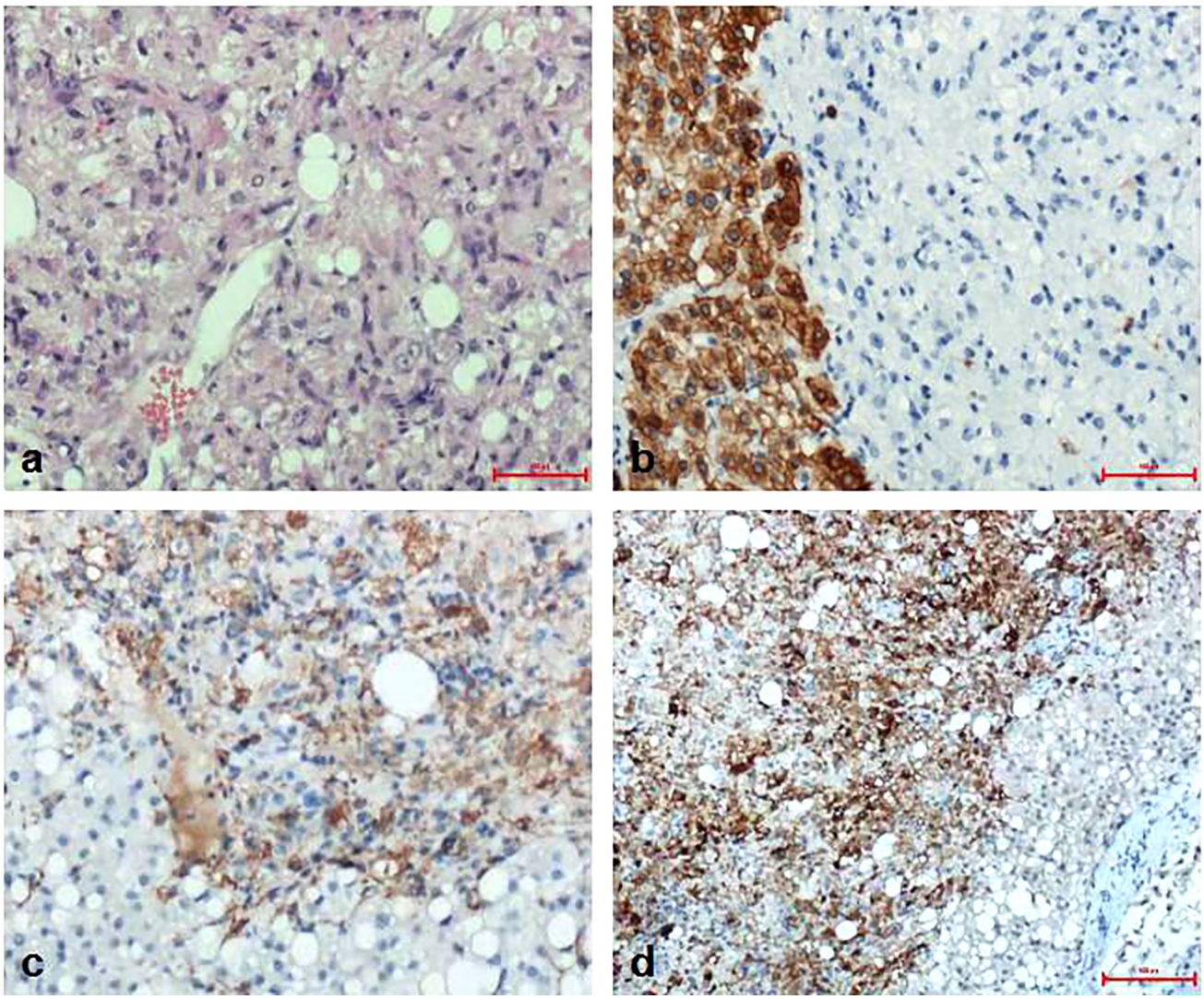


FIGURE 4 | Pathology result of the liver. **(A)** The boundary between the liver tumor and the surrounding liver tissue was unclear. The tumor was the mixture of epithelioid cells, perivascular muscle-like cells and adipocytes in different proportions; **(B)** Immunohistochemistry CK (hepatocyte+); **(C)** Immunohistochemistry Melan A(+); **(D)** Immunohistochemistry SMA (part+).

subependymal giant cell astrocytoma, out of which cortical nodules, the most common form of organic brain tissue lesion in TSC patients, is observed in over 88% of children with TSC (7). In addition, TSC involving the heart and lungs mainly manifests as cardiac rhabdomyomas and pulmonary lymphangiomyomas, which can be found in more than 80% of children with TSC (8).

The *TSC* genes are tumor suppressors and produce hamartin and tuberin proteins, which are known to be associated with a variety of tumors. TSC variant identified by sequencing is a known pathogenic variant. Mutations of TSC1 or TSC2 lead to constitutive activation of the small G protein Rheb and the mammalian target of rapamycin (mTOR) pathway, which regulates cell growth and proliferation (9). Associated to this pathway, insulin-like growth factor can cause phosphorylation of AKT and activate PI3K. In turn, activated PI3K converts PIP2 to PIP3 and recruits PDK1 and Akt, thereby activating mTOR (10). Complex of mTOR and Rheb promote the phosphorylation of downstream molecules, consequently promoting protein synthesis. TSC protein is a key regulator of this signaling pathway, which can transform the form of Rheb-GTP to Rheb-GDP, which inhibits the formation of complexes and slows down cell growth (11). Deletion of the *TSC* gene leads to dysregulation of mTOR, which is the regulator of the PI3K/Akt/mTOR signaling pathway. Their activation makes cell cycle regulation out of control and causes tumor progression. Therefore, when the *TSC* gene is mutated, the structure and function of the TSC1/TSC2 complex is abnormal. So the inhibitory effect on mTOR is

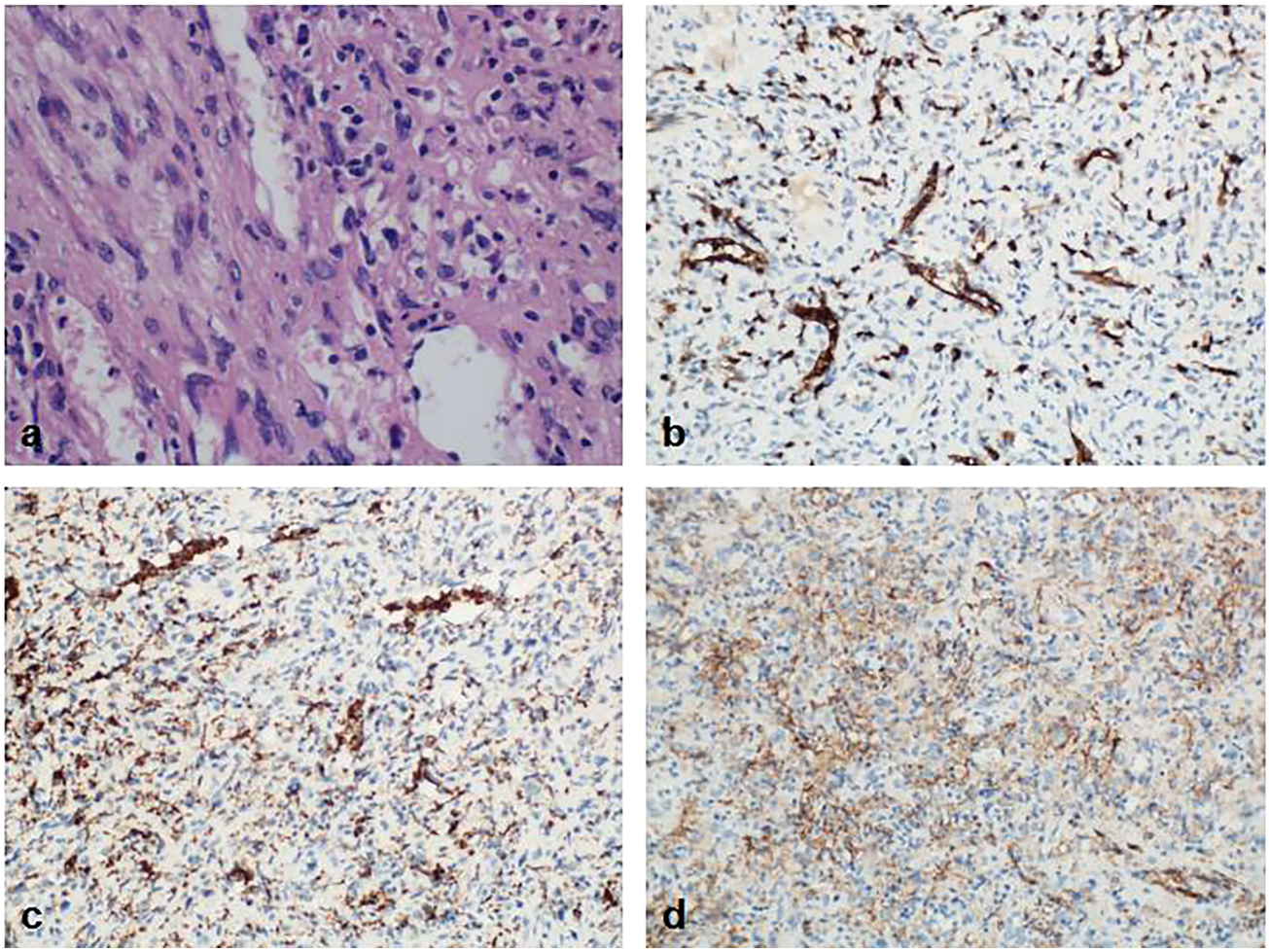


FIGURE 5 | Pathology result of the spleen. **(A)** The splenic tumor was in the shape of a round nodule, with a clear boundary with the surrounding normal spleen tissue. The tumor lost the structure of the red and white pulp of the normal spleen under the microscope. The cells were mainly composed of oval tissue-like cells and spindle-shaped muscle-like cells. Blood vessels were abundant; **(B)** Immunohistochemistry CD8(+); **(C)** Immunohistochemistry CD68(+); **(D)** Immunohistochemistry SMA (interstitial +).

weakened, resulting in growth of the tumors (12). The pathogenesis of some TSC-related benign tumors requires not only the mutation and inactivation of the *TSC* gene, but also the mutation and inactivation of their alleles, which is the "second hit" (13). These circumstances generate tumors such as facial angiofibroma, renal angiomyolipoma, renal cell carcinoma, and lymphangiomyomatosis.

What we note as unusual is that PNEN is not in the diagnostic criteria for multi-system-involved TSC. This is because most PNEN are closely related to gene such as *ATRX/DAXX*, *MEN-1*, and *MUTYH*. Only a few cases reported that PNEN and TSC are concurrent. Larson (14) retrospectively analyzed the results of 219 TSC patients, of whom 6 patients were previously diagnosed with PNEN through pathological examination. And TSC2 mutation was found in all patients. According to previous reports (15), 37% of pancreatic endocrine tumors have lost the 16p chromosome arm. This is also closely related to the PI3K-Akt-mTOR pathway. Akt activation was observed in PNEN patients. Akt/mTOR is involved in the growth and apoptosis of pancreatic β-cells. Activated mTOR can regulate downstream factors p70S6K and 4EBP-1, and affect the cell growth cycle.

Reports of TSC involving the spleen are rare, especially for splenic hamartoma. An early TSC case was reported in an infant using CT to identify the presence of bilateral renal angiomyolipomas. The splenic mass increased significantly during the 62-month follow-up period. Pathological examination after splenectomy indicated that the tumor was splenic hamartoma. Ultimately, whether it is affected by the mTOR pathway requires further verification.

In addition, PEcoma is related to TSC gene mutations (16). Specifically, 10% to 50% of patients possess TSC2 gene deletion (17). Kenerson (18) confirmed that the high expression of mTOR activity markers and the low expression of Akt in PEcoma are associated with the destruction of TSC function. Pan collected 10 sporadic and two tuberous sclerosis-related PEcomas. The phosphorylation

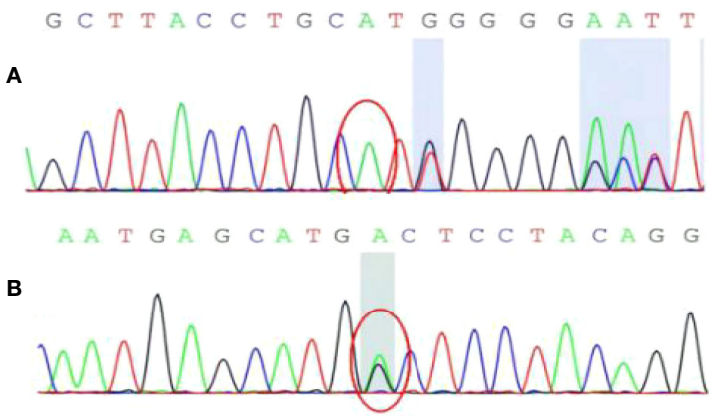


FIGURE 6 | The result of genetic mutation detection. **(A)** BAP1 gene c.1111dupA site with insertion mutation; **(B)** TSC2 gene c.4700G>A (guanine>adenine) site with heterozygous mutation.

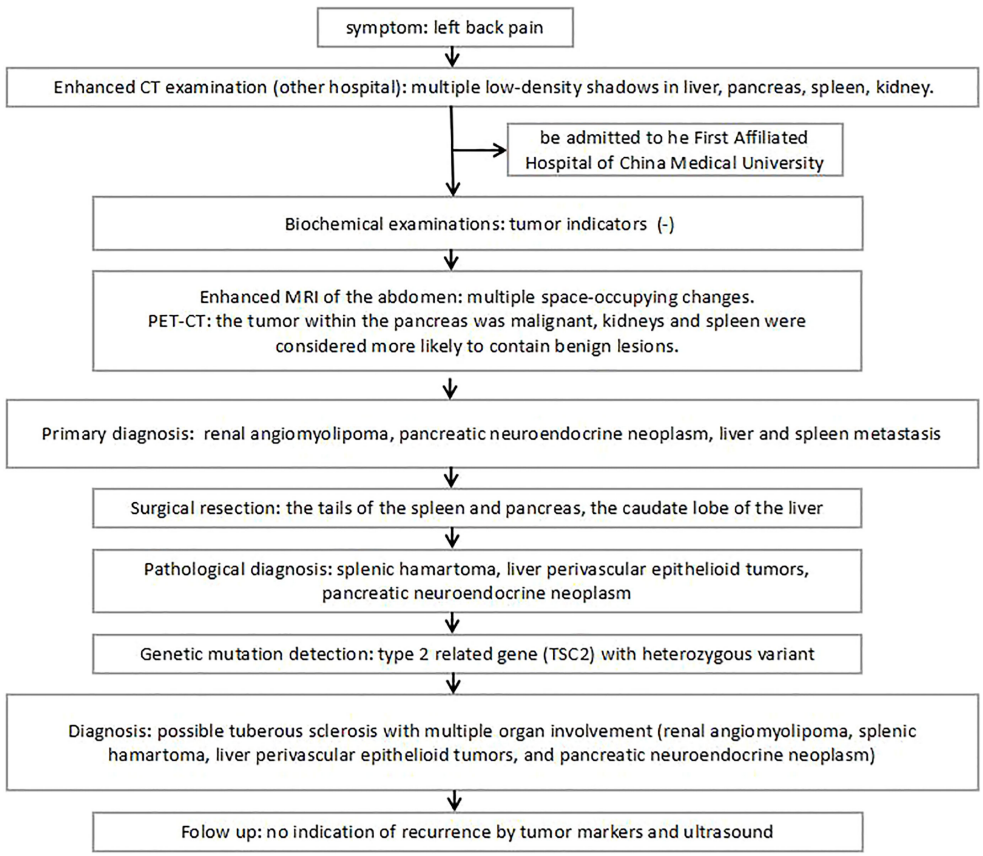


FIGURE 7 | Diagnosis and treatment algorithm based on this case report.

curve shows that the mTOR pathway is activated by the disrupted function of the TSC1/2 complex, which proves that the carcinogenic lineage of PEComa is a unique TSC2-related tumor (19). Therefore, the two-hit mutation of TSC1 or TSC2 is causally associated with the onset of TSC-PEComas. The main disease which must be distinguished from liver PEComa is primary liver cancer. Both of them can show the form of "fast in and fast out" on enhanced CT scans. However, patients with hepatocellular carcinoma usually

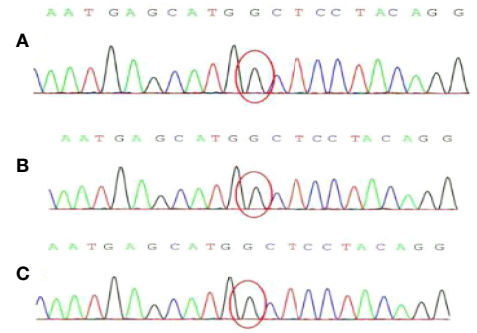


FIGURE 8 | The result of genetic first-generation sequencing detection. No TSC gene mutation was found. **(A)** Hepatocellular carcinoma; **(B)** Cholangiocarcinoma; **(C)** Neuroendocrine tumors.

have a history of liver disease such as hepatitis B and cirrhosis, and AFP level is usually elevated (20). The differences are mainly due to pathology and immunohistochemistry assessment, and the mutant genes of these two liver tumors are different. Most PEcoma patients exhibit mutations in TSC. To further verify the difference, we assessed patients from the First Hospital of China Medical University whose gender or age was similar to this case study and whose conditions included hepatocellular carcinoma, cholangiocarcinoma, and neuroendocrine tumors of the liver. First-generation sequencing of cancer tissue specimens revealed no TSC gene mutations (**Figure 8**). Therefore, genetic testing is a good tool for distinguishing between primary liver cancer and PEcoma, which is difficult to distinguish by other methods. Other diseases that need to be differentiated include liver adenocarcinoma, liver hemangioma, and liver focal nodular hyperplasia, amongst others (21).

Since TSC is a systemic disease, and treatments vary in accordance with the individual's specific manifestations. Surgical resection is the most important treatment for PEcoma (22). It has been reported that the systematic use of sirolimus, an mTOR inhibitor, can shrink tumors and facilitate surgical resection (23). There are also reports of radiofrequency ablation, chemotherapy, and other methods. But the long-term efficacy of these methods is still uncertain. In addition, because mTOR overexpression plays an important role in the development of PEcoma, mTOR inhibitors may be beneficial for PEcoma treatment. It has been reported that a 17-year-old PEcoma patient had significant reduction in tumor size after 3.5 years of sirolimus treatment (24). Based on the regulatory role of the mTOR pathways, mTORC1 inhibitors support the possibility of treating TSC patients based on the physiological pathogenesis, including renal angiomyolipoma, giant cell subependymal astrocytoma, and lymphatic vessel leiomyomatosis (25). The first generation of mTOR inhibitors, rapamycin and its derivative everolimus, can bind to FK506-binding protein-12 and form a complex, thereby inhibiting mTOR activity. Its effectiveness and safety have been confirmed clinically. A randomized, double-blind, placebo-controlled, international multi-center Phase III clinical trial study showed that 42% (33/79) of the everolimus group had reduced tumor size. The average duration of this reduction was greater than 5 months, while placebo group exhibited no reduction in tumor size (0/39) (26). The FDA approved everolimus for the treatment of TSC-RAML that does not require immediate surgery. Currently, this is the only drug that can be used to treat TSC-RAML.

CONCLUSION

The onset of tuberous sclerosis is rare. The genetic cause is mutations in the *TSC1* and *TSC2* genes, which involves the mTOR signaling pathway and lead to multiple system involvement. The clinical manifestations and types of disease are highly variable. Among them, the skin and nervous system are the most involved, causing skin nodules and epilepsy, but this condition can also rarely involve organs such as the liver, gallbladder, and spleen, causing rare symptoms and forming PEcoma or neuroendocrine tumors. Therefore, timely genetic testing should be considered when multiple organs and multiple types of space-occupying lesions occur. The accurate diagnosis of TSC disease is based on genetic testing and clinical manifestations, as proposed by the International TSC Consensus Group. Treatments should focus on symptom management, and some forms of the disease can be treated with mTOR inhibitors.

AUTHOR CONTRIBUTIONS

Writing-Original Draft Preparation, XHZ; Writing-Review and Editing, YL; Operation and case, XPZ; Image, Xd.L; Pathology, Xy.L; Gene detection, XJ; Data collection, LG, NW, HT, DL, BCYMW, JT, DH, YW, and HC. All authors contributed to the article and approved the submitted version.

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Trends in radiotherapy administration in the management of hepatocellular carcinoma: Analysis of a Korean tertiary hospital registry of hepatocellular carcinoma patients diagnosed between 2005 and 2017

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Purpose: To present the trends in radiotherapy for the management of hepatocellular carcinoma (HCC) at a single tertiary referral hospital in South Korea.

Materials and Methods: We retrospectively reviewed prospectively collected registry data of patients newly diagnosed with HCC between January 2005 and December 2017 at the Samsung Medical Center. Trends in radiotherapy, delivery techniques, tumor stage, and age were evaluated.

Results: During the study period, 9,132 patients were newly diagnosed with HCC at our institution. Of these, 2,445 patients (26.8%) received radiotherapy for all lesions, including extrahepatic metastases; 1,865 patients (20.4%) received radiotherapy for intrahepatic lesions alone, and 469 patients (5.1%) received radiotherapy as initial management. Although the proportion of patients receiving radiotherapy increased slightly over the study period (24.2% vs. 26.6%), the proportions of patients receiving radiotherapy for intrahepatic lesions (16.8% vs. 21.9%) and as initial management (0.1% vs. 12.5%) increased dramatically. The majority of patients treated between 2005 and 2008 received three-dimensional conformal radiotherapy (56.3%), whereas the majority of patients treated between 2018 and 2021 received proton beam therapy (43.6%). With the technical developments, the overall survival (OS) of patients who received radiotherapy as initial management increased significantly (5-year OS: from 5.4% to 30.1%), and the OS

difference between patients who did and did not receive radiotherapy as initial management significantly decreased (ratio of restricted mean survival time: from 0.383 to 0.544).

Conclusion: This registry-based, retrospective study indicated an increasing trend in the utilization of radiotherapy, adoption of advanced radiotherapy techniques, and OS improvements in patients with HCC.

KEYWORDS

hepatocellular carcinoma, radiotherapy, trend, three-dimensional conformal radiotherapy, intensity-modulated radiotherapy, stereotactic body radiotherapy, proton beam therapy

Introduction

Primary liver cancer is the sixth most commonly diagnosed cancer and the third leading cause of cancer-related death worldwide. Hepatocellular carcinoma (HCC) accounts for 75–85% of all cases of primary liver cancer (1). Most cases of HCC develop from chronic liver disease. Risk factors for chronic liver disease include hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, alcohol consumption, and aflatoxin exposure. In Asian countries, except Japan, and Africa, the predominant risk factor for HCC is HBV infection (70%), whereas HCV infection appears to be the key risk factor in Western countries and Japan (50–70%) (2, 3).

Curative treatments for HCC include liver transplantation, resection, and ablative therapies. However, only 30–40% of patients with HCC are diagnosed in the early stages of the disease and are eligible for curative treatment (2, 3). Even with curative therapy other than liver transplantation, more than half of patients develop recurrence (4–6). For the management of patients with intermediate- or higher-stage disease or recurrent disease, other treatment options, such as radiotherapy, trans-arterial chemoembolization (TACE), chemotherapy, and immunotherapy, should be considered.

Although HCC is a radiosensitive tumor, radiotherapy for intrahepatic HCC lesions is limited owing to the radiosensitive nature of the normal liver parenchyma (7, 8). Hence, radiotherapy has historically been administered only for palliative treatment in patients with symptomatic metastatic lesions and in patients with advanced HCC with major vascular tumor thrombi (9, 10). However, with advances in radiotherapy, stereotactic body radiotherapy (SBRT) and intensity-modulated radiotherapy (IMRT) with photon or proton beam therapy (PBT) have been introduced for the management of intrahepatic HCC lesions and have favorable outcomes with minimal toxicity (11–15). Based on these advances, several guidelines for HCC recommend that radiotherapy can be considered for patients for whom other curative therapies are not suitable (16, 17), and the role of radiotherapy in the management of HCC has changed and grown.

However, there has been no overall review on how these changes affect actual clinical practice. To better understand how the changing landscape has influenced the utilization of radiotherapy, we aimed to present the observed trends in the administration of radiotherapy for the management of HCC in a single tertiary referral hospital in South Korea.

Materials and methods

With approval of the Institutional Review Board (IRB number 2021-12-093-001), prospectively collected registry data for patients with newly diagnosed, previously untreated HCC at the Samsung Medical Center were analyzed in this study. The HCC diagnosis was confirmed either histologically or clinically, based on the guidelines of the Korean Liver Cancer Association-National Cancer Center (16, 18, 19). Clinical diagnosis of HCC was made based on the typical imaging hallmarks of HCC on multiphase CT or multiphase MRI. The major imaging feature for HCC diagnosis are defined as arterial phase hyper-enhancement with washout in portal venous, delayed or hepatobiliary phases. When imaging-based diagnosis was inconclusive or atypical features were seen, pathological diagnosis with biopsy was indicated. Data from the following patients were entered into the registry: (1) patients who were not previously treated for HCC; (2) patients who received at least one treatment or care for HCC at our institution; and (3) patients who had not been newly diagnosed or had undergone treatment for a malignancy other than HCC at the time of registration. The patients' baseline characteristics were collected, including age, sex, Eastern Cooperative Oncology Group performance status (ECOG PS), viral etiology, Child-Pugh

classification, albumin-bilirubin (ALBI) grade, Barcelona Clinic Liver Cancer (BCLC) stage, modified Union for International Cancer Control (mUICC) stage, and initial treatment method. The details of the HCC registry have been previously described (20). Data collected from patients registered between January 2005 and December 2017 were analyzed.

During the study period, as previously mentioned in other study from our institution, treatment of HCC was performed according to "Practice Guidelines for the Management of Hepatocellular Carcinoma" published by the Korean Liver Cancer Association-National Cancer Center Korea (16, 18–20). Surgical resection or radiofrequency ablation (RFA) was the primary treatment option for early-stage disease. Clinicians considered TACE and/or radiotherapy for patients with marginal liver function and HCC located in areas unsuitable for RFA. TACE was primarily considered for intermediate- or locally advanced-stage disease. Consolidative radiotherapy was added to treat hepatic lesions for patients with large tumors, macroscopic vascular/ductal invasion or an insufficient response to TACE. As atezolizumab plus bevacizumab, or lenvatinib were not available at the time of enrollment, sorafenib was prescribed for patients with metastatic or extensive tumors. When treatment of choice was evident based on the guidelines, the primary physician of the first visit decided the treatment option for the patient. If there were controversial issues in decision-making, the multidisciplinary team decided the treatment modality for the patient. The details of the multidisciplinary team approach for HCC in our institution had been described previously (21).

Registered patients were reviewed to determine whether they received radiotherapy for HCC management during follow-up. Radiotherapy was categorized into three groups based on the timing and target lesion. Radiotherapy utilization was defined as radiotherapy administered at any time during the HCC treatment period for the management of any lesion, including extrahepatic metastatic sites. Radiotherapy for intrahepatic lesions was defined as radiotherapy administered at any time during the HCC treatment period for the management of intrahepatic lesions. Radiotherapy as the initial management for intrahepatic lesions was defined as adoption of radiotherapy in the course of initial management at the time of diagnosis; this includes treatment using TACE plus consolidative radiotherapy, systemic therapy plus radiotherapy, palliative radiotherapy alone, and definitive radiotherapy alone. For patients who underwent multiple radiotherapy sessions, each session was counted individually. Radiotherapy delivery techniques were stratified into the following five subtypes: two-dimensional (2D) radiotherapy, three-dimensional conformal radiotherapy (3D-CRT), IMRT, SBRT, and PBT.

The radiotherapy utilization rate was obtained by dividing the number of patients who received radiotherapy by the total number of patients whose data were entered into the registry and was expressed as a percentage. Trends in radiotherapy utilization rates were plotted based on the year of diagnosis. Temporal trends in radiotherapy utilization were plotted based on the time from diagnosis to radiotherapy administration, with cumulative and differential counts. Trends in radiotherapy delivery technique, BCLC stage, and age at radiotherapy were plotted based on the time of radiotherapy administration.

Differences in clinical characteristics according to radiotherapy utilization were compared using the chi-square test. The Cochran–Armitage test was performed to assess the trend of radiotherapy utilization. Overall survival (OS) was calculated and compared between patients who received radiotherapy as initial treatment and those who received other treatment options for initial management. The log rank test was used to compare survival differences between groups. To observe the changing trends in the survival difference based on the initial treatment method, the 5-year estimated value of restricted mean survival time (RMST), which indicates the area under the survival curve, was analyzed (22). The RMST ratio was calculated to determine the trends of differences in OS between the groups over time. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using SPSS Statistical software ver. 27.0 (IBM, Inc., Armonk, NY, USA) and R studio ver. 1.3.1093 (R Foundation for Statistical Computing, Vienna, Austria; http://www.r-project.org).

Results

Patients

During the inclusion period, data from 9,132 patients were entered into the HCC registry. Radiotherapy was administered to 2,445 patients (26.8%; 3,570 lesions) during the entire HCC treatment period. Radiotherapy for intrahepatic lesions was administered to 1,865 patients (20.4%; 2,144 lesions), of whom 469 (5.1%; 469 lesions) received radiotherapy as the initial treatment for HCC. Supplementary Table 1 summarizes the clinical characteristics of patients based on radiotherapy status during the entire HCC treatment period. In overall HCC registry, patients with male sex (80.0%), positive for HBV (73.4%), had an ECOG PS 0 (91.6%), diagnosed clinically (94.6%), with liver function of Child–Pugh classification A (86.0%), and whose HCCs were classified as mUICC stage II (39.0%) and BCLC stage A (41.7%) were predominant. In the patients who received radiotherapy, the proportion of male sex, better underlying liver function, and worse tumor stage was higher.

Supplementary Table 2 shows the utilized radiotherapy techniques by the BCLC stage of the disease. The proportion of early disease, BLCL stages 0 and A, was higher for highly conformal radiotherapy techniques: 39.8% of 2D; 39.3% of 3D-CRT; 54.0% of IMRT; 63.5% of SBRT; 64.7% of PBT, respectively. The proportion of worse disease status, BCLC

stages C and D, was higher for conventional radiotherapy techniques: 44.5% of 2D; 47.5% of 3D-CRT; 32.6% of IMRT; 22.7% of SBRT; 18.2% of PBT, respectively.

Trends of radiotherapy utilization

The trends in radiotherapy utilization rate over the entire HCC management period based on the year of HCC diagnosis are presented in Figure 1. The overall radiotherapy utilization rate was relatively consistent throughout the study period (p=0.239). Approximately 24% of patients diagnosed in 2005 received radiotherapy, whereas 27% of patients diagnosed in 2017 received radiotherapy. The rate of radiotherapy administration for intrahepatic lesions significantly increased over the study period (p=0.006). Approximately 17% of patients diagnosed in 2005 received radiotherapy for intrahepatic lesions, whereas 22% of patients diagnosed in 2017 received radiotherapy for intrahepatic lesions. Although there were only marginal increases in the rates of radiotherapy utilization for all lesions and intrahepatic lesions (3% and 5% increases, respectively), there was a dramatic increase in the utilization of radiotherapy for the initial management of HCC (p<0.001). While only 0.5% of patients diagnosed in 2005 received radiotherapy as initial treatment, 13% of patients diagnosed in 2017 received radiotherapy as initial treatment. The results of the Cochran–Armitage test are summarized in Supplementary Table 3.

Figure 2 shows the temporal trends in radiotherapy utilization based on the interval from the date of HCC diagnosis to the date of radiotherapy for HCC management, counting each radiotherapy session individually. The slope of the graph was steeper for patients who were diagnosed more recently, indicating that the number of patients who received radiotherapy at the same time point after diagnosis gradually increased over time. Consistent trends were observed in the rates of overall radiotherapy utilization (Figure 2A) and radiotherapy for intrahepatic lesions (Figure 2B). Differential counts showed that the number of patients who received radiotherapy peaked in the first year after diagnosis and continued to decrease over time (Supplementary Figure 1).

Table 1 shows the trends in the characteristics of patients who received radiotherapy for intrahepatic lesions as the initial management. Only 37 patients (7.9%) enrolled between 2005 and 2008 received radiotherapy as initial management, whereas 165 patients (35.2%) enrolled between 2015 and 2017 received radiotherapy as initial management for intrahepatic lesions. Most patients had a relatively good performance status, with BCLC stage C and Child–Pugh classification A liver function throughout the study period. Furthermore, the adoption of advanced radiotherapy delivery techniques has increased over time. Approximately 97.3% of patients enrolled between 2005 and 2008 received 3D-CRT, whereas 21.2% of patients enrolled between 2015 and 2017 received PBT.

Supplementary Figure 2 shows the trends in radiotherapy utilization rate with patients grouped based on initial treatment: initial hepatic resection or other initial treatments. While proportion of patients receiving radiotherapy constantly increased in the patients who underwent initial other treatments, proportion of patients receiving radiotherapy after initial hepatic resection was relatively consistent throughout the period.

Trends of clinical factors

Radiotherapy delivery techniques changed dramatically during the study period. Patients who received radiotherapy in

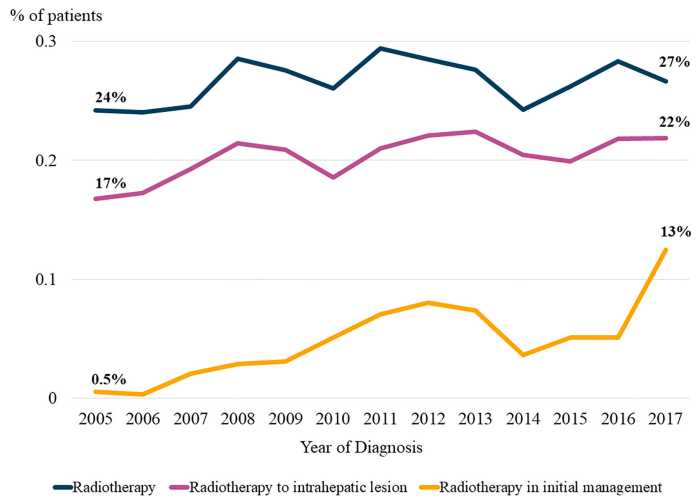


FIGURE 1
Trends in the radiotherapy utilization rate. The proportions of patients in the registry who received radiotherapy based on the year of diagnosis are plotted in line graph.

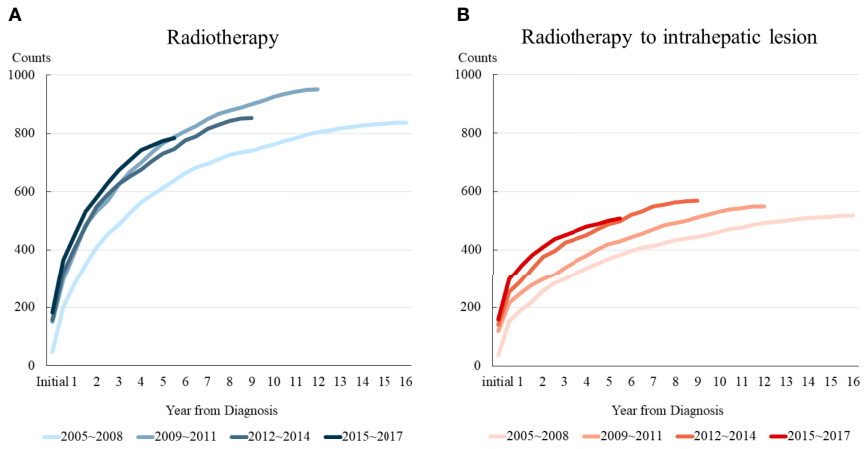


FIGURE 2
Temporal trends in radiotherapy utilization based on the time from diagnosis. Patients were divided into four groups according to the year of diagnosis. **(A)** Radiotherapy for all lesions, and **(B)** Radiotherapy for intrahepatic lesions.

2005 were treated with 2D- or 3D-CRT, whereas more than half of those treated in 2021 received PBT. Taken together, more than 80% of the radiotherapy delivery methods used for the management of HCC in 2021 were highly conformal radiotherapy, including IMRT, SBRT, and PBT (Figure 3A).

Moreover, there was a consistent change in the disease stages of patients who received radiotherapy. Patients treated in 2005 had relatively advanced disease, with no patients having BCLC stage 0, whereas more than 20% of patients treated in 2021 had BCLC stage 0 (Figure 3B). The age of patients who received radiotherapy also showed a consistent pattern, with a continuously increasing number of older patients treated with radiotherapy in recent years (Figure 3C).

Trends of overall survival

The 2-year and 5-year OS rates of the patients who received radiotherapy as initial treatment and those who did not were significantly different (2-year: 31.2% vs. 71.9% and 5-year: 16.2% vs. 56.0%; $p < 0.001$, respectively). We found a consistent improvement in OS for patients who received radiotherapy as initial treatment over time, from 13.5% and 5.4% in 2005 to 34.3% and 30.1% in 2017 for 2-year and 5-year OS, respectively. A similar change was seen for patients who did not receive radiotherapy with a change from 67.2% and 53.1% in 2005 to 78.7% and 71.6% in 2017 for 2-year and 5-year OS, respectively. The ratio of RMST increased over time (from 0.383 to 0.544), indicating that the difference in OS between patients who received radiotherapy as initial management and those who did not decreased over time. The OS curves and RMST results are summarized in Figure 4 and Table 2.

Initial radiotherapy technique was also observed to be related with OS. The OS rates of the patients who received highly conformal radiotherapy (IMRT, SBRT, or PBT) as initial treatment were significantly better than the patients who received conventional radiotherapy (2D or 3D-CRT), irrespective of the stage of patients (5-year OS of overall population: 33.0% vs. 11.4%, $p < 0.001$; BCLC stage 0 or A: 85.6% vs. 20.0%, $p < 0.001$; BCLC stage B or higher: 21.7% vs. 11.0%, $p = 0.004$, respectively). The OS curves are shown in Supplementary Figure 3.

Discussion

This retrospective study demonstrated changes in the role of radiotherapy in the management of HCC over the last 16 years at our institution. The use of radiotherapy has increased over time, particularly in regard to radiotherapy for intrahepatic lesions and radiotherapy as initial management. There has also been a dramatic increase in the use of highly conformal radiotherapy and PBT. Our results demonstrated the changing role of radiotherapy in the management of HCC.

In the past, radiotherapy played a minimal role in the management of HCC, especially in the management of intrahepatic lesions, owing to the occurrence of radiation-induced toxic events such as radiation-induced liver disease (23). Previously, radiotherapy was mainly used for palliation, management of patients with portal vein tumor thrombus, and symptomatic extrahepatic metastases. However, with recent advances in radiotherapy delivery and image-guidance techniques, radiotherapy for intrahepatic lesions is viewed as an effective and safe treatment modality. Several prospective studies

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TABLE 1 Characteristics of patients who received radiotherapy as initial management for intrahepatic lesions.

Characteristics	Year of diagnosis			
	2005–2008	2009–2011	2012–2014	2015–2017
N. of patients	37 (7.9%)	123 (26.2%)	144 (30.7%)	165 (35.2%)
Age (median, range)	53 (26–73)	54 (23–74)	55 (27–84)	57 (30–87)
ECOG PS				
0	25 (67.6%)	111 (90.2%)	140 (97.2%)	133 (80.6%)
1	11 (29.7%)	11 (9.0%)	2 (1.4%)	30 (18.2%)
2	1 (2.7%)	0 (0.0%)	1 (0.7%)	1 (0.6%)
3	0 (0.0%)	1 (0.8%)	1 (0.7%)	1 (0.6%)
4	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
BCLC stage				
O	0 (0.0%)	0 (0.0%)	2 (1.4%)	2 (1.2%)
A	2 (5.4%)	10 (8.2%)	5 (3.5%)	11 (6.7%)
B	1 (2.7%)	1 (0.8%)	3 (2.1%)	9 (5.5%)
C	34 (91.9%)	111 (90.2%)	132 (91.6%)	138 (83.6%)
D	0 (0.0%)	1 (0.8%)	2 (1.4%)	5 (3.0%)
ALBI grade				
I	11 (29.7%)	87 (70.7%)	101 (70.1%)	100 (60.6%)
II	25 (67.6%)	35 (28.5%)	41 (28.5%)	62 (37.6%)
III	1 (2.7%)	1 (0.8%)	2 (1.4%)	3 (1.8%)
Child-Pugh classification				
A	31 (83.8%)	114 (92.7%)	127 (88.2%)	141 (85.5%)
B	6 (16.2%)	9 (7.3%)	16 (11.1%)	20 (12.1%)
C	0 (0.0%)	0 (0.0%)	1 (0.7%)	4 (2.4%)
Radiotherapy technique				
2D	1 (2.7%)	1 (0.8%)	0 (0.0%)	0 (0.0%)
3D-CRT	36 (97.3%)	120 (97.6%)	134 (93.1%)	112 (67.9%)
IMRT	0 (0.0%)	2 (1.6%)	8 (5.5%)	13 (7.9%)
SBRT	0 (0.0%)	0 (0.0%)	2 (1.4%)	5 (3.0%)
PBT	0 (0.0%)	0 (0.0%)	0 (0.0%)	35 (21.2%)
Initial treatment				
TACE + consolidative radiotherapy	26 (70.3%)	118 (95.9%)	133 (92.3%)	130 (78.8%)
Systemic therapy + radiotherapy	2 (5.4%)	0 (0.0%)	1 (0.7%)	5 (3.0%)
Palliative radiotherapy alone	9 (24.3%)	5 (4.1%)	7 (4.9%)	15 (9.1%)
Definitive radiotherapy alone	0 (0.0%)	0 (0.0%)	3 (2.1%)	15 (9.1%)

ECOG PS, Eastern Cooperative Oncology Group performance status; BCLC, Barcelona Clinic Liver Cancer; ALBI, albumin-bilirubin; 3D-CRT, 3D-conformal radiotherapy; IMRT, intensity modulated radiotherapy; SBRT, stereotactic body radiotherapy; PBT, proton beam therapy; TACE, trans-arterial chemoembolization.

on SBRT as treatment for HCC patients with Child–Pugh classification A or B liver function reported good oncologic outcomes (2-year local control rates ranging from 80.9% to 97%) with acceptable toxicity (rates of grade 3 or higher gastrointestinal or liver toxicity ranging from 0% to 7%) (12, 24–27). Two prospective studies on PBT also reported good results, with 3-year local control rates of 95.2% and 94.5% and no severe toxic events (28, 29). The details of these prospective studies are summarized in Supplementary Table 4.

Previous studies have compared radiotherapy with traditional curative treatment options. Su et al. (30) compared SBRT with liver resection for patients with small HCC and Child–Pugh classification A liver function. No difference was observed in OS or progression-free survival between the two treatment methods, indicating that the local effects of SBRT were similar to those of liver resection. Kim et al. (31) conducted a multinational study to compare the effectiveness of SBRT and RFA for unresectable HCC. Their results favored SBRT over RFA, particularly for larger tumors in the subphrenic region, and for the tumors that progressed after TACE. Kim et al. (13) compared the outcomes of PBT and RFA in patients with recurrent or residual HCC in a prospective phase 3 non-

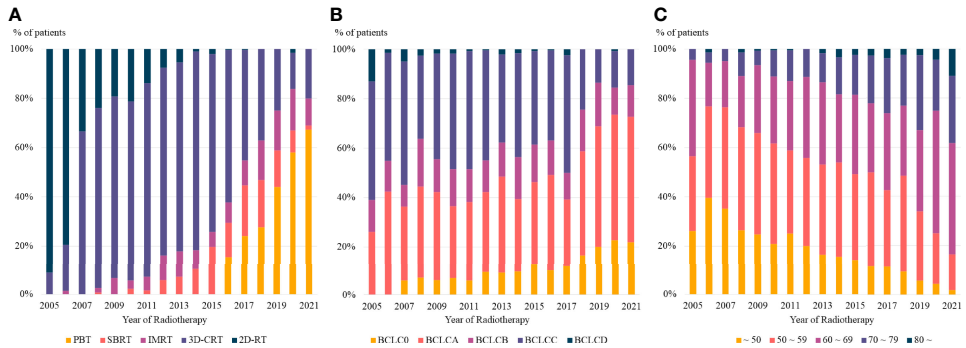


FIGURE 3
The trends in **(A)** radiotherapy delivery technique, **(B)** BCLC stage, and **(C)** age at radiotherapy based on the year of radiotherapy. PBT, proton beam therapy; SBRT, stereotactic body radiotherapy; IMRT, intensity modulated radiotherapy; 3D-CRT, three dimensional conformal radiotherapy, 2D-RT, two dimensional radiotherapy; BCLC, Barcelona Clinic Liver Cancer.

inferiority trial and reported that the local progression-free survival rate of patients undergoing PBT was non-inferior to that of patients undergoing RFA.

In addition to the aforementioned prospective studies, numerous retrospective studies have also reported the effectiveness of radiotherapy as a treatment for HCC. Based on these results, the current National Comprehensive Cancer Network guidelines recommend SBRT as an alternative to ablation/embolization techniques or when these therapies have failed or are contraindicated (32). Also, the American Society for Radiation Oncology clinical practice guideline provided evidence-based recommendation for radiotherapy in HCC recently, strongly recommending radiotherapy as a potential first-line treatment in patients with liver-confined HCC who are not candidates for curative therapy, as consolidative therapy after incomplete response to liver-directed therapies, and as a salvage option for local recurrences (33).

The baseline clinical characteristics of the patients who received radiotherapy in this study differed significantly from those of the patients who did not receive radiotherapy (Supplementary Table 1). The reason for the patients who received radiotherapy had relatively preserved liver function status and advanced disease could be owing to the indications for radiotherapy itself. Radiotherapy is relatively contraindicated for patients with poor liver function, and patients with early-stage disease are generally candidates for resection or ablative

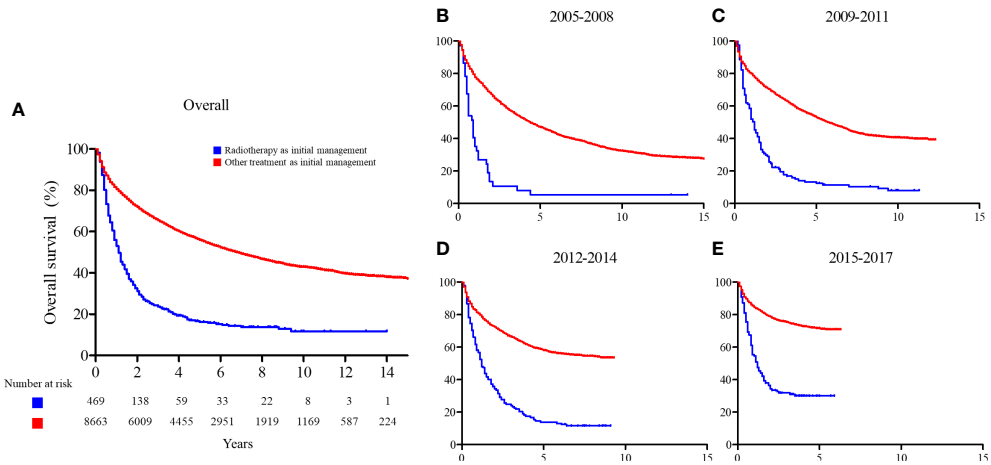


FIGURE 4
Overall survival comparison between patients who received radiotherapy as initial management and those who did not. **(A)** Entire cohort; **(B)** patients enrolled between 2005 and 2008; **(C)** patients enrolled between 2009 and 2011; **(D)** patients enrolled between 2012 and 2014; and **(E)** patients enrolled between 2015 and 2017.

TABLE 2 Overall survival and RMST values of patients over time.

		Overall survival			RMST	
		2-year	5-year	P value	Estimated	Ratio
Overall	Initial RT (+)	31.2%	16.2%	< 0.001		
	Initial RT (-)	71.9%	56.0%			
2005-2008	Initial RT (+)	13.5%	5.4%	< 0.001	1.257	0.383
	Initial RT (-)	67.2%	47.0%		3.281	
2009-2011	Initial RT (+)	29.1%	12.5%	< 0.001	1.712	0.493
	Initial RT (-)	70.9%	53.1%		3.471	
2012-2014	Initial RT (+)	34.4%	13.9%	< 0.001	1.881	0.526
	Initial RT (-)	72.5%	58.1%		3.573	
2015-2017	Initial RT (+)	34.3%	30.1%	< 0.001	2.143	0.544
	Initial RT (-)	78.7%	71.6%		3.938	

RMST, restricted mean survival time; RT, radiotherapy.

treatments. However, the reason for the increased proportion of male sex and HBV infection is difficult to define. Male sex and HBV infection are known to be poor prognostic factors for survival and recurrence (34, 35). The poor prognosis for these patients may have influenced the increased proportion of these patients in the radiotherapy arm as they may have required radiotherapy for further cancer management. However, it is difficult to draw concrete conclusions regarding the reason for this difference, and further analysis is required.

The proportion of patients who received radiotherapy as initial management for intrahepatic lesions dramatically increased over time in the current study, whereas the overall radiotherapy utilization rate and the rate of radiotherapy for intrahepatic lesions were relatively consistent. These proportions may not have noticeably increased because of the relatively short follow-up duration of the patients whose data were entered into the registry in more recent years. The data of patients entered the registry in 2005 were reviewed to determine whether they received radiotherapy within 16 years after diagnosis, whereas the data of patients entered the registry in 2017 were only reviewed to determine whether they received radiotherapy within 4 years after diagnosis. Some patients whose data were recently entered into the registry are likely to receive radiotherapy with further follow-up; therefore, the overall radiotherapy utilization rate and radiotherapy administration for intrahepatic radiotherapy are expected to increase in future.

High conformal radiotherapy techniques, PBT, IMRT and SBRT, are generally considered as main radiotherapy techniques for HCC. However, in our institution, there was marked increase in the proportion of PBT, but the proportion of IMRT and SBRT were relatively constant or even decreased over time (Figure 3A). The reason for this difference seems to be due to the treatment policy of our institution. When radiotherapy for intrahepatic lesion was required, PBT was preferred after installation of proton center. As a consequence, the proportion of SBRT and IMRT showed relatively consistent trend over time. There was ups and downs in the proportion of individual radiotherapy techniques, but it is to note that the proportion of high conformal radiotherapies increased considerably over time.

We have shown that the difference in OS between patients who did and did not receive radiotherapy as initial management for HCC decreased over time (Figure 2 and Table 4). However, the management of HCC generally involves prolonged management with multiple treatments. And as other treatment modalities also advanced over past decade, consideration of the initial management alone may not reflect the clinical outcomes. However, all patients were further managed with the most appropriate treatment options based on their status, irrespective of the initial treatment method. It seems noticeable that survival differences between groups have narrowed down in this clinical setting; however, the current results should be interpreted with caution.

The current study has some limitations. First, as a single-institutional retrospective study of HBV endemic area, the treatment policy at our institution may not be identical to that at other institutions. Also, while all HCC treatment options are covered by the National Health Insurance Service in Korea, the coverage may differ in other geographical areas. Therefore, the proportion of patients receiving radiotherapy and radiotherapy delivery methods in other institutions may not be the same as those observed in the current study. However, we believe that although the numbers may not be identical, trends in the increased utilization of radiotherapy in the management of HCC will be observed in other institutions with recent prospective trials confirming the effectiveness of RT in HCC management and recommendations of the guidelines reflecting these outcomes (11, 13, 16, 17, 24, 26, 32, 36). Second, the current study did not discuss the response to radiotherapy or

radiotherapy-related toxicity. As the aim of current study was to report the changing trends of radiotherapy utilization in the management of HCC, our study includes various treatment settings from palliative to definitive, with heterogeneous radiotherapy doses and techniques over more than 15 years. It was not possible to report the treatment response and toxicity due to the heterogeneity. However, as shown in Supplementary Table 4, in the current era of radiotherapy, radiotherapy is a safe and considerable treatment option in the management of HCC. Third, while we have addressed the changing trends of radiotherapy utilization and radiotherapy techniques, the recurrences and the managements of new recurrences were not covered in current study. Analysis of pattern of recurrences and the managements of recurrences, such as resection, liver transplantation, RFA, TACE, and chemotherapy, would have added additional depth to the study, but was not available currently. Future prospective studies might be needed to clarify these limitations.

In conclusion, the use of radiotherapy for HCC management at our institution increased dramatically from 2005 to 2017. The number and proportion of patients receiving radiotherapy for intrahepatic lesions as initial management and highly conformal radiotherapy are increasing continuously. Taken together, the findings of the current study show increased utilization of radiotherapy in the management of HCC and a shift in the paradigm of radiotherapy from palliative management of extrahepatic lesions and vascular invasions to curative treatment of intrahepatic lesions.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

This study was approved by Institutional Review Board of Samsung Medical Center (SMC IRB 2021-12-093-001). The study adhered to World Medical Association's Declaration of Helsinki for Ethical Human Research. The informed consent was waived due to the retrospective nature of the study.

Author contributions

Conceptualization, HP. Data curation, BB, HP, JY, GY, DS, MC, and JO. Formal analysis, BB, JY, and HP. Investigation, BB, JY, and HP. Methodology, BB, JY, and HP. Funding acquisition, JY. Project administration, MC, and HP. Manuscript writing—original draft, BB, and JY. Manuscript writing—review and editing, BB, HP, GY, MC, JO, and JY. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.928119/full#supplementary-material.

SUPPLEMENTARY FIGURE 1
Differential counts of radiotherapy utilization based on time from diagnosis. **(A)** radiotherapy to all lesions; **(B)** radiotherapy to intrahepatic lesions.

SUPPLEMENTARY FIGURE 2
Trends in radiotherapy utilization rate with patients grouped based on initial treatment: initial hepatic resection or other initial treatments. Trends of initial hepatic resection group is shown in solid line, and other initial treatment group is shown in dotted line.

SUPPLEMENTARY FIGURE 3
Overall survival curves of patients who received radiotherapy as initial management, categorized based on radiotherapy techniques. **(A, B)** overall patients; **(C, D)** BCLC stage 0 or A patients; **(E, F)** BCLC stage B or higher patients. 3D-CRT, 3D-conformal radiotherapy; IMRT, intensity modulated radiotherapy; SBRT, stereotactic body radiotherapy; PBT, proton beam therapy

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Mechanisms and prospects of circular RNAs and their interacting signaling pathways in colorectal cancer

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Colorectal cancer (CRC) is the leading malignant tumor in terms of morbidity and mortality worldwide, and its pathogenesis involves multiple factors, including environment, lifestyle, and genetics. Continuing evidence suggests that circular RNAs (circRNAs), as a novel non-coding RNA, constitute an important genetic variable in the pathogenesis of CRC. These circRNAs with covalently closed-loop structures exist objectively in organisms. They not only have the biological functions of regulating the expression of target genes, changing the activity of proteins, and translating proteins, but also play a key role in the proliferation, invasion, migration, and apoptosis of tumor cells. CRC is one of the most common cancers in which circRNAs are involved in tumorigenesis, metastasis, and drug resistance, and circRNAs have been demonstrated to function through crosstalk with multiple signaling pathways. Therefore, this review summarizes the biological and carcinogenic functions of circRNAs and their related PI3K/AKT, MAPK, Notch, JAK/STAT, Hippo/YAP, WNT/β-catenin, and VEGF signaling pathways in CRC. We further explore the clinical value of circRNAs and important signaling proteins in the diagnosis, prognosis, and treatment of CRC.

KEYWORDS

colorectal cancer, circular RNA, signaling pathway, biological function, clinical value

Abbreviations: CRC, colorectal cancer; CSCs, cancer stem cells; circRNAs, circular RNAs; CMS, consensus molecular subtypes; ceRNA, competitive endogenous RNA; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; IL6, interleukin-6; JAK, janus kinase; MAPK, mitogen-activated protein kinases; mCRC, metastatic CRC; mTOR, mammalian targets of the rapamycin; miRNA, microRNA; ncRNAs, non-coding RNAs; OS, overall survival; PlGF, placental growth factor; P-JAK, phosphorylation-JAK; PI3K, phosphatidylinositol 3-kinase; PFS, progression-free survival; RT-qPCR, quantitative reverse transcription-PCR; STAT, signal transducers and activators of transcription; TGF-β, transforming growth factor-beta; TME, tumor microenvironment; VEGF, vascular endothelial growth factor.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer deaths worldwide, with an estimated 1.9 million new cases and 935,000 deaths in 2020 (1). The incidence of CRC has stabilized and declined in highly developed countries as a result of national screening programs and colonoscopy (2). But with economic progress in developing countries, the number of new CRC cases worldwide is expected to reach 2.5 million by 2035 (3). At present, the 5-year survival rate of patients with early CRC is close to 90% (4). However, among newly diagnosed patients with CRC, 20% have already had metastasis, and another 25% will develop metastasis due to locally advanced tumors (5). Moreover, metastatic CRC has a poor prognosis, with a 5-year survival rate of less than 20% (6). Therefore, to seek effective biomarkers for early diagnosis of CRC and new therapeutic targets for advanced and recurrent CRC, it is necessary to further explore and clarify the molecular mechanisms underlying the development and metastasis of CRC.

Circular RNAs (circRNAs) are newly discovered non-coding RNAs (ncRNAs) that exist objectively in living organisms (7). These circRNAs have a covalently closed-loop structure, missing the 5′-3′ terminals and polyadenylate tails (8). The development of high-throughput RNA sequencing and bioinformatic tools has successfully detected thousands of circRNAs distributed in a variety of tissues, cell types, and biological fluids (9, 10). Moreover, researchers have revealed that these RNAs have cell-specific, tissue-specific, and time-specific expression patterns and are conserved across species (11, 12). Recent evidence indicates that circRNAs are not only significantly associated with neurological disorders, cardiovascular diseases, and autoimmune diseases (13–15), but also play a regulatory role in cancer-related processes such as tumorigenesis, progression, and cell apoptosis (16–18). CRC is one of the most commonly reported cancers in which circRNAs are involved in tumorigenesis and metastasis (18–20).

Signal transduction is a common way to regulate basic cellular processes in humans, and abnormal regulation of signal transduction can lead to the occurrence of pathological states such as cancer and autoimmunity (21, 22). It is widely believed that circRNA promotes cancer cell proliferation and metastasis by interacting with key components of major signaling pathways (23, 24). In CRC, the reported crosstalk signaling pathways with abnormally expressed circRNA include phosphatidylinositol 3-kinase (PI3K)/AKT (25), mitogen-activated protein kinases (MAPK) (26), Notch (27), Janus kinase/signal transducers and activators of transcription (JAK/STAT) (28), Hippo/YAP (29), WNT/β-catenin (30), and vascular endothelial growth factor (VEGF) (31). In this review, we summarized the molecular mechanism and role of circRNAs and related signaling pathways in the occurrence and progression of CRC by searching for keywords in Pubmed, Web of Science, ScienceDirect, and Springer SLCC databases. The clinical application value of circRNAs in the diagnosis, prognosis, and treatment of CRC was further discussed.

Genetic and transcriptional characterization of CRC

Recent research suggests that most CRC cells originate from stem cells or stem cell-like cells (32). The accumulation of multiple genetic and epigenetic changes produces these cancer stem cells (CSCs), which ultimately activate oncogenes and inactivate tumor-suppressor genes (33, 34). Two major precursor pathways represent multiple genetic and epigenetic events in a fairly continuous sequence. Most CRCs are chromosomal instability sequences (70-90%), also known as the traditional adenoma-carcinoma pathway (35). In this pathway, tumor development is caused by the sequential accumulation of mutations in the WNT, epidermal growth factor receptor (EGFR), P53, and transforming growth factor-beta (TGF-β) signaling pathways (36). Another is the serrated tumor pathway, which involves activating BRAF mutations or DNA mismatches to repair gene inactivation and accounts for 10-20% of CRCs (37, 38).

In 2015, the International CRC Subtype Consortium proposed a more comprehensive transcriptome classification based on gene expression profiles (39). CRC is divided into four consensus molecular subtypes (CMS): CMS1 (MSI immunity, 14%), CMS2 (canonical, 37%), CMS3 (metabolic, 13%), and the subtype with the worst prognosis, CMS4 (mesenchymal, 23%) (40). These CMS group classifications embody markedly different molecular characteristics associated with biological and clinical stratification and are the basis for targeted interventions (41). There are differences not only in embryology, anatomy, and biology but also in molecular characteristics between right colon cancer (hepatic curvature of transverse colon, ascending colon, and cecum) and left colon cancer (splenic curvature of transverse colon, descending colon, and sigmoid colon) and the rectum (42). Right colon cancer is more common in CMS1 and CMS3 subtypes, while left colon cancer is predominantly in CMS2 subtypes (43).

The biological and oncogenic functions of circRNAs in CRC

CircRNAs play different biological functions according to their localization in the cytosol or nucleus (44). First of all, circRNAs can regulate gene transcription and alternative splicing, and nuclear circRNAs can also induce parental gene expression (45). Second, some circRNAs, such as circ_0128846

(29) and circ_0106714 (46), influence the expression of target genes by competitively binding microRNA (miRNA) or acting as miRNA sponges, which is the most widely studied mechanism of circRNA in the progression of CRC (47). In addition, another biological function of circRNAs is that their interactions with RNA-binding proteins cause changes in protein activity (48). Recently, two novel circRNAs (circ-BCL2L12-1 and circ-BCL2L12-2) with different protein binding sites have been identified in CRC. Moreover, circ-BCL2L12-1 overexpression was related to shorter OS, while circ-BCL2L12-2 expression was negatively related to TNM staging in CRC (49). Finally, circRNA has the biological function of translating proteins (50), and circ-PPP1R12A is reported to have translation capabilities in CRC (51) (Figure 1).

A large number of studies have shown that circRNA can play an important role in the tumor progression of CRC by regulating tumorigenesis transcription factors or oncogene expression (52). In the study of Li's team, 448 circRNAs with abnormal expression in CRC were detected by high throughput RNA sequencing. Further *in vitro* experiments showed that the down-regulation of circDDX17 could enhance the proliferation, invasion, and migration of CRC cells (53). *In vivo* experiments on nude mice have also confirmed that some circRNAs, such as circ-ERBIN, can promote tumor growth and metastasis of CRC (54). Advanced metastatic CRC is the leading cause of cancer-related death. Epithelial-mesenchymal transition (EMT) is a cellular reprogramming process in which epithelial cells acquire a mesenchymal phenotype, which promotes the development of migrating and invading cells (55). Through GEO data set analysis, circ_101951 was found to be a novel circRNA overexpressed in CRC tissues. In-depth studies on its biological function and mechanism showed that circ_101951 could facilitate the migration and invasion of CRC cells by regulating EMT (56). CSCs are subsets of small cells in tumors that drive tumor progression and metastasis (57). Continuing studies have shown that CSCs in CRC are inherently resistant to treatment and are closely related to cancer regeneration and recurrence after conventional treatment (58). Rengganaten et al. revealed circ_0066631 and circ_0082096 as two abnormally expressed circRNAs in CSC-rich CRC globular cells that play an important role in regulating the stemness properties of CSCs (59).

The role of major signal pathways interacting with circRNAs in CRC

CircRNA/MAPK signaling axis in CRC

Compared with other intracellular signaling pathways, the MAPK pathway is more important in cell proliferation, differentiation, migration, apoptosis, and angiogenesis. The most critical signal cascade reaction in all MAPK signal

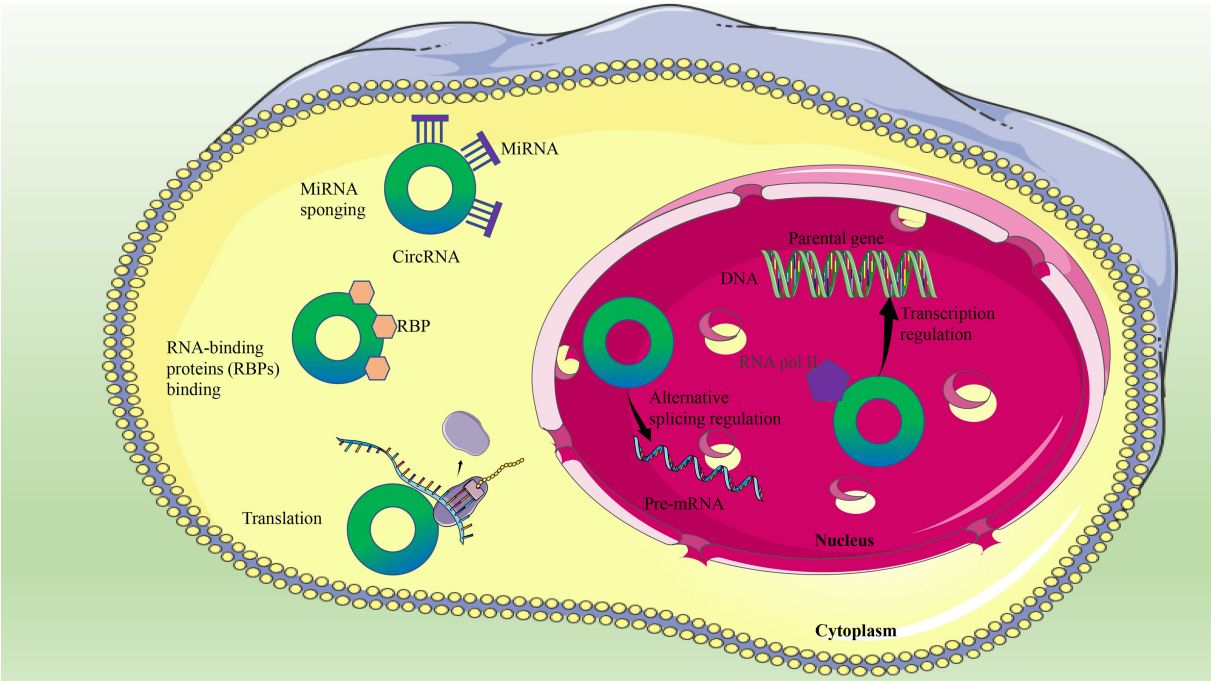


FIGURE 1
The biological functions of circRNAs in CRC.

transduction pathways is RAS/RAF/MEK/ERK (60). The pathway is initiated by activation of the RAF kinase family (ARAF, BRAF, and CRAF [RAF1]) by members of the RAS family. Activated RAF protein phosphorylates MEK1/2 and then activates and phosphorylates ERK. Finally, ERKs induce phosphorylation of a variety of substrates, such as transcription factors, which are involved in regulating a variety of cellular functions (61) (Figure 2). Abnormal activation of the MAPK signaling pathway in CRC has been reported to occur through activation mutations of RAS and BRAF (62), which are associated with treatment resistance in patients with metastatic CRC (mCRC) (63). Approximately 90% of BRAF mutations are in the V600E series, and although $BRAF^{V600E}$ mutations are rare in CRC (about 10%), their role is important (64). $BRAF^{V600E}$ series mutations in CRC are more common in older women (over 70 years old), right colon tumors, poorly differentiated tumors, and mucous subtypes, and also have a higher frequency of peritoneal metastasis (65). This also predicted poor clinical prognosis (median OS of 11 months) and poor standard treatment response in CRC with $BRAF^{V600E}$ series mutations (66). RAS family is one of the most frequently mutated families in CRC (67). About 40% of mCRCs carry KRAS mutations, mainly in exons 2 (codon 12, 13), 3 (codon 59-61) and 4 (codon 117, 146). Mutations at different points cause different clinical, pathological, and molecular features. Although mutations in NRAS account for only 4% of mCRC, they have similar clinical and pathological features to KRAS mutations. While HRAS mutations are 1%, very few studies have (65, 68).

In CRC, circRNA regulation of the MAPK signaling pathway is a topic widely discussed and worthy of study, although there are few relevant research results at present. It is generally believed that the phosphorylation of MAPK14, the core molecule of the MAPK pathway, by upstream signal kinase kinase 3/6 (MKK3/6) promotes nuclear translocation and promotes the progression of CRC (69). Based on the above results, Wang et al. further verified that circ_0131663 (circ-MAPK14) can reduce the nuclear translocation of MAPK14 by competitive binding with upstream MKK6 through a peptide encoding 175 amino acids, and ultimately inhibit the progression and metastasis of CRC (70). In addition, the functions and mechanisms of other novel circRNAs in CRC have also been preliminarily explored. Circ-ITGA7 was significantly underexpressed in CRC tissues and cell lines, and it was found by functional experiments that the expression of circ-ITGA7 prevented the growth and metastasis of CRC cells *in vitro* and *in vivo*. Further mechanistic studies have shown that

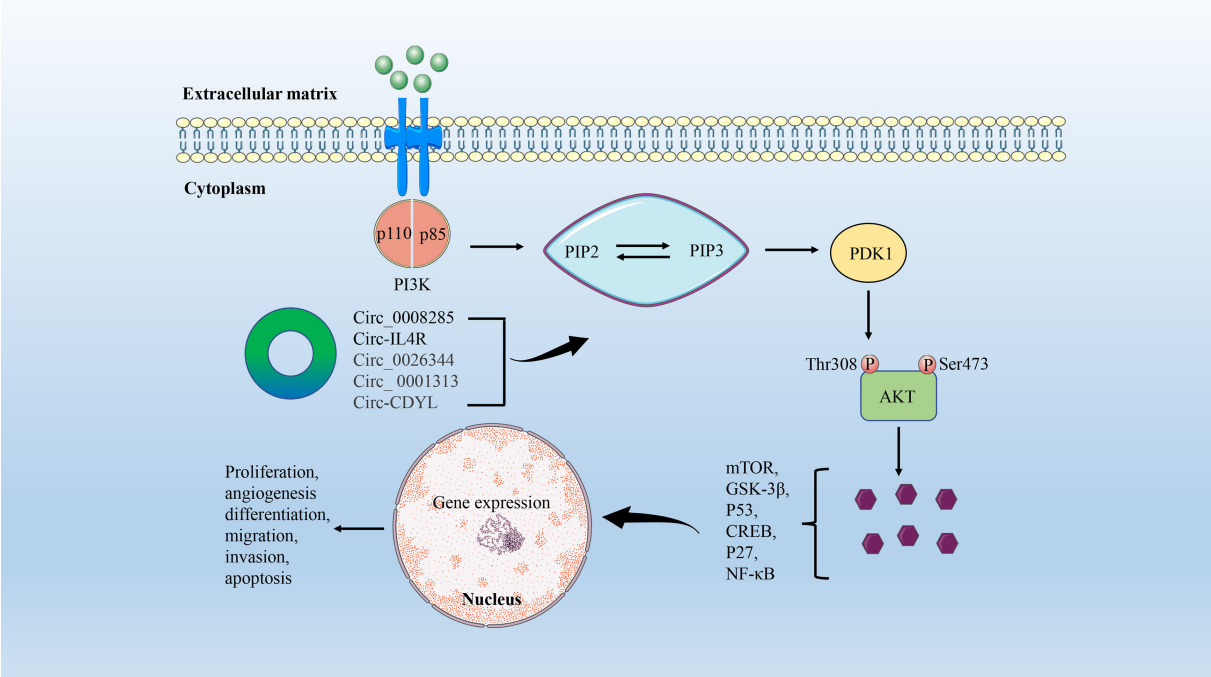


FIGURE 2
The circRNA/MAPK signaling axis in CRC. This signaling pathway is initiated by the binding of activated growth factors, such as epidermal growth factor (EGF), to tyrosine kinase receptors on the cell surface. This causes the downstream RAS to increase the GTP binding state. RAS-GTP dimers recruit RAF or RAF/MEK dimers to the plasma membrane and promote the activation of RAF and the formation of MEK homologous dimers. This is followed by activation and phosphorylation of downstream ERKs. Finally, ERKs induce phosphorylation of transcription factors and other substrates to participate in cell proliferation, differentiation, migration, and apoptosis in the nucleus.

circ-ITGA7 inhibits the growth and metastasis of CRC tumors by inhibiting RAS/RAF/MEK/ERK signaling pathways and promoting ITGA7 transcription (71). Another novel circRNA, CIRS-7, acts as a competitive endogenous RNA (ceRNA) of miR-7 to regulate EGFR/RAF1/MAPK signal transduction and plays an important role in CRC progression (72).

CircRNA/PI3K/AKT signaling axis in CRC

The PI3K/AKT signaling pathway is involved in regulating cell adhesion, growth, survival, migration, and other cellular events (73). PI3K is an intracellular lipid kinase that affects the expression levels of extracellular protein kinase and EGFR, leading to PIP2 phosphorylation to produce PIP3. PIP3 is an important messenger that recruits AKT, which in turn generates mammalian targets of rapamycin (mTOR) or GSK-3β signaling, resulting in a variety of cellular events (74). The PI3K/AKT/mTOR signaling pathway is one of the most critical abnormal regulatory pathways in CRC, and the activation of this pathway is associated with cell proliferation and transformation, tumorigenesis, progression, angiogenesis, and drug resistance (75, 76) (Figure 3).

With the continuous development of high-throughput sequencing technology, a large number of circRNAs with abnormal expression in a variety of tumors have been identified (60, 77, 78). The researchers concluded that the expression level of circ-0008285 in CRC tissues and cells was negatively correlated with tumor size and lymphatic metastasis by combining quantitative reverse transcription-PCR (RT-qPCR) and clinicopathological parameter analysis. Further functional and mechanistic studies confirmed that low expression of circ_0008285 promotes the proliferation and migration of CRC cells *in vitro* by regulating the PI3K/AKT pathway (79). In addition, Jiang et al. found a circRNA that also plays a role in the occurrence and progression of CRC by regulating the PI3K/AKT signaling pathway, named circ-IL4R. However, circIL4R is highly expressed in the serum, tissues, and tumor cell lines of CRC patients, and is positively associated with later clinical stages and poorer prognosis (80).

CircRNA/VEGF signaling axis in CRC

Generally, the most common distant metastasis site of CRC is the liver. According to statistics, about 25% of CRC patients will have liver metastasis, and the prognosis is poor (81). High

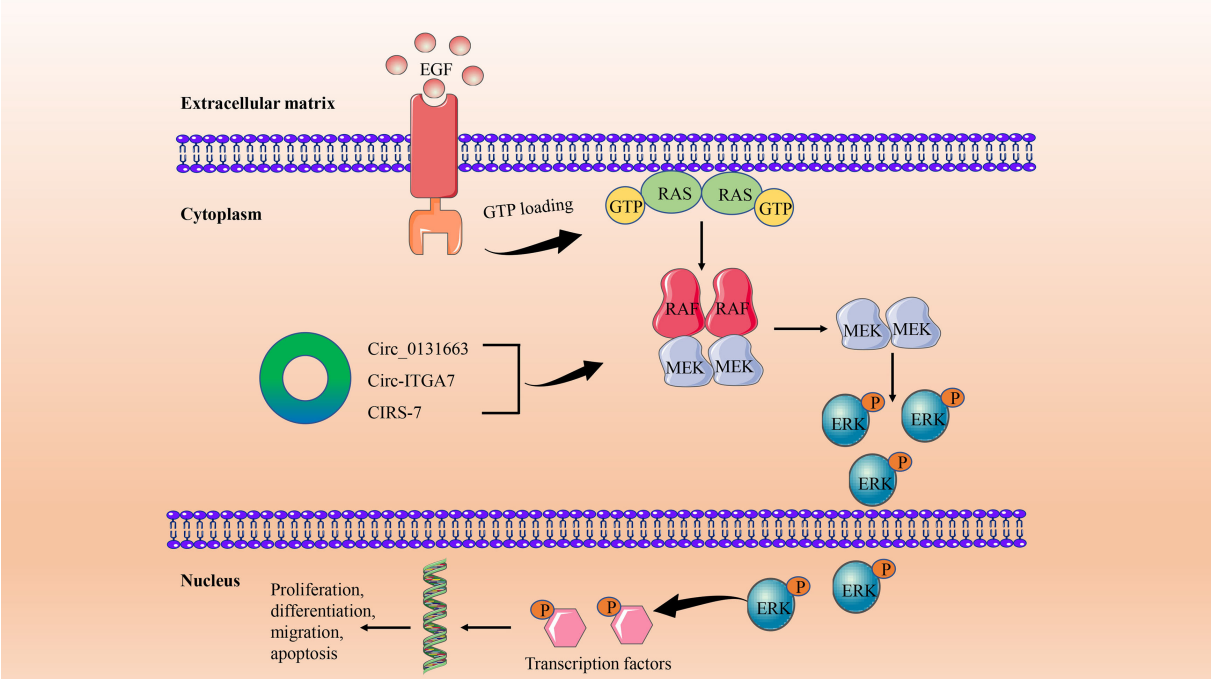


FIGURE 3
The circRNA/PI3K/AKT signaling axis in CRC. PI3K consists of a catalytic (P110) domain and a regulatory (P85) domain. PI3K is activated by a variety of growth factors and signaling complexes. Activated PI3K promotes PIP2 phosphorylation to produce PIP3, which activates PDK1. AKT is then phosphorylated at Thr308 of PDK1. Ultimately, AKT induces cell proliferation, differentiation, migration, and angiogenesis by mediating multiple signaling pathways such as mTOR, GSK-3β, P53, CREB, P27, and NF-κB.

expression of the VEGF family is often associated with the aggressiveness and metastasis of CRC (82). VEGF protein family includes VEGFA-F and placental growth factor (PlGF). VEGFs bind to tyrosine kinase cell receptors (VEGFR1-3) to activate VEGF signaling in endothelial cells, affecting cell proliferation, migration, survival, and vascular permeability during angiogenesis (83, 84) (Figure 4).

VEGFA is often overexpressed in CRC and is considered to be a key factor in inducing tumor angiogenesis, which plays an important role in tumorigenesis, tumor development, and metastasis (85). Besides the previously enumerated signaling pathways, circRNA can also play a tumorigenic role in CRC through the VEGFA signaling axis. Circ_001971 has been observed to act as a ceRNA to mitigate VEGFA inhibition by miR-29C-3p, thereby enhancing the proliferation, invasion, and angiogenesis of CRC (31). In addition, studies have shown that high expression of circ_0056618 not only produces the same effect on CRC by regulating VEGFA as mentioned above, but also is related to the poor overall survival (OS) of CRC patients (86).

CircRNA/JAK/STAT signaling axis in CRC

The JAK/STAT signaling pathway is a common intracellular signal transduction pathway that is involved in many biological processes such as cell proliferation, differentiation, apoptosis, and immune regulation (87). In CRC, the JAK/STAT signaling axis has been shown to play a key role in tumor cell genesis, progression, invasion, migration, and chemical tolerance (88). The specific molecular mechanisms by which the JAK/STAT signal transduction pathway regulates CRC progression refer to the expression of multiple proto-oncogenes, tumor suppressor genes, cytokines, and their receptors, including Ras, Src, $p27^{kip1}$, $p16^{ink4a}$, interleukin, and EGFR (88, 89). The classic JAK/STAT signaling process is that the connection between the ligand and the receptor activates JAK to form phosphorylation (P)-JAK and forms a docking site for STAT. At this docking site, P-JAK phosphorylates STAT so that it dimers with other members of the STAT family. These dimers will transfer from the cytoplasm to the nucleus and regulate the transcription of target genes (88, 90) (Figure 5).

We have listed a variety of circRNAs that play a key role in the occurrence and development of human CRC, but circRNAs alone may not be enough to promote cancer progression. In a study by Wang et al., the initial RNA sequencing found that circ_0004104 expression levels were significantly upregulated in CRC tissues and were closely related to the prognosis of CRC patients. In-depth mechanistic studies have shown that circ_0004104 modulates the JAK2/STAT3 pathway by acting as a ceRNA binding to miR-485-3p and FUS, ultimately

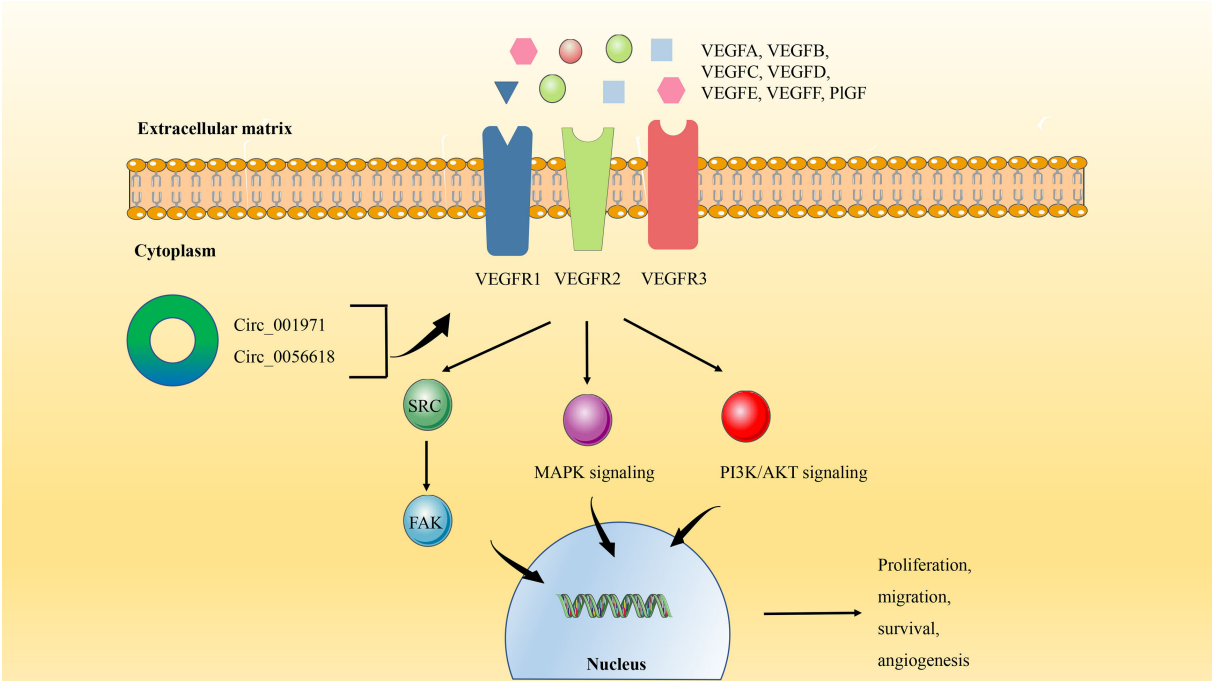


FIGURE 4
The circRNA/VEGF signaling axis in CRC. VEGF binds to tyrosine kinase cell receptors (VEGFR1-3) to activate VEGF signaling in endothelial cells. The activation of VEGF signaling can not only induce the protein expression of SRC kinase and FAK Focal adhesion kinase, but also trigger PI3K/AKT and MAPK signal transduction. Finally, it regulates the process of angiogenesis, proliferation, migration, and survival.

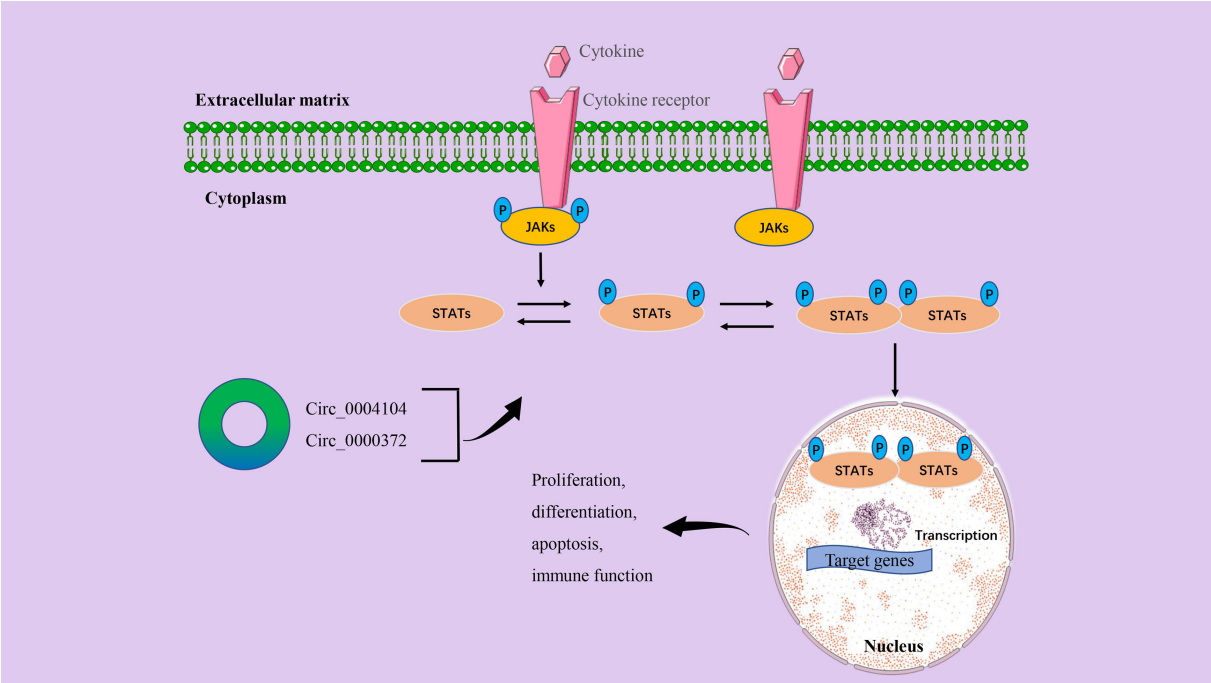


FIGURE 5
The circRNA/JAK/STAT signaling axis in CRC. The binding of the cytokine to the receptor induces receptor dimers and initiates signal transduction. After JAKs are activated and phosphorylated, STATs proteins are recruited to the phosphorylated tyrosine site. STATs are also then activated and phosphorylated. Normally, STATs reside in the cytoplasm and form phosphorylated dimers when activated by upstream signals. STAT-STAT dimers are transferred from the cytoplasm to the nucleus and regulate the transcription of target genes. Finally, it affects the proliferation, differentiation, invasion, inflammation, and immune function of cancer cells.

promoting cell proliferation and migration (19). Previous studies have demonstrated that the inflammatory cytokine interleukin-6 (IL6) can mediate the activity of the JAK2/STAT3 signaling pathway to participate in the occurrence and development of CRC (91). Recent evidence also shows that down-regulation of circ_0000372 can inhibit the protein expression of the IL6/AK2/STAT3 signaling axis. Further results confirmed that circ_0000372 may regulate IL6 expression and JAK2/STAT3 signaling pathway activity by acting on miR-495 in CRC (28).

CircRNA/Notch signaling axis in CRC

There are generally four Notch receptor subtypes (Notch-1, Notch-2, Notch-3, and Notch-4) and five Notch ligands (Dll-1, Dll-3, Dll-4, Jagged-1, and Jagged-2) in humans (92). The Notch signaling pathway, which is involved in the progression of CRC and the self-renewal and homeostasis of normal intestinal epithelium, is activated when the ligand binds to the receptor (93). Abnormal activation of Notch1 has been reported to initiate CRC and enhance its invasiveness (94). The specific mechanism attributed to Notch1 signaling creates a tumor microenvironment (TME) and promotes CRC metastasis through TGF-β-dependent neutrophil recruitment (95) (Figure 6).

Tumor metastasis is an important factor affecting the survival and prognosis of patients, and circRNA has been confirmed to be involved in the metastasis of CRC cancer. Using RNA transcriptome sequencing, Chen et al. identified a novel highly expressed circRNA, circ-NSD2, in a mouse model of liver metastasis. A series of functional and mechanistic studies revealed that circ-NSD2 may promote the migration, invasion, and metastasis of CRC cells *in vitro* and *in vivo* by targeting miR-199b-5p mediated JAG1/Notch1 signaling (27). It can also be seen that more and more experimental results reveal that many signaling pathways, including the Notch signaling pathway, are regulated by miRNA. Therefore, based on the results obtained from bioinformatics analysis, miR-101-3p has binding sites on circ-APLP2 and Notch1. Circ-APLP2 has been proven to act as a miR-101-3p sponge to regulate the Notch1 signaling pathway in CRC and activate proliferation and metastasis-related signals (c-Myc, cyclin D1, MMP-2, and MMP-9), thereby promoting the proliferation and liver metastasis of CRC (96).

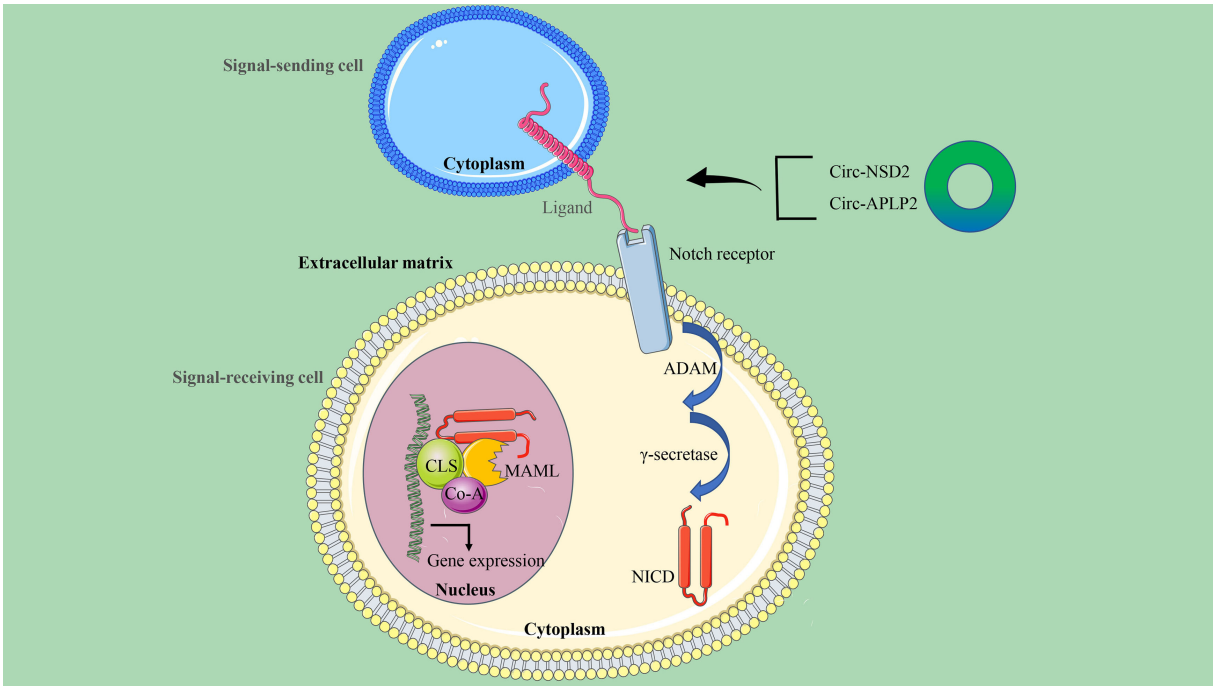


FIGURE 6
The circRNA/Notch signaling axis in CRC. This signaling is initiated by ligand-receptor binding between the signal-sending cell and the signal-receiving cell. The receptor-ligand interaction triggers a continuous cleavage mediated by ADAM metalloproteinase and γ-secretase, followed by the release of the intracellular domain NICD by Notch. When NICD is transferred to the nucleus, it recruits MAML and Co-A to CSL to initiate the expression of target genes.

CircRNA/Hippo/YAP signaling axis in CRC

CSCs are the main cause of drug resistance and disease recurrence in CRC treatment. Hippo/YAP is an important signaling pathway involved in the regulation of CSCs, and YAP1 signaling is closely associated with the proliferation and metastasis of CRC cells (58). Hippo pathway core kinases include Mst1/2, Sav1, Lats1/2, and Mob1 (97). When Hippo signaling is activated, the Mst1/2 kinase and Sav1 complex co-phosphorylate and activate Lats1/2 kinase. Subsequently, the downstream transcription coactivators YAP and TAZ are inactivated through cytoplasmic retention and protein degradation, which ultimately regulate the expression of target genes and promote tumor progression (98) (Figure 7).

Interestingly, there is growing evidence that circRNA can act as an oncogene or tumor suppressor to regulate the CSC-related Hippo/YAP signaling pathway. Recently, circ-PPP1R12A was screened for elevated expression in colon cancer cytoplasm. Circ-PPP1R12A encodes the conserved 73-aa small peptide PPP1R12A-C (but not circ-PPP1R12A itself), which promotes the proliferation, migration, and metastasis of colon cancer *in vitro* and *in vivo* by activating the Hippo/YAP signaling pathway (51). In addition, other studies have proved that circ_0128846 and circ_0106714 regulate the proliferation and migration of CRC cells through the Hippo/YAP signaling pathway mediated by miR-1184 and miR-942-5p, respectively (29, 46).

CircRNA/WNT/β–catenin signaling axis in CRC

The WNT/β-catenin signaling pathway is a key regulator of normal intestinal stem cell homeostasis (99). Abnormal activation of this pathway is associated with the invasion, migration, proliferation, and differentiation of CRC cells and is a marker of poor prognosis in CRC patients (100). WNT/β-catenin signaling is initiated by binding the WNT protein to the FZD-LRP5/6 receptor complex. This was followed by activation of Disheveled, which further induced dissociation of GSK-3β from Axin (101). This process prevents the WNT-FZD-Axin-LRP5/6 complex from phosphorylating β-catenin. The accumulation of unphosphorylated β-catenin in the cytoplasm translocates to the nucleus, where it binds to transcription factors such as the TCF/LEF family, resulting in the

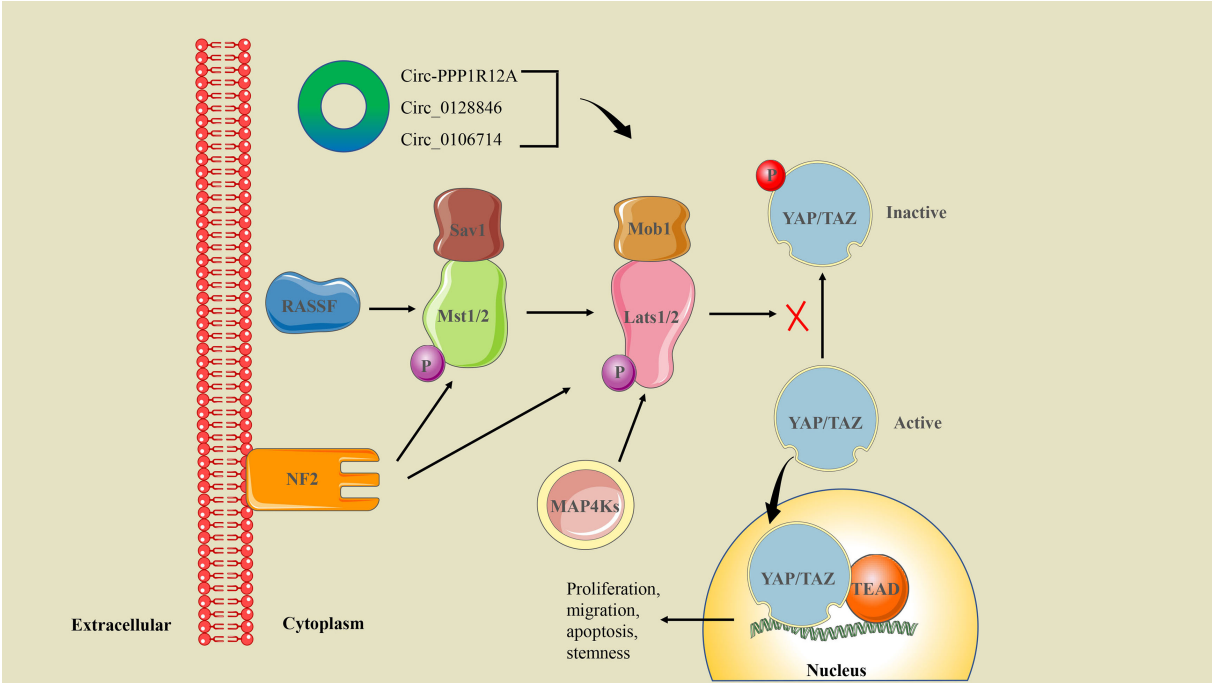


FIGURE 7
The circRNA/Hippo/YAP signaling axis in CRC. Mst1/2 is activated by upstream NF2 and RASSF family proteins. Phosphorylated Mst1/2 and MAP4Ks transmit multiple signals to activate Lats1/2, which inhibit YAP/TAZ phosphorylation. The activated YAP/TAZ enters the nucleus and binds with the transcription factor TEADs to induce gene expression. Ultimately, it regulates cell proliferation, migration, apoptosis, and the stemness properties of CSCs.

transcription of target genes that enhance CRC stemness and promote CRC progression (102, 103) (Figure 8). Cancer-related deaths in CRC patients are partly due to treatment failure due to chemotherapy resistance. WNT/β-catenin signaling has been shown to mediate chemical resistance to CRC in ncRNA, CSCs, and TME (104).

As a special type of ncRNA, circRNA can also influence tumor progression by regulating the WNT/β-catenin signaling pathway in CRC. For example, circ_0068464 (105), circ_0082182 (106), and circ_0026628 (107), which are highly expressed in CRC, can target corresponding miRNAs to regulate the activity of the WNT/β-catenin pathway, thereby promoting tumor progression. In addition, low expression of circRNA, such as circ_0000523 (108) and circ-MTO1 (109), can also participate in the proliferation and invasion of CRC cells *in vitro* by activating the WNT/β-catenin signaling pathway. 5-FU is a chemotherapeutic drug commonly used in the clinical treatment of CRC, and circRNA has been proven to be a basic regulator of cancer drug resistance (110). Chen et al. found that circ-PRKDC is up-regulated in 5-FU-resistant CRC tissues and cells, and inhibition of circ-PRKDC expression can improve the sensitivity of CRC cells to 5-FU by regulating WNT/β-catenin signaling (111).

Potential clinical application value of circRNAs signal axis in CRC

The diagnostic, prognostic, and therapeutic value of circRNAs in CRC

According to statistics, from 2009 to 2015, the 5-year survival rate of CRC in the United States reached 64%, while the survival rate in many Eastern and Southern European countries was less than 50% (112). In particular, the 5-year survival rate for advanced metastatic CRC is only 14% (113). Therefore, it is necessary to find more effective biomarkers for the early diagnosis and treatment of CRC. Studies have shown that the expression of circ_3823 in serum has high sensitivity and specificity for detecting CRC, suggesting that circ_3823 can be used as a potential biomarker for the diagnosis of CRC (114). In addition, the high expression of circ_0004104 in CRC can not only promote cell proliferation and migration but is also closely related to the prognosis of CRC patients, making it a potential therapeutic target for CRC patients (19). Similarly, high levels of circ-MYH9 predict shorter relapse-free survival and OS in CRC patients, so regulation of circ-MYH9 may lead to an effective

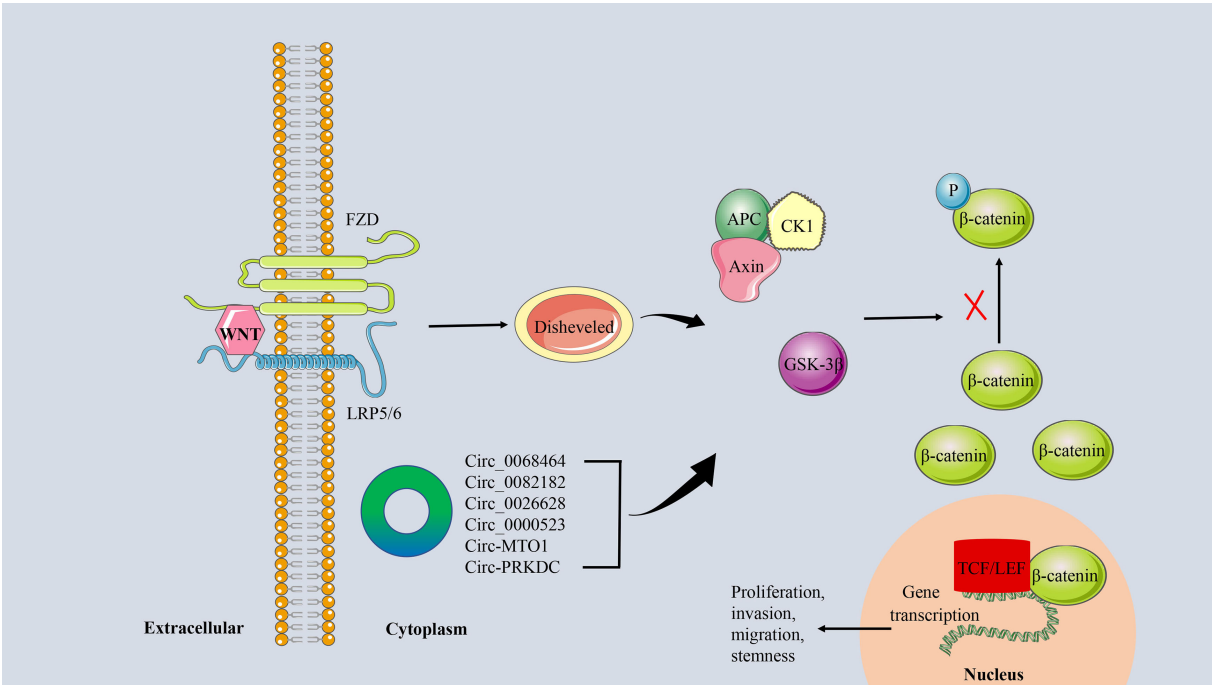


FIGURE 8
The circRNA/WNT/β–catenin signaling axis in CRC. The linking of WNT protein to the FZD-LRP5/6 receptor complex activates the downstream protein Disheveled. It further promoted the dissociation of GSK-3β from Axin, which inhibited the phosphorylation of β-catenin. Unphosphorylated β-catenin is transferred to the nucleus and binds to the transcription factor TCF/LEF to induce transcription of the target genes.

treatment for CRC (115). Chemotherapy is one of the main treatment methods for CRC, and oxaliplatin is a more commonly used chemotherapy drug. At present, resistance to CRC treatment is still an important issue for controlling the progression of the disease (116). It has been confirmed that glycolysis and resistance of drug-sensitive cells can be enhanced when exosomes present circ_0005963. The results also showed that the expression level of circ_00059633 in serum exosomes was positively related to the chemoresistance of CRC cells to oxaliplatin, and that silencing of the circRNA could reverse the resistance to oxaliplatin (117). More comprehensive clinical application data of circRNAs in CRC is summarized in Table 1.

Clinical application of circRNAs related signaling pathways in CRC

Early CRC patients are still mainly treated with surgery, and mCRC patients are based on chemotherapy and targeted therapy. Activation of RAS and BRAF mutations in MAPK signaling is one of the most common mutations in human tumors, and the presence of $BRAF^{V600E}$ mutations is considered to be a marker of poor prognosis in mCRC patients (66). Dabrafenib, encorafenib, and vemurafenib (inhibiting BRAF signaling), and trametinib and binimetinib (inhibiting MEK signaling) have previously been developed for the treatment of mCRC by blocking the MAPK pathway (118). However, some mutations such as KRAS and BRAF make mCRC resistant to these therapies (61). Some preclinical data suggest that abnormal activation of EGFR, PI3K signaling, and WNT signaling pathways may be responsible for resistance to BRAF inhibitor monotherapy in patients with $BRAF^{V600E}$ mCRC (35, 119, 120). Therefore, preclinical studies have been conducted, such as BRAF inhibitor and anti-EGFR antibody dual therapy (vemurafenib + panitumumab) (121), BRAF inhibitor and MEK inhibitor dual therapy (dabrafenib + trametinib) (122), and BRAF inhibitor, anti-EGFR antibody with PI3K or MEK inhibitor triple therapy (encorafenib, cetuximab, and alpelisib or binimetinib) (123, 124). Moreover, these combination therapies involving MAPK pathway blocking have achieved certain efficacy in $BRAF^{V600E}$ mCRC patients. However, the three-drug combination therapy brought more adverse reactions to patients, such as hyperglycemia, nausea, diarrhea, and so on (123). In addition, studies have shown that circ_0131663 (70), circ-ITGA7 (71), and CIRS-7 (72) affect the progression and metastasis of CRC by regulating the MAPK signaling pathway. We believe that the targeted therapies have certain efficacy in specific circRNA patients. Further research is expected. The above data is presented in detail in Table 2.

TABLE 1 Potential clinical application value of circRNAs in CRC.

CircRNA	Expression	Effect	Potential application	Reference
Circ_3823	Upregulated	Promote growth, metastasis, and angiogenesis	Diagnostic marker or therapeutic target	(114)
Circ_0004104	Upregulated	Promote proliferation, and migration	Diagnostic and prognostic biomarker, and therapeutic target	(19)
Circ-MYH9	Upregulated	Promote proliferation	Therapeutic target	(115)
Circ_0005963	Upregulated	Promote glycolysis and oxaliplatin resistance	Therapeutic target of drug-resistant patients	(117)
CircRNA_0001178	Upregulated	–	Diagnosing liver metastases from CRC	(18)
CircRNA_0000826	Upregulated	–	Diagnosing liver metastases from CRC	(18)
Circ-HERC4	Upregulated	Promote proliferation and migration/Induce liver and lung metastasis	Prognostic biomarker and therapeutic target	(20)
Circ-MAPK14	Downregulated	Block progression and metastasis	Therapeutic target	(70)
Circ-ITGA7	Downregulated	Suppress growth and metastasis	Therapeutic target	(71)
CiRS-7	Upregulated	Promote proliferation, migration, and invasion	Prognostic biomarker and therapeutic target	(72)
Circ_0008285	Downregulated	Inhibit proliferation and migration	Therapeutic target	(79)
Circ-IL4R	Upregulated	Promote proliferation, migration, and invasion	Diagnostic and prognostic biomarker, and therapeutic target	(80)
Circ_001971	Upregulated	Promote proliferation, invasion, and angiogenesis	Therapeutic target	(31)
Circ_0056618	Upregulated	Promote proliferation, migration, and angiogenesis	Therapeutic target	(86)
Circ-SPARC	Upregulated	Promote proliferation and migration	Diagnostic and prognostic biomarker, and therapeutic target	(19)
Circ_0000372	Upregulated	Promote proliferation, migration, and invasion	Prognostic biomarker and therapeutic target	(28)
Circ-NSD2	Upregulated	Promote migration and metastasis	Prognostic biomarker and therapeutic target	(27)
Circ-APLP2	Upregulated	Promote proliferation, migration, and invasion/Induce tumor growth and liver metastases	Therapeutic target	(96)
Circ-PPP1R12A	Upregulated	Promote proliferation, migration, and invasion	Therapeutic target	(51)
Circ_0128846	Upregulated	Promote proliferation, migration, invasion, and cell cycle progression/Inhibit apoptosis	Therapeutic target	(29)
Circ_0106714	Downregulated	Inhibit proliferation, migration, and invasion/Promote apoptosis	Prognostic biomarker and therapeutic target	(46)
Circ_0068464	Upregulated	Promote proliferation, migration/Induce tumor growth and lung metastasis	Diagnostic biomarker and therapeutic target	(105)
Circ_0082182	Upregulated	Promote proliferation, cell cycle progression, and metastasis/Inhibit apoptosis.	Diagnostic biomarker and therapeutic target	(106)
Circ_0026628	Upregulated	Promote proliferation, migration, EMT, and stemness	Therapeutic target	(107)
Circ_0000523	Downregulated	Inhibit proliferation/Promote apoptosis	Therapeutic target	(108)
Circ-MTO1	Downregulated	Inhibit proliferation and invasion	Therapeutic target	(109)
Circ-PRKDC	Upregulated	Enhance 5-FU resistance	Therapeutic target of 5-FU-resistant patients	(111)

Currently, compounds targeting the PI3K/AKT signaling axis are mainly divided into four types: PI3K inhibitors, AKT inhibitors, mTOR inhibitors, and dual PI3K/mTOR inhibitors. Class Ia PI3K pan-inhibitors PX-866 and BKM120 (buparlisib) have shown good anti-tumor effects in preclinical studies of a variety of tumors (126, 134) and some clinical trials in CRC patients are ongoing. These included PX-866 in combination with cetuximab (NCT01252628), BKM120 in combination with panizumab (NCT01591421), and BKM120 in combination with paclitaxel (NCT04338399). However, the results are not satisfactory. In patients with mCRC, the addition of PX-866 to cetuximab failed to improve OS, progression-free survival (PFS), and objective response rates, but resulted in greater toxicity (125). The BURAN Study (NCT04338399) is scheduled for completion in December 2023. Other PI3K inhibitors are specific subtype inhibitors with strong targeted inhibition and low toxicity properties, such as MEN1611 and BYL719. Phase I trials are being conducted in mCRC patients with BRAF mutations (BYL719 and LGX818 [BRAF inhibitor] with cetuximab) (NCT01719380) and CRC patients with PIK3CA mutations (MEN1611 and cetuximab) (NCT04495621) (135). Some AKT inhibitors, such as KRX-0401, have been proven to be effective in patients with mCRC (127), while others, such as MK-2206, are still in clinical trials (NCT01802320) (NCT01333475). In addition, dual PI3K/mTOR inhibitors also showed preliminary tumor regression ability in CRC patients, among which representative ones were PF-05212384 (128), PF-04691502 (130), and NVP-BEZ235 (129). At present, the most common mTOR inhibitors in clinical trials are temsirolimus and everolimus, but the overall efficacy of mTOR inhibition in

TABLE 2 Clinical trials of circRNAs related signaling pathways in CRC.

Drug	Signaling pathway	Therapeutic targets	Phase	Reference/NCT number	Identified circRNAs
Encorafenib	MAPK	BRAF inhibitor	Phase I	(118)	Circ_0131663 (70) Circ-ITGA7 (71) CIRS-7 (72)
Vemurafenib	MAPK	BRAF inhibitor	Phase II	NCT00405587	
Trametinib + panitumumab	MAPK	MEK inhibitor and anti-EGFR antibody	Phase II	NCT02399943	
Dabrafenib + trametinib	MAPK	BRAF inhibitor and MEK inhibitor	Phase I/II	(122)	
Dabrafenib + panitumumab	MAPK	BRAF inhibitor and anti-EGFR antibody	Phase I	NCT01750918	
Vemurafenib + cetuximab/panitumumab	MAPK	BRAF inhibitor and anti-EGFR antibody	Phase I/Phase II	(121)	
Encorafenib + cetuximab	MAPK	BRAF inhibitor and anti-EGFR antibody	Phase III	NCT02928224	
Encorafenib + cetuximab + binimetinib	MAPK	BRAF inhibitor, anti-EGFR antibody with MEK inhibitor	Phase III	NCT02928224	
Dabrafenib + trametinib + panitumumab	MAPK	BRAF inhibitor and MEK inhibitor with anti-EGFR antibody	Phase I	NCT01750918	
PX-866 + cetuximab	PI3K/AKT	PI3K pan-inhibitor and anti-EGFR antibody	Phase II	(125)	Circ_0008285 (79) Circ-IL4R (80)
BKM120 (buparlisib)	PI3K/AKT	PI3K pan-inhibitor	Phase I/Phase II	(126)/NCT01833169	
BKM120 + irinotecan/docetaxel	PI3K/AKT	PI3K pan-inhibitor	Phase I/Phase I	NCT01304602/ NCT01540253	
BKM120 + panitumumab/paclitaxel/ everolimus	PI3K/AKT	PI3K pan-inhibitor and anti-EGFR antibody/mTOR inhibitor/mTOR inhibitor	Phase I/II/ Phase III/ Phase I	NCT01591421/ NCT04338399/ NCT01470209	
GDC-0941	PI3K/AKT	PI3K pan-inhibitor	Phase I	NCT00876109	
GDC-0941 + erlotinib	PI3K/AKT	PI3K pan-inhibitor and anti-EGFR antibody	Phase I	NCT00975182	
MEN1611	PI3K/AKT	PI3K Selective-inhibitor	Phase I/Ib	NCT04495621	
MEN1611 + cetuximab	PI3K/AKT	PI3K Selective-inhibitor and anti-EGFR antibody	Phase I	NCT04495621	
KRX-0401	PI3K/AKT	AKT inhibitor	Phase I	(127)	
MK-2206	PI3K/AKT	AKT inhibitor	Phase II	NCT01802320	
GDC-0068	PI3K/AKT	AKT inhibitor	Phase I	NCT01090960	
PF-05212384	PI3K/AKT	PI3K/mTOR inhibitor	Phase I	(128)	
BEZ235	PI3K/AKT	PI3K/mTOR inhibitor	Phase I	(129)	
GDC-0980	PI3K/AKT	PI3K/mTOR inhibitor	Phase I	NCT00854152	
DS-7423	PI3K/AKT	PI3K/mTOR inhibitor	Phase I	NCT01364844	
PKI-587	PI3K/AKT	PI3K/mTOR inhibitor	Phase I	NCT00940498	
XL-765	PI3K/AKT	PI3K/mTOR inhibitor	Phase I	NCT00485719	
XL-765 + erlotinib	PI3K/AKT	PI3K/mTOR inhibitor and anti-EGFR antibody	Phase I	NCT00777699	
Temsirolimus + irinotecan/cetuximab	PI3K/AKT	mTOR inhibitor/anti-EGFR antibody	Phase II/Phase I	NCT00827684/ NCT00593060	
Everolimus	PI3K/AKT	mTOR inhibitor	Phase II	NCT00419159/ NCT01387880/ NCT00337545	
Everolimus + BEZ235	PI3K/AKT	mTOR inhibitor + PI3K/mTOR inhibitor	Phase I/II	NCT01508104	
AZD2014 + paclitaxel	PI3K/AKT	mTORC1/mTORC2 inhibitor	Phase I	NCT02193633	
Encorafenib + cetuximab + alpelisib	MAPK and PI3K/AKT	BRAF inhibitor, anti-EGFR antibody with PI3K inhibitor	Phase I/IIb	(123)	Circ_0131663 (70) Circ-ITGA7 (71) CIRS-7 (72) Circ_0008285 (79) Circ-IL4R (80)
BKM120 + binimetinib	PI3K/AKT and MAPK	PI3K pan-inhibitor and MEK inhibitor	Phase Ib	NCT01363232	
BYL719 + LGX818 + cetuximab	PI3K/AKT and MAPK	PI3K Selective-inhibitor, BRAF inhibitor with anti-EGFR antibody	Phase I	NCT01719380	

(Continued)

TABLE 2 Continued

Drug	Signaling pathway	Therapeutic targets	Phase	Reference/NCT number	Identified circRNAs
MK-2206 + AZD6244	PI3K/AKT and MAPK	AKT inhibitor and MEK inhibitor	Phase II	NCT01333475	
PF-04691502 + PD-0325901	PI3K/AKT and MAPK	PI3K/mTOR inhibitor and MEK inhibitor	Phase I	(130)	
BEZ235 + binimetinib	PI3K/AKT and MAPK	PI3K/mTOR inhibitor and MEK inhibitor	Phase I	NCT01337765	
XL147 + pimasertib/erlotinib	PI3K/AKT and MAPK	PI3K/mTOR inhibitor and MEK inhibitor/anti-EGFR antibody	Phase I	NCT01357330/ NCT00692640	
Bevacizumab	VEGF	anti-VEGFA antibody	FDA approved	(131)	Circ_001971 (31) Circ_0056618 (86)
Regorafenib	VEGF	anti-VEGFR antibody	FDA approved	(131)	
Ramucirumab	VEGF	anti-VEGFR antibody	FDA approved	(131)	
Ziv-aflibercept	VEGF	anti-VEGF antibody	FDA approved	(131)	
Napabucasin	JAK/STAT	STAT3 inhibitor	Phase III	(132)	Circ_0004104 (19) Circ_0000372 (28)
Napabucasin + bevacizumab + FOLFIRI (5-FU, leucovorin, and irinotecan)	JAK/STAT and VEGF	STAT3 inhibitor and anti-VEGFA antibody	Phase I	NCT02641873	Circ_001971 (31) Circ_0056618 (86)
Ruxolitinib + regorafenib	JAK/STAT and VEGF	JAK1/2 inhibitor and anti-VEGFR antibody	Phase II	NCT02119676	Circ_0004104 (19) Circ_0000372 (28)
OMP-52M51	Notch	anti-Notch1 antibody	Phase I	(133)	Circ-NSD2 (27)
RO4929097	Notch	Gamma secretase inhibitor	Phase I	NCT01116687	Circ-APLP2 (96)
OMP131R10	WNT/β-catenin	Wnt-receptor complex inhibitor	Phase I	NCTO2482441	Circ_0068464 (105) Circ_0082182 (106)
PRI-724	WNT/β-catenin	Wnt-receptor complex inhibitor	Phase I/II	NCT01764477	Circ_0026628 (107) Circ_0000523 (109)
Foxy 5	WNT/β-catenin	Wnt-receptor complex inhibitor	Phase I/	NCTO2655952	Circ-MTO1 (109) Circ-PRKDC (111)
LGK974	WNT/β-catenin	Wnt-receptor complex inhibitor	Phase I/II	NCTO2278133	
ETC-159	WNT/β-catenin	Wnt-receptor complex inhibitor	Phase I	NCTO2521844	

clinical application is limited except for certain disease-stabilizing effects in patients with refractory mCRC (76). However, these targeted inhibitors may have a better therapeutic effect in circ_0008285 (79) and circ-IL4R (80) patients because these two circRNAs can promote the proliferation and migration of CRC cells by regulating the PI3K/AKT signaling pathway. These clinical applications are also listed in Table 2.

The VEGFA-targeted monoclonal antibody bevacizumab is the first targeted agent to be approved for the treatment of patients with mCRC. After that, three anti-angiogenic drugs, regorafenib, ramucirumab, and ziv-aflibercept, were also approved in the mCRC (131). Antiangiogenic agents do not directly target cancer cells, but rather target the TME like immune checkpoint inhibitors (136). Due to the inevitable problem of drug resistance, it is also a hot topic to explore the combination therapy of antiangiogenic drugs and immune checkpoint inhibitors in addition to developing new antiangiogenic drugs. On the other hand, the combination of targeted regulation of circ_001971 and circ_0056618 may reverse drug resistance. Clinical reports of JAK inhibitors or STAT inhibitors in the treatment of CRC are rare. In a Phase III trial of patients with mCRC, there were no significant differences in OS, PFS, or disease control between the STAT3 inhibitor (napabucasin) group and the placebo group. However, OS was significantly prolonged in py-STAT3-positive patients treated with napabucasin (132). Several natural and small-molecule inhibitors that inhibit Notch signaling have been shown to induce apoptosis in CRC cells *in vitro*, but they lack target specificity and efficacy in clinical evaluation (94). In addition, monoclonal antibodies targeting Notch1, such as OMP-52M51, did not show significant antitumor efficacy in phase I dose-escalation trials (NCT01778439) (133). Similarly, CRC therapies targeting WNT/β-catenin signaling include natural compounds, small molecules, and biological agents. The drugs currently in clinical trials include vitamin D3, curcumin, genistein, resveratrol, LGK974, and ETC-159 (100). Targeting WNT signaling pathways in CRC seems to be a long and difficult process, and no drugs targeting WNT pathways have been approved at present. These targeted therapies may be

beneficial for CRC patients in some identified circRNA patients. The clinical data and potentially identified circRNAs are summarized in Table 2.

Conclusions and perspectives

In conclusion, CRC is still a disease with high morbidity and mortality worldwide. Although with the improvement of people's health awareness and the continuous improvement of diagnosis and treatment technology, some patients can be detected and treated early with a good prognosis. However, the current treatment methods for patients with advanced metastatic CRC are limited and the prognosis is poor. Therefore, human beings have never stopped exploring the mechanism of CRC occurrence and metastasis. Excitingly, a novel ncRNA, circRNAs, has become the focus of CRC research due to its critical regulatory role in cancer-related processes such as tumorigenesis, development, and apoptosis. To improve patients' OS, many circRNAs are being developed as potential biomarkers for clinical diagnosis and prognosis of CRC, as well as effective therapeutic targets. More and more studies have shown that circRNA usually promotes the proliferation and metastasis of CRC cells by regulating several important signaling pathways, including PI3K/AKT, MAPK, Notch, JAK/STAT, Hippo/YAP, WNT/β-catenin, and VEGF. Currently, there are very few targeted drugs based on these signaling pathways for the clinical treatment of mCRC patients. This may be related to the lack of a deeper and comprehensive understanding of the biological functions and carcinogenic mechanisms of circRNAs and related signaling pathways in CRC. Therefore, based on the comprehensive elaboration and exploration of the molecular mechanism of CRC occurrence and progression in this review, more circRNA signal axes can be developed as effective targets for clinical diagnosis, prognosis, and treatment of CRC in the future to serve patients.

Author contributions

SW and LC were mainly responsible for literature review and manuscript writing. HW and GL completed the construction pictures and tables. SW designed the ideas of this paper and modified the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Neoadjuvant chemoradiotherapy for resectable gastric cancer: A meta-analysis

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Objectives: To evaluate the clinical curative effects and toxicity of neoadjuvant chemoradiotherapy for resectable gastric cancer compared to those of neoadjuvant chemotherapy.

Methods: A systematic review and meta-analysis of the randomized controlled trials (RCTs) of neoadjuvant chemoradiotherapy versus neoadjuvant chemotherapy were performed in patients with resectable gastric cancer.

Results: Seven RCTs were included (601 patients; 302 in the neoadjuvant chemoradiotherapy group and 299 in the neoadjuvant chemotherapy group). The neoadjuvant chemoradiotherapy group had an increased number of patients with a complete response [odds ratio (OR) = 3.79, 95% confidence interval (CI): 1.68–8.54, p = 0.001] and improved objective response rate (OR = 2.78, 95% CI: 1.69–4.57, p < 0.0001), 1-year (OR = 3.51, 95% CI: 1.40–8.81, p = 0.007) and 3-year (OR = 2.14, 95% CI: 1.30–3.50, p = 0.003) survival rates, R0 resection rate (OR = 2.21, 95% CI: 1.39–3.50, p = 0.0008), and complete pathologic response (OR = 4.39, 95% CI: 1.59–12.14, p = 0.004). Regarding the incidence of adverse effects after neoadjuvant therapy, only the occurrence rate of gastrointestinal reaction in the neoadjuvant chemoradiotherapy group was higher than that in the neoadjuvant chemotherapy group (OR = 1.76, 95% CI: 1.09–2.85, p = 0.02), and there was no significant difference in other adverse effects. There was no difference in the incidence of postoperative complications between the two groups.

Conclusion: Neoadjuvant chemoradiotherapy for resectable gastric cancer has several advantages in terms of efficacy and safety compared to neoadjuvant chemotherapy. Therefore, neoadjuvant chemoradiotherapy has great potential as an effective therapy for resectable gastric cancers.

Systematic Review Registration: https://inplasy.com/inplasy-2022-3-0164, registration number INPLASY202230164.

KEYWORDS

resectable gastric cancer, gastrointestinal cancers, neoadjuvant chemoradiotherapy, neoadjuvant chemotherapy, meta-analysis

Introduction

Gastric cancer is a malignant tumor with high morbidity and mortality (1). Epidemiological statistics indicate that there were more than one million new cases of gastric cancer and 760,000 deaths in 2020, which rank fifth and fourth, respectively, in the incidence and mortality of cancer worldwide; for patients with advanced gastric cancer, the median survival rate is less than 12 months (2). The incidence is twice as high in men as in women, and the number of new cases continues to increase in younger patients (3). Gastric cancer remains a global health problem.

Surgery is known to play a crucial role in the treatment strategy of gastric cancer, and the prognosis and survival of patients are improved when surgery achieves R0 resection. Preoperative neoadjuvant therapy is the key to achieve R0 resection and has been proven to be effective for potentially resectable gastric cancer (4, 5). Theoretically, an effective preoperative approach can downgrade the tumor stage, facilitate R0 resection, and reduce local relapses and is imperative for patients with potentially resectable gastric cancer (6).

However, it is not clear whether neoadjuvant chemotherapy (NACT) is superior to neoadjuvant chemoradiotherapy (NACRT) in terms of efficacy and safety in potentially resectable gastric carcinoma (7). In 2004, J. A. et al. conducted a multi-institutional trial of NACRT in patients with potentially resectable gastric carcinoma that showed that NACRT caused a substantial pathologic response that resulted in durable survival (8, 9). NACRT followed by surgery and postoperative adjuvant therapy has been clinically recommended for esophageal and gastric junction cancer (10). However, the treatment strategy for non-esophagogastric junction cancer has been controversial, and the application of NACRT for gastric cancer has thus far only been tested in a small number of phase II studies (9). Therefore, in this study, we compared the efficacy and safety of NACRT with those of NACT in resectable gastric cancer through a meta-analysis to provide an evidence-based approach for the treatment of resectable gastric cancer.

Materials and methods

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses were followed as closely as possible for this systematic review and meta-analysis, and the protocol for this systematic review was registered on the International Platform of Registered Systematic Review and Meta-analysis Protocols (202230164) and is available in full on inplasy.com (https://doi.org/10.37766/inplasy2022.3.0164).

The inclusion criteria of the study were as follows:

i. Randomized controlled trials (RCTs) published worldwide
ii. Patients confirmed by histopathological or cytological examination and assessed by gastroscopy, computed tomography (CT), and magnetic resonance imaging (MRI) to meet the diagnostic criteria for operable gastric cancer
iii. Patients in the experimental group received NACRT, whereas those in the control group received NACT
iv. The objective response rate (ORR), pathologic complete response (pCR), and R0 resection rate were used as primary efficacy outcomes. We evaluated the efficacy of neoadjuvant therapy in the two groups according to the Response Evaluation Criteria in Solid Tumours. Complete response (CR): the disappearance of all target lesions. Partial response (PR): at least a 30% decrease in the sum of diameters of target lesions. Progressive disease (PD): at least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on the study. Stable disease (SD): neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. ORR: the proportion of patients whose tumors shrank to a certain extent and remained there for a certain time, including CR + PR cases. The secondary indicators were survival rate and incidence of adverse reactions, including nausea and vomiting, myelosuppression, anemia, and digestive tract reactions.

The exclusion criteria of the study were as follows:

(i) Review articles, systematic evaluations, animal based experiments, or case reports
(ii) Non-RCTs, observational studies, or retrospective studies
(iii) Repeated articles, studies reporting incomplete or inconsistent outcomes, or having unreasonable trial designs
(iv) Some ongoing clinical trials with no published results
(v) Violation of any of the above inclusion criteria

Search strategy and study selection

Two investigators (JC and YG) independently searched PubMed, EMbase, Cochrane Library, Web of Science, Chinese National Knowledge Infrastructure, Chinese Biological Medicine Database, Wanfang Database, and VIP Database; we simultaneously searched for related trials in the International Clinical Trial Registry Platform and the Chinese Clinical Registry up to 1 October 2021. We used the following medical subject headings to search for the terms: stomach neoplasms, neoadjuvant chemoradiotherapy, and neoadjuvant chemotherapy. Two investigators filtered the searched articles

according to the inclusion and exclusion criteria, and when they had differences, a third researcher determined whether the article would be included.

Data extraction and quality assessment

Two investigators (JC and YG) independently reviewed the entire articles for all the eligible studies and extracted relevant data, including the author, year of publication, number of patients, age of patients, interventions, radiotherapy dose, and chemotherapy regimen. Two reviewers (MF and YY) evaluated the quality of the selected articles using the Cochrane Collaboration's tool for RCTs and assessed the items in three categories according to the risk of bias (low, unclear, and high risk of bias), including random sequence generation (selection bias), allocation concealment (selection bias), the blinding of participants and personnel (performance bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other biases.

Statistical analysis

All meta-analyses were performed using Cochrane RevMan version 5.3 and Stata (version 13). The results were reported as pooled odds ratios (ORs) with 95% confidence intervals (95% CIs). We used Cochran's Q test and I^2 statistics to evaluate the heterogeneity of all the studies. If the heterogeneity was significant ($p < 0.1$, $I^2 > 50.0\%$), the random effects model was adopted; otherwise, the fixed effects model was used. Potential publication bias was assessed using funnel plots, Egger's test, and Begg's test. All p-values were two sided, and statistical significance was set at $p < 0.05$.

Results

Characteristics of studies

We identified 256 articles for review of the title and abstract (Figure 1) and retrieved the full text of potentially eligible articles for a particular assessment after the initial screening. Seven studies were included in the meta-analysis. A total of 601 patients were enrolled, including 302 in the experimental group and 299 in the control group. The particular characteristics of each enrolled article are summarized thoroughly in Tables 1–3.

Quality assessment

We evaluated the quality of all meta-analyses using the Cochrane Collaboration's tool for assessing the risk of bias, as

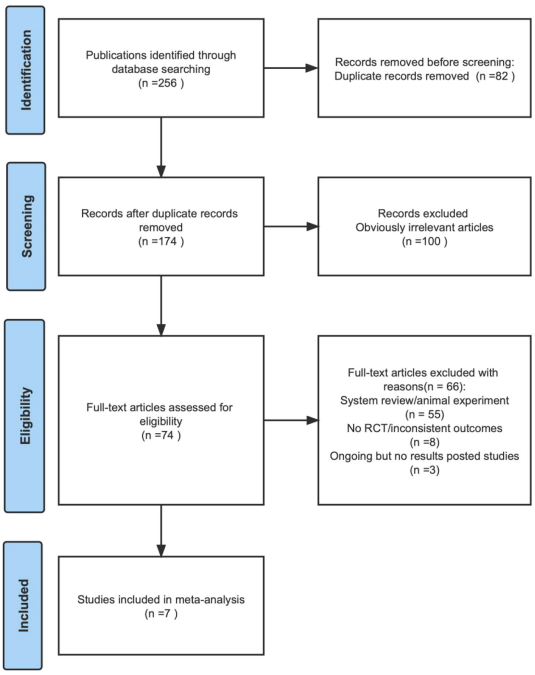


FIGURE 1
Flow diagram of study selection process for the meta-analysis.

TABLE 1 Characteristics of studies enrolled.

Study	Study design	Country	(Experiment/ Control) N	Gender (male/ female)/N		Age (years)		Tumor location
				Experiment	Control	Experiment	Control	
Cao MF 2019 (11)	RCT (phase II)	China	29 (49)/30(51)	40(68)/19(32)		60.6 ± 7.1		Stomach
Jiang Y 2019 (12)	RCT (phase II)	China	42(50)/42(50)	24(29)/18(21)	25(30)/17 (20)	53.14 ± 8.72	53.14 ± 8.72	Stomach (fundus, body, and antrum)
He ZR 2017 (13)	RCT (phase II)	China	25(50)/25(50)	14(28)/11(22)	13(26)/12 (24)	46.6 ± 4.5	47.7 ± 4.6	Gastroesophageal junction and the lower and upper third of the stomach
Leong T 2017 (14)	RCT (phase III)	Australia	60(50)/60(50)	45(37)/15(13)	46(38)/14 (12)	58 ± 13	56 ± 13	Gastroesophageal junction and the lower and upper third of the stomach
Stahl M 2017 (15, 16)	RCT (phase III)	Germany	60(51)/59(49)	54(45)/6(5)	54(45)/5 (4)	Median age 60.6	Median age 56	Gastroesophageal junction
Zhang XT 2016 (17)	RCT (phase II)	China	64 (51)/62(49)	78(62)/48(38)		Median age 55	Median age 57	Stomach (fundus, body, and pylorus)
Wang X 2016 (18)	RCT (phase II)	China	22(51)/21(49)	–	–	–	–	Gastroesophageal junction and the stomach

Data are expressed as n (%).

shown in Figures 2, 3. Through our assessment, we concluded that all the included articles were randomized controlled trials, of which one article followed allocation concealment and other articles included trials carried out using the method of informed consent. There were no errors in that all the eligible studies adopted random numbers to decide the final treatment and all had completed data, no selective reports, or other deviations.

Efficiency

Complete response

Four of the included articles reported the CR. Because there was no heterogeneity between the studies ($p = 0.95$, $I^2 = 0\%$), we adopted the fixed effects model for meta-analysis, which showed that the CR rate in the NACRT group was higher than that in the

TABLE 2 Therapeutic regimen of studies enrolled.

Study	RT regimens	Interventions		D-stage resection
		NACRT group	NACT group	
Cao MF 2019 (11)	IMRT (40 Gy/20f/4w)	TC(paclitaxel + carboplatin)+ 40 Gy	TC	D2
Jiang Y 2019 (12)	IMRT (47–50 Gy/24–25f/5–6w)	46.8–50.4 Gy concurrently with capecitabine	Oxaliplatin + capecitabine	–
He ZR 2017 (13)	3D-CRT (45 Gy/25f/5w)	(5-fluorouracil + folinic acid + oxaliplatin) or capecitabine + 45 Gy	(5- fluorouracil + folinic acid + oxaliplatin) or capecitabine	–
Leong T 2017 (14)	3D-CRT or IMRT or VMAT (45 Gy/25f/5w)	(Epirubicin + cisplatin + 5-fluorouracil/ capecitabine) + 45 Gy concurrently with 5-fluorouracil/ capecitabine	Epirubicin + cisplatin + 5-fluorouracil/ capecitabine	D2 recommended, D1 is the minimum approach
Stahl M 2017 (15, 16)	3D-CRT (30 Gy/15f/3w)	5-fluorouracil + folinic acid + cisplatin + 30 Gy with cisplatin and etoposide	5- fluorouracil + folinic acid + cisplatin	D2
Zhang XT 2016 (17)	IMRT (45 Gy/25f/5w)	S-1 + docetaxel + 45 Gy	S-1 + docetaxel	D2
Wang X 2016 (18)	IMRT (45 Gy/22f)	40.04–45.1 Gy concurrently with S-1	SOX (S-1 + oxaliplatin)	–

TABLE 3 The irradiation volumes of studies enrolled.

Study	The irradiation volumes (CTV)
Cao MF 2019 (11)	–
Jiang Y 2019 (12)	–
He ZR 2017 (13)	➢ Tumors of the proximal third of the stomach or cardiac esophagogastric junction: primary tumor, 3–5 cm of the lower esophagus, the left hemidiaphragm, and the adjacent pancreatic body, with high-risk lymph node areas including the adjacent peri-e,sophageal, perigastric, suprapancreatic, celiac trunk, splenic artery and splenic hilar lymph node areas. ➢ Tumors of the middle third of the stomach or the body of the stomach: primary tumor and the pancreatic body, with the lymph node area including the adjacent perigastric, suprapancreatic, truncal and splenic hilar, hepatic, and duodenal lymph node areas. ➢ Tumors of the distal third of the stomach: if the gastroduodenal junction is involved: primary tumor, the head of the pancreas, the first and second segments of the duodenum, with the lymph node area including the perigastric, suprapancreatic, celiac trunk, hilar, and pancreaticoduodenal lymph nodes.
T.Leong 2017 (14)	➢ The entire stomach, any perigastric tumor extension, and regional lymph nodes.
M.Stahl 2017 (15, 16)	➢ The pretherapeutic extension of the primary tumor with a transversal margin of 2 cm and a both- sides longitudinal margin along the mucosa of the gastro-oesophageal junction (GEJ) of 5 cm in Siewert type 1 tumors. ➢ Suspicious lymph nodes with a 1-cm margin and the regional lymph nodes with a margin of 1.5 cm around the cardia, along the left gastric artery and the minor curvature to the incisura angularis, the celiac artery, the proximal part of the commune hepatic artery, and along the first 2 cm of the splenic artery.
Zhang XT 2016 (17)	–
X.Wang 2016 (18)	–

NACT group (OR = 3.79, 95% CI: 1.68–8.54, p = 0.001) and that the results were statistically significant (Figure 4A).

Partial response

Four of the included articles reported the PR. Because there was no heterogeneity between the studies (p = 0.73, I^2 = 0%), we adopted the fixed effects model for meta-analysis, which showed that the results were not statistically significant (OR = 1.55, 95% CI: 0.96–2.51, p = 0.07) (Figure 4B).

Objective response rate

There were four studies that reported the ORR. There was no heterogeneity between the studies (p = 0.68, I^2 = 0%); we therefore adopted the fixed effects model for meta-analysis, which showed that the ORR rate in the NACRT group was higher than that in the NACT group (OR = 2.78, 95% CI: 1.69–4.57, p < 0.0001) and that the results were statistically significant (Figure 4C).

Pathologic complete response rate

There were three studies among the included articles that reported the pCR. We adopted the fixed effects model for meta-analysis because there was no heterogeneity between the studies (p = 0.64, I^2 = 0%), which showed that the pCR rate in the NACRT group was higher than that in the CRT group (OR = 4.39, 95% CI: 1.59–12.14, p = 0.004) and that the results were statistically significant (Figure 5A).

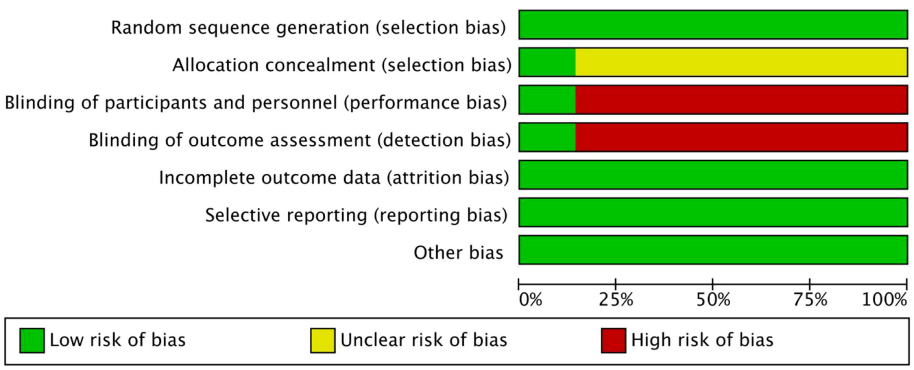


FIGURE 2
Risk of bias graph for seven randomized controlled trials (RCTs).

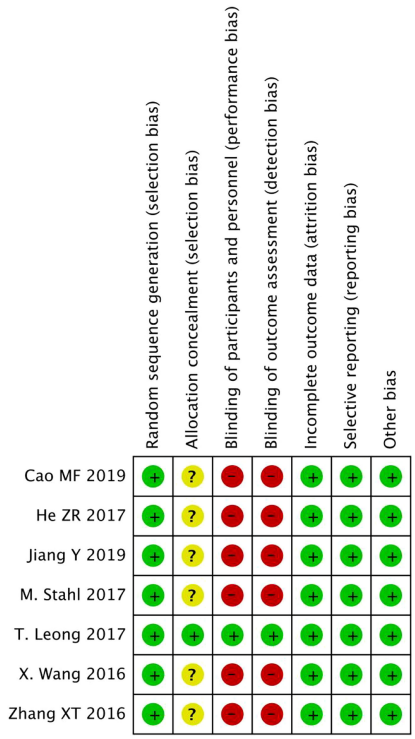


FIGURE 3
Risk of bias summary for seven RCTs.

R0 resection rate

Of the included articles, five studies reported R0 resection rates. No heterogeneity was observed between the studies ($p = 0.29$, $I^2 = 19\%$); we therefore adopted the fixed effects model for meta-analysis, which showed that the R0 resection rate in the NACRT group was higher than that in the NACT group (OR = 2.21, 95% CI: 1.39–3.50, $p = 0.0008$) and that the results were statistically significant (Figure 5B).

1-year and 3-year survival rates

Two studies reported the 1-year survival rate, and three studies reported the 3-year survival rate. Due to the lack of heterogeneity between the studies ($p = 0.41$, $I^2 = 0\%$ and $p = 0.31$, $I^2 = 15\%$), we adopted the fixed effects model for meta-analysis, which showed that the 1-year survival rate in the NACRT group was higher than that in the NACT group (OR = 3.51, 95% CI: 1.40–8.81, $p = 0.007$), and the 3-year survival rate in the NACRT group was also higher than that in the NACT group (OR = 2.14, 95% CI: 1.30–3.50, $p = 0.003$). The results were all statistically significant (Figures 5C, D).

Postoperative complications

Two of the included articles reported anastomotic leak, and two studies reported abdominal infection. Because no heterogeneity was found between the studies ($p = 0.80$, $I^2 = 0\%$ and $p = 0.53$, $I^2 = 0\%$), we adopted the fixed effects model for meta-analysis, which showed that there was no difference in the incidence of anastomotic leak and abdominal infection between the two groups (Figure 6A).

Adverse effects after neoadjuvant therapy

There were five studies that reported gastrointestinal reaction, four studies reported leukocytopenia, four studies indicated thrombocytopenia, four studies reported anorexia, three reported anemia, three indicated diarrhea, two studies mentioned liver damage, two studies reported mucositis, and two studies indicated dysphagia. The results showed that there was no statistical significance in the incidence of adverse reactions, except gastrointestinal reactions that were higher in the NACRT group than in the NACT group (OR = 1.76, 95% CI: 1.09–2.85, $p = 0.02$), and this result was statistically significant (Figure 6B).

Sensitivity and publication bias evaluation

Sensitivity analyses were performed by excluding one study at a time, to assess the influence of each study on the overall

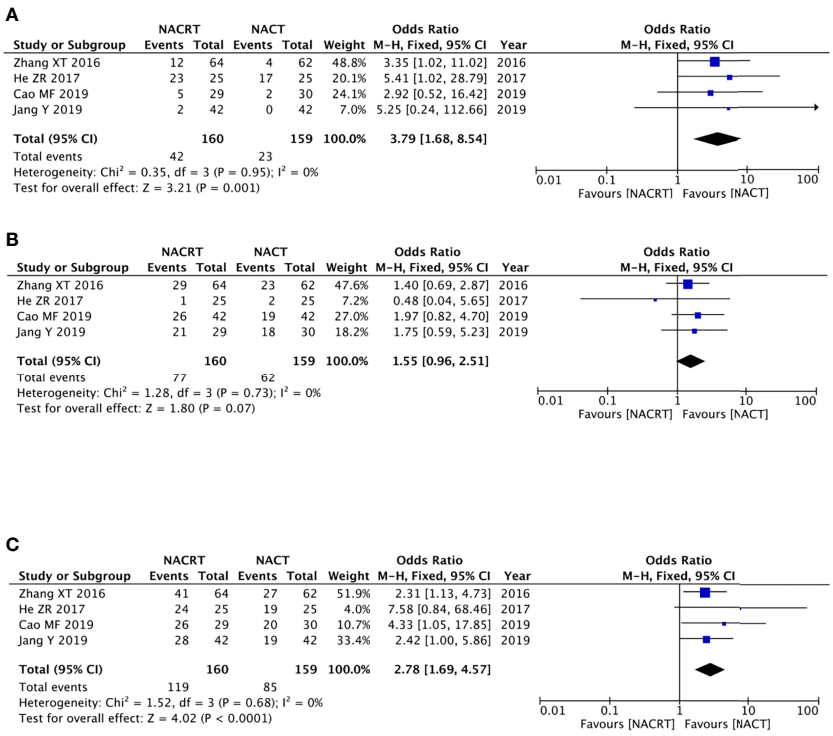


FIGURE 4
Forest plot for the complete response (CR) **(A)**, partial response (PR) **(B)**, and objective response rate (ORR) **(C)** of the neoadjuvantchemoradiotherapy (NACRT) group and neoadjuvant chemotherapy (NACT) group.

results. The results showed that the deletion of any one study had no significant effect on the results (Figures 7B–11B), indicating that the results of this meta-analysis are relatively stable. The publication bias analysis of the seven included articles showed that there was no obvious publication bias in the CR, PR, ORR, pCR rate, and R0 resection rate. Begg's funnel plot indicated no significant publication bias (Figures 7A–11A).

Discussion

Our study supports the efficacy and safety of NACRT compared to NACT for resectable gastric cancer. Neoadjuvant therapy is effective in reducing the volume of the primary tumor, tumor stage, and lymph node involvement to narrow the range of surgical resection, improve the R0 resection rate, and prolong the survival cycle (19, 20). In addition, neoadjuvant therapy can reduce or eliminate the risk of residual tumor cells and distant metastasis, which are considered to be closely associated with postoperative recurrence and metastasis. Some studies have also shown that pathological reactions after neoadjuvant therapy are closely associated with a reduction in the recurrence rate and overall survival (21–27). Neoadjuvant chemoradiotherapy + surgery + postoperative adjuvant chemotherapy has become the standard treatment for resectable esophagogastric junction cancer (10). However, the choice of preoperative neoadjuvant therapy for non-esophagogastric junction cancer remains controversial (28, 29). Whether neoadjuvant chemotherapy should be combined with radiotherapy requires more clinical studies to prove its efficacy and safety.

This systematic review included seven RCTs involving 601 patients. The results of our study showed that the NACRT group had an increased number of patients with CR, ORR, and pCR; improved R0 resection rate; and 1-year and 3-year survival rates. In our meta-analysis, the average ORR rate of the NACRT group in the four enrolled articles was 79.1%, compared to 57.9% in the NACT group, and the highest ORR rate was 96% in the study by He ZR (13). Of the seven studies, five reported R0 resection rates; the average R0 resection rate was 83.28% in the NACRT group and 66.31% in the NACT group. In terms of 1-year and 3-year survival rates, the NACRT group had higher survival rates than the NACT group, and the results were statistically significant. Two of the included studies reported the median survival time; the NACRT group had a significantly longer median survival time [27.5 m *vs.* 22.5 m in the study by Zhang XT (17), and 30.8 m *vs.* 21.1 m in the study by Stahl M (15)] These results

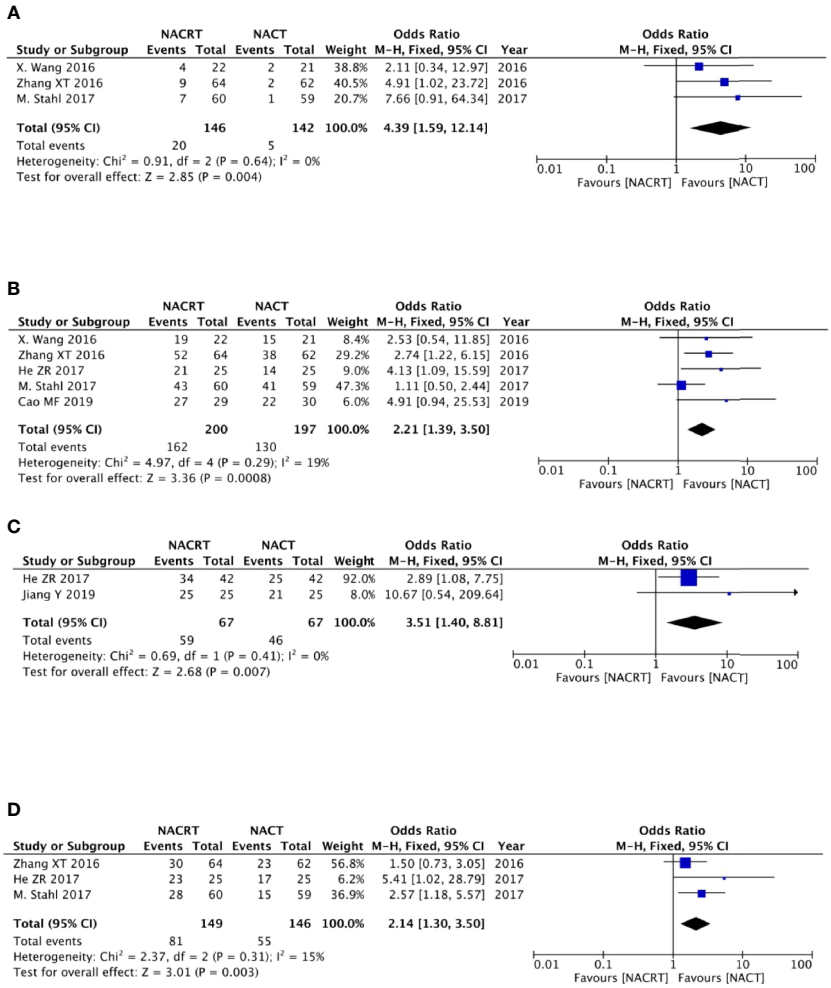


FIGURE 5
Forest plot for the pathologic complete response (pCR) rate **(A)**, R0 resection rate **(B)**, and 1- and 3-year survival rates **(C, D)**.

provide sufficient evidence for the efficacy of NACRT in resectable gastric cancer. Moreover, there was no difference in the incidence of adverse effects (except for the occurrence rate of gastrointestinal reactions) and postoperative complications between the two groups after neoadjuvant therapy. In conclusion, it stands to reason that the patients of resectable gastric cancer benefit from NACRT.

Some challenges remain before NACRT can become a standard treatment strategy. First, the adjuvant and neoadjuvant therapies have always been complementary. Results from the CRITIC study of chemotherapy versus chemoradiotherapy after surgery and preoperative chemotherapy for resectable gastric cancer showed that postoperative chemoradiotherapy did not improve overall survival (30). However, in the current analysis, only patients who started their allocated postoperative treatment were included, and the per-protocol (PP) analysis of patients who started the allocated postoperative treatment showed that the chemotherapy group had a significantly better 5-year overall survival than the chemoradiotherapy group (31). This study was based on adjuvant therapy administered after neoadjuvant chemotherapy. If neoadjuvant chemoradiotherapy is widely used, the choice of postoperative adjuvant therapy should be explored. Second, there are likely biological differences between Eastern and Western countries. Most of our studies were from China, and whether NACRT works for Westerners remains unknown (32). Furthermore, as mentioned above, NACRT is proven to be effective for resectable esophagogastric junction cancers, and the current debate is only about non-esophagogastric junction cancers. Some of our enrolled studies did not clearly define non-esophagogastric junction cancer as the inclusion criteria that might have caused some discrepancy in our research.

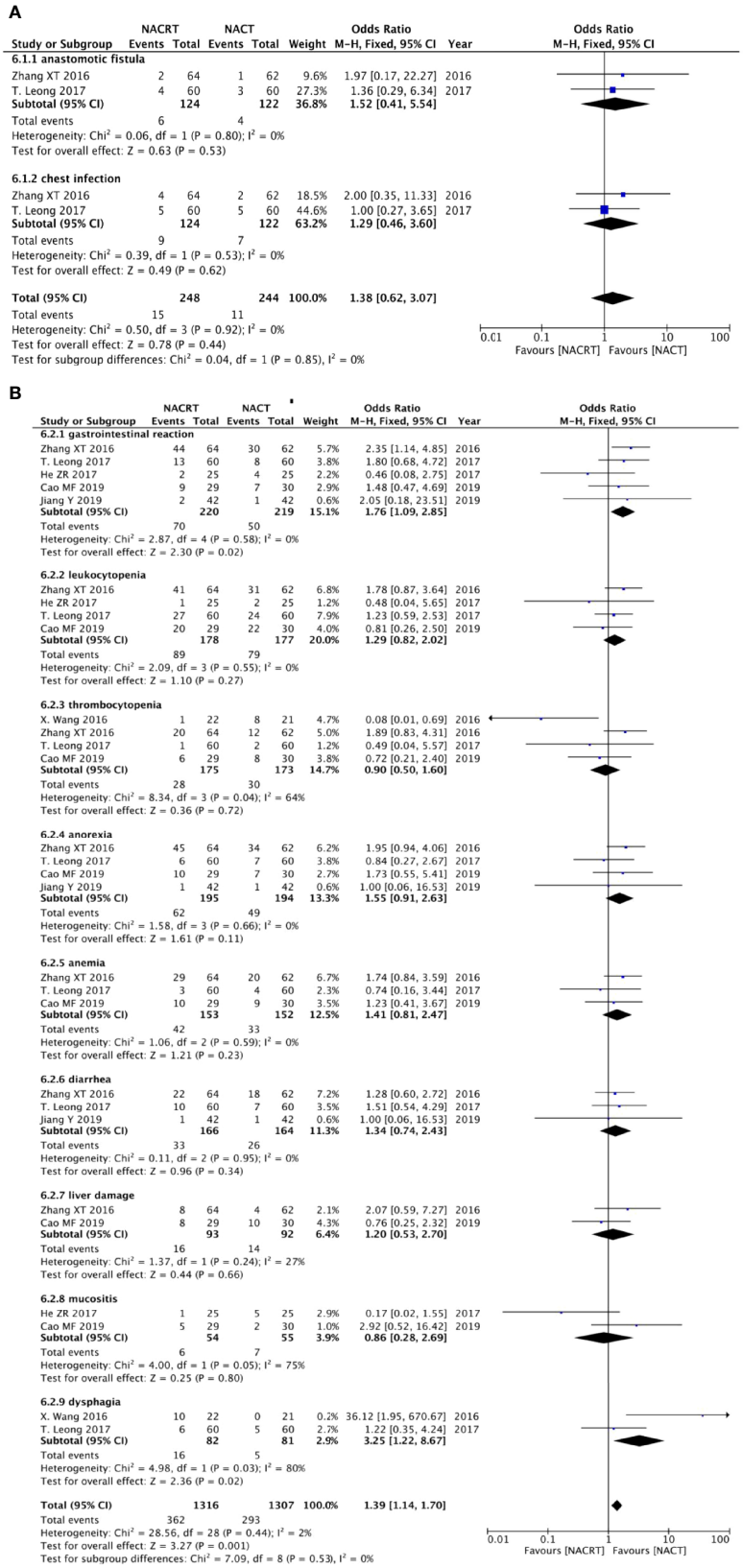


FIGURE 6
Forest plot for postoperative complications **(A)** and adverse effects after neoadjuvant therapy **(B)**.

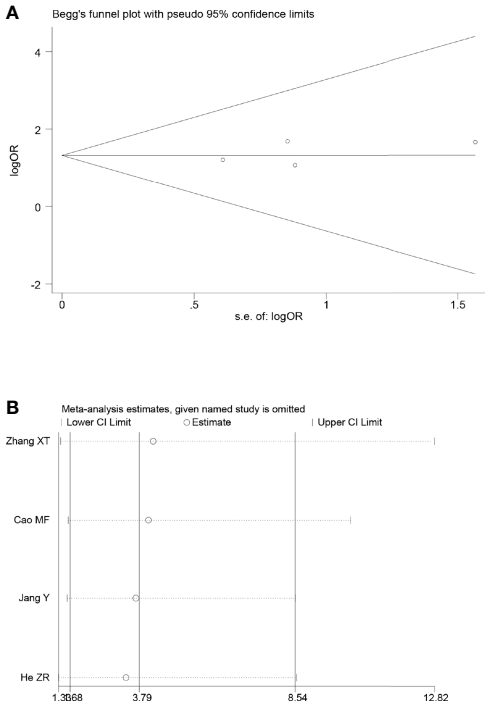


FIGURE 7
Begg's funnel plot **(A)** and sensitivity analysis **(B)** of all the included studies for the analysis of CR.

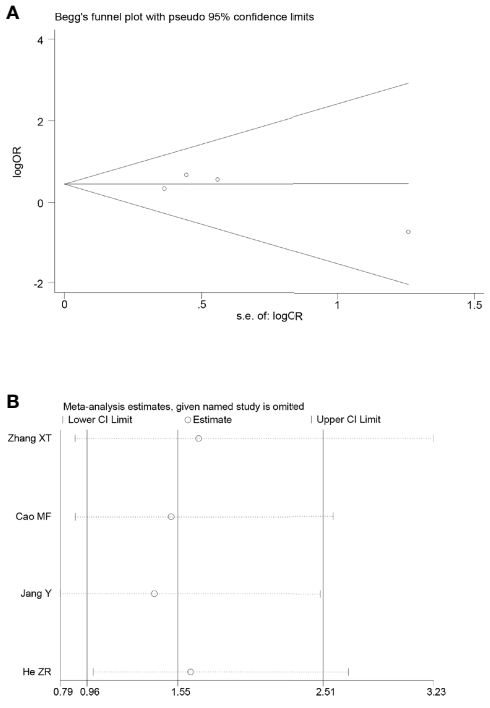


FIGURE 8
Begg's funnel plot **(A)** and sensitivity analysis **(B)** of all the included studies for the analysis of the R0 resection rate.

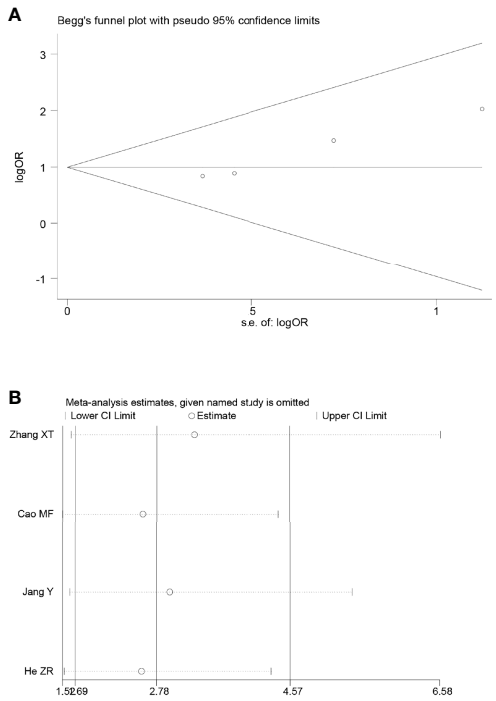


FIGURE 9
Begg's funnel plot **(A)** and sensitivity analysis **(B)** of all the included studies for the analysis of PR. .

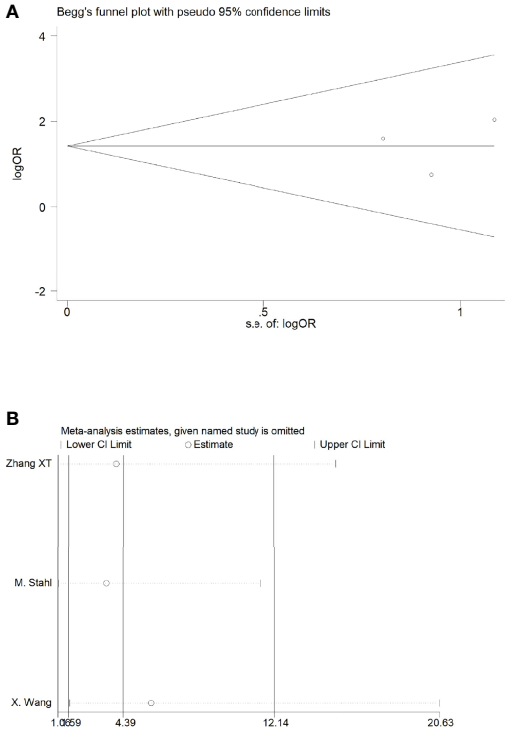


FIGURE 10
Begg's funnel plot **(A)** and sensitivity analysis **(B)** of all the included studies for the analysis of ORR. .

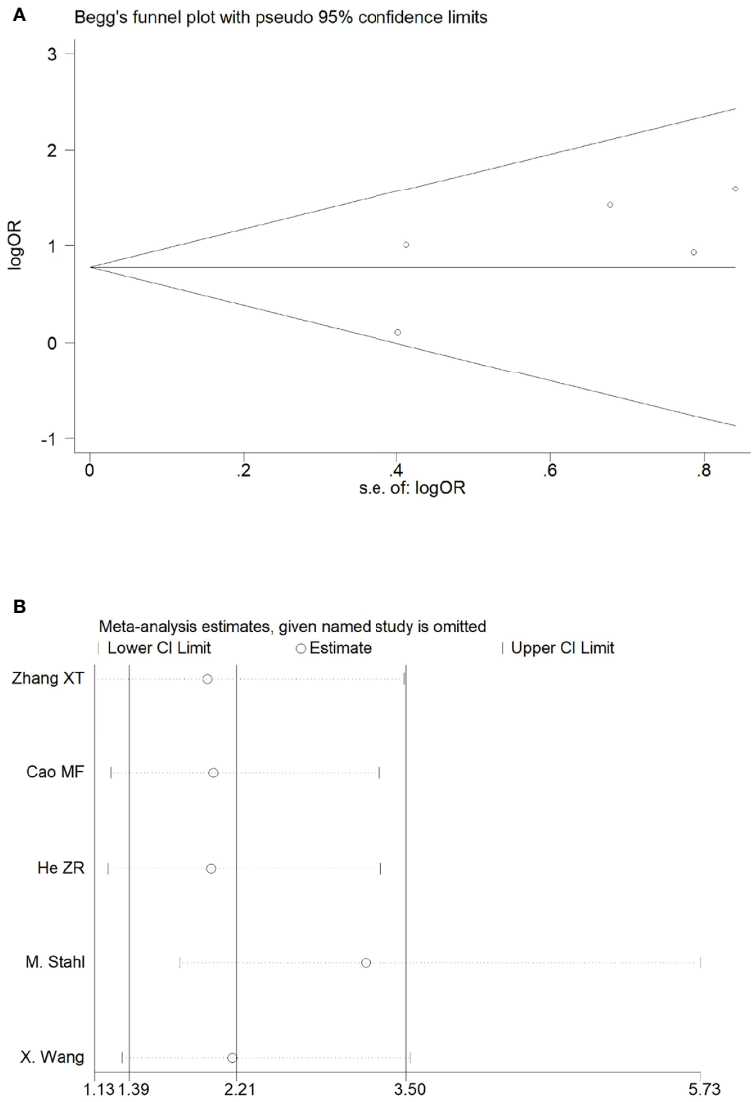


FIGURE 11
Begg's funnel plot **(A)** and sensitivity analysis **(B)** of all the included studies for the analysis of the pCR rate.

Limitations

This meta-analysis has certain limitations. First, although the included studies were all RCTs, the sample size of some studies was small. Second, the interventions of the enrolled studies, the chemotherapy regimen, or the recommended dose of radiotherapy were inconsistent, which may have caused some degree of bias. The outcome indicators mentioned in this article are not identical. Jiang Y regarded the ORR as the primary efficacy outcome and not the R0 resection rate (12). Leong T [the Trial Of Preoperative therapy for Gastric and Esophagogastric junction AdenocaRcinoma (TOPGEAR)] only reported the interim results regarding adverse effects after neoadjuvant therapy and postoperative complications, whereas we expected the final results of this randomized, phase III trial (14). Several ongoing studies have not published their results (such as the PREACT trial), and we believe that their final results will help our research (33).

Conclusion

In conclusion, our meta-analysis demonstrated the efficacy and safety of NACRT for resectable gastric cancer, providing clinical support for its wide application. However, since some clinical trials have not yet reached their end points, the long-term outcomes and toxicity must be examined to confirm this conclusion.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

JC, YX, and LZ contributed to the conception and design of the study. JC and YY organized the databases and provided methodological support. YG, MF, and YZ performed the statistical analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.927119/full#supplementary-material

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Pro-tumorigenic role of type 2 diabetes-induced cellular senescence in colorectal cancer

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Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide. The disease still remains incurable and highly lethal in the advanced stage, representing a global health concern. Therefore, it is essential to understand the causes and risk factors leading to its development. Because age-related cellular senescence and type 2 diabetes (T2D) have been recognised as risk factors for CRC development, the recent finding that type 2 diabetic patients present an elevated circulating volume of senescent cells raises the question whether type 2 diabetes facilitates the process of CRC tumorigenesis by inducing premature cell senescence. In this review, we will discuss the mechanisms according to which T2D induces cellular senescence and the role of type 2 diabetes-induced cellular senescence in the pathogenesis and progression of colorectal cancer. Lastly, we will explore the current therapeutic approaches and challenges in targeting senescence.

KEYWORDS

senescence, diabetes, colon cancer, fibroblast, endothelial cells, tumour microenvironment

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality worldwide and the third most common type of cancer (1). In 2020, global CRC cases increased by 6%, with a mortality rate of 29 per 100,000 people in men and 20 per 100,000 people in women (1). In the UK, there are around 42,900 new colorectal cancer cases every year (2). However, CRC incidence and mortality rates have decreased by 6% and 12% respectively. In regard to survival, around 52.9% of patients diagnosed with CRC survive the disease for ten years or more (2). Despite the decline in CRC incidence and mortality over the last decade, mainly attributed to the improvement in early screening

methods, CRC still remains incurable and highly lethal in the advanced stage (3). Because of its magnitude, CRC represents a global health concern and therefore it is essential to understand the causes and risk factors leading to its development.

Several risk factors and pathologies, including ageing, smoking, obesity and diabetes have been associated with poor prognosis in colorectal cancer patients. Ageing represents the major risk factor for CRC development. Brenner et al. (2007) showed that the transition rates from advanced adenoma to CRC strongly increase with age, from 2.6% in age groups 55-59 years to 5.6% in age group ≥ 80 years among women, and from 2.6% in age group 55–59 years to 5.1% in age group ≥ 80 years among men (4). In addition, Siegel et al. (2020) showed that CRC incidence rates increase by 30% every 5 years of age in individuals aged 50 and over (5). The ageing process is thought to contribute to tumorigenesis *via* aberrant genome maintenance and systemic inflammation that result in tissue damage and occurrence of unfavourable genome modifications (6). More recently, cellular senescence has been considered as an additional cause of age-related tumorigenesis. Senescence is a stress-response cellular state characterised by proliferative arrest but active metabolism (7). Over lifetime, due to the action of several stressors such as DNA damage and telomere shortening, senescent cells accumulate in the organism and release a variety of pro-inflammatory cytokines responsible for low-grade inflammation.

This age-related inflammation, also referred to as inflammaging, increases the risk for tissue damage and genetic aberrations that cause cellular transformation and cancer development (8, 9). However, cellular senescence is not exclusive to ageing. Age-related and metabolic diseases such as type 2 diabetes (T2D) represent a source of cellular stress due to their disruptive effect on normal physiological processes and, therefore, can induce premature senescence (10). In fact, several studies have shown that T2D induces senescence in multiple types of cells, including fibroblasts and endothelial cells (11, 12). T2D has also been recognised as a risk factor for CRC development. For example, Xiao et al. (2022) showed that diabetes was associated with increased risk of both right-sided colorectal cancer (Relative risk [RR] = 1.35, 95% CI = 1.24 - 1.47) and left-sided colorectal cancer (RR = 1.18, 95% CI = 1.08 - 1.28) using data regarding 1,642,823 individuals and 17,624 colon cancer patients (13). Ma et al. (2018) also suggested that T2D is associated with increased risk of CRC development (Hazard ratio [HR]: 1.42; 95% CI: 1.12 - 1.81) (14). The peculiar association between CRC, senescence and T2D raises the question whether the T2D-induced premature senescence facilitates the process of CRC tumorigenesis in T2D patients (15–17).

In addition, the recent finding that T2D patients present a higher circulating volume of senescent T cells compared to their age-matched healthy counterparts supports the hypothesis that T2D pathophysiology is also implicated in premature immunosenescence (18). T-cells also play a key role in immune control of the colorectal carcinoma microenvironment (19). Considering the importance of T cells in the response against cancer, T cell senescence would be a detrimental factor for the organisms because of its tumorigenic potential as well as reduced anti-cancer response. The distinctive association between CRC, senescence and T2D is intricate due to several aspects such as the anatomical location and complexity of the tumour microenvironment. However, the lack of early CRC diagnosis reflects the partial understanding of some of the processes leading to the disease (20). Thus, cellular senescence could represent a novel mechanism. Despite the supporting evidence, the correlation between CRC, senescence and T2D still remains unclear. In this review, we will discuss the mechanisms according to which T2D induces cellular senescence, and the potential role of T2D-induced senescence in the development of colorectal cancer. Particularly, we will focus on the tumorigenic activity of senescent fibroblasts, endothelial cells and T cells within the tumour microenvironment. Lastly, we will explore the current therapeutic challenges, approaches and future perspectives in targeting senescence.

Cellular senescence

Cellular senescence is a stress-response process characterised by changes in gene expression that ultimately lead to the alteration of cellular phenotype and metabolism (7). The concept of cellular senescence was discovered approximately 60 years ago by Hayflick and Moorhead (1961) but its roles in physiological processes and diseases have recently emerged (21, 22).

Senescent cells are denoted by proliferative arrest, alteration of morphology and secretome, and resistance to apoptosis (7, 23). The arrest in cell division, which is the major hallmark of senescence, prevents the progression of damaged cells into malignancy (7). The altered secretome results in the secretion of pro-inflammatory cytokines, proteases and growth factors that are collectively referred to as the senescence-associated secretory phenotype (SASP). SASP exerts paracrine action on the surrounding environment and is involved in the attraction of immune cells, stimulation of angiogenesis and cell proliferation in a process that mimics a wound healing response. Consequently, cellular senescence is a key mechanism in wound healing and tissue repair (23–26). Most senescent cells also express high levels of the enzyme β-galactosidase at pH 6. This enzymatic activity, initially described by Dimri et al. (1995), enabled identification of senescent fibroblasts and keratinocytes in biopsies of aged human skin, and subsequently the enzyme became known as senescence-associated β-galactosidase (SA-β-Gal) (27).

The stressors responsible for the induction of cellular senescence are categorised into acute and chronic stressors, each determining a different outcome: acute stressors stimulate

tissue repair and wound-healing response whereas chronic stressors can induce persistent senescence activation and accumulation of senescent cells, leading to continuous SASP release. As a result, chronic SASP generates low-grade inflammation, excessive cell proliferation and angiogenesis, causing tissue damage and potentially contributing to the promotion of a pro-tumorigenic environment (8, 23, 28, 29). Therefore, whilst acute senescence seems to be a programmed process, the switch from a temporary to a persistent senescent state appears to be unscheduled in nature (8). Some of the cellular stressors are endogenous and part of the normal cell cycle such as telomere shortening during DNA replication, DNA damage and reactive oxygen species (ROS) produced by mitochondria during normal metabolism (7, 30, 31). These events arise with age and so does cellular senescence. Other stressors are environmental factors, obesity, and ongoing pathologies such as T2D and hypertension (32–35).

In the context of cancer, the senescence-induced proliferative arrest is an important tumour-suppressive mechanism. However, the SASP released by senescent cells possesses both pro- and anti-tumorigenic abilities on the surrounding environment: acute senescent cells present anti-tumorigenic potential because the pro-inflammatory component of their SASP is associated with the recruitment of immune cells at the site of the tumour, therefore promoting a tumour-specific immune response. However, acute SASP action is limited and should be considered as an anti-cancer mechanism against pre-tumorigenic cells rather than malignant cells. On the other hand, chronic senescent cells do not present anti-tumorigenic properties but can instead contribute to the generation of a pro-tumorigenic environment due to the prolonged inflammatory state caused by their SASP (9, 29, 36, 37).

Despite the common characteristics, the phenotype of senescent cells vary depending on the cell type and senescence-inducing stressor (38). For example, senescent T cells are characterised by specific surface markers that allow to detect their differentiation and senescent state. These cells lack CD27 and CD28 surface markers but express markers such as CD57 and KLRG-1 which determine a decrease in cellular proliferation (39–41). Among senescent T cells there are the effector memory T cells re-expressing the surface receptor CD45RA, referred to as EMRA T cells. EMRA T cells have reduced proliferative capacity and display SASP secretion (42, 43). However, whether these cells can be considered fully senescent cells or not is still under debate (44, 45).

Type 2 diabetes induces premature cellular senescence

Chronic hyperglycaemia is the major contributing factor to T2D-associated cardiovascular complications such as retinopathy, nephropathy and hypertension (46). Along with these complications, hyperglycaemia has been shown to induce cellular senescence in fibroblasts, endothelial cells and more recently in mesenchymal stem cells such as umbilical cord-derived mesenchymal stem cells (11, 12, 47). These senescent stem cells are characterised by multipotentiality loss in addition to the hallmarks of senescence (47). Here, we propose a general mechanism according to which hyperglycaemia activates senescence-inducing pathways.

Intracellular hyperglycaemia induces oxidative stress, proteostasis alteration and dysregulation of protein kinase C (PKC) signalling (48–54) (Figure 1). These pathways are integrated and promote the establishment of the major characteristics of senescence: cell cycle arrest, changes in cellular morphology, SASP secretion and SA-β-Gal activity. It should be emphasised that these pathways are mechanistically similar in both age-related and T2D-induced cellular senescence, but the inducing stimuli and time span are different: during ageing, senescence-inducing stimuli such as telomere erosion occur over a long period of time while in T2D senescence is accelerated by stimuli such as hyperglycaemia and hypertension (55, 56). A pivotal pathway involved in senescence activation is the p38 mitogen-activated protein kinases (MAPK) pathway. The p38 MAPK is a stress-response pathway activated by several stressors among which are ROS and transforming growth factor β (TGF-β) (54, 57). P38 activation leads to cell cycle arrest and SASP secretion whereas endoplasmic reticulum (ER) stress contributes to the changes in cellular morphology and expression of SA-β-Gal (58–60). However, the process that leads to intracellular hyperglycaemia is still not fully understood. For example, activated T cells express insulin receptors and, unlike naive T cells, insulin signalling increases GLUTs expression on the plasma membrane (61, 62). As a result, the T2D hyperinsulinemic environment may induce GLUT transporters overexpression, leading to intracellular glucose concentration rising in parallel with serum hyperglycaemia (48). By contrast, other studies have suggested that vascular endothelial and smooth muscle cells downregulate GLUTs expression in response to hyperglycaemia (63, 64).

Oxidative stress

As a part of their normal metabolic activity, mitochondria produce ROS and reactive nitrogen species by-products *via* the complexes of the electron transport chain (30). In a hyperglycaemic environment, the excessive glucose oxidation causes mitochondrial overload, resulting in ROS overproduction and increased ATP/ADP ratio (51, 53). In addition, intracellular hyperglycaemia promotes polyol pathway activation. In this pathway, the enzyme aldose reductase converts glucose to sorbitol, which is then oxidised to fructose. However, in the conversion from glucose to sorbitol, aldose reductase consumes

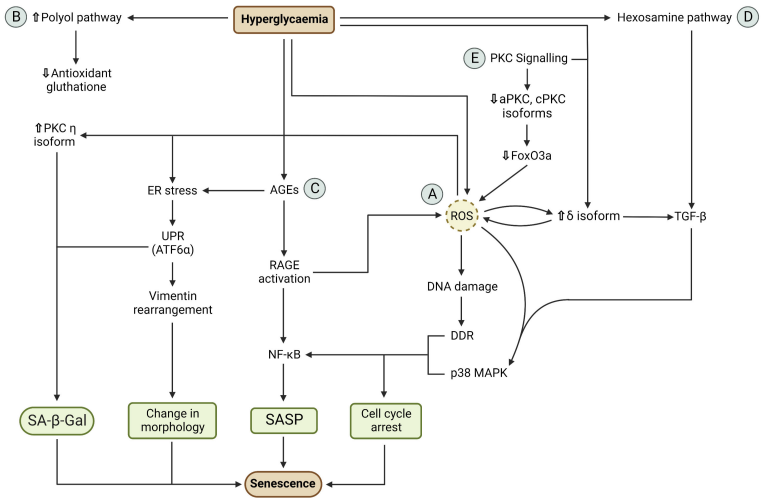


FIGURE 1
Mechanism of hyperglycaemia-induced cellular senescence. **(A)** Hyperglycaemia induces ROS overproduction *via* mitochondria overload which results in oxidative stress. ROS causes DNA damage response (DDR) activation, due to DNA oxidative damage, and p38 MAPK pathway activation. DDR and p38 MAPK determines cell cycle arrest and NF-κB upregulation. NF-κB activation results in SASP secretion. ROS also generates ER stress *via* chemical modification of ER proteins. ER stress activates the unfolded protein response (UPR). The activation of the ATF6α branch of the UPR causes expression of SA-β-Gal and changes in cellular morphology *via* cytoskeletal vimentin rearrangement. **(B)** Hyperglycaemia increases polyol pathway activity, causing reduced antioxidant glutathione synthesis due to reduce NADPH availability. Glutathione deficiency contributes to the inability of the cell to counteract oxidative stress. **(C)** Hyperglycaemia causes advance glycation end products (AGEs) *via* glycation of intracellular and extracellular proteins. Intracellular AGEs cause ER stress which results in SA-β-Gal activity and change in cellular morphology. Extracellular ages cause AGE receptor (RAGE) activation which results in ROS production and NF-κB activation. This ultimately results in cell cycle arrest and SASP secretion. **(D)** Hyperglycaemia results in increased hexosamine pathway activity due to increased glucose-6-phosphate production. This pathway produces N-acetyl glucosamine (GlcNAc) which induces TGF-β expression. TGF-β activates the p38 MAPK which results in cell cycle arrest and SASP secretion. **(E)** PKC signalling contributes to the activation of senescence pathways. Hyperglycaemia results in increased diacyl glycerol production and activation of PKC δ isoform. PKC δ activation causes TGF-β expression and ROS production which, in turn, activate PKC δ in a positive feedback loop mechanism. In addition, ROS also activate PKC η which induces SA-β-Gal activity. Downregulation of aPKC and cPKC results in inactivation of FoxO3a which results in ROS production. Cell cycle arrest, SASP secretion, change in cellular morphology and SA-β-Gal activity are the major characteristics of senescence.

NADPH which is also a cofactor used for the generation of the antioxidant glutathione (49). Therefore, high glucose concentration shifts the equilibrium towards the polyol pathway, resulting in reduced glutathione synthesis. The reduction in this antioxidant levels reduces the ability of the cell to counteract ROS overproduction as well as other free radicals.

The oxidative stress caused by this ROS imbalance and reduced antioxidant determines mitochondrial DNA oxidative damage, leading to mitochondrial dysfunction and, as a consequence, impairment of cellular metabolism (50, 65). Oxidative stress also causes nuclear DNA oxidative damage and activation of the DNA-damage response (DDR) pathway. DDR results in cell cycle arrest *via* the $p16^{INK4a}$/Rb pathway and upregulation of the CDK-inhibitor p21 (66–69). DDR is also involved in the generation of SASP *via* the activation of the kinases ataxia-telangiectasia mutated (ATM) and ATM- and Rad3-Related (ATR) which belong to the phosphatidylinositol-3 kinase-related kinases (PIKK) family (70). ATM and ATR have been shown to activate the transcription factor GATA4, a novel positive regulator of senescence that activates NF-kB, ultimately leading to SASP formation (71).

Increased cellular ROS also activate the p38 MAPK pathway. Similarly to the DDR, p38 MAPK activation results in cell cycle arrest *via* $p16^{INK4a}$/Rb and p21 pathways (59). In addition, p38 MAPK induces SASP production through NF-kB activation (57, 69). Conversely, p38 MAPK inhibition by the selective inhibitor SB203580 effectively collapses the senescence-associated cytokine network, preventing the SASP paracrine effects of senescent cells (69). The p38 MAPK pathway can also be activated by intracellular hyperglycaemia *via* the hexosamine pathway. In fact, high intracellular glucose levels determine increased levels of the glycolytic intermediate fructose-6-phosphate (F6P). Part of this sugar is used in the hexosamine pathway. In this pathway, F6P is converted into uridine diphosphate (UDP) and N-acetyl glucosamine (GlcNAc). The latter can interact with transcription factors that induce the expression of TGF-β1 which, in turn, activates the p38 MAPK pathway (49, 72).

Proteostasis alteration

Proteostasis can be defined as the ability of the cell to maintain a functional cellular proteome (50). Loss of proteostasis due to protein misfolding has detrimental effects on cellular function. One of the causes of such event is a non-programmed chemical modification, such as glycation or carbonylation, of cellular proteins (50).

Glycation is a non-enzymatic reaction of glucose with proteins that leads to the formation of molecular products known as advanced glycation end products (AGEs) involved in diseases and ageing (48). AGEs are formed both intracellularly and extracellularly and alter cellular proteostasis in T2D. After cytoplasmic glucose-6-phosphate (G6P) is transported into the ER *via* the transporter G6PT, glucose then glycates the proteins present in the ER lumen, causing protein misfolding and ER stress. ER stress results in the activation of the unfolded protein response (UPR) pathway (65, 68, 73, 74). Extracellular AGEs precursors interact and modify connective tissue components, such as collagen and plasma proteins, and bind to the AGE receptor (RAGE) present on the cell surface (75, 76). RAGE activation has been shown to activate the transcription factor NF-kB by degradation of the IkB proteins and to induce the production of cytosolic ROS (75, 77).

Carbonylation is an irreversible reaction of ROS with proteins. Carbonylation of ER enzymes involved in protein folding, such as protein disulphide isomerase (PDI) and calreticulin proteins, causes proteins misfolding and aggregation into structures known as lipofuscin that are resistant to proteolytic degradation (50, 78). These events cause accumulation of misfolded proteins and ER stress, resulting in the activation of UPR. The ATF6α branch of the UPR pathway has been shown to be involved in cellular senescence by increasing SA-β-Gal activity and by altering the cellular morphology *via* changes in cytoskeletal vimentin (60). It should be noted that ER stress involves complex signalling pathways and the cell may reinstate its normal proliferative activity by resolving the proteostasis alteration rather than activating senescence-inducing pathways (79). However, it remains unclear whether UPR downstream signalling results in either proliferation or senescence as this depends on the nature and intensity of the stimuli involved (79). It is plausible that UPR senescence-inducing pathways arise from stimuli that cause sustained damage which is, however, insufficient to trigger apoptosis.

PKC signalling

Activation or downregulation of certain PKC isoforms has been shown to promote senescence. Hyperglycaemia determines an increase in diacyl glycerol (DAG) production, which is responsible for the activation of DAG-sensitive PKC isoforms such as cPKC, PKC δ and PKC η (54, 80). PKC δ activation promotes expression of TGF-β and induces ROS production which, in turn, activates PKC δ in a positive feedback loop mechanism (54, 81). PKC η activation has been shown to induce SA-β-Gal expression. For instance, PKC η-knockdown MCF-7 cells showed SA-β-Gal expression reduced by 2-fold compared to the scrambled controls ($p \leq 0.0001$). Importantly, PKC η is also activated by ROS (82). On the other hand, aPKC or cPKC downregulation also promotes senescence. The downregulation of these PKC isoforms has been shown to inhibit the nuclear import of the transcription factor FoxO3a *via* AKT-mediated phosphorylation. Specifically, aPKC or cPKC inhibition in HCT116 cells resulted in a 10-fold increase in SA-β-Gal expression compared to the control ($p < 0.05$). In these cells, the levels of phosphorylated FoxO3a were increased by two-fold compared to the controls ($p < 0.05$) (80). FoxO3a inactivation has been shown to induce ROS production, therefore contributing to cellular oxidative stress (83).

Senescent cells modulate the tumour microenvironment through SASP release

The tumour microenvironment (TME) is a complex network of cellular and molecular components consisting mainly of tumour-infiltrating cells, blood vessels, extracellular matrix (ECM) and other matrix-associated molecules (84). Persistent inflammation in the TME has been recognised as a tumour initiator and promoter because it serves as a chemoattractant for the recruitment of tumour-infiltrating cells that support tumour growth and metastasis (85). Consequently, the chronic systemic inflammation that characterises T2D represents a risk factor for cancer development (16). T2D interferes with the normal colon tissue physiology in multiple processes. Firstly, AGEs accumulation and TGF-β upregulation determine ECM accumulation, cross-linking of collagen, thickening of basement membrane, loss of elasticity and fibrosis [76]. These events result in colon wall remodelling and change in its biomechanical properties (86, 87). Secondly, increased levels of insulin determine activation of the insulin-like growth factor 1 (IGF-1) receptor, which stimulates cell growth and proliferation (88). For instance, Teng et al. (2016) showed that the treatment of MC38 cells with 50 ng/mL of insulin and 50 ng/mL of IGF-1 determined a 2-fold increase in cell proliferation compared to the negative control ($p < 0.001$). In mouse models, the tumour growth of MC38 cells was doubled in mice that had serum insulin and serum IGF-1 6 times and 5 times higher than the control, respectively ($p < 0.001$) (88). Therefore, the extracellular matrix remodelling and the proliferative stimulation *via* IGF-1 activation may alter the

colon tissue into a pro-tumorigenic environment (89). An additional and less explored role of T2D on CRC tumorigenesis is represented by T2D-induced cellular senescence. Chronic senescent cells may have the ability to modulate and enhance the inflammatory state of the tumour microenvironment *via* the release of pro-inflammatory SASP. In support to this hypothesis, multiple studies demonstrated the ability of senescent fibroblasts, endothelial cells and T cell to induce tumour growth and metastasis.

Fibroblasts

Fibroblasts represent the major cell type within the connective tissue and are involved in ECM production and maintenance (90). Following fibroblast senescence, SASP components such as growth factors can promote neoplastic alterations that initiate and support metastatic development (Figure 2). The ability of senescent fibroblasts to induce tumour cell hyperproliferation has been observed in breast and prostate tumours (91, 92). In regard to colorectal cancer, a study by Guo et al. (2019) showed that senescent fibroblasts accumulate in individuals with advanced adenomas and colon cancer compared to healthy individuals. *In vitro*, senescent fibroblasts promoted the proliferation of both adenoma and colon cancer cells *via* the secretion of the SASP component growth differentiation factor 15 (GDF15) (93). GDF15, also termed macrophage-inhibiting cytokine-1 (MIC-1), is a growth factor belonging to the TGF-β superfamily which has been revealed to accelerate G1-S phase transition, to stimulate angiogenesis and to promote colon cancer metastasis *via* epithelial-to-mesenchymal transition (94–97). Study investigating co-culture of LT97, AA/C1, Caco-2, and HT-29 cells with senescent CCD-18Co fibroblasts determined a 2-fold increase in cell migration and invasion compared to the co-culture with non-senescent CCD-18Co fibroblasts ($p < 0.01$) (93). Real-time PCR analysis of the senescent CCD-18Co cells revealed that GDF15 mRNA levels were increased by 10-fold compared to non-senescent CCD-18Co cells ($p < 0.05$). Western blot analysis of GDF15 in senescent CCD-18Co cells showed that GDF15 protein concentration was increased by 2-fold compared to non-senescent CCD-18Co cells ($p < 0.001$) (93). In addition, co-culture of LT97, AA/C1, Caco-2 and HT-29 cells with senescent CCD-18Co cells which presented GDF15 knockdown *via* short-hairpin GDF15 (shGDF15) resulted in a 2-fold decrease in cell migration and invasion compared to the controls ($p < 0.05$) (93).

In support to the hypothesis that T2D, senescence and cancer are related, T2D patients present circulating GDF15 levels that are three times higher compared to the healthy controls ($p < 0.001$) (98). Although the upregulation of the SASP component GDF15 has been associated with many types of cancers, including colon cancer, its pro-tumorigenic role remains unclear and context-dependent (99). Yang et al.

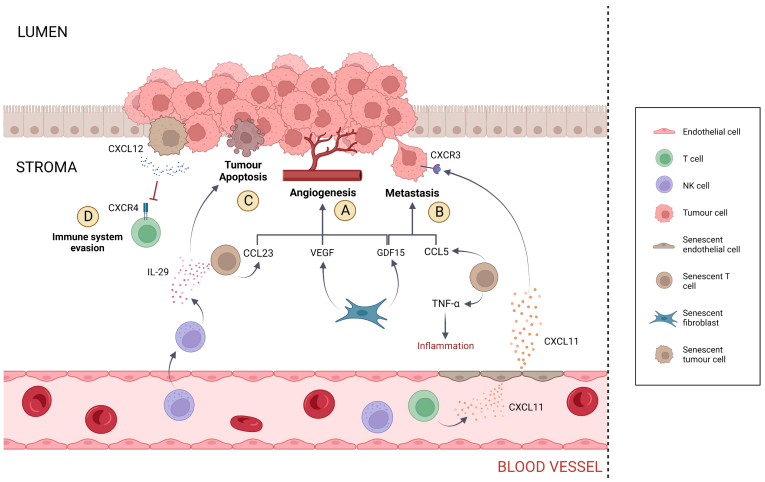


FIGURE 2
SASP activity within the tumour microenvironment. **(A)** Angiogenesis is stimulated by CCL23, VEGF and GDF15. CCL23 is secreted by senescent T cells, while VEGF and GDF15 are secreted by senescent fibroblasts. **(B)** Metastasis is promoted by GDF15 and CCL5, which are secreted by senescent fibroblasts and T cells, respectively. Senescent T cells contribute to tumorigenesis by inducing inflammation *via* the release of TNF-α. Metastasis is also induced in tumour cells that express the receptor CXCR3 *via* the SASP component CXCL11 secreted by senescent endothelial cells. However, CXCL11 also presents anti-tumorigenic activity by recruiting T cells and NK cells at the site of tumour. **(C)** Tumour apoptosis is induced by IL-29 secreted by senescent T cells. In addition, IL-29 contributes to cancer-specific immune response *via* the recruitment of NK cells. **(D)** Senescent tumour cells evade the immune system *via* the secretion of elevated CXCL12 levels, inducing CXCR4 internalisation in T cells and impairing T cell directional migration.

(2020) showed that senescent fibroblasts expressing galactosylceramidase (GALC) enhance the migration ability of CRC cells. Specifically, the co-culture of LoVo cells with GALC-expressing fibroblasts determined a 2-fold increase in LoVo cells migration ability compared to the controls ($p < 0.05$) (100). Coppé et al. (2006) showed that WI-38 senescent fibroblasts secrete high levels of vascular endothelial growth factor (VEGF) and increase tumour vascularization. The VEGF mRNA and protein levels in senescent WI-38 cells were increased by 2-fold and 5-fold, respectively, compared to the pre-senescent WI-38 cells ($p < 0.05$). *In vivo*, treatment of tumour-bearing nu/nu mice with senescent fibroblasts determined a 3-fold increase in the number of large blood vessels compared to mice treated with pre-senescent fibroblasts ($p < 0.05$) (101).

Endothelial cells

Research concerning the effect of endothelial senescence on cancer has been scarce. Nevertheless, there is some evidence that endothelial senescence promotes tumour growth and metastasis. Borovski et al. (2013) showed that senescent tumour microvascular endothelial cells (tMVEC) favour the growth of glioblastoma (GBM) cells (102). Specifically, co-culture of GBM cells with senescent tMVEC resulted in the 10-fold increase in GBM cell number compared to the negative control ($p < 0.05$) (102). Hwang et al. (2020) showed that senescent human umbilical vein endothelial cells (HUVEC) secrete the chemokine CXCL11, which promotes breast tumour migration both *in vitro* and *in vivo* (103). Specifically, the co-culture of MDA-MB-231 cells with senescent HUVEC cells transfected with CXCL11 small interfering RNA (siRNA) determined a 10-fold decrease in MDA-MB-231 cell migration compared to the negative control ($p < 0.001$). *In vivo*, mice xenografts were treated with conditioned medium containing CXCL11 secreted from senescent HUVEC. The tumour volume of MDA-MB-231 cells was increased by 4-fold compared to the volume of MDA-MB-231 cells treated with conditioned medium from senescent HUVEC transfected with CXCL11 siRNA ($p < 0.05$) (103). Significantly, CXCL11 is involved in CRC growth and metastasis. Gao et al. (2018) showed that CXCL11 downregulation inhibits cell growth and invasion in CRC. Specifically, the cell growth of SW480 cells transfected with CXCL11 siRNA was reduced by 6-fold compared to the negative control ($p < 0.01$). In addition, the migration of SW480 cells transfected with CXCL11 siRNA was reduced by 5-fold compared to the negative control ($p < 0.01$) (104). CXCL11 is among the ligands that activate the CXCR3 receptor, which is mainly expressed on effector T cells and NK cells and promotes infiltration into an inflammatory site (105). Although CXCL11 has anti-tumour activity *via* the recruitment of innate and adaptive immune cells at the site of tumour, some colorectal tumours express CXCR3 receptors that function as a metastatic mediator (106). Kawada et al. (2007) showed that CXCR3 activation on Colo205 cells resulted in approximately a 2-fold increase in cell migration compared to the controls ($p < 0.01$) (107). In addition, Cambien et al. (2009) showed that the treatment of C26 cells with CXCL11 and the CXCR3 inhibitor AMG487 resulted in almost a 2-fold decrease in cell migration compared to the negative control ($p < 0.001$) (108). Therefore, CRC is plausibly affected by endothelial senescence (Figure 2).

T lymphocytes

Similar to endothelial cells, the effect of T cell senescence on CRC has been scarcely investigated. In support of the idea that T2D-induced T cell senescence is implicated in cancer, Broadway et al. (2021) suggested that T2D-associated T cell senescence has a potential tumorigenic role in ovarian cancer metastasis (109). Because the intestine contains the largest number of immune cells in the human body, including T cells, it is probable that T cell senescence also affects this anatomical location (110). The presence of senescent cells such as EMRA T cells in the TME can contribute to the generation of a pro-inflammatory environment that supports tumorigenesis, therefore contrasting the action of non-senescent tumour-infiltrating cells (TILs) against the tumour as a part of the normal host's immunity (111). EMRA $CD4^+$ and $CD8^+$ cells, which have been found to be elevated in T2D patients, possess a unique inflammatory SASP repertoire – proteases, chemokines, interleukins, growth factors and insoluble factors such as extracellular matrix components (29, 43). Particularly, the gene expression of CCL5, CCL23, tumour necrosis factor α (TNF-α) and IL-29 and are upregulated in the EMRA $CD8^+$ subset (43). Cambien et al. (2011) showed that CCL5 promotes cell migration and invasion in colon cancer: *in vitro* experiments showed that treatment of CT26 and HT29 cells with 50 ng/ml of CCL5 determined a 2-fold increase in cell proliferation compared to the controls treated with base medium only ($p < 0.01$). *In vivo*, treatment of CT26-inoculated mice with anti-CCL5 resulted in the 2-fold decrease in tumour incidence ($p < 0.05$) (112). Hwang et al. (2005) showed that CCL23 has pro-tumorigenic potential *via* induction of angiogenesis. Specifically, in chick chorioallantoic membrane (CAM) assay, injection of 10 ng per egg of CCL23 determined a 3-fold increase in blood vessels number compared to the negative control ($p < 0.01$) (113). The role of TNF-α in tumour growth has been controversial: Carswell et al. (1975) showed that TNF-α was capable of inducing tumour necrosis (114). However, over the years multiple studies demonstrated the pro-tumorigenic activity of TNF-α *via* inflammation (115). For instance, treatment of HCT-116 cells with 20 μg/L of TNF-α determined a 2-fold increase in cell number compared to the control ($p = 0.001$) (115). In addition, the IL-6 and IL-8 levels in HT-29 cells treated with 5ng/ml of TNF-α were approximately 3

times and 5 times higher, respectively, compared to the controls ($p < 0.05$) (116).

By contrast, other SASPs such as IL-29 have tumour inhibitory effects *via* the induction of caspase-mediated apoptosis and increase in NK cell activity (117). Specifically, Sato et al. (2006) showed that the caspase activity of B16/F0 cells transfected with IL-29 was doubled compared to both the control and transfected B16/F0 cells treated with the caspase inhibitor Z-VAD-fmk ($p < 0.05$). In addition, following hepatic injection with IL-29 in two independent experiments, the $CD3^-$ $NK1.1^+$ cell number increased from 10.9% to 26.9%, whereas the $CD3^+$ $NK1.1^+$ cell number increased from 1.0% to 11.0% (117). Therefore, the overall outcome of senescent T cell activity on TME depends on the type and amount of SASP released. At present, however, the degree of infiltration of senescent T cells and their influence on the tumour microenvironment is still unclear (118, 119).

The tumorigenic action of EMRA T cells is not limited to the colon/rectum. In fact, the enhanced expression of the protease ADAM28 and the receptor CX3CR1 has the potential to alter the migration of these cells to other tissues (43). Shimoda et al. (2007) showed that ADAM28 binds to the P-selectin glycoprotein ligand-1 (PSGL-1) and enhances PSGL-1/P-selectin-mediated cell adhesion to endothelial cells *in vitro*. Specifically, immunolocalization of HL-60 cells with the recombinant ADAM28 protein rpro-ADAM28s determined the 3-fold increase in P-selectin binding compared to the control ($p < 0.01$) (120). As a result, the ability of these cells to adhere to the endothelial wall in the absence of stimulation increases the possibility of cell migration to other tissues. CX3CR1 expression in EMRA T cells is three times higher than the control ($p < 0.001$) (43). CX3CR1 allows cell adhesion to fractalkine-expressing endothelial cells: in two independent experiments, Imai et al. (1997) showed that expression of CX3CR1 in K562 cells determined a 5-fold increase in adhesion to fractalkine-expressing ECV304 endothelial cells compared to the control (121).

Tumour cells

Cellular senescence is not restricted to only healthy cells but has been observed in several tumour cells: primary neoplastic cells from different types of cancer, including colon cancer, appear to be senescent *in-vitro* and express high levels of SA-β-gal (122, 123). Choi et al. (2021) showed that senescent colorectal cancer cells generate a cytokine shield through their SASP that inhibits intratumoral $CD8^+$ T cell infiltration. This is achieved *via* the secretion of high CXCL12 concentration, which induces internalisation of the CXCR4 receptor and results in impaired directional migration (124). Specifically, the CXCL12 mRNA levels in senescent SW480 cells were increased by 6-fold compared to the control ($p < 0.01$). When $CD8^+$ T cells were co-cultured with senescent SW480 cells overexpressing CXCL12, the number of migrated T cells was reduced by almost 2-fold compared to the $CD8^+$ T cells cultured with non-senescent SW480 cells ($p < 0.001$). In order to demonstrate that CXCL12 causes downregulation of CXCR4 expression on the plasma membrane, Jurkat cells were treated with 1 mg/mL of recombinant human CXCL12 (rhCXCL12) for 30 minutes and then analysed *via* fluorescence-activated cell sorting (FACS). As a result, the number of CXCR4-expressing cells was reduced from 10^4-10^5 cells to 10^3-10^4 cells (124). Therefore, tumour senescence represents an additional ability of tumour cells to evade and suppress the host's immune response (125) (Figure 2).

Detection and therapies

As previously mentioned, CRC still remains incurable and highly lethal in the advanced stage. Thus, improvements in therapies are required to reduce the disease burden. In this section, we will discuss the limitations regarding the traditional chemotherapy and will review some of the recent therapeutic approaches that have shown promising outcomes in targeting both senescent and cancer cells.

Current chemotherapy and its challenges

The use of conventional CRC chemotherapeutic drugs such as leucovorin and 5-Fluorouracil (5-FU) poses multiple problems in that a patient can develop severe side-effects such as nausea, alopecia, diarrhoea and neutropenia (126, 127). In addition, cytotoxic drugs that provide a cure to metastasis are effective in only a few types of tumours. In tumours such as colorectal, gastric, ovarian and breast cancer, chemotherapy limits to prolong patient's survival but does not provide a definitive cure. The phenomenon of tumour drug resistance is also observed, suggesting the emergence of mechanisms that counteract the cytotoxic drugs action, leading to the lack of tumour cells sensitivity despite optimal exposure to the drugs (128, 129). An additional effect of chemotherapy is the induction of senescence on the tumour and surrounding cells, a phenomenon referred to as chemotherapy-induced senescence or therapy-induced senescence (TIS) (130, 131). Although TIS is beneficial because it restricts tumour growth, it also has negative consequences. Firstly, the SASP released by senescent tumour cells support tumour growth in the TME, as discussed before. Secondly, senescent tumour cells can escape the senescent state, increasing the risk of tumour relapse (131).

Importantly, tumour cells that escape senescence manifest an increased malignancy, drug resistance and a stem-like phenotype. This increased malignancy seems to be associated with the activation of Wnt signalling as a result of TIS (131, 132). As a consequence, while TIS gives an advantage in restricting tumour growth, this advantage is limited to a short period. Therefore, improvements in cancer therapeutic approach are required.

Senolytic therapy

Chronic senescent cells and tumour senescent cells have great potential to favour tumour growth and metastasis. Senescent cells can be targeted and killed by senolytic drugs. There are several types of senolytics and some of them have been shown to decrease the number of senescent cells in human clinical trials (133). For this reason, senolytics represent a promising therapeutic approach. However, because senescent cells possess a large variety of phenotypes, it has been challenging to find a biomarker that is consistent across cellular senescence in all organ systems (134). One potential biomarker for senescence as well as senolytic drug target is SA-β-Gal (135, 136). Cai et al. (2020) developed the prodrug "senescence-specific killing compound 1" (SSK1) that is specifically cleaved by lysosomal β-gal into cytotoxic gemcitabine (137). SSK1 effectively induced apoptosis and cleared senescent cells in different tissues. Specifically, SSK1 reduced the viability of senescent human umbilical vein endothelial cells (HUVECs) and mouse lung fibroblasts by 2-fold and 8-fold, respectively, compared to the non-senescent controls ($p < 0.0001$) (137). Importantly, SSK1 eliminated mouse and human senescent cells independently of the senescence inducers. In fact, compared to non-senescent controls, SSK1 determined a 2-fold decrease in the viability of senescent human oesophageal fibroblasts in which senescence has been induced by replication, etoposide, H_2O_2, and oncogene-induced senescence ($p < 0.0001$) (137). In addition, SSK1 determined at least the 2-fold decrease in serum levels of SASP such as IL-6 and CXCL1 in mice ($p < 0.01$). Therefore, SSK1 has the ability to attenuate SASP-associated inflammation (137). Despite the promising results, currently there are no potent and specific markers of senescent cells (138). In fact, the staining method for SA-β-Gal detection proposed by Dimri et al. (1995) presents some limitations due to its time consumption and lack of sensitivity (27, 139). Cahu et al. (2013) proposed a faster and more sensitive senescence detection method based on flow cytometry. This technique is based on the detection of green fluorescence emitted from the hydrolysis of the molecule 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG) by the SA-β-Gal (140).

Another potential biomarker characterising both colorectal cancer cells and senescent cells is the mitochondrial enzyme glutaminase 1 (GLS1) which is involved in the conversion of glutamine into glutamic acid (141, 142). Importantly, cancer cells depend on glutamine metabolism for metabolic and anabolic processes – glutamic acid is involved in ATP production and biosynthesis of amino acids, nucleotides and lipids *via* the conversion of glutamate to α-ketoglutarate (143, 144). Senolytics that inhibit GLS1 represent an alternative therapeutic approach compared to the SA-β-Gal-associated senolytics. Huang et al. (2014) showed that expression of GLS1 is increased in human colorectal cancer tissues and that GLS1 inhibition decreases tumour growth rate, suggesting that GLS1 may be associated with the progression of colorectal cancer. Specifically, GLS1 was strongly expressed in approximately 54% of T3 colorectal tumour tissues ($p < 0.001$, $N = 128$) and approximately 46% of T4 colorectal tumour tissues ($p < 0.001$, $N = 78$). On the other hand, GLS1 was strongly expressed only in approximately 23% of T1/T2 colorectal tumour tissues ($p < 0.001$, $N = 31$). After treatment with the GLS1 inhibitor 6-diazo-5-oxo-L-norleucine (DON) for 48 hours, the growth rate of HT29 cells was reduced by 2-fold ($p < 0.0001$) (142).

Since the current chemotherapy for cancer is responsible for TIS in several cases, it has been reasoned that senolytics should be used to avoid tumour relapse and to reduce the undesired tumour-promoting effects deriving from the SASP. This led to the proposal of the one-two punch therapeutic strategy for cancer: a first compound is used to induce senescence in cancer cells followed by the use of senolytic drugs to specifically kill those senescent cancer cells (131). However, it is necessary to acknowledge that TIS is not limited to tumour cells: TIS in non-malignant cells has been associated with dysfunction of the heart, kidneys, bone, bone marrow and nervous system, which contribute to the adverse effects of cancer therapy (132). Therefore, while the adoption of senolytics in cancer therapy carries potential promise, there are still several concerns regarding the lack of senolytic action universality, the potential for systemic toxicity, senolytic drug resistance and damage to healthy senescent cells like those contributing to wound-healing (132).

CAR T cell immunotherapy

Cancer immunotherapy is an innovative therapeutic approach that modulates the host's immune system to specifically target cancer cells (145). Immunotherapy is particularly important considering that cancer cells use several mechanisms to evade immune surveillance such as downregulation of MHC molecules, Fas ligand-induced apoptosis and upregulation of immune checkpoint molecules (146–148). As such, CAR T cell therapy has been in development to target tumours in various settings with successes (149–151), including in CRC (149, 152–156) (Figure 3).

A novel therapeutic approach has been proposed by Amor et al. (2020), who suggested to use CAR T cells as a senolytic

therapy. These CAR T cells have been engineered to target the receptor urokinase-type plasminogen activator receptor (uPAR) (157) (Figure 3). uPAR is a regulator of ECM proteolysis and it is upregulated in senescence as well as many human cancers (157, 158). In two independent experiments, uPAR-specific CAR T cells completely lysed uPAR-expressing NALM6 cells when the E:T ratio was 1:1. By contrast, with the same E:T ratio, the negative control lysed less than 20% of uPAR-expressing NALM6 cells. In addition, uPAR-specific CAR T cells lysed 80% of senescent KP cells when the E:T ratio was 25:1 (157). *In vivo*, senescence was induced in hepatocytes of immunodeficient NSG mice *via* oncogene-induced senescence, detected through bioluminescence imaging. The treatment with uPAR-specific CAR T cells resulted in the 3-fold decrease in bioluminescence signal compared to the negative control ($p = 0.0182$). This suggests that uPAR-specific CAR T cells effectively cleared the senescent hepatocytes in NSG mice. Importantly, in support to the one-two punch therapeutic strategy, the authors evaluated the combination of TIS and CAR T cell therapy. Mice with KP lung adenocarcinomas were treated with a combination of MEK and CDK4/6 inhibitors with the purpose to induce KP cell senescence. Treatment for 7 days with uPAR-specific CAR T cells determined a 2-fold increase in the number of infiltrated $CD69^+$ $CD45.1^+$ $CD8^+$ T cells compared to the control ($p = 0.0021$). This suggests that CAR T cell treatment enhances activated $CD8^+$ T cell infiltration within a senescent tumour (157). Thus, CAR T cell immunotherapy against CRC showed favourable results both *in vitro* and *in vivo*, representing a potential candidate for combination therapy.

Future directions

Further research assessing the efficacy and cost-effectiveness of combination therapy with senolytics and/or CAR T cells is required. These therapies are preferable compared to those focussing exclusively on tumour senescence induction: although Wang et al. (2018) showed that treatment of HCT116 cells with 40 μM of the compound Baicalin reduced the colony survival by 5 fold ($p < 0.001$), senescent cells have been shown to be resistant to apoptosis (159–161). As shown in Figure 3, chemotherapy-treated tumour cells could become senescent and that some could escape and relapse. Moreover, there is a risk that these cells could become resistant to apoptosis, conferring senescent tumour cells a pro-survival advantage and therefore could promote tumour resistance to therapy. Therefore, it may be a better approach to utilise senolytics as a therapeutic strategy rather than tumour senescence induction. However, further investigation is needed to confirm this.

Further research should also assess the use of metformin to treat CRC patients with T2D. Metformin has been associated with decreased risk of CRC in diabetic patients and prognosis improvement in CRC patients with diabetes (162). For instance, Tarhini et al. (2020) showed that the use of metformin in patients with both CRC and T2D is associated with improved overall survival (adjusted hazard ratios [aHR] = 0.45, 95% CI = 0.21 - 0.96) and disease-free survival (aHR = 0.31; 95% CI = 0.18 - 0.54) (163). Furthermore, metformin can be considered as a senolytic drug: metformin treatment has been associated with increased tumour cell apoptosis and inhibition of SASP secretion

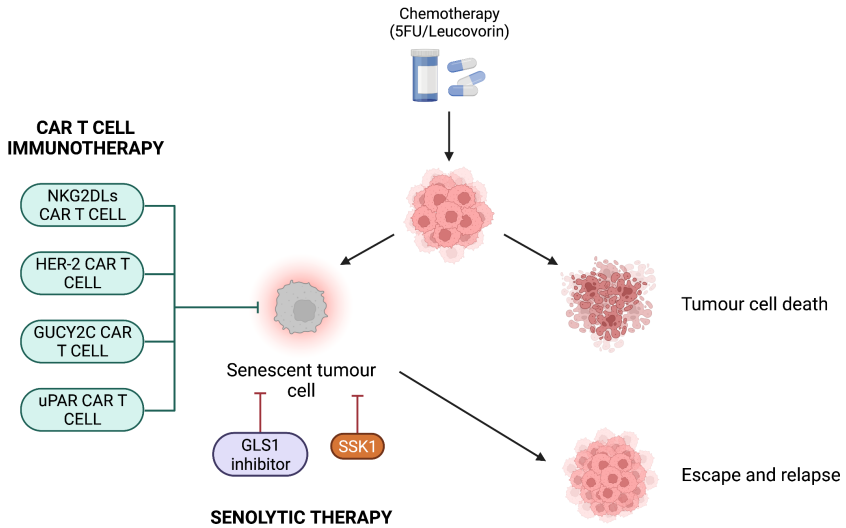


FIGURE 3
Potential combination therapies for colorectal cancer. After chemotherapeutic treatment, tumour cells are either killed or become senescent. CAR T cell immunotherapy or senolytic therapy can be used to avoid escape from the senescent state and tumour relapse. CAR T cell immunotherapy targets antigens present on colorectal cancer cells such as NKG2DLs, HER-2, GUCY2C and uPAR. Senolytic therapy targets the SA-β-Gal *via* the compound SSK1 or inhibits the mitochondrial enzyme GLS1, which is important for tumour cell metabolism.

(164–166). Thus, should metformin be considered as a therapeutic choice in patient with CRC and T2D?

Another area to explore is CRC immunology. Previously, we showed that tumour infiltrating T-, B- and IgA+ plasma cells play key roles in rectal cancer tumour microenvironment (19). For instance, $CD20^+$ TIL-B and IgA^+ cells demonstrated significant associations with long-term survival of patients with rectal cancer. Although we did not investigate T2D, in the future, it would be important to understand whether these immune cells are present in the tumour microenvironment of CRC patients with T2D. Additionally, Saito et al. (2020) reported that CRC and T2D patients treated with metformin induced structural changes and immune cell profile in the tumour microenvironment. It has been shown that metformin increases the immune cell ($CD3^+CD8^+$) infiltration and reduces the rate of M2-type tissue associated macrophages, and promotes stromal fibrosis in human CRC, which may result in an immunocompetent microenvironment from an immunosuppressive one (167). Thus, along with its senolytic activity, metformin could be utilised as a pro-immunogenic anti-tumour agent.

Conclusions

Cellular senescence represents a risk factor for colorectal cancer development in type 2 diabetic patients. The pathophysiological events occurring in type 2 diabetes contribute to the generation of premature cellular senescence. Research shows that senescent fibroblasts, endothelial cells and T cells release proinflammatory SASP that favours tumorigenesis, tumour growth and metastasis. However, some SASP have tumour inhibitory effect and therefore the overall outcome of senescence activity on the tumour microenvironment depends on the type and amount of SASP released. Senescence also occurs in tumour cells and enhances the ability of colorectal cancer cells to suppress and evade the host's immune response. Because conventional chemotherapy increases the risk of tumour senescence and relapse, new therapeutic approaches are required. Senolytic drugs showed favourable results in their ability to clear senescent cells, reducing the likelihood of tumour relapse and cancer immune resistance. In addition, CAR T cell immunotherapy against colorectal cancer showed promising outcomes in their tumour-killing ability both *in vitro* and *in vivo*. Thus, senolytic compounds and CAR T cells represent a potential candidate for combination therapy. Further research with the purpose to expand the knowledge in the field of senescence and cancer is required, allowing improvements for the diagnosis and treatment of senescence-associated diseases.

Author contributions

FM, PU and DZ drafted the manuscript. NA contributed with discussions and critical revision of the manuscript. MP, DZ and NA conceptualised the project. MP revised, edited, supervised and administered the project. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Efficacy and safety of immune checkpoint inhibitors-combined antiangiogenic drugs in the treatment of hepatocellular carcinoma: A systematic review and meta analysis

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Background: Hepatocellular carcinoma is a pathological type of liver cancer and accounts for the majority of primary liver cancers. We conducted a meta-analysis to evaluate the efficacy and safety of immune checkpoint inhibitors in combination with antiangiogenic drugs in the treatment of hepatocellular carcinoma.

Methods: We searched scientific literature databases and clinical trials databases through May 2022 for required studies. Progression-free survival was taken as the main outcome, and overall survival, response rate and adverse events as secondary outcomes. These data were extracted, combined and used for meta-analysis to compare the treatment effect and safety of immune checkpoint inhibitors combined with antiangiogenic drugs in patients with advanced/unresectable/metastatic hepatocellular carcinoma.

Results: This study included 3 randomized controlled trials and 6 single-arm trials of immune checkpoint inhibitors in combination with antiangiogenic drugs in hepatocellular carcinoma. Meta-analysis showed that compared with single use, combination of the two can significantly improve PFS (HR=5.93, 95% CI=5.41, 6.45) and OS (HR=15.84, 95% CI=15.39, 16.28). The ORR and DOR of patients with combination therapy were HR=19.11, 95% CI=15.99, 22.22 and HR=12.26, 95% CI=10.32, 14.21, respectively. Common adverse reactions to combination therapy included hypertension (26.8%), diarrhea (23.6%), fatigue (23.8%), decreased appetite (22.8%), hypothyroidism (9.9%), and rash (14.5%).

Conclusion: In the treatment of advanced/unresectable/metastatic hepatocellular carcinoma, immune checkpoint inhibitors combined with antiangiogenic drugs achieved better survival benefits than alone. In addition, the combination therapy has tolerable safety.

KEYWORDS

immune checkpoint inhibitors, antiangiogenic drug, combination therapy, hepatocellular carcinoma, tumor treatment

Introduction

Primary liver cancer is a malignant tumor of the digestive system with high incidence worldwide, and most patients are already in the advanced stage of cancer when they are found to have liver cancer. According to the new data released by GLOBOCAN 2020, the annual number of new cases of liver cancer in the world reached 906,000, ranking sixth among malignant tumors and 830,000 deaths, ranking third among malignant tumors (1). The main pathiogyical type of primary liver cancer is hepatocellular carcinoma (HCC), accounting for 85%-90% (2). HCC occurs in the liver which is severely damaged by chronic injury or inflammation (3–5).

Immune checkpoint inhibitors (ICIs) are new types of monoclonal antibodies. It works by inhibiting the function of inhibitory immune receptors and by stimulating the immune system's antitumor response (6). Cancer immunotherapy against antibodies against programmed cell death-1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis shows excellent effect in the treatment of liver cancer (7). Immune checkpoint inhibitors of PD-1/PD-L1 are important anti-tumor immunotherapy drugs, representing a major breakthrough in the treatment of advanced HCC. Tumor immunotherapy with PD-1 blockers shows a good effect in the treatment of liver cancer. The factors that affect the clinical outcome of PD-1 inhibitors include specific receptors, signal pathways and inflammatory genes. The findings of these factors suggest that researchers can use combination therapy to reduce the impact of other factors on the treatment effect of PD-1/PD-L1 inhibitors (8). At present, the National Drug Administration (NMPA) in China has approved the PD-1 antibodies for HCC indications: Camrelizumab, Tislelizumab, Sintilimab; and the PD-L1 antibody for HCC indications: Atezolizumab. PD-1 antibodies for FDA approval of HCC indications: Nivolumab, Pembrolizumab; PD-L1 antibodies for approval of HCC indications: Atezolizumab. The effect of immunologic drug monotherapy for unresectable HCC is unsatisfactory, and the ORR of PD-1 monotherapy for HCC is 17%-20%. But so far, the survival superiority of monotherapy with ICIs including PD-1 has not been demonstrated in randomized studies (9).

Tumor angiogenesis is a complex process because multiple signaling pathways are involved. Among them, vascular endothelial growth factor (VEGF)/VEGF receptor 2 (VEGFR2) signal pathway is one of the important pathways of tumor angiogenesis and plays an important role in regulating immune response. By disrupting blood vessel supply and starving tumors of nutrients and oxygen, antiangiogenic drugs are also a promising treatment. This is primarily achieved by blocking the VEGF/VEGF receptor VEGFR signaling pathway that is active in the tumor microenvironment under hypoxic conditions (10). Therefore, inhibition of this pathway can promote vascular normalization, increase lymphocyte infiltration in tumor, and attenuates the function of inhibitory immune cell phenotype. Currently, the global standard first-line systemic regimen for unresectable or metastatic HCC is VEGFR tyrosine kinase inhibitors. Although these drugs have a certain degree of survival benefit, they are accompanied by considerable toxicity. According to RECIST1.1, the objective response rate (ORR) of lenvatinib and sorafenib for unresectable HCC was 19% and 7%, respectively (9).

Various types of immune cells are present in the liver, and they produce different cytokines and growth factors in response to local stimuli. Thus, these immune cells establish an immune microenvironment to maintain a balance between immune tolerance and hepatic immune activation (11). The high efficacy of combination therapy with ICIs and antiangiogenic drugs is not only due to their additive effects on tumor growth, but also because both focus on targeting the tumor microenvironment, reprogramming the immunosuppressive microenvironment into an immunostimulatory one. Among them, the reason why VEGF inhibitors can reprogram the immunosuppressive tumor microenvironment into an immunostimulatory environment is that such drugs can increase the antigen presentation of dendritic cells, promote the activation of T cells in the priming phase, and improve T cells. Migration from lymph nodes to tumor sites. Furthermore, these drugs inhibit the generation of Tregs, TAMs and MDSCs at tumor sites and negatively regulate the expression of immunosuppressive cytokines. Therefore, the combination of ICIs and antiangiogenic agent exhibits a potential synergistic anti-tumor effect (12, 13). At present, there are a number of clinical trials to verify whether the addition of antiangiogenic drugs can improve the efficacy of immune checkpoint inhibitors in tumor treatment. And when used in the comprehensive treatment of liver cancer, impressive anti-tumor effects have been observed (14–16). For example, the median survival time involved in the natural progression of disease in patients with advanced HCC is about 8 months, while the combination of anti-PD-L1 antibodies-atrazumab and anti-VEGF antibodies-bevacizumab more than doubled this life expectancy and improved patient reported outcome indicators (17).

Although the combination therapy of ICIs and antiangiogenic drugs has shown stronger antitumor activity in hepatocellular carcinoma, the clinical trials of the related combination have been reported in small population sizes and with specific series of Adverse events are not fully defined, including evidence of increased risk of gastrointestinal, cutaneous, and vascular events. Importantly, there has been no systematic attempt to synthesize data on the efficacy and safety of combination therapy with these drugs, and considering that the combination therapy of ICIs and antiangiogenic drugs offers new hope in the treatment of HCC, we believe that it is crucial to clarify the efficacy and safety of these drug combinations for cancer treatment. Therefore, we conducted this systematic review and meta-analysis after searching extensive literature to analyze the treatment efficacy and safety of ICIs-combined antiangiogenic drugs in advanced/unresectable/metastatic HCC.

Materials and methods

Search strategy

We have carried out a systematic literature search in electronic databases (Pubmed, Web of Science, Cochrane Library), and the final search time is up to May 2022. The retrieval is carried out by the combination of subject headings and free words, and adjusted according to the characteristics of each database. The search strategy mainly includes three parts: (1) Words related to 'immune checkpoint inhibitors': (ie 'immune checkpoint inhibitors', 'PD-1 inhibitors', 'PD-L1 inhibitors', 'nivolumab', 'pembrolizumab', 'sintilimab', 'camrelizumab', 'toripalimab', 'tislelizumab', 'atezolizumab', 'durvalumab', 'avelumab'). (2) Words related to 'liver cancer': (ie 'Liver cancer', 'Hepatocellular carcinoma'). (3) The search filter is set to 'Clinical Trial'.

Inclusion and exclusion criteria

Potential trials were screened on the basis of: (1) expected Phase I, II and III clinical trials and expanded access (i.e., external clinical trials) programs; (2) clinical investigations of patients with hepatocellular carcinoma and the fact that some participants have been assigned to PD-1/PD-L1 in combination with antiangiogenic drugs; and (3) recording adverse event-related events or efficacy comparisons.

Data extraction and definitions

In order to understand all the baselines included in the study, we extracted the following information: the first author, the number of patients involved in the study, treatment scheme, and the basic characteristics of the participants. The main outcome is progression-free survival (PFS), the secondary outcome is overall survival (OS), objective response rate (ORR), and duration of response (DOR). The response criteria of the above extracted data are all RECIST1.1. Based on the safety results of a combination of immune checkpoint inhibitors and antiangiogenic drugs, we considered the following adverse events of grade 1-2 as clinical endpoints: hypertension, diarrhea, fatigue, decreased appetite, hypothyroidism and rash.

Evaluation of quality

The collected RCTs and nonrandomized studies were assessed using the Jadad scoring tool (18) and the nonrandomized study methodological index (19), respectively. RCTs with scores ≥4 and nonrandomized studies with scores ≥8 were considered high-quality reports; RCTs with scores ≤3 and nonrandomized studies with scores <8 were considered low-quality reports. All included studies were assessed to have a low risk of bias (20). The results of quality evaluation are illustrated in Table 1. As shown in the table, 3 RCTs and 6 nonrandomized controlled studies were of high quality.

Statistical analysis

Stata16.0 and Revman 5.4 of 64-bit Windows were used for statistical analysis. The difference between combination therapy and single treatment was estimated by combining effect size or HR (hazard ratio) and 95% confidence interval (CI). Effect sizes, HR and OR estimates were summarized using random or fixed-effects models, and heterogeneity between studies was assessed by P-value and I^2 statistic, with a threshold of $p<0.1$. Homogeneous data ($I^2<50\%$) were pooled with a fixed-effects model, and heterogeneous data ($I^2\geq50\%$) were pooled with a random-effects model. The symmetry of visual observations of funnel plots and the Egger's test were used to assess publication bias.

Result

Study selection

We searched the database for 1438 studies (including 315 in PubMed, 179 in Web of Science, and 944 in Cochrane Library). After eliminating duplicates (n=769), browse and filter the titles and abstracts. The remaining 24 studies were screened in full text, and 7 articles were finally included according to the inclusion criteria. The flow chart of the following search strategy is shown in Figure 1.

TABLE 1 Basic characteristics of studies included in this meta-analysis.

Author (issuing time)	Trial design	Patients, N	Age, years	Characteristics of hepato-cellular carcinoma	Interventions		Qualityscore (Jadad/ MINORS)
					Experiment group	Control group	
Yung-Jue Bang, 2020 (21)	ST phase Ia/ b	28	63 (27~87)	advanced / metastatic	Durvalumab+ Ramucirumab	None	10 (full score: 16 points)
Hoffmann-La Roche Ltd 2020 (22)	RCT phase III	558	(18~85+)	advanced / metastatic	Atezolizumab + Bevacizumab	Sorafenib	4 (full score: 7 points)
Zhenggang Ren 2020 (23)	ST Phase II	24	(18+)	unresectable / metastatic	Sintilimab + Bevacizumab biosimilar	None	9 (full score: 16 points)
	RCT phase III	571	Sintilimab–bevacizumab biosimilar group: 53 (21~82) Sorafenib group: 54 (28~77)	unresectable / metastatic	Sintilimab + Bevacizumab biosimilar	Sorafenib	5 (full score: 7 points)
Michael S Lee, 2020 (24)	ST phase Ib	104	62 (23~82)	unresectable	Atezolizumab + Bevacizumab	None	10 (full score: 16 points)
	RCT phase Ib	119	Atezolizumab plus bevacizumab (n=60): 60 (22~82); Atezolizumab monotherapy (n=59): 63 (23~85)	unresectable	Atezolizumab + Bevacizumab	Atezolizumab	5 (full score: 7 points)
Masatoshi Kudo, 2021 (25)	ST phase Ib	22	68.5 (20~84)	advanced / metastatic	Avelumab + Axitinib	None	9 (full score: 16 points)
Richard S. Finn, 2020 (26)	ST phase Ib	100	66.5 (47~86)	unresectable	Pembrolizumab + Lenvatinib	None	9 (full score: 16 points)
Jianming Xu, 2019 (27)	ST phase Ia/ b	18	49 (29-64)	advanced	SHR-1210 + Apatinib	None	10 (full score: 16 points)

(RCT, randomized-controlled trial; ST, single-arm trial).

Study characteristics

This systematic review includes 9 studies, including 3 randomized controlled trials and 6 single-arm trials, all published between 2019 and 2021. The clinical trial patients in the searched literature were advanced/unresectable/metastatic hepatocellular carcinoma patients. Among them, patients received PD-1/PD-L1 inhibitors including avelumab, pembrolizumab, durvalumab, atezolizumab, SHR-1210 (anti-PD-1 antibody), sintilimab and antiangiogenesis inhibitors including axitinib, lenvatinib, ramucirumab, bevacizumab, apatinib, bevacizumab biosimilar. The basic characteristics of the studies included in this meta-analysis are shown in Table 1.

Publication bias test

Publication bias was assessed for 8 clinical trials in the included seven articles. The funnel plot shows that most studies are in the upper part of the 'inverted funnel' and fewer studies are in the base, and the left and right are basically symmetrical. Egger's test showed that P=0.780>0.05, so the included study could not be considered to have publication bias. See Figure 2 funnel plot and Figure 3 Egger's test result chart for details.

Median progression-free survival and overall survival

Three studies (21–23) involving 610 patients with advanced/ unresectable/metastatic HCC reported median OS. The OS of the included studies were HR=15.84, 95% CI=15.39, 16.28, I^2= 68.3%, p=0.043. Clinical trials of F. Hoffmann-La Roche Ltd (22) showed that the OS of PD-1 combined with antiangiogenic drugs and without PD-1 were HR=19.22, 95% CI=17.02, 23.66 and HR=13.40, 95% CI=11.37, 16.85.

All studies involving 1,520 patients with advanced/ unresectable/metastatic HCC reported median PFS. The PFS of the included studies were HR=5.93, 95% CI=5.41, 6.45, I^2=

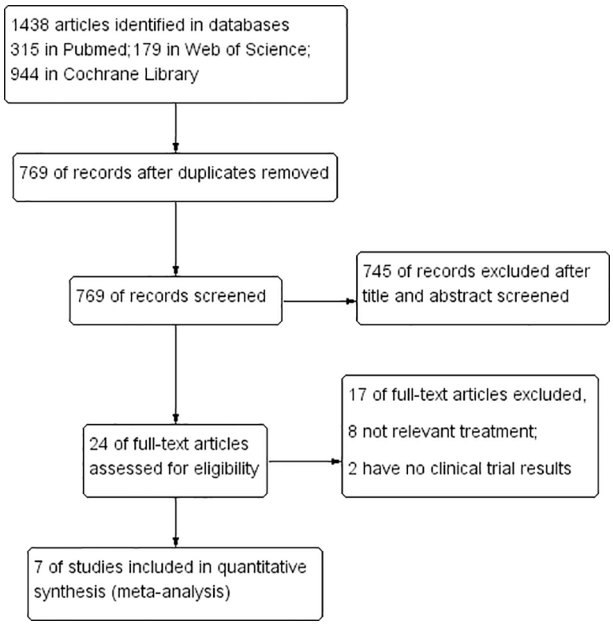


FIGURE 1
The Prisma search strategy flowchart followed in article search and selection in this study. This systematic review includes 7 studies, including 3 randomized controlled trials and 6 single-arm trials..

76.3%, p=0.000. Research by Michael S Lee et al. (24) showed that the PFS of PD-1 combined with antiangiogenic drugs and without antiangiogenic drugs were HR=5.6, 95% CI=3.6, 7.40 and HR=3.40, 95% CI=1.90, 5.20. Clinical trials of F. Hoffmann-La Roche Ltd (22) showed that the PFS of PD-1 combined with antiangiogenic drugs and without PD-1 were HR=6.83, 95% CI=5.75, 8.28 and HR=4.27, 95% CI=3.98, 5.55. Research by Zhenggang Ren et al. (23) showed that the PFS of PD-1 combined with antiangiogenic drugs and without PD-1 were HR=4.60, 95% CI=4.10, 5.70 and HR=2.80, 95% CI=2.70, 3.20.

A pooled analysis of OS and PFS in HCC patients treated with immune checkpoint inhibitors combined with antiangiogenic drugs is shown in Figures 4, 5.

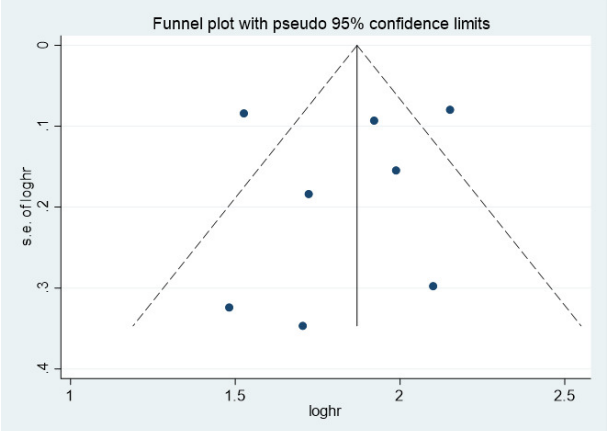
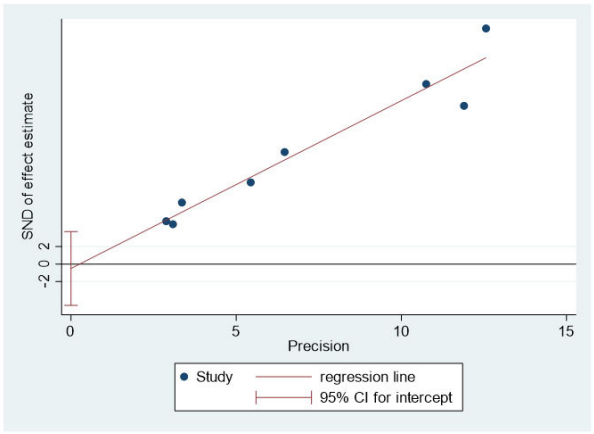


FIGURE 2
The funnel plot of the risk of bias. SE, standard error.



Number of studies = 8 Root MSE = 2.331

Std_Eff	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
slope	1.925011	.2152929	8.94	0.000	1.398209	2.451814
bias	-.5056488	1.728186	-0.29	0.780	-4.734368	3.723071

FIGURE 3
Egger's test results.

Response rate

The meta-analysis showed that the combined ORR (22, 25–27) and DOR (25, 27) of PD-1 combined with antiangiogenic inhibitors in hepatocellular carcinoma was HR=19.11, 95% CI=15.99, 22.22, I^2= 92.7%, p=0.000 and HR=12.26, 95% CI=10.32, 14.21, I^2= 95.7%, p=0.000. Clinical trials of F. Hoffmann-La Roche Ltd (22) showed that the ORR of PD-1 combined with antiangiogenic drugs and without PD-1 were HR=27.30, 95% CI=22.54, 32.48 and HR=11.90, 95% CI=7.35, 18.03.

A pooled analysis of ORR and DOR in advanced/unresectable/metastatic HCC patients treated with immune checkpoint inhibitors combined with antiangiogenic drugs is shown in Figures 6, 7.

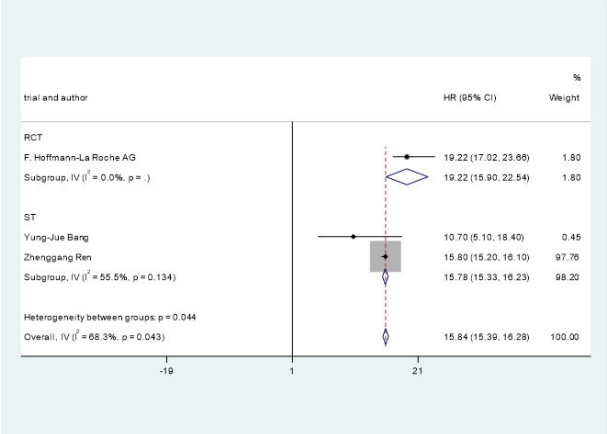


FIGURE 4
Pooled analysis of OS in patients with HCC treated with immune checkpoint inhibitors combined with antiangiogenic drugs..

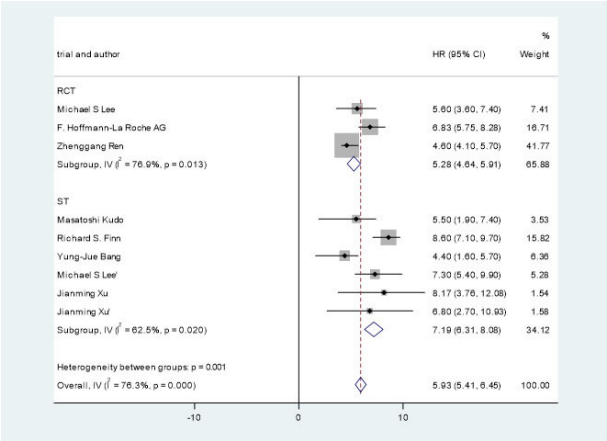


FIGURE 5
Pooled analysis of PFS in patients with HCC treated with immune checkpoint inhibitors combined with antiangiogenic drugs.

Adverse events

Adverse events of grade 1-2 common to ICIs in combination with antiangiogenic drugs in the treatment of hepatocellular carcinoma are shown in Table 2, including hypertension (26.8%, 95% CI=15.3%, 38.2%), diarrhea (23.6%, 95% CI=15.3%, 31.8%), fatigue (23.8%, 95% CI=19.3%, 28.3%), decreased appetite (22.8%, 95% CI=14.4%, 31.2%), hypothyroidism (15.1%, 95% CI=9.7%, 20.4%), rash (14.5%, 95% CI=9.2%, 19.8%). Common adverse reactions of immune checkpoint inhibitors combined with antiangiogenic drugs in the treatment of HCC are shown in Table 2.

The 3 RCTs mentioned above were included in the meta-analysis. Meta-analysis indicated that compared with monotherapy, PD-1/PD-L1 inhibitor combined with antiangiogenic drugs had a higher incidence of grade 1-2 hypertension (OR=1.56, 95%CI 1.11-2.19, I^2= 21%, P=0.01), as illustrated in Figure 8. In addition, the analysis results showed that compared with monotherapy, PD-1/PD-L1 inhibitor combined with anti-angiogenic drugs had lower incidences of diarrhea (OR=0.43, 95%CI 0.29-0.64, I^2= 65%, P<0.0001), as illustrated in Figure 9. Moreover, there was no statistical difference in the incidence of decreased appetite and rash between the single drug and the combined drug, respectively

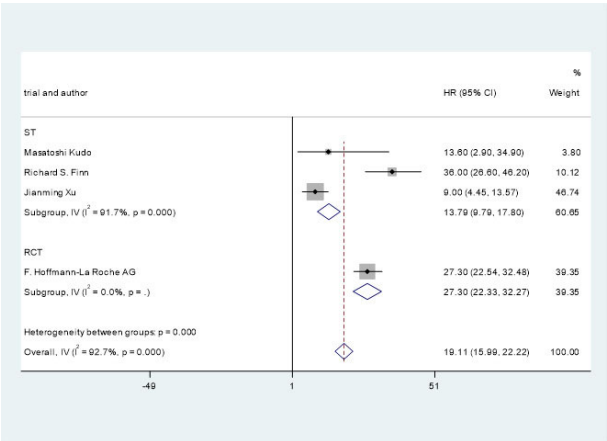


FIGURE 6
Pooled analysis of ORR in patients with HCC treated with immune checkpoint inhibitors combined with antiangiogenic drugs.

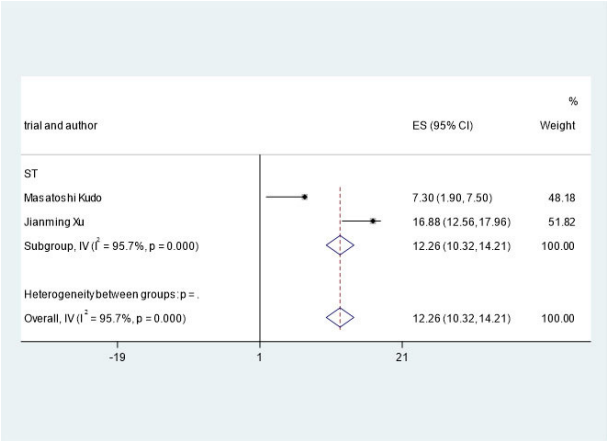


FIGURE 7
Pooled analysis of DOR in patients with HCC treated with immune checkpoint inhibitors combined with antiangiogenic drugs.

(OR=0.83, 95%CI 0.63-1.09, I^2= 0%, P=0.18), (OR=0.88, 95%CI 0.48-1.62, I^2= 66%, P=0.69), as shown in Figures 10, 11.

Discussion

Hepatocellular carcinoma as one of the most common fatal tumors in the world, its fatality rate ranks the third in the world, the incidence ranks sixth, and is increasing year by year. The treatment of hepatocellular carcinoma needs to be based on the stage of the lesion, whether it is metastatic, the extent of metastasis, the patient's physical condition, and their willingness to accept treatment methods. Targeted therapy for hepatocellular carcinoma first needs to identify the genetic mutation of this cancer. Based on mutations in the driver genes of hepatocellular carcinoma found by second-generation genetic testing, it is still unclear whether selective inhibition of these mutations can produce better clinical efficacy. For hepatocellular carcinoma, a major bottleneck is that there are no drugs that inhibit the most common genetic mutations in hepatocellular carcinoma, such as the TERT promoter, TP53, CTNNB1, AXIN1, ARID1A, or ARID2. However, the current clinical trial design of targeted therapy for hepatocellular carcinoma seldom considers genome-directed stratification, so this aspect needs urgent attention (28).

The existence of various types of immune cells in the liver establishes an immune microenvironment that strongly affects the occurrence and development of tumors. Using single-cell RNA sequencing, some studies have identified subsets of immune cells that may have distinct immune functions, and patients with certain subsets of features have significantly better outcomes. Therefore, the efficacy of immunotherapy is determined in part by the individual's immune microenvironment. By specifically inhibiting PD-1, PD-L1 and CTLA-4, immunotherapy breaks the tumor immune tolerance mechanism and effectively delays tumor progression (11, 16). In recent years, as an important member of T lymphocyte immune deficiency related immunoglobulin superfamily costimulatory molecules, PD-1/PD-L1 contribute significantly to tumor immune escape. Anti-PD-1/PD-L1 therapy has become the most promising immunotherapy for hepatocellular carcinoma. The PD-1/PD-L1 inhibitors used in the studies included in this meta-analysis include avelumab, pembrolizumab, durvalumab, atezolizumab, SHR-1210 (anti-PD-1 antibody), and sintilimab. Among them, Avelumab, Atezolizumab, Durvalumab are humanized anti-PD-L1 IgG1 monoclonal antibodies. While

TABLE 2 The common adverse events of immune checkpoint inhibitors combined with antiangiogenic drugs in the treatment of HCC.

Adverse events (Any grade)	ES	95% CI	Model	I^2	P
Hypertension	26.8%	15.3%, 38.2%	Random-effects	95.2%	0.000
Diarrhea	23.6%	15.3%, 31.8%	Random-effects	89.0%	0.000
Fatigue	23.8%	19.3%, 28.3%	Fixed-effects	32.2%	0.000
Decreased appetite	22.8%	14.4%, 31.2%	Random-effects	90.4%	0.000
Hypothyroidism	15.1%	9.7%, 20.4%	Random-effects	72.2%	0.006
Rash	14.5%	9.2%, 19.8%	Random-effects	80.1%	0.000

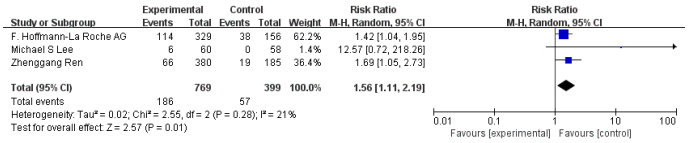


FIGURE 8
Forest plot of the incidence of hypertension (grade 1-2) in patients with HCC treated with ICIs and antiangiogenic drugs in combination or alone.

Pembrolizumab and sintilimab are humanized anti-PD-1 IgG4 monoclonal antibodies. The efficacy and safety of PD-1/PD-L1 inhibitors from different sources alone and in combination in the treatment of hepatocellular carcinoma need to be further studied and discussed.

In the process of tumor growth and metastasis, angiogenesis is vital. Due to abnormal perfusion and increased permeability, newborn tumor vessels will lead to tissue hypoxia, lactic acid increase and necrosis, and then activate immunosuppression and inhibit the function of effector T cells. Antiangiogenic drugs can disrupt the vascular supply by blocking the VEGF/VEGFR signal pathway, the tumor is deficient in nutrients and oxygen. However, its effect on the overall survival rate of cancer patients is limited, and it rarely produces a lasting response (29). Additionally, crosstalk and VEGFR signaling downstream of the immune checkpoint axis may lead to synergistic effects of combination therapy on tumor cells (13). The antiangiogenic drugs used in the trials included in this Meta-analysis include axitinib, lenvatinib, ramucirumab, bevacizumab, apatinib, and bevacizumab biosimilar. Among them, axitinib, lenvatinib, and apatinib are small-molecule multi-target angiogenesis inhibitors, while ramucirumab and bevacizumab are macro-molecule single-target angiogenesis inhibitors. Axitinib is particularly specific for VEGFR, PDGFRβ and c-Kit. Lenvatinib inhibits angiogenesis by inhibiting the activity of VEGFR1, VEGFR2, VEGFR3, FGFR1-3, KIT, PDGFRα and RET. Apatinib targets include VEGFR1, VEGFR2, VEGFR3, PDGFR-β, C-KIT, FGFR1 and FLT3. Bevacizumab is a recombinant humanized monoclonal IgG antibody that can specifically bind to VEGF, block the binding of VEGF and its receptors, reduce angiogenesis, induce the degeneration of existing blood vessels, and thereby inhibit the tumor growth. Ramucirumab is a fully humanized IgG1 monoclonal antibody that selectively binds to the extracellular region of VEGFR2 and prevents the phosphorylation of VEGFR2. It is the only VEGFR2 monoclonal antibody that has been marketed in the world. Bevacizumab is an anti-vascular endothelial growth factor monoclonal antibody that specifically binds to VEGF-A and blocks angiogenic cell pathways. It is the world's first approved anti-tumor angiogenesis targeted drug and the first recombinant humanized anti-VEGF monoclonal antibody. Different targets of the above anti-angiogenic drugs may exert different therapeutic effects, which also requires in-depth research in order to obtain the best treatment plan.

Both immunotherapeutic drugs and antiangiogenic drugs act on tumor microenvironment, and they have synergistic effect in theory. The mechanism of immune combined antiangiogenic therapy may include the following four aspects: a) antiangiogenic drugs reduce the activity of myelogenous suppressor cells and regulatory T cells and reshape the tumor microenvironment; b) antiangiogenic drugs block the VEGF-mediated inhibition of dendritic cell maturation, which makes T cells binding to tumor antigens start and activate more effectively; c) antiangiogenic drugs normalize tumor vascular structure and promote T cell infiltration into the tumor; d) antiangiogenic drugs restore anti-tumor immune function by killing tumor cells mediated by T cells. The combination of PD-1/PD-L1 and VEGF antibodies works well not only because of their additive effect on tumor growth inhibition, but also because they reprogram the immunosuppressive microenvironment to immunostimulatory microenvironment (30). In addition, studies have found that antiangiogenic drugs can induce the formation of high endothelial venules (HEV), and HEV is generally considered to be involved in lymphocyte homing.

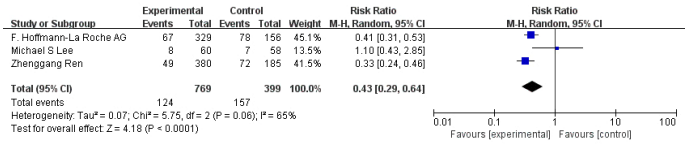


FIGURE 9
Forest plot of the incidence of diarrhea (grade 1-2) in patients with HCC treated with ICIs and antiangiogenic drugs in combination or alone.

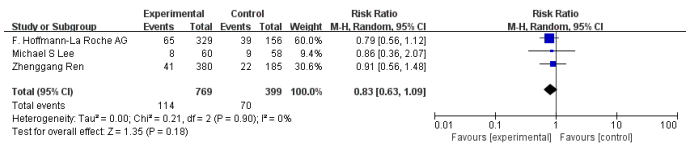


FIGURE 10
Forest plot of the incidence of decreased appetite (grade 1-2) in patients with HCC treated with ICIs and antiangiogenic drugs in combination or alone.

Therefore, researchers speculate that intratumor HEV will similarly promote T cell infiltration of tumors, tumor-associated high endothelial venules (TA-HEVs) are the main pathway for lymphocytes to enter tumors (31–33). In summary, immune checkpoint inhibitors combined with anti-angiogenic drugs have a synergistic mechanism in tumor treatment.The combined use of antiangiogenic drugs and immune checkpoint inhibitors still has some challenges to be solved. First, the normalization of tumor blood vessels induced by anti-tumor angiogenesis has a window period, and how to define the window period is still inconclusive. Second, how to optimize the dosage and administration frequency of anti-angiogenic drugs in combination therapy to avoid excessive inhibition of angiogenesis and bring maximum survival benefit to patients. In addition, although the expression level of PD-1/PD-L1, tumor mutation load, etc. can screen for dominant patients to a certain extent, more evidence shows that other components of the tumor microenvironment also play a role in determining the effectiveness of tumor immunotherapy. However, there are currently no biomarkers to guide the use of anti-angiogenic drugs, so combination therapy requires a more systematic evaluation method to pinpoint the benefit population.

This study shows that ICIs combined with antiangiogenic drugs can potentially improve OS and PFS in patients with advanced/unresectable/metastatic HCC. The OS and PFS of the patients treated with immune checkpoint inhibitors combined with antiangiogenic drugs were HR=15.84, 95% CI=15.39, 16.28, I^2= 68.3%, p=0.043 and HR=5.93, 95% CI=5.41, 6.45, I^2= 76.3%, p=0.000, respectively. Studies by Michael S Lee et al. (24) have shown that immune checkpoint inhibitors combined with antiangiogenic drugs can improve PFS from 3.4 months to 5.6 months. F.Hoffmann-La Roche Ltd (22) studies have indicated that immune checkpoint inhibitors combined with antiangiogenic drugs can improve PFS from 4.27 months to 6.83 months. The study by Zhenggang Ren et al. (23) showed that the combination of ICIs with antiangiogenic drugs improve PFS from 2.8 months to 4.6 months. The study by F. Hoffmann-La Roche Ltd (22) showed that the combination of immune checkpoint inhibitors with antiangiogenic drugs can improve OS from 13.40 months to 19.22 months.

The ORR and DOR of the patients treated with immune checkpoint inhibitors combined with antiangiogenic drugs were HR=19.11, 95% CI=15.99, 22.22, I^2= 92.7%, p=0.000 and HR=12.26, 95% CI=10.32, 14.21, I^2= 95.7%, p=0.000, respectively. The study by F. Hoffmann-La Roche Ltd et al (22) showed that the combination of immune checkpoint inhibitors with antiangiogenic drugs improved ORR from 11.9 months to 27.3 months.

The incidence of adverse events (grade 1-2) in patients with advanced/unresectable/metastatic HCC treated with immune checkpoint inhibitors combined with antiangiogenic drugs included hypertension (26.8%), diarrhea (23.6%), fatigue (23.8%), decreased appetite (22.8%), hypothyroidism (15.1%) and rash (14.5%). At the same time, the analysis results of RCTs may indicate that the combination therapy can reduce the incidence of diarrhea to a certain extent, and the incidence of decreased appetite and rash is not significantly different from that of single therapy. For other adverse reactions of combination therapy, clinicians and pharmacists can reduce the impact on patients' medication compliance through medication monitoring and timely treatment. Therefore the above data reflects the tolerable safety of the combination of the two. This is presumably due to the normalization of blood vessels by antiangiogenic drugs, which improves the delivery of

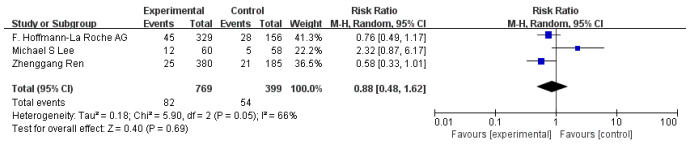


FIGURE 11
Forest plot of the incidence of rash (grade 1-2) in patients with HCC treated with ICIs and antiangiogenic drugs in combination or alone.

therapeutic drugs to the tumor, thereby reducing the dose of ICIs and reducing the risk of immune-related adverse effects.

Based on the funnel plot and Egger's publication bias test, it is evident that the included article is not biased by publication.

The limitations of this study include: (1) there are few relevant randomized controlled trials; (2) the treatment cycles of the trials included are different; (3) the regimens of ICIs combined with antiangiogenic drugs are not uniform; (4) some of the I^2 values in this meta-analysis were large, implying heterogeneity between studies; (5) subgroup analyses were not included in this meta-analysis; (6) there is currently a lack of sensitive and effective biomarkers for predicting antiangiogenic drugs combined with ICIs, hindering the adjustment of regimens in certain conditions. After overcoming these problems, the efficacy and safety of immune checkpoint inhibitors combined with antiangiogenic drugs in the treatment of advanced/unresectable/metastatic hepatocellular carcinoma may be clearer.

Conclusion

To sum up, in the treatment of advanced/unresectable/metastatic hepatocellular carcinoma, the combination of immune checkpoint inhibitors and antiangiogenic drugs achieved better survival benefits than single use. In addition, the combination therapy has tolerable safety. This meta-analysis involves advanced/unresectable/metastatic hepatocellular carcinoma patients, thus providing new treatment options for patients with advanced HCC and new hope for the treatment prospects in this field. However, more RCTs are needed for further research. And the timing or sequence of each drug in combination and optimal regimens is unclear, nor is the optimal dose of each drug. In the future, basic research on the mechanism of the positive feedback loop between ICIs and antiangiogenic drugs should be increased and strengthened to help develop new prescriptions and design clinical studies. At the same time, biomarkers should be tested concurrently in more clinical trials. With the increase of effective evidence, the clinic can better decide the timing and sequence of administration of the combination to improve the efficacy and reduce the toxic and side effects.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

The corresponding author SL is responsible for proposing research directions and innovations, the first author YZ completes the main research work and the writing of the paper, and the second and third authors assist in literature retrieval and article revision. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Primary hepatopancreatobiliary lymphoma: Pathogenesis, diagnosis, and management

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Primary hepatopancreatobiliary lymphoma (PHPBL) is extremely rare, which is defined as a lympho-proliferative disease confined to the hepatobiliary system and pancreas without any involvement of lymph nodes, bone marrow, or other organs. The clinical and imaging manifestations of PHPBL are variable and non-special, which are akin to those of tumors of the hepatobiliary and pancreatic systems. The overall prognosis and management of PHPBL differ from those of other tumors in the hepatobiliary system and pancreas. Proper diagnosis and prompt treatment are essential for improving clinical outcomes. Due to its rarity, the optimal treatment has not been issued. However, combination chemotherapy is considered as a standard treatment for them. This review provides an overview of the pathogenesis, diagnosis, pathology, and management of PHPBL and offers clinicians the diagnosis and management schedule for PHPBL.

KEYWORDS

primary hepatic lymphoma, primary biliary lymphoma, primary pancreatic lymphoma, diagnosis, treatment

Introduction

Lymphoma accounts for about 4.8% of newly diagnosed cases of cancer in the United States, 90% of which is non-Hodgkin lymphoma (NHL), and about 3.6% of all cancer deaths (1). Lymphoma is traditionally classified as Hodgkin lymphoma (HL) and NHL. They can both locate in sites except for the lymphatic system, which is commonly found

in NHL (2). The most common site of extranodal involvement is the gastrointestinal tract, particularly stomach and small bowel (3). Involvement of hepatobiliary and pancreatic system is rare and is divided into primary and secondary lymphoma (4). Primary hepatopancreatobiliary lymphoma (PHPBL) is a rare entity, which has a lower incidence than secondary hepatopancreatobiliary lymphoma (SHPBL), commonly presented in widespread lymphoma.

The commonly accepted diagnosis criteria for PHPBL, as defined by previous studies, include a mass in the hepatobiliary and pancreatic systems, no enlargement of superficial and mediastinal lymph nodes, no involvement of other organs, and normal leukocyte count in the peripheral blood smear (5, 6).

Primary hepatic lymphoma (PHL), primary biliary lymphoma (PBL), and primary pancreatic lymphoma (PPL) are rare and account for 0.4%, 0.4%, and 1% of extranodal non-Hodgkin's lymphomas, respectively (7–9). PHL and PPL both affect middle-aged people, with a marked male preponderance, whereas PBL often affect elderly individuals, with a sight female preponderance (10–12). Due to their non-specific clinical manifestations and radiological features, PHPBL is always confused with other diseases of the hepatobiliary system and pancreas. The discrepancy in their treatment and prognosis makes it important to achieve a proper diagnosis.

In this article, we will discuss the pathogenesis, epidemiology, clinical presentation, imaging feature, pathological finding, and treatment of PHPBL (Tables 1–3). We aim to raise clinicians' awareness of the possibility of PHPBL, when they meet a patient with a mass in the hepatobiliary system and pancreas, and offer them the diagnosis and management schedule for PHPBL.

Primary hepatic lymphoma

Risk factors and pathogenesis

The exact risk factors are not clear and several studies have suggested that chronic liver diseases play an etiological role in the development of PHL, including Epstein–Barr virus (EBV), hepatitis B or C virus infection, liver cirrhosis, and primary biliary cirrhosis (13–17). Like gastric mucosa-associated lymphoid tissue (MALT) lymphoma, primary MALT lymphoma of liver may also be associated with *H. pylori* infection (18). In addition, PHL could present in patients with autoimmune diseases (19, 20), those with acquired immune deficiency syndrome (AIDS), and immunosuppressive drug-treated transplant recipients (21–23).

In all conditions, chronic inflammation of the liver represents the common steps in the pathogenesis of PHL, which induce lymphocyte migration to liver, mediated by some adhesion molecules, and cause B lymphocyte chronic proliferation, eventually leading to hepatic lymphoma (24, 25). Lack of T-cell surveillance is also cited as an inciting factor.

Among all these conditions, hepatitis C virus (HCV) infection is strongly associated with PHL (26), which causes malignant transformation by indirect methods. HCV, a lymphotropic virus, brings about chronic stimulation of B-cell and polyclonal proliferation, eventually leading to hepatic lymphoma. It may also induce a t (14, 18) translocation resulting in overexpression of BCL-2, an anti-apoptotic factor, and rearrangement of monoclonal IgH. Furthermore, through the viral core proteins, it can downregulate the transcription of tumor suppressor genes like p21, p53, and Ras (24).

TABLE 1 Summary of the main features of primary hepatic lymphoma.

Histotype	Clinical manifestations	Imaging findings	Pathology	Immunophenotype	Behavior	Treatment
DLBCL	Abdominal pain; B symptom; nausea, vomiting; jaundice	A solid mass/ multiple masses	Diffuse infiltration of large lymphocytes with prominent nucleoli and increased or atypical mitotic figures	CD20+, CD79a+, CD19+, CD3- GC group: CD10+ or/ and BCL-6+, MUM-1- Non-GC group: MUM-1+, BCL-6-, CD10-	Aggressive	Surgery+ chemotherapy (CHOP/R-CHOP) +/- radiation; chemotherapy; surgery
BL			Diffuse infiltration of medium-sized lymphocytes, with distinct nucleoli, scant cytoplasm, and increased mitotic figures; starry-sky appearance	Monotypic sIgM+, CD20+, CD10+, CD43+, BCL-6+; CD3-, CD5-, CD23-, BCL-2-		
MALT		A solid mass/ multiple masses in portal fields of the liver	Diffuse infiltration of small-sized lymphocytes, with mildly irregular nucleoli, dense chromatin and scant cytoplasm, without germinal center differentiation; lymphoepithelial lesions in bile duct	CD20+, CD79α+, Bcl-2+, CD21+ CD3-, CD10-, CD5-, CD23-, CD43-, and cyclinD1-	Mostly indolent	
FL		A solid mass/ multiple masses	Distinct follicles, formed by small-to-medium-sized centrocytes; follicular meshworks	CD20+, CD10+, BCL-2+, BCL-6+, CD23+, CD21+		

*DLBCL, diffuse large B-cell lymphoma; BL, Burkitt lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma.

TABLE 2 Summary of the main features of primary biliary lymphoma.

Histotype	Clinical manifestations	Imaging findings	Pathology	Immunophenotype	Behavior	Treatment
DLBCL	Obstructive jaundice; abdominal pain; B symptom; nausea, vomiting	A solid mass/ multiple masses	Diffuse infiltration of large cell with prominent nucleoli, numerous mitoses	CD20+, CD79a+, CD19 +, CD3-, CD45- GC group: CD10+ or/ and BCL-6+, MUM-1- Non-GC group: MUM-1 +, BCL-6-, CD10-	Aggressive	Surgery+ chemotherapy (CHOP/R-CHOP) +/- radiation; chemotherapy; surgery
MALT		An irregular thickening in the gallbladder or bile duct wall	Diffuse infiltration of small cell with intact mucosal layer and occasional lymphoid follicles; lymphoepithelial lesions	CD20+, CD79a+, CD19 +, BCL-6-, CD10-, cyclin D1-	Mostly indolent	
FL			Infiltration of small or medium cleaved lymphocytes, forming follicles; follicular meshworks	CD20+, CD10+, BCL-2+, CD23+, CD3-, CD5-		

*DLBCL, diffuse large B-cell lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma.

TABLE 3 Summary of the main features of primary pancreatic lymphoma.

Histotype	Clinical manifestations	Imaging findings	Pathology	Immunophenotype	Behavior	Treatment
DLBCL	Abdominal pain; jaundice; B symptoms; acute pancreatitis; duodenal obstruction; nausea, vomiting;	A solid mass/multiple masses/ diffuse enlarged pancreas without marked dilation of pancreatic duct and vascular infiltration	Diffuse infiltration of large atypical B cells with round nuclei, dispersed chromatin, some marked nucleoli, and scant cytoplasm	CD20+, CD79a+, CD19+, CD5-, cyclin D1-, CD30-, CD56- GC group: CD10+ or/ and BCL-6+, MUM-1- Activated GC group: CD10+ or/and BCL-6+, MUM-1+ Activated non-GC group: CD10-, BCL-6-, MUM-1+	Aggressive	Surgery+ chemotherapy (CHOP/R-CHOP) +/- radiation; chemotherapy; surgery
FL			Infiltration of the small or medium-sized cleaved cells forming uniform and dense follicles	CD20+, CD10+, CD19+, BCL-2+, BCL-6+; CD3-, CD5-	Mostly indolent	
MALT			Infiltration of morphologically heterogeneous small B cells	CD20+, CD79α+, Bcl-2+, CD21+ BCL-6-, CD3-, CD10-, CD5-, and cyclinD1-		
BL			Diffuse infiltration of medium-sized lymphocytes, with round-to-irregular nucleoli, scant cytoplasm; starry-sky appearance	Monotypic sIgM+, CD20+, CD10+, and Bcl-6+; CD3-, CD5-, TdT-, Bcl-2-	Aggressive	

*DLBCL, diffuse large B-cell lymphoma; BL, Burkitt lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma; FL, follicular lymphoma.

In primary hepatic MALT lymphoma, the most frequent translocation is t (14, 18) (q32; q21), which brings the mucosa-associated lymphoid tissue 1 (MALT1) gene to the downstream of the IgG enhancer and makes MALT1 overexpressed, thus resulting in activation of the NF-κB pathway. Meanwhile, activated MALT1 has protease activities, causing hydrolyzation of tumor necrosis factor alpha inducible protein 3 (TNFAIP3), which act as a NF-κB negative regulator, further promoting NF-κB activation. Overexpression of MALT1 and BCL-10 can upregulate the BAFF expression, thus enhancing the activation of the non-canonical NF-κB pathway (Figure 1) (27).

TNFAIP3 plays a key role in the regulation of several inflammation signaling pathways, which can negatively regulate the NF-κB pathway by inhibiting signals from various surface receptors to activate the signaling pathway. Therefore, the inactivating mutation or deletion of TNFAIP3 gene can downregulate its expression and reduce the repression of NF-kB

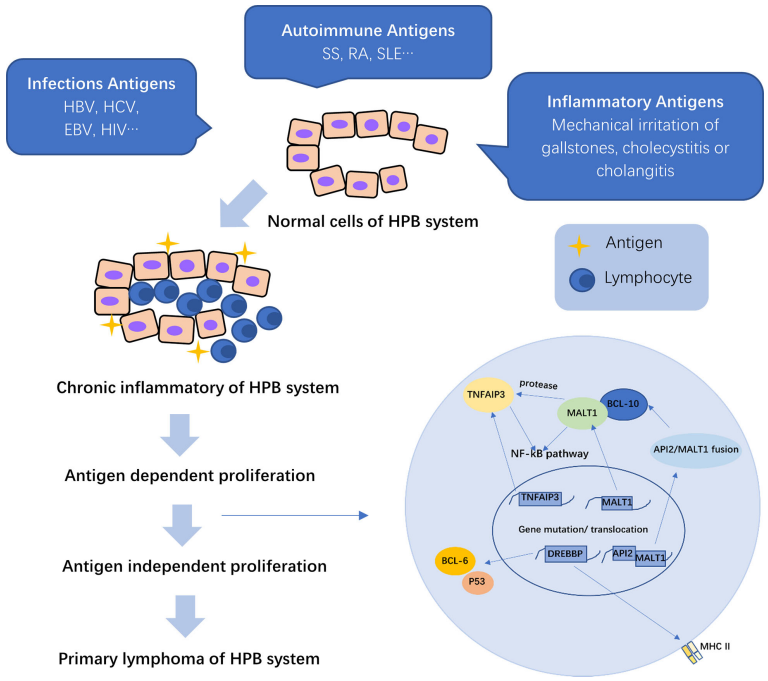


FIGURE 1
The pathogenesis of primary hepatopancreatobiliary lymphoma (PHPBL). As a result of the prolonged antigen exposure, chronic inflammation of the HBP (hepatopancreatobiliary) system induces lymphocyte migration to the site of inflammation and antigen-dependent proliferation. Prolonged inflammatory stimulation can cause irreversible chromosomal translocations and induce lymphocytes to antigen-independent proliferation, thus leading to primary lymphoma of HBP. MALT1, mucosa-associated lymphoid tissue 1; TNFAIP3, tumor necrosis factor alpha-inducible protein 3; CREBBP, cyclic adenosine monophosphate (cAMP) response element binding protein; API2, apoptosis inhibitor 2; MHC II, major histocompatibility complex II; NF-kB, nuclear factor-kappa B.

activation (Figure 1) (18, 27). Such genetic abnormalities play an essential role in lymphomagenesis.

Cyclic adenosine monophosphate response element binding protein (CREBBP) is a transcriptional coactivator, which is involved in several signaling pathways. Due to the lack of acetylation, the mutant CREBBP protein can activate BCL-6 and decrease the tumor suppressor activity of p53, thus protecting atypical lymphocytes from apoptosis. Deletion of CREBBP gene can downregulate the expression of MHC-II gene, leading to immune escape of malignant cells for proliferation and invasion (Figure 1) (18, 28).

Epidemiology and clinical features

PHL is extremely rare, which makes up only 0.4% of extranodal NHL, and 0.016% of all NHL (7). PHL affects individuals of varying age, but it is essentially a disease of middle-aged people (median age: 50 years old). Men are affected approximately twice as women (10).

Although the clinical features of PHL are wide-ranging, patients with PHL usually present with a complaint of upper right abdominal pain (29). The typical B symptoms of fever and weight loss can be found in one-third of all cases (24). Other symptoms include fatigue, anorexia, nausea, jaundice, and vomiting (30–33). On the physical examination of abdomen, tenderness in the upper right quadrant and hepatomegaly are the common presenting features. Splenomegaly occasionally can be observed in a few cases, as a consequence of hepatic dysfunction and portal hypertension (24, 31, 34).

Typical serum findings include variably elevated lactic dehydrogenase (LDH), bilirubin levels, as well as elevated liver enzyme levels (35, 36). The level of LDH, as a prognostic marker, increases in 30%–80% of all cases (24). The significantly increased LDH and normal tumor markers are useful clues in the diagnosis of PHL (37). Full blood counts are usually within the normal range unless the bone marrow or spleen is involved (38). Other occasional laboratory findings include monoclonal paraproteinemia and hypercalcemia, which is possibly the result of the secretion of calcitriol by lymphoma cells (39, 40).

Imaging

The imaging features of PHL are wide-ranging, and are commonly evaluated by abdominal ultrasound (US), CT, and

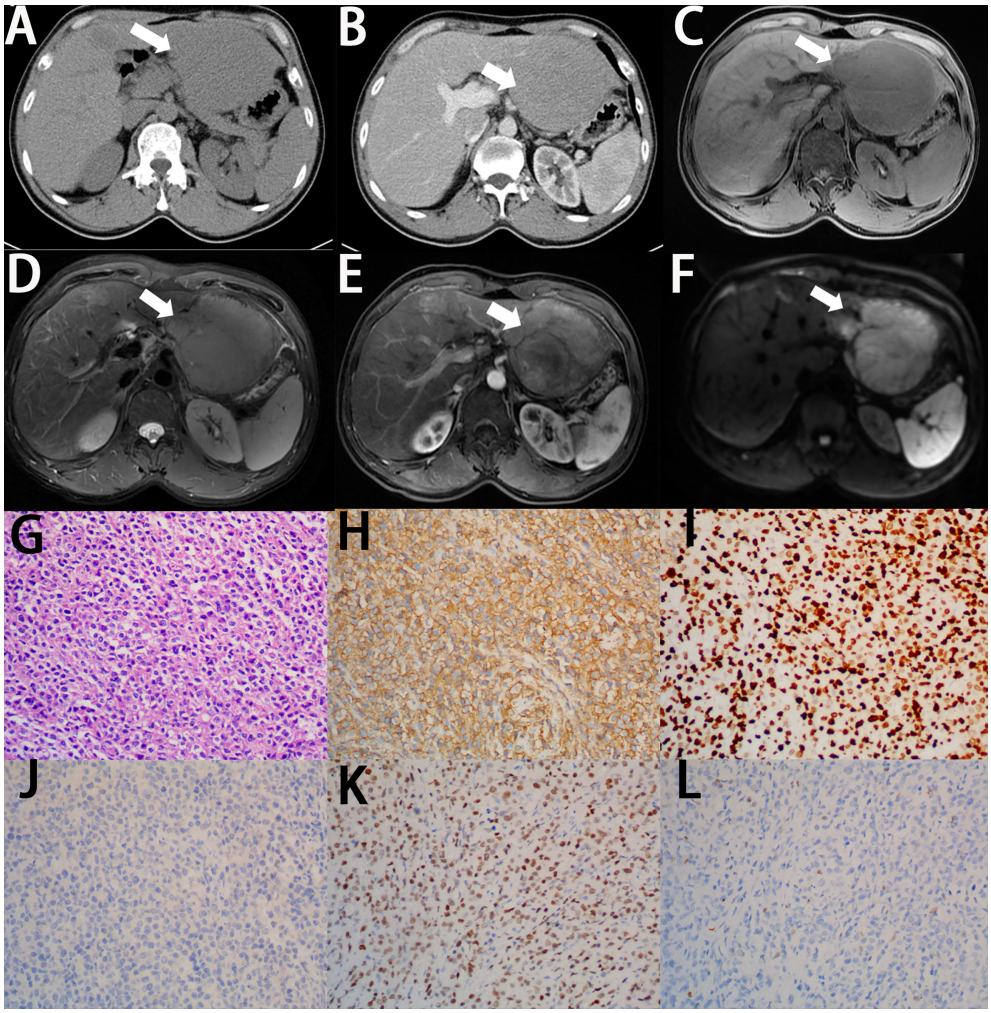


FIGURE 2
Imaging finding and histology of primary hepatic diffuse large B-cell lymphoma. **(A)** On unenhanced CT, the lesion shows hypoattenuation mass (white arrow). **(B)** On contrast-enhanced CT, the lesion shows a poor enhancement, lower than the normal parenchyma (white arrow). **(C)** On T1-weighted images, the lesion shows lower signal intensity (white arrow). **(D)** On T2-weighted images, the lesion shows higher signal intensity (white arrow). **(E)** On enhanced MRI, the lesion shows significant enhancement (white arrow). **(F)** On DWI, the lesion shows significant signal restriction (white arrow). **(G)** A diffuse infiltrate of large lymphocytes replaces the liver parenchyma (H&E stain, 20×). **(H)** Lymphocytes are positive for CD20 (CD20 immunostain, 20×). **(I)** The Ki-67 proliferative index is high in the lymphocytes (Ki-67 immunostain, 20×). **(J)** Lymphocytes are negative for CD10 (CD10 immunostain, 20×). **(K)** Lymphocytes are positive for BCL6 (BCL-6 immunostain, 20×). **(L)** Lymphocytes are negative for MUM-1 (MUM-1 immunostain, 20×).

MRI. At imaging, PHL can appear as a solitary mass, multiple lesions, or diffuse infiltration (41), mimicking other liver diseases, such as hepatocellular carcinoma, cholangiocarcinoma, metastatic disease of liver, and hepatitis.

On US, PHL mostly appears as homogeneous hypoechoic lesions confined in the liver (30, 42). Anechoic lesion can occasionally be seen, which may be confused with cyst (43). If there is a mass in the porta hepatis, dilation of the intra- and extra-hepatic bile ducts can also be found (44). The manifestations of contrast-enhanced US show mild heterogeneous enhancement in the arterial phase and washout in the portal and late phases (45).

On CT, PHL always presents as hypoattenuating lesions, with or without distinct margins (Figures 2A, 3A) (46, 47), the center of which may have a lower intensity, suggesting necrosis (2). However, it also presents as homogeneous or heterogeneous hepatomegaly without definite hepatic masses (48). Unlike hepatic carcinoma, PHL shows that hepatic vessels passed through the lesions without evidence of compression, or infiltration (48). On contrast-enhanced CT (CECT), the hepatic lesions show mild enhancement in the

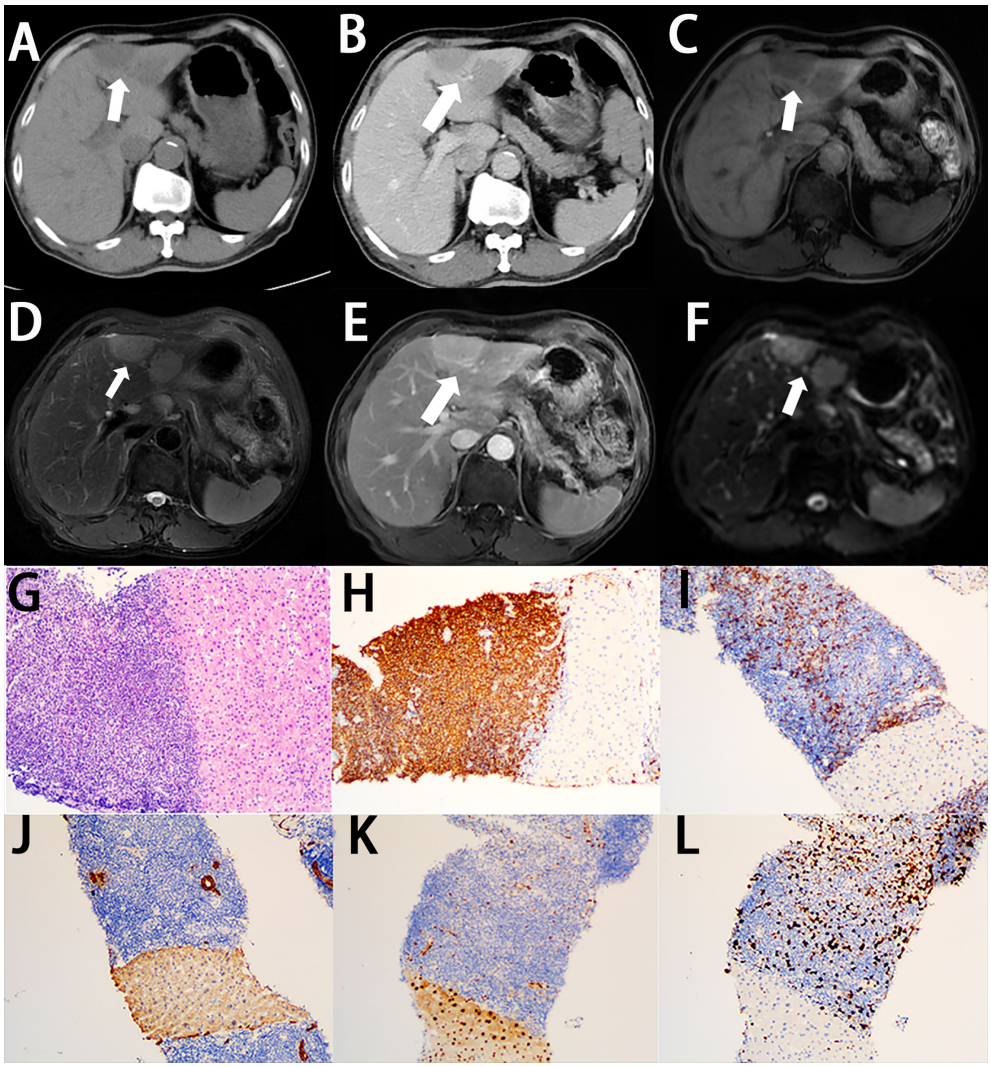


FIGURE 3
Imaging finding and histology of primary hepatic MALT lymphoma. **(A)** On unenhanced CT, the multiple lesions show hypoattenuation lesions (white arrow). **(B)** On contrast-enhanced CT, the multiple lesions show a poor enhancement, lower than the normal parenchyma (white arrow). **(C)** On T1-weighted images, the multiple lesions show lower signal intensity (white arrow). **(D)** On T2-weighted images, the multiple lesions show higher signal intensity (white arrow). **(E)** On enhanced MRI, the multiple lesions show significant enhancement (white arrow). **(F)** On DWI, the lesions show significant signal restriction. **(G)** A diffuse infiltrate of small-sized lymphocytes replaces the liver parenchyma (H&E stain, 10×). **(H)** Lymphocytes are positive for CD20 (CD20 immunostain, 10×). **(I)** A minor population of reactive T cells are positive for CD3 (CD3 immunostain, 10×). **(J)** Lymphocytes are negative for CKp (CKp immunostain, 10×). **(K)** Lymphocytes are negative for Cyclin D1 (Cyclin D1 immunostain, 10×). **(L)** The Ki-67 proliferative index is low in the lymphocytes (Ki-67 immunostain, 10×).

arterial phase (Figures 2B, 3B) and progressive enhancement in the venous phase, which is vital to differentiate from hepatocellular carcinoma. Rim enhancement can also be noted (46, 47, 49).

On MRI, the lesions of PHL tends to show hypointense signal on the T1-weighted images (T1WI) and hyperintense signal on the T2-weighted images (T2WI) (Figures 2C, D, 3C, D) (50, 51). Some PHLs show a heterogeneous signal because of necrosis or fibrosis within the mass (52). Nearly half of PHLs show enhancement after the intravenous administration of the contrast agent, which is lower than that of normal liver parenchyma (Figures 2E, 3E), whereas 40% of PHLs are hypointense. PHL patients mainly present as a significant signal restriction in diffusion-weighted imaging (DWI), with a lower ADC value (median ADC value: 0.83×10^{-3} mm^2/s) than other malignant hepatic diseases (Figures 2F, 3F) (53). Like CT features of PHL, we can also observe no distortion of the blood vessels and bile ducts

passing through hepatic lesions on MRI, which is called "insinuative growth" (54).

PET/CT has been an excellent modality for the diagnosis, staging, and follow-up of tumor, which can evaluate the involvement of other sites in patients with lymphoma and differentiate primary liver lesions from metastatic diseases (55, 56). The hypermetabolic lesions with a maximum Standardized Uptake Value (SUVmax, mainly ranging from 4.5 to 33.5) might be observed, with tumor confined in the liver without abnormal uptake in any other tissues or organs (57).

Pathology and immunohistochemistry

The clinical and radiological findings of PHL are non-special, and a definite diagnosis can only be made after pathological analysis. Liver tissue can be obtained by fine needle biopsy (FNB), image-guided percutaneous biopsy, laparoscopic biopsy, or open biopsy (2, 58).

As the most common subtype of PHL, diffuse large B-cell lymphoma (DLBCL) accounts for up to 80% of all PHL cases (10), followed by MALT lymphoma, follicular lymphoma, Burkitt lymphoma, and T-cell lymphoma (24, 59–62).

Microscopically, on low-power magnification, the lymphoid cells infiltrate the hepatic parenchyma and form solitary, multiple nodules or diffuse infiltration (63). On high-power magnification, the histological features vary, depending on the subtypes of PHL. In primary hepatic DLBCL, the lesions are formed by diffuse large-sized lymphoid cells with prominent nucleoli and increased or atypical mitotic figures (Figure 2G) (64–66). In primary hepatic MALT lymphoma, the atypical lymphoid cells are small in size, with mildly irregular nucleoli, dense chromatin, and scant cytoplasm, without germinal center differentiation (Figure 3G) (64, 67). Because MALT lymphomas commonly involve the portal fields of the liver, lymphoepithelial lesions are always presented in the bile ducts, which is a typical accompanying feature of hepatic MALT lymphoma (68). In primary hepatic follicular lymphoma, it mainly presents as distinct follicles, formed by small- to intermediate-sized centrocytes, with germinal center differentiation (69). The presence of follicular meshworks is a vital feature in the diagnosis of follicular lymphoma (64). In primary hepatic Burkitt lymphoma, diffuse medium-sized lymphocytes, with distinct nucleoli, scant cytoplasm, and increased mitotic figures, form the lesions (64). A typical starry-sky appearance has been reported, which is formed by the dispersal of numerous tangible-body macrophages among malignant cells (62, 70).

Immunohistochemistry is always required for classification of lymphoma, which is important to obtain the correct diagnosis. Most PHLs are of B-cell origin, which are always positive for part of or all the B-cell markers, usually including CD19, CD20, and CD79a, and negative for CD3 (Figures 2H, 3H, I) (71–74). However, different immunophenotypes have their own characteristics, which are essential for the classification of PHL. In DLCBL, they are often positive for CD45, PAX5, and BCL-2 with a high Ki-67 index (Figure 2I) (30, 75, 76). Patients with DLBCL are always subclassified into the germinal center B-cell (GCB) group, expressing CD10 and/or BCL-6 without MUM-1, and the non-GCB group, expressing MUM-1 without CD10 and BCL-6 (Figures 2J–L) (37, 64). In MALT lymphoma, they are also positive for IgM and CD21 and negative for CD5, CD10, and cyclin D1, with a low Ki-67 index (Figures 3J–L) (68, 77–79). In Burkitt lymphoma, they are also positive for monotypic surface IgM, CD10, BCL-6, and MYC and negative for BCL-2, with a high Ki-67 proliferative index, which is nearly 100% (64, 80). In follicular lymphoma, they are also positive for CD10, BCL-2, and BCL-6, with a low Ki-67 proliferative index. Immunostaining with CD21 and CD23 can highlight the follicular meshworks (64, 81, 82). The characteristics of pathology and immunophenotype of PHL are summarized in Table 1.

Differential diagnosis

Due to non-special symptoms and imaging features, a number of liver diseases should be considered during differential diagnosis of PHL including hepatocellular carcinoma, metastatic disease of liver, cholangiocarcinoma, hepatitis, or systemic NHL with secondary hepatic involvement. Some clinical and imaging features are helpful in differentiating between these entities.

Hepatocellular carcinoma appears as marked enhancement in late arterial phase, which becomes both progressive washout of contrast compared to healthy liver tissue in portal venous or delayed phases (83). The "bulls-eye" sign is a characteristic feature of metastatic carcinoma of liver. It always appears as a thick rim-like enhancement in the arterial phase and a hypo-enhancement in portal venous and delayed phases (84). Patients with acute hepatitis appear to have a thickening of the gallbladder wall and hepatic periportal lucency, which can favor a diagnosis of acute hepatitis (85). SHL typically presents as multifocal or diffuse lesions along with extrahepatic involvements, which can be detected by CT, MRI, or PET/CT (86).

Although some features are useful clues in diagnosis, it is difficult to make a definite diagnosis without histological analysis, which is the gold standard of diagnosis.

Prognosis and treatment

PHL with different infiltration types has a different prognosis. The 1-year and 3-year survival of patients with nodular infiltration are 70% and 57%, respectively, whereas those of patients with diffuse infiltration are 38% and 18%, indicating a poorer prognosis (87). In addition, the prognosis of indolent lymphoma, like follicular lymphoma and MALT

lymphoma, is better than that of aggressive diseases like DLBCL, Burkitt lymphoma, and T-cell lymphoma (88). Major prognostic factors associated with survival are the large size of the tumor, high proliferation of cells, old age, systemic symptoms, unfavorable histologic subtype, disease stage, and complications (44, 68). An elevated level of LDH, β2 microglobulin, or serum calcium, as prognostic markers, suggests a worse prognosis (41).

Because of the rarity of PHL, no consensual recommendation for treatment has been issued. The choices of management for PHL contains surgery, chemotherapy, radiotherapy, or combinations of the above modalities (49). Liver transplantation has also been used in PHLs (68). The isolated case report and case series studies of PHL in the recent 10 years are summarized in Supplementary Table 1. As shown in Table S1, systemic combination chemotherapy is commonly used as the main therapeutic approach, which could usually achieve disease remission (89–91). Moreover, chemotherapy with CHOP-based regimens (cyclophosphamide, doxorubicin, vincristine, and prednisone) is the first-line treatment (92, 93). The combination of rituximab with conventional chemotherapy can prolong survival of PHL patients who are positive for CD20 (69, 94–96). The role of surgery is not clear, but some studies have found that surgical resection can offer a good outcome (97–102). Solitary lesions could be considered as the best candidates for hepatectomy and another indication for surgery is related to an uncertain diagnosis (30). In a review of 72 patients with PHL, Avlonitis et al. confirmed that surgical resection followed by adjuvant chemotherapy and radiotherapy is the optimal treatment for PHL. Patients treated with surgery and chemotherapy might have better prognosis than those treated with chemotherapy alone (38). However, due to the rarity of PHL, the definite role of surgery still needs to be further confirmed. Lymphomas also respond to radiotherapy, while radiotherapy is not as effective as chemotherapy, which is always used as an adjunct to chemotherapy (93, 103–106). Radiofrequency ablation (RFA), a new option for the treatment of PHL, has good efficacy in the short term. When the preoperative diagnosis is clear and the mass measures less than 2 cm, RFA can be selected. However, the effect of RFA in the long term needs more relevant cases (77).

Primary biliary lymphoma

Risk factors and pathogenesis

Due to the rarity of PBL, the exact etiological factor is obscure. Several studies found that half of all reported cases had gallstone, and this implies that PBL may be related to inflammation such as chronic cholecystitis or cholangitis associated with cholelithiasis or infected bile (107). Furthermore, mechanical irritation of gallstones is more responsible for the pathogenesis of PBL (108). PBL can also be found in patients with hepatitis virus infections and immunosuppression, such as HIV infection and organ transplantation (109, 110).

Whatever the specific cause of inflammation, cholecystitis or cholangitis induces lymphocyte migration to the mucosa of the biliary tract and prolonged lymphoid reactive proliferation, which is antigen-dependent, and accumulation in the site of inflammation, thus leading to irreversible chromosomal translocations, which can inhibit apoptosis and cause antigen-independent proliferation (111–113). Bisig et al. (108) detected a specific chromosomal translocation t (11, 18) (q21; q21) in primary biliary MALT lymphoma, leading to the expression of a transcript fusing the apoptosis inhibitor 2 (API2) gene to MALT1 gene. API2/MALT1 fusion can reduce the inhibition of API2 on apoptosis response to antigen stimulation, thus leading to MALT lymphoma of the biliary tract (108). Its production can also induce expression of BCL-10 and activation of the NF-κB pathway, leading to cell proliferation (Figure 1) (27). Another possible mechanism is that prolonged chronic inflammation causes irreversible genetic rearrangements, thus disabling the response of cells to IL-2 regulation, eventually helping in the development of MALT lymphoma (114).

In addition, another possible pathogenesis was reported by Angelopoulou and his colleagues. In PBL, the malignant transformation of the original clone could have occurred outside the biliary system with subsequent homing by an adhesion molecule mechanism (115).

Epidemiology and clinical features

PBL is an extremely rare entity, which could be divided into intrahepatic bile duct and the extrahepatic biliary system. The clinical and radiological findings of lymphoma of the intrahepatic duct resemble those of hepatic lymphoma. Hence, we only discuss primary lymphoma of the extrahepatic biliary system here. Extrahepatic biliary non-Hodgkin's lymphoma (EBNHL) constitutes 0.6% of malignant biliary tumors, including lymphoma of gallbladder and extrahepatic bile duct (8). Primary lymphoma of gallbladder has a higher prevalence than extrahepatic bile duct lymphoma. PBL can occur in various age groups and commonly affect elderly individuals with a mean age of 75.8 years old, with a slight female preponderance (11).

Patients with primary gallbladder lymphoma commonly present with symptoms of acute or chronic cholecystitis, and the most common symptom is upper right abdominal pain (11, 116). The common clinical manifestation of primary bile duct lymphoma is obstructive jaundice (8). Fever, night sweats, weight loss, nausea, and vomiting can also be present in these patients (117). The physical examination of these patients is often normal, while yellowing of skin and sclera with itching marks on the skin can be found in PBL patients with biliary

obstruction (118). Slight tenderness in the upper right quadrant is also be observed in some cases.

The common abnormalities revealed by laboratory test results associated with PBLs include variably elevated bilirubin and liver enzyme levels, which suggest cholestasis (119). Interleukin (IL)-2 receptor, a serum marker of lymphoma, increases in patients with PBL (107, 120). Tumor markers are often within normal range, which can differentiate PBL from carcinoma of the biliary system, while mildly elevated CA19-9 can be observed in some cases (117). Full blood counts are usually normal at the early stage; however, the number of erythrocytes, leukocytes, and platelets might decrease when bone marrow or spleen is involved (8). In addition, other rare laboratory abnormalities include elevated serum and urine amylase level (121).

Imaging

Radiological characteristics of PBL depend on their pathological classifications. Their imaging appearances can be divided into two morphological groups (1): a solid mass, seen in high-grade lymphomas, such as DLBCL; and (2) an irregular thickening in the gallbladder or bile duct wall, seen in low-grade lymphomas, such as MALT and follicular lymphoma (107, 111).

US is the modality of choice in the initial evaluation of gallbladder and biliary diseases (122). The common US features of primary gallbladder lymphoma present as thickening of the gallbladder wall or a soft tissue mass located in gallbladder with or without gallstone (107, 109), which has lower echo compared to gallbladder carcinoma (123). In addition, patients with primary bile duct lymphoma usually present with thickening of the bile duct or a hypoechoic mass with dilation of the proximal bile duct (124). The imaging features of endoscopic ultrasound (EUS) are similar to those of typical US.

The common CT feature of primary gallbladder lymphoma is a thickened gallbladder wall with intact mucosa and layered enhancement after administration of the contrast agent (Figure 4A) (107, 125). The other common CT feature presents as a focal mass confined in the gallbladder with slight enhancement (120, 126). Patients with primary bile duct lymphoma present with segmental circumferential wall thickening of the bile duct or a bile duct mass, with proximal bile duct dilatation and smooth mucosal layer (127).

On MRI, the gallbladder or bile ductal lesion shows lower and slightly higher signal intensity on the T1WI and T2WI, compared to the surrounding normal liver parenchyma (111, 127). Homogeneous enhancement of biliary lesion can be observed on contrast-enhanced images. The dilation of upstream bile duct can also be seen in most lymphomas of bile duct on MRI (128). MR cholangiopancreatography (MRCP) has rarely been applied to gallbladder lymphoma, and thus, the MRCP findings of gallbladder lymphoma are rarely reported. Moreover, MRCP features of primary bile duct lymphoma are similar to those of cholangiocarcinoma, commonly presenting as a segmental luminal narrowing of bile duct without mucosal irregularity and with dilation of the proximal bile duct (128). Endoscopic retrograde cholangiopancreatography (ERCP) can observe similar features (Figure 4B).

On PET/CT, the location and size of PBL are similar to other imaging modalities. The common imaging feature with PET/CT demonstrates hypermetabolic activity in gallbladder or bile duct without other metastatic areas (129, 130). PET/CT is a preferred choice for staging lymphoma and helpful for

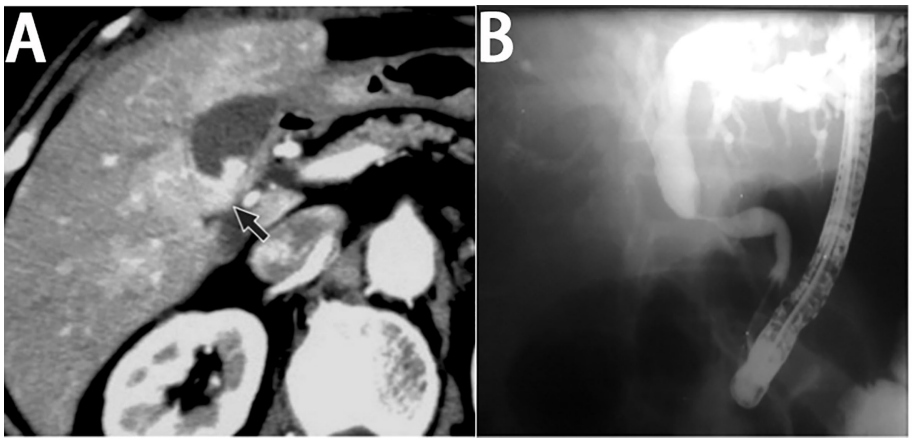


FIGURE 4
Imaging finding of primary biliary lymphoma. **(A)** On enhanced CT, the lesion of primary gallbladder lymphoma shows laminar enhancement on the mucosal surface of the gallbladder wall (black arrow). (with the permission of BIR publication). **(B)** ERCP of primary bile duct lymphoma shows a segmental luminal narrowing of mid common bile duct with marked dilation of proximal bile duct (with the permission of Elsevier).

differentiating PBL from secondary lymphoma and other diseases, particularly cholecystitis.

Pathology and immunohistochemistry

Like other lymphoma, adequate biopsy is needed for definitive diagnosis. Most PBL patients obtain pathologic specimens by surgery, while it can also be obtained under CT, EUS, or ERCP guidance (131).

Grossly, low-grade PBL presents as thickening of the bile duct and gallbladder with intact mucosal layer; however, in high-grade PBL, it shows a mass defined in the biliary system. Histologically, a dense and diffuse infiltration of atypical lymphoid cells in the gallbladder or bile duct wall, with intact mucosal layer and occasional lymphoid follicles, could be observed in patients with low-grade lymphoma [127, 132], whereas in patients with high-grade lymphoma, it demonstrated diffuse infiltration of large-sized lymphocytes with prominent nucleoli and abundant mitoses [36].

As summarized in Table 3, the most common subtypes of primary lymphoma of gallbladder or bile duct are DLBCL and MALT, followed by follicular lymphoma and B-lymphoblastic lymphoma (11). The immunohistochemical features and molecular findings of different subtypes of PBL are summarized in Table 3, which are similar to those of PHL (83, 112, 123–128, 132, 133).

Differential diagnosis

PBL is a rare disorder that can be present as a focal mass or a thickening in the gallbladder or bile duct wall and imitate the characteristics of biliary tumors such as adenocarcinoma or inflammatory process such as sclerosing cholangitis and cholecystitis (119).

Patients with cholangiocarcinoma presented as concentric or eccentric wall thickening without intact mucosa, resulting in varying degrees of luminal stenosis (134). Gallbladder carcinoma always appears as a mass or thickening of the gallbladder wall, with disruption of the mucosal layer (135). Primary sclerosing cholangitis (PSC), associated with inflammatory bowel disease (IBD), presents as multifocal biliary strictures (136). Radiographical findings of cholecystitis show a thickened gallbladder wall (>4 mm), often accompanied by gallstones and pericholecystic fluid (137). Besides imaging features, patient's disease history, physical examination, and laboratory studies are essential for differentiation diagnosis (122).

The prognosis and treatment of PBL are different from other biliary diseases, and the proper diagnosis of the disease is important. The histological analysis is indispensable for a correct diagnosis.

Prognosis and treatment

PBL has a better prognosis than cholangiocarcinoma and gallbladder carcinoma (118). Like other NHLs, the prognosis of PBL may be associated with age, tumor stage, subtype, and treatment. Due to lack of sufficient case series, this association has not been confirmed; more studies are needed to prove this.

Due to the low incidence of PBL, there is no consensus on the optimal treatment. As shown in Table S2, the management consists of surgery, chemotherapy, radiotherapy, or a combination of all. Because of the difficulty of acquiring pathological diagnosis, surgical resection is commonly performed in most PBL patients (Table S2) (120). Surgery can also be a therapeutic option for patients with complicating biliary obstruction, or who fail to respond to chemotherapy (119). Surgical intervention is proven curative in many cases (121, 138). Chemotherapy is considered as the predominant management modality and an integral part of the postoperative treatment (124). Radiotherapy is often considered an adjunct to chemotherapy, which might increase the survival of patients with residue after chemotherapy (128). From reviewing previous PBL case studies, surgical resection, combined with chemotherapy with or without radiotherapy may be a treatment regimen for improved survival rate, which need further studies to confirm.

Primary pancreatic lymphoma

Risk factors and pathogenesis

Due to the rarity of PPL, the consensus of pathogenesis is still not issued. It is commonly associated with immunosuppression, related to HIV infection or solid organ transplantation (139). A few studies have implied that HCV or HBV infection is associated with PPL (140, 141).

However, to our knowledge, HBV and HCV are hepatotropic and are main etiologies of liver cancer. The possible reasons that hepatitis virus can cause pancreatic damage are the proximity of the liver to the pancreas and shared blood vessels and ducts. The possibility is further supported by findings of hepatitis B surface antigen (HBsAg) in pancreatic juice and hepatitis virus replication in pancreatic cells among patients with HBV infection (142, 143).

Like PHL, HBV or HCV infection may indirectly give rise to inflammation-associated lymphomagenesis in the pancreas. It may induce cytokine and cytokine growth, perhaps affecting various genes and causing proliferation of lymphoid cells (140). Moreover, it may also reduce the threshold of antigen response or result in DNA mutations by binding to surface receptors of B lymphocytes, thus leading to lymphoproliferation (141).

Epidemiology and clinical features

PPL is a rare disease and accounts for approximately 1% of extranodal lymphoma and 0.5% of pancreatic cancers (9). PPL commonly affects middle-age individuals with a median age of 53 years old and has a male predilection (male:female ratio of 1.5:1) (12).

Clinical features of PPL are non-specific and include epigastric pain, abdominal mass, weight loss, obstructive jaundice (144–146), intestinal obstruction, and rarely acute pancreatitis (147), which are similar to those of pancreatic adenocarcinoma (148, 149). However, the typical symptoms of NHL, such as fever, chills, and night sweats, are rare in PPL (150). A physical examination was significant for epigastric tenderness, abdominal mass, and jaundice, but not for organomegaly or lymphadenopathy.

The tumor marker levels in PPL patients, such as CA19-9 and CEA, are commonly normal or slightly elevated in the case of biliary obstruction, which are apparently increased in most pancreatic adenocarcinoma patients (151). An elevated LDH level and serum beta-2-microglobulin are often presented in patients with pancreatic lymphoma. Although their elevations are not necessarily required for the diagnosis of PPL, they are useful markers, high levels of which are indicators of a poor prognosis for patients with PPL (152). Elevated liver enzymes and bilirubin levels are found in PPL patients with biliary obstruction.

Imaging

Radiologically, the lesions of PPL appear as focal and well-defined lesions or diffuse infiltration of pancreas (153), as summarized in Table 2. The tumor is most commonly located in the head of the pancreas, though it can be found in other portions of the pancreas, such as the body and tail (149, 154). PPL almost presents as bulky masses with a median size of 7.9 cm (155).

The most common US finding is a bulky homogeneous hypoechoic mass confined to the pancreas, with or without dilatation of bile ducts. Peripancreatic vessels are encompassed by mass, but are always not infiltrated, which can be distinguished from pancreatic adenocarcinoma (12, 156). The appearances of EUS are consistent with those of typical US; however, it has a higher sensitivity (157).

Boninsegna et al. found that PPLs have the following common CT features: a large and hypo-attenuation mass with mild enhancement (Figures 6A–C), peri-pancreatic fat stranding, vessel encasement without infiltration, rare pancreatic duct dilatation, and absence of necrosis (158, 159). These findings are in agreement with some studies reported by other teams (12, 160, 161). The "sandwich sign", in which a mesenteric mass surrounds the mesenteric vessels, without vascular infiltration, is frequently observed in patients with PPL (161, 162).

The MRI characteristics of PPL appear as a bulk lesion with mild enhancement, without significant pancreatic duct dilatation, which has a lower signal intensity on T1WI and a higher signal on T2WI (Figures 5A, B) (12, 163, 164). Unlike CT, MRI shows a slightly heterogeneous character of the lesion, especially on T2WI (165). In some cases, PPL may present with diffuse enlargement of pancreas, mimicking pancreatitis (166, 167). Occasionally, PPL may initially present only as acute pancreatitis and can be diagnosed only on follow-up imaging (167). MPD dilatation can rarely be found in PPL patients. However, mildly upstream MPD dilatation is another feature of PPL with diffuse pancreatic enlargement (165).

The most common PET/CT feature is a solitary hypermetabolic lesion in the pancreas, with SUVmax ranging from 7.4 to 26.5 (the mean SUVmax is 13.2) (168–171).

Pathology and immunohistochemistry

Pancreatic tissue can be obtained by percutaneous/endoscopic FNA or biopsy (172), exploratory laparotomy, or resection surgery. EUS-guided FNA or biopsy is an optimal method to obtain preoperative diagnosis (132, 157, 173–175).

As confirmed in Table S3, the most predominant PPL subtype is DLBCL (occupying nearly 77%), followed by follicular lymphoma (occupying 14%) (155). Furthermore, Burkitt lymphoma, small lymphocytic and T-cell lymphoma, or Hodgkin's lymphoma can also be present in PPL (176–180).

The specific pathologic and immunohistochemical findings of PPL are demonstrated in Table 2 and in Figures 6D–I (181–194).

Differential diagnosis

The clinical manifestations of PPL may mimic those of other neoplastic or inflammatory pancreatic diseases, such as pancreatic adenocarcinoma, pancreatic neuroendocrine tumor, acute pancreatitis, and autoimmune pancreatitis (AIP) (195). Although PPL has overlapped symptoms and imaging findings with other pancreatic diseases, some characteristic findings can help in the diagnosis of PPL.

In contrast to PPL, pancreatic ductal dilatation, and ductal and peripancreatic vascular invasion were frequently shown in pancreatic adenocarcinoma (196, 197).

Both PPL and AIP might appear as diffuse enlargement, a focal lesion, or multifocal lesions, making differential diagnosis difficult. Ishigami et al. (166) observed 8 patients with pancreatic lymphoma and 21 patients with AIP to identify the point of imaging discrimination for the two diseases. They concluded that patients with AIP present with delayed enhancement with a capsule-like rim on CT and MRI, which was absent in the PPL

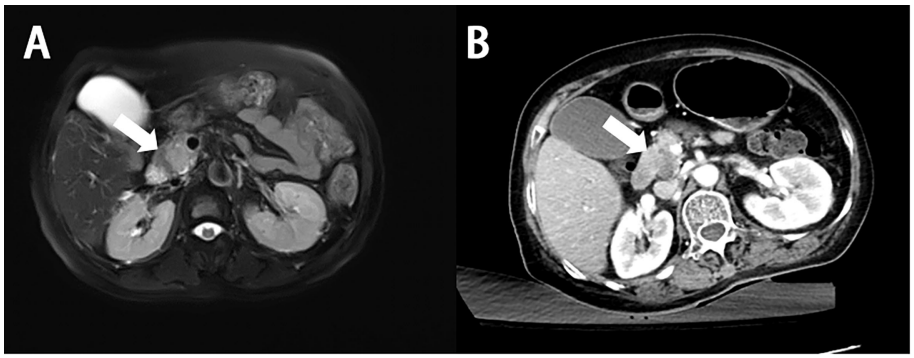


FIGURE 5
Imaging finding of primary pancreatic diffuse large B-cell lymphoma. **(A)** MR shows a lesion with hyperintense signal on the T2-weighted images (white arrow). **(B)** On enhanced CT, the lesion shows a mild enhancement, lower extent than the normal parenchyma (white arrow).

imaging feature (166, 198). In addition, elevated serum c-globulin levels, particularly immunoglobulin G, can be observed in almost all of the patients with AIP, but not in PPL patients (199).

Even if some findings can help with the diagnosis, histological analysis is still required for a definitive diagnosis.

Prognosis and treatment

The prognosis for PPL is much better than that of other pancreatic malignant tumors. A cohort study showed a median overall survival of 53 months (200). Patients with advanced age

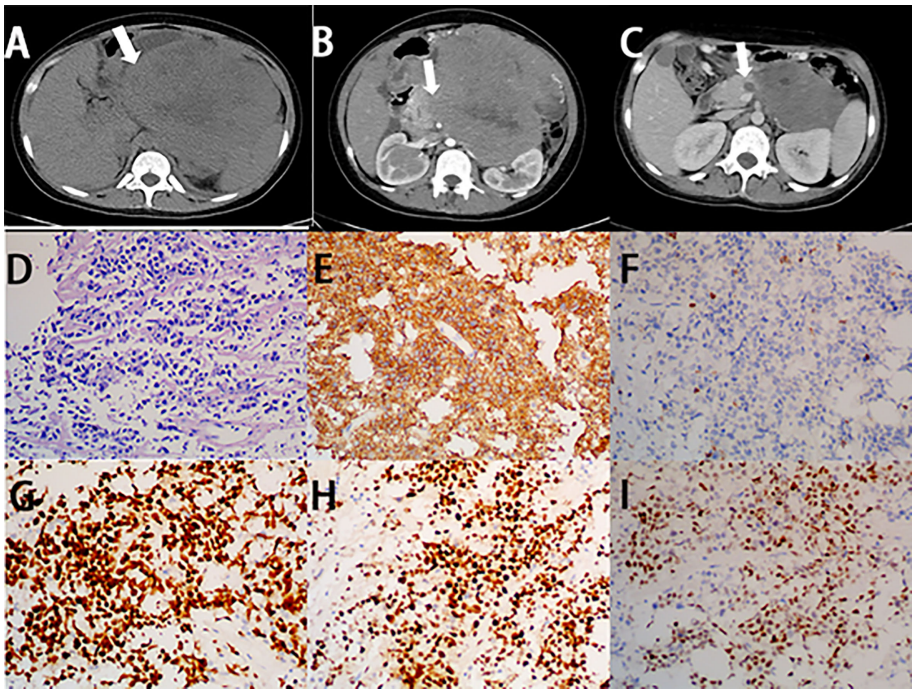


FIGURE 6
Imaging finding and histology of primary pancreatic Burkitt lymphoma. **(A)** Unenhanced CT of PPL shows a bulky and heterogeneous mass, with irregular margin (white arrow). **(B)** On the horizontal plane of enhanced CT, the lesion shows mild enhancement (white arrow). **(C)** After three cycles of chemotherapy, the size of the lesion significantly decreased. **(D)** A diffuse infiltrate of medium-sized lymphocytes replaces the pancreas parenchyma with starry-sky appearance (H&E stain, 20×). **(E)** Lymphocytes are positive for CD20 (CD20 immunostain, 20×). **(F)** Lymphocytes are negative for CD3 (CD3 immunostain, 20×). **(G)** The Ki-67 proliferative index is high in the lymphocytes (Ki-67 immunostain, 20×). **(H)** Lymphocytes are positive for BCL-6 (BCL-6 immunostain, 20×). **(I)** Part of lymphocytes are positive for Myc (Myc immunostain, 20×).

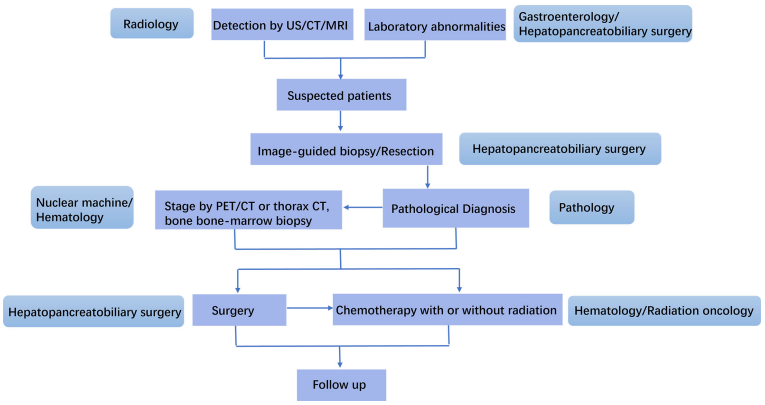


FIGURE 7
Management schedule for an experienced MDT for primary hepatopancreatobiliary lymphoma (PHPBL). Patients with characteristic laboratory abnormality are suspected as PHPBL after imaging finding of HPB mass. They will receive image-guided biopsy or resection. The pathological diagnosis will be made by the Department of Pathology. PET/CT or thorax CT, and bone/bone marrow biopsy are used to lymphoma stage. After that, the precise treatment will be discussed by hepatopancreatobiliary surgeons, hematologists, and radiation oncologists. After the treatment, the follow-up of patients will be made.

have a worse overall survival. Similarly, patients with stage IV or aggressive subtype have a worse prognosis, with a median survival of only 13 months, whereas those with an earlier stage have a longer survival of 80 months. Undergoing chemotherapy is also significantly associated with better overall survival, while location of tumor, race, and sex are not associated with overall survival (200).

The rarity of PPL patients makes it difficult to draw a definite conclusion about the optimal treatment, which is mainly determined by the histological subtype. As summarized in Table S3, the strategy for PPL includes surgery, chemotherapy, radiotherapy, or a combination of all (9). Considering that the pathology of most PPL patients is DLBCL, chemotherapy is the standard treatment (12, 155, 201). The most commonly used regimen is CHOP or R-CHOP (176). The majority of patients with PPL receiving only chemotherapy can achieve long-term disease remission (12, 148, 149, 163, 202). Vijungco et al. (203) found that early-stage NHL patients treated with radiotherapy alone have a high overall cure rate. However, in the last decade, radiotherapy has often been used as an adjunct to chemotherapy, rather than used alone (144, 151, 175, 188, 204). Some studies demonstrated that patients treated with chemotherapy with radiotherapy had a high overall response rate (144, 188). However, the role of radiotherapy has also not yet been well defined (204). Surgical intervention is not adopted as the primary treatment for PPL, which is considered when the diagnosis of a mass in pancreas remains uncertain or patients present symptoms caused by obstruction of the biliary tract (155). Behrns et al. (205) reported that the combination of surgery and chemotherapy has a better survival benefit than chemotherapy alone. Interestingly, a study by Facchinelli et al. (188) showed different results. Thus, surgical intervention remains controversial and needs to clarify its benefits through numerous PPL study series (188).

Conclusion

PHPBL often overlaps with other diseases of hepatobiliary and pancreas in clinical and radiological features, resulting in misdiagnosis and delayed treatment. Since early diagnosis depends on the alertness of clinicians, mainly radiologists, gastroenterologists, and hepatopancreatobiliary surgeons, it is important for them to know more clinical and imaging features of PHPBL, in order to obtain a proper diagnosis and management. Clinical manifestation, imaging findings, and laboratory studies could provide helpful clues for diagnosis, whereas histological analysis is the gold standard for accurate diagnosis and subtype analysis. Due to the rarity of the disease, there is no consensus on treatment options. The optimal therapeutic choice, including surgery, chemotherapy, radiotherapy, immunotherapy, or other treatments, either alone or in combination, needs further investigation. The management of PHPBL needs an experienced multidisciplinary team, involving radiologists, gastroenterologists, hepatopancreatobiliary surgeons, pathologists, hematologists, and radiation oncologists, to provide individualized therapy and better prognosis (Figure 7).

Author contributions

Conceptualization, BZ. Literature search, QW, YL, and ZS. Investigation, KW and XZ. Project administration, BZ and SW.

Writing—original draft, QW. Data curation, KW, XZ, and SW. Formal analysis, YL and ZS. Writing—review and editing, BZ. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.951062/full#supplementary-material

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Advances in the research of the mechanism of secondary resistance to imatinib in gastrointestinal stromal tumors

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Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract. At present, surgery is the first-line treatment for primary resectable GISTs; however, the recurrence rate is high. Imatinib mesylate (IM) is an effective first-line drug used for the treatment of unresectable or metastatic recurrent GISTs. More than 80% of patients with GISTs show significantly improved 5-year survival after treatment; however, approximately 50% of patients develop drug resistance after 2 years of IM treatment. Therefore, an in-depth research is urgently needed to reveal the mechanisms of secondary resistance to IM in patients with GISTs and to develop new therapeutic targets and regimens to improve their long-term prognoses. In this review, research on the mechanisms of secondary resistance to IM conducted in the last 5 years is discussed and summarized from the aspects of abnormal energy metabolism, gene mutations, non-coding RNA, and key proteins. Studies have shown that different drug-resistance mechanism networks are closely linked and interconnected. However, the influence of these drug-resistance mechanisms has not been compared. The combined inhibition of drug-resistance mechanisms with IM therapy and the combined inhibition of multiple drug-resistance mechanisms are expected to become new therapeutic options in the treatment of GISTs. In addition, implementing individualized therapies based on the identification of resistance mechanisms will provide new adjuvant treatment options for patients with IM-resistant GISTs, thereby delaying the progression of GISTs. Previous studies provide theoretical support for solving the problems of drug-resistance mechanisms. However, most studies on drug-resistance mechanisms are still in the research stage. Further clinical studies are needed to confirm the safety and efficacy of the inhibition of drug-resistance mechanisms as a potential therapeutic target.

KEYWORDS

gastrointestinal stromal tumor, imatinib, secondary imatinib resistance, drug-resistance mechanism, therapeutic targets

1 Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the digestive tract, originating from gastrointestinal pacemaker cells (interstitial cells of Cajal, ICC) or related stem cells (1, 2). GISTs are typically driven by mutations of the receptor tyrosine kinase oncogene (C-KIT) or the platelet-derived growth factor receptor α (*PDGFRα*), which account for more than 80% and 5%–10% of all cases of GIST, respectively (3–5). GISTs without *KIT* or *PDGFRα* mutations are known as wild-type GISTs (WT-GISTs), which account for 10%–15% of all cases of adult GISTs and up to 85% of all cases of pediatric GISTs (6–8). In this category, 20%–40% are characterized by the loss of succinate dehydrogenase complex (SDH-deficient GISTs), approximately 15% carry *BRAF*/*RAS* or *NF1* mutations, and the remainder is referred to as KIT/PDGFRA/SDH/RAS-P WT-GISTs (or quadruple WT-GISTs) (9, 10). A careful examination for germline mutations is of great significance for all patients with WT-GISTs (11). Analyses conducted using tissue microarrays have shown that the *DOG1* gene is relatively specifically expressed in GISTs, regardless of the *KIT* or *PDGFRA* mutation status (12). A monoclonal antibody against *DOG1* has been proven to be a highly sensitive specific marker for the diagnosis of GISTs, and its sensitivity is higher than that of *KIT* (13).

Imatinib mesylate (IM) is a selective tyrosine kinase inhibitor (TKI) that targets *KIT* and *PDGFRα* for the treatment of unresectable or metastatic GISTs, which significantly improves the 5-year survival of patients (14, 15). The efficacy of IM varies among different *KIT* and *PDGFRA* mutation types, depending on the exons involved (16, 17). Approximately 14% of patients with GISTs are initially resistant to IM (18), whereas approximately 50% of patients develop resistance after 2 years of treatment, the so-called secondary resistance to IM (19). Therefore, it is important to clarify the mechanisms of secondary resistance to IM through research and develop new therapeutic targets and regimens to improve the long-term prognoses of patients with GISTs. A recent study demonstrated that IM specifically increases the expression of the complex II (SDHB) protein in oxidative phosphorylation (OXPHOS) proteins by downregulating miR-483-3p (Figure 1A). This study demonstrated the molecular mechanism of increased OXPHOS protein expression induced by IM and confirmed the biological role of miR-483-3p in regulating energy metabolism after IM treatment (20).This review will focus on the discussion and summary of research on the mechanisms of secondary resistance to IM conducted in the last 5 years from the aspects of abnormal gene mutation, energy metabolism, non-coding RNA, and key proteins.

2 Mechanisms of secondary resistance to imatinib

2.1 Gene mutation and resistance to imatinib

Secondary *KIT* and *PDGFRA* mutations are the main causes of secondary resistance to IM in non–wild-type GIST (21, 22). In most cases of GIST, secondary KIT mutations reactivate *KIT*

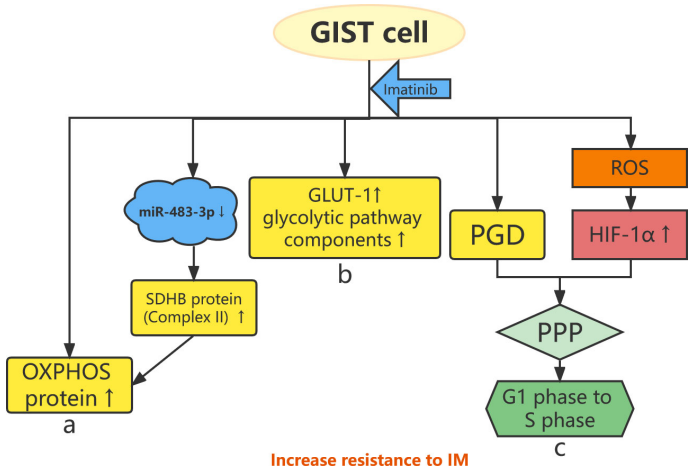


FIGURE 1
Abnormal energy metabolism and resistance to imatinib. **(A)** OXPHOS protein expression is increased in IM-resistant GIST cells, and IM specifically increases the expression of complex II (SDHB) protein by downregulating miR-483-3p. **(B)** GLUT-1 and glycolytic pathway components increase in IM-resistant GIST cells. **(C)** The HIF-1α–PGD–PPP axis and IM-induced ROS stimulate GIST cells from the G1 phase to the S phase, leading to drug resistance.

downstream signaling pathways, such as the PI3K/AKT/mTOR pathway, and continue to drive GIST proliferation and survival, leading to acquired IM resistance (23–27). KIT T670I is one of the most common types of secondary KIT mutations (24). Cassier et al. found that *PDGFRA* exon 18 D842V gene subtype mutation is associated with primary resistance to IM (16). Secondary *PDGFRA* mutations are less common in IM-resistant GISTs than secondary *KIT* mutations (28, 29). Secondary *KIT* mutations or *PDGFRA* mutations do not occur in wild-type IM-resistant GISTs (30).

In a previous study of 210 Chinese patients with IM-resistant GIST who underwent next-generation sequencing for the identification and characterization of secondary *KIT* mutations, the results showed that 63.81% of the patients had mutations on exon 13, 4.76% had mutations on exon 14, and 31.43% had mutations on exon 17. All secondary *KIT* mutations were missense mutations, mostly located in the kinase domain (31). Zhao et al. obtained consistent results in an analysis of the distribution of the most common Kit mutation forms in 2,273 Chinese patients with GIST. The results showed that KIT exon 13 V654A and exon 17 N822K were the most common secondary mutations in GISTs with primary mutations in exon 11 (32). These two secondary *KIT* mutations induce resistance to IM by activating the PI3K/AKT/mTOR pathway (33). Inhibition of PI3K induces massive apoptosis in IM-resistant GISTs (34). Interestingly, *KIT* is overactivated in IM-resistant GISTs with secondary *KIT* mutations; however, the expression levels are not significantly increased. Secondary *PDGFRA* mutations are mostly located in exon 18 (24, 35).

In addition to secondary *KIT* and *PDGFRA* mutations, several additional genetic mutations have been associated with secondary resistance to IM in GISTs. Additional mutations of *RB1*, *SMARCB1*, and *MAX* (myc-related protein) are important causes of resistance to IM. Notably, GISTs caused by different gene mutations show different clinicopathological characteristics (31). Genome-scale CRISPR-Cas9 knockout (GeCKO) screening classifies *TP53* and *SOCS6* as candidate genes for resistance to IM owing to their presence in multiple signaling pathways, such as the apoptosis pathway, Wnt signaling pathway, and JAK-STAT signaling access (36).

Identifying the abovementioned types of gene mutation is a key supplement to the existing GIST risk assessment model. In addition, the discovery of new potential candidate therapeutic targets for different genetic mutations will be beneficial in delaying the progression of GISTs. Individualized therapy based on the identification of types of genetic mutation will also provide new adjuvant treatment options for patients with IM-resistant GIST. Furthermore, the type of gene mutation may be used as a biomarker to help identify patients who can benefit more from adjuvant therapy and to predict the risk of recurrence of GISTs.

2.2 Abnormal energy metabolism and resistance to imatinib

An important feature of cancer cells is abnormal energy metabolism, which is characterized by strong aerobic glycolysis and reduced mitochondrial energy metabolism. This feature is called the Warburg effect (37). Metabolic reprogramming of cancer cells sets the stage for rapid growth and metastasis (38, 39). Drug-resistant cancer cell subsets depend on the enhancement of mitochondrial function and OXPHOS (40, 41). Moreover, the metabolic adaptation of cancer cells to the toxic effects of targeted drugs contributes to drug resistance (42–45). GIST cells exhibit high levels of glucose uptake and aerobic glycolytic activity, and metabolic reprogramming induced by IM stress enhances mitochondrial function and OXPHOS (46).

IM alters the metabolic phenotype of GISTs (46) and increases the expression of several OXPHOS proteins, including complexes II, III, and V (40). Huang et al. found that IM-resistant GIST cells show increased OXPHOS protein expression compared with IM-sensitive GIST cells (Figure 1A) (40). In addition, IM-resistant GIST cells show higher OXPHOS levels and glycolysis rates than IM-sensitive cells and are more susceptible to glycolysis inhibition. Inhibition of OXPHOS increases the sensitivity of GISTs to IM. OXPHOS protein expression is increased in IM-sensitive GIST cells after IM treatment but not in IM-resistant GIST cells (47). Notably, there is a heterogeneity of metabolic phenotypes in IM-resistant GIST (40). Glucose transporter 1 (GLUT-1) is a key component of the glycolytic pathway and is associated with secondary resistance to IM in GIST cells. IM downregulates the expression of GLUT-1 and the glycolytic pathway components hexokinase 2, pyruvate kinase M2, and lactate dehydrogenase in IM-sensitive GIST cell lines. In contrast, the expression of GLUT-1 and these glycolytic pathway components increases after the treatment of IM-resistant GIST cell lines using IM (Figure 1B). This indicates that IM-resistant GIST cells have a higher glycolysis rate than IM-sensitive GIST cells (48).

Following chronic IM induction, energy metabolism in GIST cells shifts from the tricarboxylic acid cycle to the pentose phosphate pathway (PPP) (47). On one hand, the expression of phosphate glucose dehydrogenase (PGD), one of the rate-limiting enzymes of the PPP, is significantly upregulated in IM-resistant GIST cell lines. Overexpression of PGD promotes GIST cell proliferation and inhibits cell apoptosis. On the other hand, the level of hypoxia-inducible factor 1α (HIF-1α) is elevated under prolonged stimulation of reactive oxygen species generated by IM (47). HIF-1α leads to changes in metabolic pathways as follows: the HIF-1α–PGD–PPP axis stimulates GIST cells from the G1 phase to the S phase, inhibits GIST cell apoptosis through metabolic reprogramming, and ultimately leads to IM resistance (Figure 1C) (47).

Most of the research viewpoints on energy metabolism in GIST cells have reached a consensus, which provides a theoretical basis for overcoming resistance to IM from the perspective of abnormal energy metabolism. Therapy involving the inhibition of the energy metabolism pathway combined with IM, such as VLX600 combined with IM and WZB117 combined with IM, requires further preclinical validation (46, 48).

2.3 Non-coding RNAs and resistance to imatinib

2.3.1 Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides with no or limited protein-coding capacity (49–51). LncRNAs play key roles in several important biological processes, including regulation of epigene expression, as well as transcriptional and posttranscriptional regulation (52). Numerous studies have demonstrated that lncRNAs play key regulatory roles in the disease course of human cancers, including cancer cell proliferation, apoptosis, and drug resistance (53–55). In addition, recent studies have shown that lncRNAs can modulate the sensitivity of patients to anticancer drugs and thus have the potential to be therapeutic targets in the treatment of drug-resistant tumors (56, 57). Moreover, lncRNAs may promote the progression and metastasis of GISTs, and the expression of many lncRNAs in primary GIST tissue differs from that in recurrent GIST tissue (58, 59). Furthermore, lncRNAs are associated with secondary resistance to IM in GISTs, and the resistance mechanisms are mostly related to signaling pathways (60–63). LncRNAs, such as the HOX antisense intergenic RNA (HOTAIR), can also promote IM resistance by activating autophagy in GIST cells (62).

The lncRNA coiled-coil domain-containing 26 (*CCDC26*), located on chromosome 8q24.21, is a retinoic acid–dependent regulator of myeloid differentiation, also known as RAM (64). *CCDC26* interacts with C-KIT and regulates its transcription. In addition, *CCDC26* downregulates the expression of c-Kit in GISTs, whereas *CCDC26* knockout induces IM resistance in GIST cells by upregulating the expression of C-KIT (Figure 2A) (60). *CCDC26* knockout also upregulates the expression of insulin-like growth factor 1 receptor (*IGF-1R*) (Figure 2A). *IGF-1R* induces drug resistance by participating in the apoptosis pathway (Figure 2C), whereas inhibition of *IGF-1R* reverses *CCDC26* knockout–induced drug resistance (59, 65, 66). These findings suggest that treatment targeting the CCDC26 or

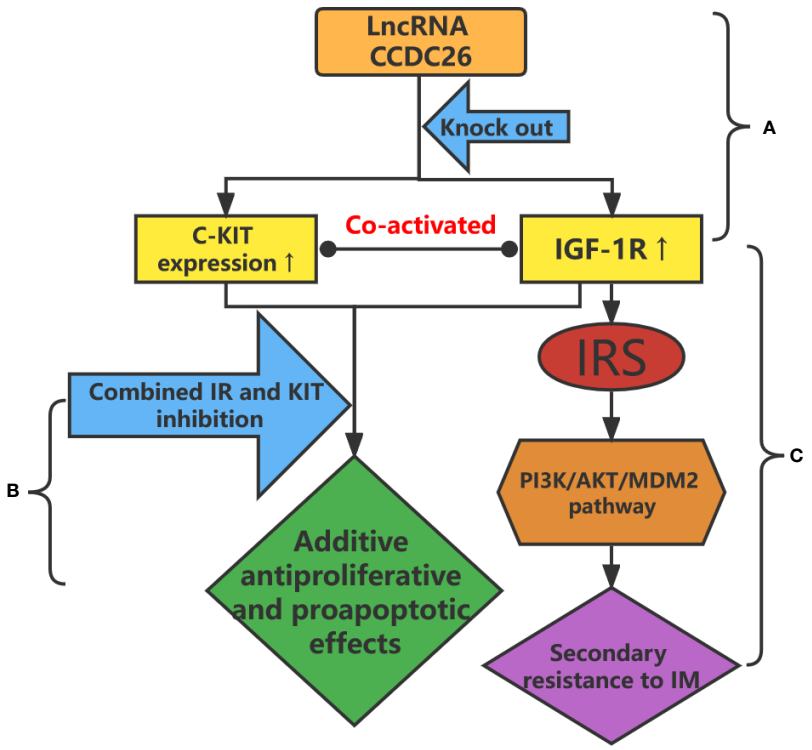


FIGURE 2
(A) *CCDC26* knockout upregulates the expression of C-KIT and IGF-1R in IM-resistant GISTs. **(B)** Additive antiproliferative and proapoptotic effects are obtained after the combined inhibition of IR and KIT in IM-resistant GIST cells. **(C)** Upregulation of IGF-1R leads to drug resistance through the PI3K/AKT/MDM2 signaling pathway.

CCDC26-IGF-1R axis may improve sensitivity to IM in patients with IM-resistant GISTs.

Yan et al. identified a set of dysregulated lncRNAs in IM-resistant GISTs using chip technology and found that lncRNAJC6-2 is associated with the HIF-1α pathway, which links lncRNAs to energy metabolism (61). Using high-throughput RNA sequencing, Shao et al. found that GIST samples express 40% of all annotated lncRNAs in humans. Notably, the number of downregulated lncRNA expressions was greater than the number of upregulated expressions, irrespective of the presence or absence of resistance to IM. The expression of RP11616M22.7 is significantly increased in IM-resistant samples than that in non-resistant samples and is closely related to the Hippo pathway. Overexpression of RP11-616M22.7 induces resistance to IM in GIST cells, whereas RP11616M22.7 gene knockout enhances IM resistance in GIST cells both *in vitro* and *in vivo* (62).

Some studies have demonstrated that the expression and dysregulation of lncRNAs are more cancer-specific than those of protein-coding genes (67). Therefore, specific lncRNAs in GISTs are likely to be involved in unique biological functions related to treatment and drug resistance. With an in-depth exploration of specific lncRNAs and their mechanisms, our understanding of the non-coding transcriptome of GISTs will become more comprehensive, which will, in turn, accelerate the development of new effective therapeutic targets.

2.3.2. MicroRNAs

MicroRNAs (MiRNAs) are 22-nucleotide non-coding small ribonucleic acids that control tumor cell growth by regulating the expression of multiple gene products and the function of cellular pathways (68). MiRNAs play important roles in the pathogenesis, invasion, and drug resistance of tumors and are thus identified as targets for cancer diagnosis, therapy, and prognosis (69–72). Akçakaya et al. analyzed miRNA expression profiles to study the miRNA expression signatures associated with response to IM and *KIT* mutation status in patients with GIST. They found that miR-125a-5p and its target gene, tyrosine-protein phosphatase non-receptor type 18 (*PTPN18*), play important roles in IM resistance. The mechanism behind this is that overexpression of miR-125a-5p downregulates the level of *PTPN18* expression in GISTs and promotes resistance to IM (73). Subsequent studies demonstrated that the effects of miR-125a-5p and *PTPN18* on IM resistance are mediated through phosphorylated FAK levels (Figure 3) (28). By comparing two groups of IM-resistant GIST samples with and without secondary mutations, Amirnasr et al. detected 22 significantly differentially expressed miRNAs and almost completely separated the two groups of samples. Three of these miRNAs, namely, miR-92a-3p, miR-99a-5p, and miR-101-3p, are potential effectors of IM resistance. This suggests that the distribution of miRNA biomarkers may be related to the presence of secondary mutations (74). Zhang et al. used

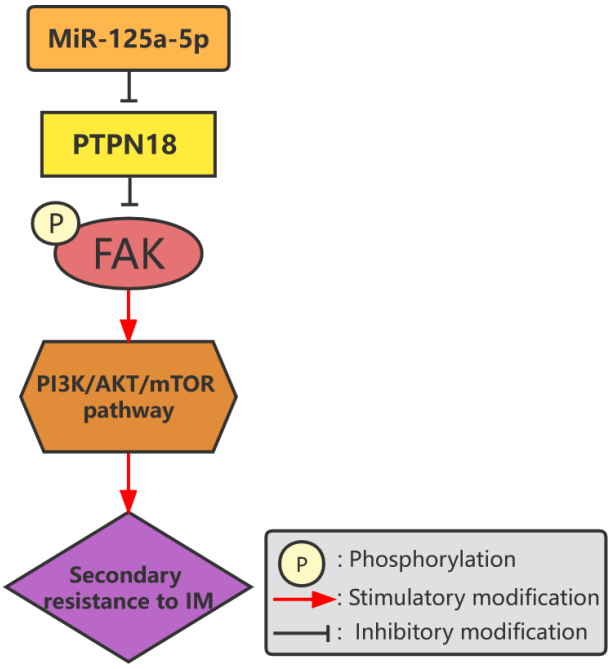


FIGURE 3
Overexpression of miR-125a-5p downregulates the expression of *PTPN18* and promotes IM resistance in GISTs mediated by phosphorylated FAK levels.

themicroarray data preserved by Akçakaya et al. to identify five key miRNAs in the lncRNA–miRNA target gene regulatory network, confirming that overexpression of miR-28-5p and miR-125a-5p is significantly related to secondary resistance to IM (75). Kou et al. studied the miRNA expression profiles in the serums of patients with GIST and found that the levels of miR-518e-5p and miR-548e in the serums of the patients in the IM-resistant group were significantly higher than those of the patients in the IM-sensitive and healthy control groups. This indicates that the serum level of miR-518e-5p can distinguish IM-resistant patients from IM-sensitive patients or healthy individuals (76).

Studies have demonstrated that miRNAs can regulate resistance to chemotherapy by inducing autophagy in GIST cells (77, 78). Chen et al. found that miR-30a sensitizes GIST cells to IM by inhibiting autophagy and confirmed that the autophagy marker Beclin-1 is a target gene of miR-30a (79). Zhang et al. found that HOTAIR targeting the autophagy-related protein 2 homolog B inhibitor miR-130a promotes resistance to IM by upregulating the level of autophagy (63).

Information regarding most miRNAs associated with secondary resistance to IM is still in the discovery stage; thus, the resistance mechanisms need to be studied further. Because miRNAs are closely related to the pathogenesis, invasion, metastasis, and drug resistance of tumors, research ideas should be broadened rather than limited to one aspect. Several studies have confirmed that lncRNAs can regulate other non-coding RNAs, especially miRNAs, and that miRNAs also have regulatory effects on lncRNAs (80, 81). Therefore, improving the regulatory network of miRNAs and lncRNAs in IM-resistant GISTs is also a promising research direction.

2.4. Several key proteins and resistance to imatinib

From a protein perspective, approximately 10% of *KIT*-positive GISTs lose the expression of *KIT* oncoproteins and become resistant to TKIs owing to the transition to a *KIT*-independent state (*KIT*-negative) during TKI treatment (82). Tu et al. found that Axl in TKIs is highly expressed in KIT-negative GISTs and that *Axl* gene knockout or silencing can inhibit the proliferation of *KIT*-negative GISTs. This information provides a new perspective regarding the Axl/P53 signaling axis as a therapeutic target for a subset of *KIT*-negative GISTs (83).

Cyclin D1 can regulate the cell cycle through the activation of the cyclin-dependent kinase (CDK), activation of transcription factors, *RAD51* co-regulation of DNA repair, and activation of the AMPK-LKB1 signaling pathway (84). Cyclin D1 is highly expressed in each *KIT*-independent GIST cell subline. In addition, inhibition of cyclin D1 has antiproliferative and proapoptotic effects in *KIT*-independent GISTs, which are associated with Rb activation and p27 upregulation. Notably, *PRKCQ* is a negative regulator of cyclin D1 expression, whereas the Jun and Hippo pathway effector molecules YAP and TAZ are positive regulators of cyclin D1 expression. The *PRKCQ*, Jun, and Hippo pathways synergistically regulate cyclin D1 expression in GISTs (85). Using GeCKO screening, (85) found that *CDK1* is highly expressed in advanced and IM-resistant GISTs in three patient cohorts. *CDK1* is the founding member of the CDK family (86). It can promote the proliferation and progression of GISTs by binding to substrate protein kinase B (Akt) and regulating its phosphorylation (87). In most solid tumors, Aurora kinase A (*AURKA*) promotes cell cycle progression by regulating cell cycle checkpoints (88). A clinical analysis has demonstrated that *AURKA* can be an independent prognostic factor for GISTs. In addition, experiments have shown that overexpression of *AURKA* can promote the proliferation of GIST-T1 cells, inhibit cell apoptosis, and enhance the resistance of cells to IM (89).

Several multidrug transporters play key roles in secondary drug resistance by regulating drug concentrations in tumor cells. Multidrug resistance–related protein 1 (MRP1) is one of the major multidrug transporters (90). Intracellular IM level plays an important role in the development of IM resistance in patients with chronic myeloid leukemia (91). Studies have confirmed that MRP1 and breast cancer resistance protein are highly expressed in IM-resistant GIST cell lines and that IM-resistant patients with GIST show significantly lower intracellular IM levels than IM-sensitive patients (92). This suggests that drug transporters may play an important role in IM resistance. Xu et al. proposed the following mechanism for this: the methyltransferase *METTL3* mediates 6-methyladenosine (M6A) to modify the 5'end non-coding region of the multidrug transporter MRP1 mRNA and promotes the translation of MRP1 mRNA, leading to drug resistance in GISTs (93). M6A is a common mRNA modification that regulates mRNA stability, splicing, and translation (94, 95). These findings suggest that drug transporters may be potential therapeutic targets in the treatment of IM-resistant GISTs.

The insulin receptor (IR) is a member of the tyrosine kinase family, including homologous types 1 and 2 (*IGF-1R* and *IGF-2R*) (96). IR and *IGF-1/2R* play important roles in energy metabolism and cell growth, division, and differentiation (97). Chen et al. showed that IR and Kit are co-activated in IM-resistant GIST cells and biopsy samples but not in IM-sensitive GIST cells (Figure 2A). They also found that additive antiproliferative and proapoptotic effects were obtained after the combined inhibition of IR and *KIT* in IM-resistant GIST cells (Figure 2B) (98). Thus, the inactivation of IR increases the sensitivity of resistant cells to IM, suggesting that the combined inhibition of IR and *KIT* is a promising therapeutic strategy in the treatment of IM-resistant GISTs.

Serrano-Candelas et al. found that the linker molecule SH3-binding protein 2 (*SH3BP2*) is expressed in non–wild-type GISTs. *SH3BP2* is involved in the regulation of the expression

and cellular activity of *KIT* and *PDGFRA* in GISTs. They also found that silencing of *SH3BP2* is accompanied by downregulation of oncogenic *KIT* and *PDGFRA* and significant promotion of apoptosis in IM-sensitive and resistant GIST cells (99).

The relationship between various key proteins and IM resistance mechanisms is intricate and interconnected. However, there is no clear comparison of the role of each protein network in the mechanism of resistance to IM. Targeted therapy that involves a single protein network may not solve the problem of secondary resistance to IM. The combined inhibition of multiple protein networks may become a new research direction for the treatment of IM-resistant GISTs.

2.5. Mutation and other gene aberrations and resistance to imatinib

2.5.1. Oncogenic *KIT* signaling on the Golgi apparatus

The Golgi apparatus may serve as a platform for oncogenic *KIT* signaling (100, 101). Moreover, oncogenic *KIT* signaling on the Golgi apparatus is essential for the autonomous proliferation of GIST cells (101). In IM-resistant GISTs with secondary *KIT* mutations, oncogenic *KIT* signaling is predominantly localized to the Golgi apparatus (100). This KIT activates the PI3K/AKT/mTOR pathway, MEK-Erk pathway, and signal transducer and activator of transcription 5 (Figure 4) (100). Blocking KIT biosynthetic transport from the endoplasmic reticulum to the Golgi apparatus suppresses oncogenic signaling, suggesting that Kit autophosphorylation is spatiotemporally regulated (100, 101). In an analysis of this mechanism, Obata et al. discovered a biosynthetic protein, 2-methylcopropylamide (M-COPA; also known as "AMF-26"), which blocks the transport of KIT from the endoplasmic reticulum to the Golgi apparatus by inhibiting the autophosphorylation of *KIT* at Y703/Y721/Y730/Y936 and ultimately inhibits oncogenic *KIT* signaling (Figure 4) (101). M-COPA inhibits the activation of Kit kinase domain mutants, thereby inhibiting the proliferation of IM-resistant GISTs (101). A novel heat shock protein 90 inhibitor, TAS-116, also inhibits the growth of drug-resistant cells and induces their apoptosis by reducing *KIT* autophosphorylation in the Golgi apparatus (102). Notably, the effect of TAS-116 has been validated in an animal study conducted using a xenograft mouse model (102).

Oncogenic *KIT* signaling on the Golgi apparatus provides new insights into not only the pathogenesis of KIT but also the treatment of IM-resistant GISTs that express mutant *KIT*. However, further studies are needed to confirm the clinical efficacy of drug therapies that target this carcinogenic signal.

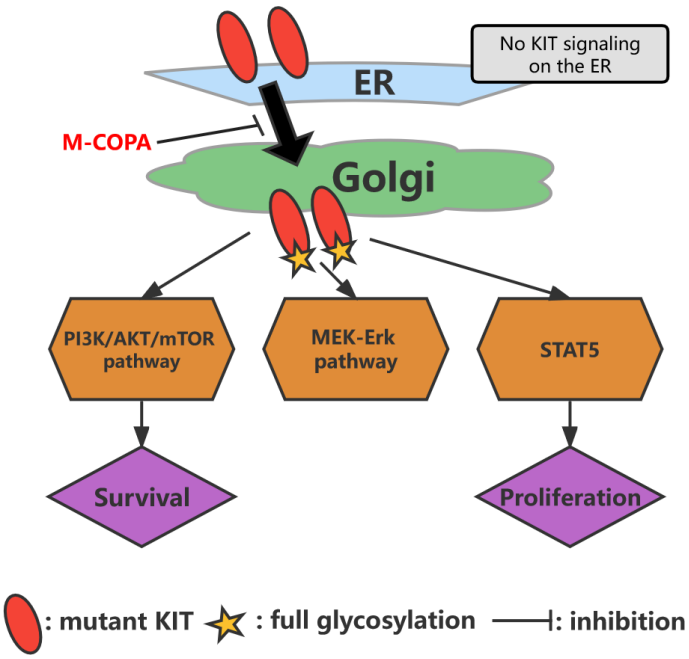


FIGURE 4
Model of oncogenic *KIT* signaling on intracellular compartments in GISTs. *KIT* is normally transported from the endoplasmic reticulum to the Golgi apparatus, followed by full glycosylation. After reacting with the Golgi apparatus, *KIT* can activate the PI3K/AKT/mTOR pathway, MEK-Erk pathway, and *STAT5*. M-COPA inhibits oncogenic signaling by blocking the transport of *KIT* from the endoplasmic reticulum to the Golgi because *KIT* activates downstream molecules only on the Golgi apparatus.

2.5.2. KIT^{low} cell subsets

KIT^{low} cell subsets may be a cell bank that mediates the progression and recurrence of GISTs (103). Bardsley et al. detected a precursor cell of ICCs in the stomach wall of a mouse that possesses stem cell properties, including the ability to self-renew and differentiate into mature ICCs. This ICC precursor cell–derived cell line was able to spontaneously transform to form GIST-like tumors. Notably, the expression of Kit in this ICC precursor cell was lower than that in mature ICC precursor cells (104, 105).

Inherently, IM-resistant *CD34* KIT^{low} cells are a distinct subset of GIST cells. KIT^{low} cells have stronger replication ability and clonogenic potential than KIT^{High} cell subsets. This subpopulation has tumor stem cell–like expression characteristics and behaviors and can self-renew and differentiate into IM-sensitive *CD34* KIT^{High} progeny. Notably, TKI treatment results in the enrichment of this KIT^{low} cell subset, which may be mediated by cell-associated transcription factors (OCT4 and NANOG) (103). The KIT^{low} cell subset represents a novel mechanism of primary resistance to TKIs and a targetable subpopulation in the treatment of GISTs. This provides valuable therapeutic ideas for overcoming the persistence and recurrence of GISTs after TKI therapy.

3 Conclusions

In this review, the findings of studies on mechanisms of secondary resistance conducted over the last 5 years are summarized from the aspects of abnormal energy metabolism, gene mutations, non-coding RNA, and key proteins. These previous studies provide theoretical support for solving the problem of the mechanism of resistance to IM. However, the available data on most drug-resistance mechanisms are still in the research stage. Further clinical studies are needed to confirm the safety and efficacy of utilizing drug-resistance mechanisms as potential therapeutic targets.

Addressing the problem of secondary resistance to IM has always been the key to improving the treatment outcomes and prognoses of patients with GISTs. Different resistance mechanisms are closely linked and interact with each other; thus, using a single resistance mechanism as a therapeutic target should be avoided. The combined inhibition of drug-resistance mechanisms with IM therapy and the combined inhibition of multiple drug-resistance mechanisms are expected to become new options in the treatment of GISTs. Implementing individualized therapy based on the identification of resistance mechanisms will provide new adjuvant treatment options for patients with IM-resistant GISTs, thereby delaying the progression of GISTs.

Author contributions

Conceptualization: ZW. Article collection and analysis: YK, XH, PS, and QZ. Manuscript writing: XH and QZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Molecular biology of pancreatic neuroendocrine tumors: From mechanism to translation

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Pancreatic neuroendocrine tumors (pNETs) are a group of heterogeneous tumors originated from progenitor cells. As these tumors are predominantly non-functional, most of them display asymptomatic characteristics, making it difficult to be realized from early onset. Therefore, patients with pNETs are usually diagnosed with metastatic disease or at a late disease stage. The relatively low incidence also limits our understanding of the biological background of pNETs, which largely impair the development of new effective drugs. The fact that up to 10% of pNETs develop in patients with genetic syndromes have promoted researchers to focus on the gene mutations and driver mutations in *MEN1*, *DAXX/ATRX* and mTOR signaling pathway genes have been implicated in disease development and progression. Recent advances in sequencing technologies have further enriched our knowledge of the complex molecular landscape of pNETs, pointing out crucial roles of genes in DNA damage pathways, chromosomal and telomere alterations and epigenetic dysregulation. These novel findings may not only benefit early diagnosis of pNETs, but also help to uncover tumor heterogeneity and shape the future of translational medical treatment. In this review, we focus on the current molecular biology of pNETs and decipher how these findings may translate into future development of targeted therapy.

KEYWORDS

pancreatic neuroendocrine tumors (pancreatic NETs), molecular biology, epigenetic modulation, targeted therapies, signaling pathway

Introduction

Neuroendocrine tumors are tumors originating from progenitor cells with neuroendocrine functions, which are a large group of cells that have a neuroendocrine phenotype in the body with the capacity of producing a variety of hormones. Pancreatic neuroendocrine tumors (pNETs) are types of neuroendocrine tumors with significant

heterogeneity originating from hormone-producing cells (islet cells), accounting for about 4% to 5% of pancreatic primary tumors (1). Although neuroendocrine tumors can arise from any location of the gastrointestinal tract and bronchopulmonary system, with small intestinal NETs being firstly discovered in the early 20th century, increasing evidence has shown that pNETs are actually a distinct biological disease possessing unique genetic phenotypes (2). pNETs are further classified into functional (hormone producing) and nonfunctional tumors based on the secretion of hormones, and nonfunctional pNETs account for 85% of pNETs, with a significantly worse prognosis compared to functional pNETs (2). Therefore, these asymptomatic pNETs are often detected at advanced stages when patients have missed the best opportunity for treatment, resulting in the overall poor prognosis of pNETs.

In the past 30 years, with the application of imaging, endoscopy, and biomarker detection technologies, the detection rate of pNETs have increased significantly (3), whereas overall survival has remained relatively unchanged during the past several decades (1, 2). Similar to other solid tumors, pNETs also display tumor heterogeneity which leads to the difficulty in targeted therapy development. The tumor heterogeneity is mediated by different molecular biological mechanisms, which is also an important reason for the different tumor behaviors and symptoms of different types of pNETs. According to the different pathological behavior, the World Health Organization (WHO) proposed a common framework to describe the classification of pNETs (Table 1) (4). Furthermore, according to the different genetic and epigenetic signatures, the pNETs can be divided into six subtypes, each corresponding to different prognosis and pathological features (5). The genetic characteristics of well-differentiated nonfunctional-pNETs and low-differentiated pancreatic neuroendocrine carcinoma (pNEC) are largely different, which lead to differences in the formation, growth and progression of tumors. Poorly differentiated pNEC often has mutations in the *TP53* and *RB1* genes, resulting in pNEC with strong invasion and metastasis ability, and genetic changes such as *ATRX*/*DAXX* and *MEN1* are more common in patients with highly differentiated nonfunctional-pNETs (6, 7). Since deep insights on alteration in molecular biology stand a chance to provide novel strategies on targeting therapy towards malignancies, our research group has conducted a series of great exploration on malignant lesions (8–10). However, relatively limited understanding of the molecular control of pNETs development also slows down the pace to new effective therapies. With the rapid development of next generation sequencing (NGS), our understanding of the molecular mechanism and genotyping of pNETs has accelerated (11). A large number of studies have found that the occurrence and development of pNETs are associated with epigenetic abnormalities such as gene mutations, DNA damage repair, DNA methylation, histone modification, chromosome remodeling, and activation of alternative lengthening of telomeres (ALT) mechanisms and related signal pathways. This review summarizes current advances in molecular biology of pNETs tumorigenesis and provides future prospects in targeted therapy development based on these molecular alterations.

Molecular control of pNETs in genetic syndromes

A total of 10% pNETs occurs in genetic syndromes. In-depth research on genetic syndromes provides a basis for further understanding of genetic changes during tumor formation (1, 12). Multiple endocrine tumor syndrome type I (MEN1): Hereditary pNETs is the most common in MEN1 syndrome, seen in about 30% to 80% of patients with MEN1. Most of pNETs in MEN1 are small multiple non-functional tumors with a well-differentiated phenotype. MEN1 syndrome is caused by *MEN1* tumor suppressor gene germline inactivation mutation and normal allele somatic cell loss. The mutation can lead to the loss of the expression of its coding protein-menin protein. As a tumor suppressor, menin protein can interact with more than 40 proteins, and participate in gene transcription regulation, chromosome stability, DNA repair, epigenetic regulation and other processes to regulate cell proliferation (13). In pNETs patients with multiple endocrine tumors, the menin protein may be modified by the histone modification of the cyclin B2 promoter region, which affects histone H3 acetylation and H3K4me3 methylation levels and thus plays a regulatory role (14). The *MEN1* gene is also the most common mutant gene in sporadic pNETs, which plays an important role in tumorigenesis (2, 6). VHL disease (Von Hippel-Lindau disease, VHL): The incidence of pNETs in VHL disease is around 15%. Generally, it is a small non-functioning tumor with multiple occurrences. Liver metastasis is rare, and the prognosis is

TABLE 1 The World Health Organization (WHO) Gastrointestinal and pancreatobiliary tract neuroendocrine neoplasms classification.

Neuroendocrine neoplasm type	Classification	Diagnostic Criteria
Neuroendocrine tumor (NET)	NET, grade 1	< 2 mitoses/2 mm^2 and/or Ki67 < 3%
	NET, grade 2	2-20 mitoses/2 mm^2 and/or Ki67 3-20%
	NET, grade 3	> 20 mitoses/2 mm^2 and/or Ki67 > 20%
Neuroendocrine Carcinoma (NEC)	Small cell NEC	> 20 mitoses/2 mm^2 and/or Ki67 > 20%, with small cell cytomorphology
	Large cell NEC	> 20 mitoses/2 mm^2 and/or Ki67 > 20%, with large cell cytomorphology

better than sporadic cases. *VHL* gene inactivation mutations can inhibit the ubiquitination of hypoxia-inducible factor transcription factors, leading to increased expression of target genes related to hypoxia-driven angiogenesis pathways, and promoting angiogenesis and tumor growth. *VHL* gene mutation is extremely rare in sporadic pNETs, but it can cause similar gene mutation effects through gene censorship or gene promoter hypermethylation (3, 15). Neurofibromatosis type I (NF1): The incidence of pNETs in NF1 patients is less than 10%. *NF1* gene encodes neurofibrillin, which is a negative regulator of RAS/MAPK and PI3K/AKT/mTOR signal transduction network. Inactivation mutation of *NF1* gene causes an increase in the activity of related pathways, which may promote tumor progression to malignancy (15). (4) Tuberous sclerosis (TSC): pNETs is extremely rare in TSC, only occurs in 1% of patients, and is mainly a non-functional tumor. The proteins encoded by the *TSC1* and *TSC2* genes can form a complex and function together. *TSC1* or *TSC2* gene inactivation mutations make the TSC complex lose its inhibitory effect on the mTOR pathway and promote cell proliferation (15). In sporadic pNETs, down-regulation and mutation of *TSC1*/*TSC2* genes were also found (6).

Molecular alteration in sporadic pNETs

Most pNETs are sporadic with a long onset. During the onset of pNETs, molecular alteration including typical gene mutations occurs. Chromosomal changes and gene fusion has also been suggested to play an important role in tumorigenesis. Owing to the development of sequencing technologies, the discovery of various genetic events with molecular heterogeneity provides new spectrum of disease development and progression (11). Related genetic changes in pNETs formation involve several critical signaling pathways and chromatin remodeling, with telomere maintenance and DNA damage also being identified.

MEN1 mutations

The *MEN1* gene is located at 11q13, and it is highly conserved during evolution. The coding protein of *MEN1* gene is called menin. Menin participates in the regulation of transcription and maintains the homeostasis of the gene group. 90% of patients with germline mutations in the *MEN1* gene will eventually develop multiple endocrine tumor syndrome type I as discussed above. In addition, *MEN1* mutations have been discovered in both functional and non-functional pNETs (6, 7). Loss of menin expression or abnormal nuclear translocation caused by *MEN1* gene mutations will cause a series of signaling pathway disorders, and then result in systemic endocrine diseases including pNETs. Menin interacts with many transcription factors in the cell nucleus, directly or indirectly participates in epigenetic regulation processes such as histone methylation modification and chromosome remodeling, and plays a key regulatory role in the normal transcription of target genes and the maintenance of cell phenotype. Tumor chromosome translocation causes the loss of important domains of chromatin binding protein, leading to the loss of its ability to recruit SWI/SNF complexes, and thus destroying the transcriptional function of chromatin binding protein regulating genes (7). Menin can also recruit protein arginine methyltransferase 5 (PRMT5) to the promoter region of *Gas1* gene, a key factor of the Hedgehog signaling pathway, and strengthen the inhibition of histone arginine methylation, thereby inhibiting the Hedgehog signaling pathway to achieve the anti-tumor effect (16). Interestingly, in different tissues, menin can regulate the transcription of different target genes through the same histone modification mechanism to exert different biological function (17). In addition, menin and DAXX use histone (H3K9me3) modification mechanism to regulate the endopeptidase promoter of membrane metal and affect the occurrence of pNETs (18). The *ATRX*/*DAXX* and *MEN1* genes can maintain the integrity and stability of the somatic cell genome by regulating the structure of chromatin. If there is a problem with these regulatory genes, it will promote the occurrence and development of pNETs. Studies have sequenced 38 well-differentiated pNETs specimens, and also found that *MEN1* gene, *ATRX*/*DAXX* gene and PI3K/AKT/mTOR signaling pathway are the most common hotspot mutations (19).

ATRX/DAXX mutations in chromatin remodeling and telomere maintenance

Chromatin remodeling related gene mutations are very common in pNETs. The menin protein can recruit the MLL1 histone methyltransferase complex to play an important role in chromatin remodeling and gene expression (13). The discovery of the role of *ATRX* and *DAXX* gene mutations in pNETs is of great significance. DAXX is a specific histone chaperone that can guide histone H3.3 to deposit on the inter-arm and telomere heterochromatin, and ATRX is the chromatin remodeling adenosine triphosphate enzyme in the SWI/SNF family (20). Related proteins encoded by *ATRX*/*DAXX* genes can form complexes and combine with histone H3.3 to deposit them in specific areas such as telomeres and centromeres, and participate in apoptosis and chromatin remodeling (7). In addition, the ATRX-DAXX complex may form nucleosomes containing H3.3 through replication-independent methods, such as defective nucleosomes. It can lead to DNA damage and genome instability. At the same time, at the end of the chromosome, the ATRX/DAXX complex also needs to inhibit the defective DNA for repair, and the complex formed by the mutation cannot inhibit the repair of the defective DNA, so that

telomere fusion occurs. *ATRX* gene mutations can also cause chromatin plate assembly, chromatin condensation, and centromeric dysfunction during chromosome mitosis. These abnormal changes can cause chromosomal mutations and lead to the occurrence of pNETs (21).

Tumors maintain the length of telomeres in cells without relying on telomerase, and the mechanism by which cells continue to proliferate is called alternative lengthening of telomeres (ALT) (22). *ATRX*/*DAXX* gene mutations are related to the ALT phenotype, and those with gene mutations have a better prognosis. In tumors> 2 cm, *ATRX*/*DAXX* gene mutations are common, but no related protein expression loss was found in microadenomas, indicating that the loss of ATRX/DAXX protein expression and the appearance of ALT phenotype may occur at the progression phase of pNETs (23). Consistent with these, immunohistochemistry staining on 192 metastases of 52 patients with pNETs showed that 52% metastases had *ATRX*/*DAXX* gene expression loss, with both ATRX/DAXX and ALT abnormalities and a shortened survival (24). Another study found that the frequencies of ATRX/DAXX inactivation and ALT activation in pNETs patients were 19.3% and 20.8%, respectively, which were related to a high-grade tumor, neurovascular invasion, liver metastasis and other malignant clinical pathological features, as well as a prognostic survival (25). Moreover, recent study has shown that loss of *ATRX*/*DAXX* and presence of ALT are able to predict distant metastasis and are correlated to inferior overall survival and relapse-free survival in ≤2.0cm non-functional pNETs (26). Hence, *ATRX*/*DAXX* status and ALT detection are recommended as prognostic biomarkers for pNETs, especially for non-functional pNETs. *ATRX*/*DAXX* somatic mutations are common in extended telomerase. The shortened telomerase has fewer *ATRX*/*DAXX* mutations, but more chromosome fragmentation and gene fusion. Research has also shown that non-functional pNETs also has copy number abnormalities, accompanied by *DAXX*/*ATRX* gene mutations, and both can accurately predict the risk of postoperative recurrence (27). Most of the frameshift mutations and nonsense mutations of *ATRX* and *DAXX* in pNETs can cause complete loss of protein expression. More importantly, *ATRX*/*DAXX* gene mutations are related to the alpha cell origin of non-functional pNETs, and the prognosis of such patients is worse than that of wild-type gene mutations (28).

PI3K/AKT/mTOR signaling pathway

Phosphatidylinositol 3-kinase (PI3K) belongs to the lipid kinase family, which can be activated by extracellular growth factor signals to phosphorylate and activate protein kinase B (AKT) and participate in protein synthesis, cell growth, and many other cellular processes. Mammalian target of rapamycin (mTOR) is the most important downstream factor in the PI3K/AKT/mTOR pathway. It can combine with a variety of proteins to form mTOR1/2 complexes through the integration of growth factors and nutritional molecules and other intracellular and extracellular signals (29). In sporadic tumors, multiple mTOR-pathway related genes including *TSC2*, *PTEN* and *PIK3CA* genes were widely altered. The menin protein also binds to AKT, which can further inhibit intracellular positional transfer of AKT and lead to the inhibition of mTOR signaling pathway. The PTEN gene encodes a product that inactivates PI3K through dephosphorylation, and the mutation of this gene can activate mTOR signaling. Recent study showed that about 37% of the samples of sporadic pNETs had *MEN1* gene mutations, and about 13% of the samples had mutually exclusive gene mutations related to the mTOR pathway, including *PTEN*, *TSC1*/*TSC2*. In addition to gene mutations, chromosomal changes and gene fusions related to this pathway were also identified, including the repeated amplification of chromosomal loci of the PI3K activator *PSPN* gene and mTOR regulator *ULK1* gene, and the fusion of *EWSR1* gene with *BEND2* and *FLI1* genes, which can activate mTOR signal and promote tumor formation (6). (Figure 1)

The frequency of mutations in genes related to the mTOR signaling pathway in pNETs patients is extremely high (6), and there is usually activation of the mTOR pathway, that is, p-mTOR overexpression, accompanied by loss or down-regulation of PTEN expression, both of which are usually in close relation to biological behavior and poor prognosis (30). These evidences all indicate that mTOR-related signaling pathways might be one of the key targets of pNETs therapy. Everolimus is an mTOR inhibitor. In the RADIANT-2 study, compared with placebo and long-acting octreotide (LAR), taking everolimus and LAR can prolong the median disease free survival of patients with pNETs (31). The RADIANT-3 study showed that the everolimus group prolonged the median disease-free survival of advanced pNETs by 6.4 months compared with the placebo group, which is of great clinical significance (32). Somatostatin analogues (SSA) have been used for the treatment of pNETs. Studies showed that the somatostatin signal functions on the insulin-like growth factor 1/PI3K/mTOR pathway, thereby inhibiting the secretion and proliferation activity of pNETs (33). Studies also suggested that the synergistic mechanism of everolimus and LAR was related to the inhibition of mTOR by everolimus and the down-regulation of insulin-like growth factor 1 by octreotide (33). Taken together, both everolimus and octreotide can inhibit tumor growth through the mTOR signaling pathway.

DNA damage and repair

ATM suppressor genes are involved in a variety of cellular processes related to DNA damage. Bersani et al. found ATM gene mutations in 5.5% of the samples (34), indicating that there were changes related to DNA damage and repair during the

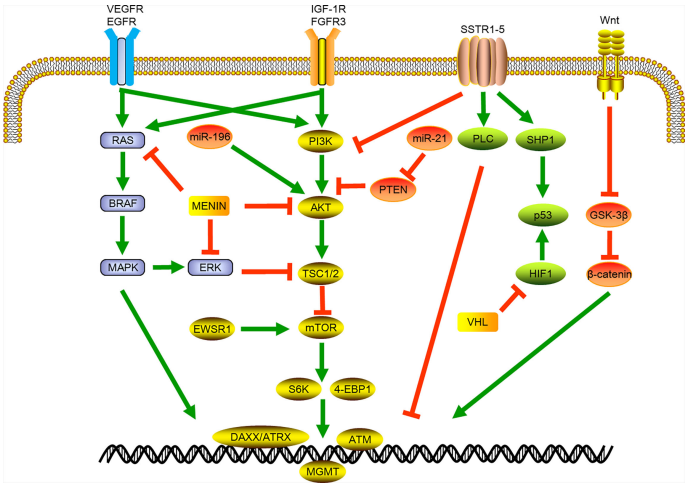


FIGURE 1
Schematic of signaling pathways involved in pancreatic neuroendocrine tumor development and progression. VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; BRAF, B-Raf Proto-oncogene; MAPK, mitogen-activated protein kinase; miR, micro RNA; ERK, Extracellular-signal-regulated kinase; EWSR1, EWS RNA binding protein 1; IGF-1R, insulin-like growth factor receptor 1; FGFR3, fibroblast growth factor receptor 3; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; TSC1/2, Tuberous sclerosis 1; mTOR, mammalian target of rapamycin; S6K,; 4-EBP1, Eukaryotic translation initiation factor 4E-binding protein 1; DAXX, death domain-associated protein; ATRX, X-linked mental retardation and a-thalassemia syndrome protein; ATM, ataxia telangiectasia; SSTR, somatostatin receptor; PTEN, phosphatase and tensin homolog deleted; PLC, phospholipase C; SHP1, Src homology region 2 domain-containing phosphatase-1; HIF1, hypoxia-inducible factor 1; VHL, Von Hippel-Lindau.

occurrence of pNETs. Germline mutations in DNA damage repair related genes *MUTYH*, *CHEK2* and *BRCA2* were also discovered (6), indicating that DNA damage repair related genes may be the initiation event of some genetic syndromes. A study of exome sequencing of sporadic insulinomas found that 30% of the samples had functional mutations in the YY1 (YinYang 1, YY1) transcription factor gene *T372R*, which could increase the transcription activity of YY1 and induce the target genes expression such as mitochondrial genes *IDH3A* and *UCP2* (35). Studies have found that other genes related to changes in DNA expression in insulinomas including *H3F3A*, *KDM6A* and *ATR* genes (36). Therefore, these results suggested targeting DNA damage and repair genes may be a new potential therapy for the treatment of pNETs.

Sporadic pNECs

According to the histological differentiation characteristics of tumors, G3 tumors were further divided into well differentiated pNETs and poorly differentiated pNECs, which usually have *TP53* or *RB1* gene mutations. Among pNETs, pNECs accounts for about 7.5% of the total, and the 5-year survival rate of patients is less than 7%. The survival time of patients with metastasis was even worse, with a usually less than 1 year survival time (37). Yachida et al. analyzed 19 pNECs samples and found that 57% and 71% of samples had mutations in *TP53* and *RB1* genes, respectively (38). A recent large-sample study of 123 pNECs also found the above changes, but this change was very rare in well-differentiated pNETs (39), which further illustrates that poorly differentiated pNECs were not developed from the progression of well-differentiated tumors to poorly differentiated states. Different genetic changes may occur in the development and progression of pNECs, which needs further studies to illustrate.

Epigenetic control of pNETs

The occurrence of tumors is a process in which genetic and epigenetic changes are intertwined, which together promote the occurrence and progression of tumors. In pNETs, less than half of the tumors caused by genetic changes such as gene mutations indicating that epigenetic changes have a non-negligible role in tumorigenesis (6). Epigenetic control of pNETs includes CpG methylation, histone modification, and non-coding RNA molecules, which have all been studied extensively in the development and progression of pNETs.

DNA methylation

DNA methylation mostly occurs in the nucleotides of gene CpG islands and is mediated by DNA methylation transferase.

In cancer cells, downregulated gene expression due to promoter hypermethylation is the hallmark of dysregulated CpG methylation which leads to silencing of tumor suppression genes and tumor development. Genome-wide hypomethylation which result in DNA instability is also a typical characteristic during tumor progression. There are many gene methylation changes in pNETs, among which the *RASSF1* (Ras association domain family 1 isoform A) gene, regulated by p53 and DAXX, is the most common hypermethylated gene in metastatic tumors (40). Hypermethylation of *VHL*, *HOPX* and *TIMP3* gene promoter is also related to metastatic tumors with poor prognosis (12, 41, 42). *CDKN2A* gene promoter methylation is associated with early tumor recurrence and shortened overall survival (43). *MGMT* promoter hypermethylation has been found in 40-44% of pNETs with more aggressive tumors and worse prognosis (42). Furthermore, the methylation status of the *MGMT* gene promoter can also be used to predict the therapeutic response of temozolomide (44). Not only hypermethylation is involved in pNETs development and progression, but also global hypomethylation has been shown to contribute to different clinical outcome in pNETs. Choi et al. reported for the first time that there was hypomethylation of *LINE1* and *ALU* gene sequences in pNETs (45). Consistent with these results, further studies showed that hypomethylation was related to poor prognosis and progression of tumors (46).

Histone modification

Histone is an important part of chromatin, which can package DNA to form nucleosomes. After gene translation, histone modification can regulate the binding ability of genes and transcription factors and maintain chromatin structure. Menin protein regulates histone methylation status by recruiting the MLL1 histone methyltransferase complex, and participates in various cellular processes and chromatin remodeling (47). Menin also mediates H3K9 methylation through recruiting SUV39H1 (Suppressor of variegation 3-9 homolog protein family), which is reduced through introduction of patient-derived *MEN1* mutations into the SUV39H1 interaction domain (48). Loss of menin also leads to H3K4me3 loss, which results in MEN1-like sporadic pancreatic tumors (49). Therefore, menin also controls the development of pNETs through regulating methylation process. The protein encoded by *SETD2* gene is involved in the regulation of histone methylation status and chromatin activity. Disruption of SETD2 function was identified in 81% of primary pNETs with distant metastases (24). *ATRX*/*DAXX* gene encoding protein can bind to histones, change the histone deposition area, and participate in chromatin remodeling (12). Loss of *ATRX* and *DAXX* was also associated with shorter survival in patients with pNETs (24). Furthermore, recent studies also found that phosphorylated histone H3 can be used as an ideal prognostic factor to better predict the survival of pNETs patients (50).

MicroRNAs

Micro-RNA (miRNAs) are small non-coding RNAs that regulate targeted mRNA, affect the expression of downstream effector proteins, and also regulate post-transcriptional gene expression. Along with long non-coding RNAs (lncRNAs), miRNAs are post-transcriptional gene regulators implicated in tumor development and progression (51). miRNAs functions through their imperfect 5'-base pairing with target mRNA sequences to induce gene silencing, and they also have the capacity of upregulating target genes under specific circumstances, indicating a duel properties in both oncogenic and tumor suppressing (52). miRNAs are relatively stable in circulation and their expression correlates with various clinical characteristics not only in different tumor types but also in subtypes of the same tumor. Therefore, since most pNETs are non-functional and asymptotic, the detection of miRNAs in the peripheral may be promising candidates as diagnosis/prognosis tools.

miR21, which targets and downregulates *PTEN*, has been suggested to be in close relation to higher proliferation and metastatic disease. It also targets *PDCD4*, which is a tumor suppressor, to induce the downregulation of *PDCD4* mRNA in metastatic tumors (53). In addition, miR21 was overexpressed in the circulation of patients with pNETs when compared to that of chronic pancreatitis (54), although information in respect to detailed type and grade of pNETs lacked. Similarly, miR210 can be induced by hypoxia-induced stress (55), which may further facilitate the metastasis of pNETs (56). The expression of miR-103 and miR-107 associated with lack of expression of miR-155 discriminated tumors from normal, and miR204 and miR211 further distinguished functional and non-functional pNETs, as they were restricted to insulinomas (57). Regarding to the distinguish between pancreatic ductal adenocarcinoma and pNETs, miR1290 was identified to differentiate pancreatic ductal adenocarcinoma from pNETs with an AUC of 0.80 (56). Other miRNAs involved in pNETs compared to pancreatic ductal adenocarcinoma were miR584, miR1285, miR550a-5P, and miR1825 (58). As mentioned above, the function of miRNAs largely depends on their molecular control of target genes. Therefore, miRNAs involved in the regulation of pNETs related genes might display different functional properties and may guide future studies based on their target gene characteristics. Consistent with these, three clusters of miRNAs were identified recently, with each cluster displaying distinct gene mutational rates in *mTOR*, *MEN1*/*DAXX*/*ATRX* and DNA damage pathways (59). miR-Cluster-1 tumors were enriched in the MEN1 mRNA subtype, and were composed of non-functional pNETs with a moderate metastatic

potential. miR-Cluster-2 tumors were enriched in the metastasis-like primaries mRNA subtype with high metastatic potential, and miR-Cluster-3 tumors were mainly composed of insulinomas.

Long non-coding RNAs

Studies on the molecular control of pNETs by long non-coding RNAs (lncRNAs) are limited. The HOX antisense intergenic RNA (HOTAIR) and the metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) were newly identified lncRNAs in the control of pNETs development and progression. HOTAIR is a chromatin-modifying lncRNA involved in prostate cancer neuroendocrine differentiation (60), and overexpression of HOTAIR led to increased metastasis of breast cancer cells through manipulating H3K27me (61). Therefore, targeting HOTAIR has been shown to reduce cell invasiveness through the induction of cell cycle arrest and apoptosis (62). However, controversial results were discovered recently in which upregulation of HOTAIR in pNETs resulted in less aggressive disease and better prognosis (63). Similar results were also discovered for MALAT1, which was associated with favorable clinical outcome in pNETs. Detailed functional studies are urged for understanding the related mechanisms. Other dysregulated lncRNAs include MEG3 and lncNEN885 (64, 65). MEG3 was shown to be regulated by menin in cell line model and epigenetic activation of MEG3 was also thought to be an attractive therapeutic approach for potential future clinical use (64). MEG3 directly targets the proto-oncogene c-MET (hepatocyte growth factor receptor) to block cell proliferation and delay cell cycle progression, and it also displays an anti-tumor effect through manipulating the p38/ERK/Akt and Wnt/β-catenin pathway in a MEG3/miR183/BRI3 dependent manner (66).

Therapy implication

For patients with advanced stage and distant metastasis, comprehensive treatment is the main approach to prolong survival. With a better understanding in molecular biology of pNETs, developing new therapeutic targets based on molecular alterations has been a new promising field. According to the characteristics of the patient's molecular changes, individualized treatment plans may also better extend the patient's survival time.

Somatostatin analogues

Therapy against somatostatin receptor (SSR) is currently most widely used target therapy in pNETs, as around 70% of tumors express somatostatin receptor (67). SSR2 has a high affinity with somatostatin analogues (octreotide or lanreotide, etc.), which can lead to the inhibition of tumor secretion, cell proliferation and angiogenesis. Somatostatin analogs can also be coupled with radionuclide nuclide yttrium or lutetium to treat patients in advanced stage or metastasis. According to reports from clinical trials, the objective response rate of this treatment can reach 20% ~60%, with an overall survival period of 53 months (68).

mTOR inhibitors

As mentioned above, the mTOR pathway is very important in the occurrence of pNETs. Everolimus, an mTOR inhibitor, has been used in patients with advanced tumors (69). However, there is still no reliable diagnostic method for early screening of patients who can benefit from mTOR inhibitor treatment. Since the expression of genes after mutations can be affected by epigenetic changes, screening for mTOR pathway-related proteins or mRNA may help select appropriate patients.

Anti-angiogenic drugs

The pathological feature of pNETs is characterized by a rich blood supply, and targeted therapy of angiogenic molecules thus may help to inhibit tumor growth. Tyrosine kinase receptor inhibitors have been used for patients with pNETs (70). However, anti-angiogenic drugs may promote tumor progression and distant metastasis, which may be related to tumor hypoxia and the expression of vascular endothelial growth factor A, fibroblast growth factor, ephrin A1, and the activation of the proto-oncogene c-Met (71). Therefore, the combined use of c-Met inhibitors can simultaneously inhibit tumor growth and metastasis. At present, this treatment program has achieved certain effects in animal models, but further clinical studies are still needed. Nitric oxide synthase inhibitors and thrombospondin analogs have also been found in mouse models to inhibit angiogenesis and tumor growth, with potential benefits in clinic in the future (72).

MEN1 gene replacement therapy

MEN1 gene is the most common mutant gene in pNETs, and *MEN1* gene replacement therapy for pNETs with a lack of menin protein expression is gradually emerging. As a tumor suppressive protein, menin, encoded by MEN1, has long been proved to play an anti-tumor role in various tumor when over-expressed in tumor (73, 74). At present, the overexpression of menin protein has been found to inhibit tumor growth in *in vitro* cell line experiments and has been confirmed in animal models of pituitary tumors (75). *MEN1* gene transgene therapy is an emerging and effective treatment method for pNETs with a lack of menin protein expression, but it still needs further confirmation and clinical research.

Epigenetic regulatory factor

The reversibility of tumor epigenetic changes has a great research value in treatment. Epigenetic regulatory factor is an emerging anti-cancer drug that has been approved for use in diseases such as leukemia (76). In pNETs, patients with methylation of the *MGMT* gene promoter have better therapeutic effects with temozolomide, but this result still needs to be confirmed (77). Studies on histones have found that histone deacetylase (HDACs) inhibitors can enhance the expression of somatostatin receptor mRNA and the uptake of radionuclide-labeled octreotide in pNETs cell lines (QGP1 and BON1), enhancing the effectiveness of related treatment (78). A recent study found that JQ1 (a histone inhibitor) can inhibit tumor growth in the pNETs mouse model (79). At present, most of the apparent regulatory factor drugs for pNETs are in the stage of *in vitro* experiments or animal models, and a large number of clinical studies are still needed to evaluate clinical efficacy.

Hypoxia-inducible factor

VHL gene mutation can result in pNETs occurrence. It has been found that pNETs caused by VHL disease possibly progress through hypoxia-inducible factor 2a (HIF-2α) and hypoxia related biological process (80). Similarly, a study also found that hypoxia-inducible factor expression was elevated in the pNETs tissue compared to non-tumor samples (81). According to the findings, Belzutifan, a specific HIF-2α inhibitor, has been developed to treat VHL disease and achieved great success, which makes it approved by Food and Drug Administration (FDA) for VHL disease and its related tumor treatment (82).

Novel molecular for targeting therapy

By using unbiased phage display screening technique, construction of tumor-specific peptides or antibody fragments targeting tumor-associated antigens have become true, thus providing a novel potential therapeutic strategy for malignancies (83). Researchers have found that CDH17, which can be detected in the intestinal epithelial cells, is also highly expressed in pNETs (84). Unbiased phage display screening technique further screened and identified a single variable-domain antibody (VHH) targeting CDH17 in non-functional pNETs, with superiority of better permeability in tissues and reduced toxicity triggered by complement system (85). Based on this finding, the researchers developed chimeric antigen receptor (CAR) T cells targeting CDH17, which achieved eradication in pNETs and showed reliable security in the drug administration (85).

In a word, although pNETs exhibit highly variable clinical characteristics and prognosis, with the increasing awareness of the molecular characteristics of pNETs, more and more precision and translational treatment strategies are being developed. For instance, research into somatostatin receptor and VHL gene mutation have led to application of somatostatin analogues and belzutifan in clinical treatment. The translation of basic research findings into clinical treatment strategies is expected to improve the prognosis of patients with pNETs.

Conclusion

With the development of genomic sequencing technology, molecular characteristics and tumor heterogeneity of pNETs have been gradually identified, which also help to further classify pNETs and better understand the potential clinical outcomes for patients with different pNETs subtypes. The genetic characteristics of well-differentiated non-functional pNETs and poorly-differentiated pNEC are different. Functional pNETs such as insulinomas also differ in genetic background when compare to non-functional pNETs. In addition, epigenetic changes also play an important role in tumor development and progression, which have an impact on tumor diagnosis, treatment and prognosis. How to integrate various molecular information to fully address disease heterogeneity may not only benefit the diagnosis and classification of pNETs, but also provide promising strategy to develop powerful personalized treatment.

Author contributions

XS, XW and XL drafted the manuscript. YZ and WG raised the concept and suggested the topic, and YZ illustrated the figures. YZ, WG and XS made contributions in funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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The role of neoadjuvant chemotherapy in patients with locally advanced colon cancer: A systematic review and meta-analysis

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Background: Controversy persists about neoadjuvant chemotherapy (NAC) within the field of locally advanced colon cancer (LACC). The purpose of this study was to assess the existing and latest literature with high quality to determine the role of NAC in various aspects.

Methods: A comprehensive literature search of the PubMed, Embase, Web of Science, and the Cochrane Library databases was conducted from inception to April 2022. Review Manager 5.3 was applied for meta-analyses with a random-effects model whenever possible.

Results: Overall, 8 studies were included in this systematic review and meta-analysis, comprising 4 randomized controlled trials (RCTs) and 4 retrospective studies involving 40,136 participants. The 3-year overall survival (OS) (HR: 0.90, 95% CI: 0.66-1.23, P = 0.51) and 5-year OS (HR: 0.89, 95% CI: 0.53-1.03, P = 0.53) were comparable between two groups. Mortality in 30 days was found less frequent in the NAC group (OR: 0.43, 95% CI: 0.20-0.91, P = 0.03), whereas no significant differences were detected concerning other perioperative complications, R0 resection, or adverse events. In terms of subgroup analyses for RCTs, less anastomotic leak (OR: 0.51, 95% CI: 0.31-0.86, P = 0.01) and higher R0 resection rate (OR: 2.35, 95% CI: 1.04-5.32, P = 0.04) were observed in the NAC group.

Conclusions: NAC is safe and feasible for patients with LACC, but no significant survival benefit could be demonstrated. The application of NAC still needs to be prudent until significant evidence supporting the oncological outcomes is presented.

Systematic review registration: https://www.crd.york.ac.uk/prospero, identifier (CRD42022333306).

KEYWORDS

neoadjuvant chemotherapy, locally advanced colon cancer, overall survival, R0 resection, perioperative complication

Introduction

According to the report by the American Cancer Society in 2022 (1), approximately 151,030 individuals of both sexes would be newly diagnosed with colorectal cancer (CRC), of which 70.3% would be colon cancer. The prognosis of patients with CRC has been greatly improved following advances in surgical concepts and techniques. The CONCORD-2 trial (2) revealed that 5-year cancer-specific survival of localized, regional, or metastatic colon cancer was 90%, 70%, and 14% respectively in the United States. In recent years, neoadjuvant therapy has been widely and well applied in solid tumors such as mammary, esophageal, gastric, and rectal cancers. Notably, preoperative concurrent chemoradiotherapy has been considered the preferred standard treatment for locally advanced rectal cancer (3, 4), while neoadjuvant treatment remains controversial in the field of locally advanced colon cancer (LACC). The National Comprehensive Cancer Network (NCCN) Guideline for colon cancer has provided recommendations since 2016 that neoadjuvant chemotherapy (NAC) such as FOLFOX or CAPEOX could be an alternative primary treatment for clinical T4b findings, aiming to improve R0 resection rate, postoperative recovery, and survival outcome. On the other hand, the only two published randomized controlled trials including the FOXTROT (5) and PRODIGE 22 (6) trials focusing on survival outcomes both failed to demonstrate the oncological benefit of NAC in patients with LACC. Additionally, the safety and feasibility of NAC should also be cautiously assessed. Therefore, we performed this systematic review and meta-analysis of the existing and latest literature with high quality to determine the role of NAC in patients with LACC.

Material and methods

Study selection

This systematic review and meta-analysis was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) (7, 8) and Meta-analysis Of Observational Studies in Epidemiology (MOOSE) (9) reporting guidelines. This study has been registered in the International Prospective Register of Systematic Reviews (PROSPERO). A comprehensive literature search of the PubMed, Embase, Web of Science, and the Cochrane Library databases was conducted from inception to April 2022.

Following were the inclusion criteria for this study: (a) studies concerning patients with locally advanced colon cancer, (b) studies comparing outcomes between NAC and upfront surgery with adjuvant chemotherapy, (c) studies reporting survival outcomes, perioperative complications, adverse events of chemotherapy, or tumor characteristics on pathological examination and, (d) RCTs or cohort studies. Exclusion criteria were as follows: (a) cases with distant metastasis, (b) cases with perioperative radiotherapy or intraoperative chemotherapy, (c) studies concerning the non-human subject, (d) articles of letter, case report, review, editorial, comment or only protocol, (f) without adequate data for analysis and, (g) non-English publications.

Data extraction and types of outcomes

Demographics extracted from included studies consisted of year, country, study design, Union for International Cancer Control (UICC) tumor stage, number of participants, age, sex, chemotherapy regimen, and chemotherapy completion.

The primary outcomes of this study assessed were survival outcomes. Secondary outcomes included perioperative complications, adverse events of chemotherapy, and tumor characteristics on pathological examination. Eventually, variables capable for meta-analyses consisted of OS, anastomotic leak, wound infection, abscess, ileus, re-operation, stoma, R0 resection, 30-day mortality, and grade 3 or higher adverse events of chemotherapy. Additionally, cases diagnosed with T3 with extramural depth ≥ 5 mm or T4 and RCTs would be respectively selected for subgroup analyses.

Two authors (Liang and Li) independently extracted and cross-checked all relevant data from included studies. In case of discrepancies, a third author (Yang) was asked to discuss until a consensus was reached.

Quality assessment

A measurement tool for the 'assessment of multiple systematic reviews' (AMSTAR) (10) consisting of 11 items with good face and content validity for measuring the methodological quality of systematic reviews was used. We carefully read the original literature and the details from the clinical trials registry (https://clinicaltrials.gov/) if available and then, evaluated the quality of literature. The modified Jadad quality scale (11) ranging from 0-7 points was used for bias assessment of RCTs and the Newcastle-Ottawa scale (NOS) (12) ranging from 0-9 points was for non-RCTs in this systematic review, with higher scores indicating better quality. Studies scoring greater than or equal to 4 points of the modified Jadad scale or 5 points of the NOS were considered high quality and therefore eligible.

Quality assessment was rated by two review authors (Liang and Li). In case of disagreements, a third author (Yang) was asked to participate in discussion until a consensus was reached.

Statistical analysis

The major demographic characteristics of all the included studies were summarized by a basic descriptive statistical method. Chi-squared and Fisher's exact tests were used for categorical and continuous variables, respectively. A systematic review and meta-analysis were performed following accumulation of sufficient research data. The software Review Manager, version 5.3 (https://community.cochrane.org/help/tools-and-software/revman-5) was used to analyze the data and a random-effects model was used to calculate the pooled effect estimates. Survival outcomes were presented as hazard ratios (HRs) with 95% confidence intervals (CIs). If HRs of included studies were not reported directly, an estimated HR was derived from Kaplan-Meier curves based on the method raised by Tierney et al. (13). In addition, continuous variables were analyzed by weighted mean differences (WMDs) with 95% CI, and odds ratios (ORs) with 95% CIs were used to assess dichotomous variables. All results compared were considered statistically significant at a two-sided $P < 0.05$.

The heterogeneity was evaluated by the Cochrane Q test and Higgins I^2 test. A sensitivity analysis or subgroup analysis would be conducted once the heterogeneity was considered high ($P < 0.1$ or $I^2 > 50\%$).

Results

Study selection and quality assessment

Our literature search yielded 4444 potential studies after duplicates were removed (Supplementary Table 1-4). Of these, 40 full-text articles were considered for inclusion. Eventually, a total of 8 published studies were included in this systematic review and meta-analysis. The flow diagram of the inclusion & exclusion process was presented in Figure 1 and also, reasons as well as references for full-text articles excluded were presented in Supplementary Table 5. The modified Jadad and NOS scale were used for quality assessment, shown in Supplementary Table 6.

Demographic characteristics

The major demographic characteristics of all the included studies consisting of 4 RCTs (5, 6, 14, 15) and 4 retrospective research (16–19) were summarized in Table 1. Notably, the FOXTROT trial (14) and Morton (5) independently reported results from the same registered clinical trial but different centers. Karoui et, al reported short-term outcomes in 2020 (15) and survival outcomes in 2021 (6) among the same

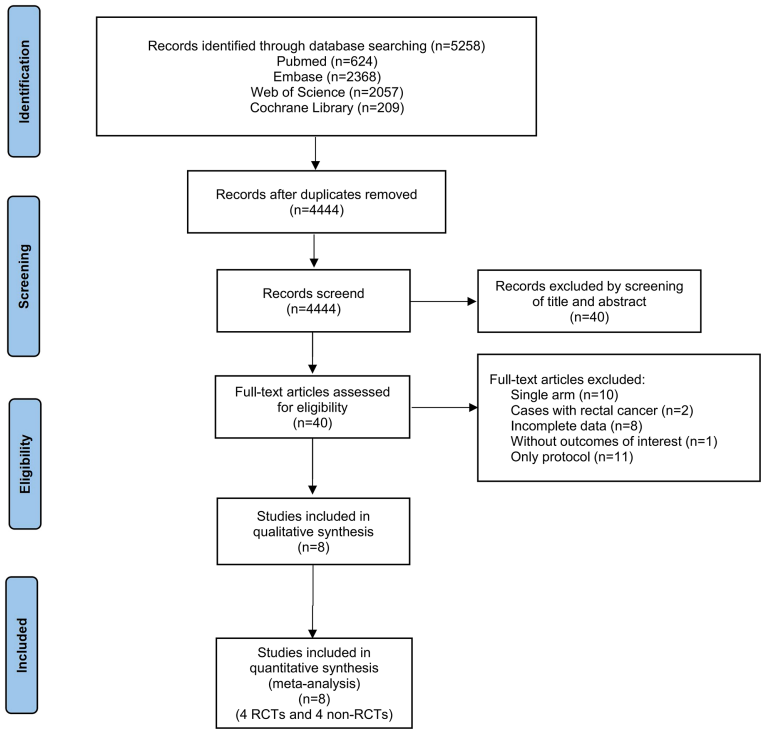


FIGURE 1
PRISMA selection flow diagram.

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TABLE 1 Baseline characteristics of studies included.

Study	Design	Stage	Number of participants	Number of nCT (%)	Median age (range),years old		Female / Male		Chemotherapy regimen
					nCT	pCT	nCT	pCT	
Foxtrot, UK,2012	RCT, Multi-center	T3 with extramural depth ≥5 mm or T4	150	99 (66%)	64 (31-82)	65 (38-78)	34/65	19/32	OxMdG
Dehal, USA,2018	Retrospective, Multi-center	T3 or T4	27575	921 (33%)	58.4 (12.1)*	61.5 (12.8)*	388/533	13384/13270	NA
Morton, UK,2019	RCT, Multi-center	T3 with extramural depth ≥5 mm or T4	1053	699 (66%)	NA		NA		OxMdG
Gooyer, NLD,2020	Retrospective, Multi-center	T4	2146	192 (9%)	64 (29-84)	64 (25-88)	91/101	961/993	NA
Karoui, France,2020	RCT, Multi-center	T3 with extramural depth ≥5 mm or T4	104	52 (50%)	65 (46-79)	62 (30-75)	22/30	19/33	simplified FOLFOX-4
Karoui, France,2021	RCT, Multi-center	T3 with extramural depth ≥5 mm or T4	104	52 (50%)	65 (46-79)	62 (30-75)	22/30	19/33	simplified FOLFOX-4
Silva, USA,2021	Retrospective, Multi-center	T3 or T4	7694	599 (8%)	60 (12)*	68 (13)*	228/371	3533/3562	NA
Laursen, Denmark,2022	Retrospective, Multi-center	T3 with extramural depth ≥5 mm or T4	1310	179 (14%)	67 (60-73)†	73 (67-80)†	80/99	586/545	NA

nCT, neoadjuvant chemotherapy; pCT, postoperative chemotherapy; RCT, randomized controlled trial; NA, not available.
*Presented with median (standard deviation).
†Presented with mean or mean (standard deviation).

participants. All of the included studies were conducted in multiple centers from Europe or America. In total, 40136 patients diagnosed as T3 or T4according to UICC tumor stage were included in the pooled analysis, with 2793 (7%) undergoing NAC. Among the retrospective studies included except for Gooyer, et al. (17), NAC was more likely to be administrated to younger patients. Only RCTs recorded the regimen and completion rate of chemotherapy.

Primary outcomes

Four studies reported survival outcomes, but only three could be pooled in meta-analysis. The 3-year OS (HR: 0.90, 95% CI: 0.66-1.23, P = 0.51) and 5-year OS (HR: 0.89, 95% CI: 0.53-1.03, P = 0.53) were comparable with low heterogeneity between the NAC and non-NAC groups (Figure 2). In addition, Morton (5) reported that no

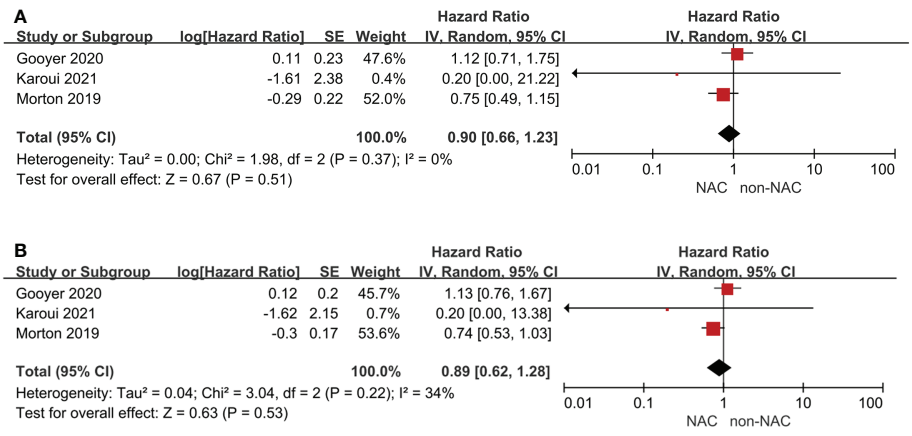


FIGURE 2
Primary outcomes: **(A)** 3-year overall survival, **(B)** 5-year overall survival.

statistically significant difference of cancer-specific mortality or recurrence was observed. Furthermore, the PRODIGE 22 phase II (6) also indicated that disease-free survival, recurrence-free survival, and time to recurrence were all comparable between two groups. Notably, Dehal, et al. (16) founded that no difference of OS was found in the NAC group among patients with T3 (HR: 1.03, 95% CI: 0.85–1.24, P = 0.79) or T4a (HR: 0.97, 95% CI: 0.62-1.53, P = 0.90), but significant benefit could be demonstrated for T4b patients (HR: 0.7, 95% CI: 0.56-0.87, P = 0.002).

Secondary outcomes

There were six studies reporting perioperative complications, adverse events of chemotherapy, or tumor characteristics on pathological examination. No evidence of differences in anastomotic leak (OR: 1.18, 95% CI: 0.62-2.25, P = 0.62), wound infection (OR: 1.08, 95% CI: 0.72-1.62, P = 0.72), abscess (OR: 1.74, 95% CI: 0.51-5.95, P = 0.38), re-operation (OR: 0.68, 95% CI: 0.40-1.14, P = 0.14), stoma (OR: 1.38, 95% CI: 0.92-2.07, P = 0.12), or R0 resection (OR: 0.98, 95% CI: 0.62-1.56, P = 0.94) was found between the NAC and non-NAC groups (Figures 3A-3F). Karoui, et al. (15) also reported comparison of postoperative ileus, suggesting that there was no difference between two groups (P = 0.68). However, the NAC group had a significantly lower 30-day mortality than the non-NAC group (OR: 0.43, 95% CI: 0.20-0.91, P = 0.03) (Figure 3G). On the other hand, Karoui, et al. (15) indicated that no difference was found 60 days after surgery (P = 1.00). Additionally, adverse events of chemotherapy were reported by two RCTs (14, 15), presenting grade 3 or higher adverse events. A meta-analysis was conducted, and no advantage in the NAC group could be found (OR: 0.73, 95% CI: 0.42-1.29, P = 0.28) (Figure 3H).

Subgroup analyses

Cases diagnosed with T3 with extramural depth ≥ 5 mm or T4 were separated as a subgroup for further analyses (Figure 4). Studies reported OS outcomes all met this standard. Anastomotic leak and R0 resection remained comparable and high heterogeneity, but 30-day mortality showed no difference between two groups, which was different from the result of primary outcomes.

In addition, RCTs were also be selected as a subgroup (Figure 5). The results of the 3-year and 5-year OS outcomes remained unchanged and had low heterogeneity. Anastomotic leak and R0 resection remained comparable and notably, the heterogeneity (I^2) decreased from 76% to 0% and from 83% to 46%, respectively.

Discussion

This systematic review identified 8 available studies with high quality, investigating the effects of NAC for patients with LACC from multiple perspectives. The meta-analysis indicated that NAC was not associated with better survival outcomes, even though it was safe and feasible in perioperative management. It is worth noting that the side effects caused by NAC cannot be ignored. In certain cases, these adverse events even led to the failure of some patients to complete the operation on schedule. On the other hand, there were also patients achieving tumor remission due to preoperative treatment, so they were able to avoid receiving surgery with pain and recovery.

No heterogeneity of the primary outcomes was detected, but the that of anastomotic leak and R0 resection in secondary outcomes was considered high. Sensitivity analysis may be achieved through subgroup analysis. Majority of the studies defined LACC as T3 with extramural depth ≥ 5 mm or T4 determined by computed tomography, and therefore, a subgroup was set according to this criterion. All outcomes showed no evidence supporting the use of NAC in these cases but could not solve the homogeneity. In the subgroup of RCTs, all subgroup remained unchanged, and also, the decrease of I^2 concerning anastomotic leak and R0 resection helped explain that the source of heterogeneity might be the study design of non-RCTs.

Compared with the two previous studies of meta-analysis (20, 21), more studies were included in the comparison of the feasibility and safety of NAC. Besides, more indicators were evaluated in this study for further assessment, such as abscess, re-operation, stoma, mortality and adverse events of chemotherapy. Regarding the survival outcomes in this study, only two RCTs and one non-RCT were included without a huge quantity of participants yet, which might bring bias. However, due to the addition of new literature in recent years and the elimination of low-quality Chinese documents not recruited in the world-wide database, this study overturned the conclusion that NAC has a survival benefit suggested by the previous meta-analysis (20).

Some patients with LACC cannot be radically cured even with combined organ resection. In addition, neoadjuvant therapy could help judge the biological behavior of tumors. For patients who still have disease progression during neoadjuvant chemotherapy, the significance of surgical resection is very limited. Although the use of NAC for colon cancer has increased significantly over time (16, 17, 22) in certain countries and regions, it is still infrequently as a common practice all over the world yet. According to previous published studies, the major obstacle to promotion might be the uncertainty regarding its potential benefit (23). Further RCTs concerning oncological outcomes is warranted to identify a subgroup of patients who could benefit from neoadjuvant

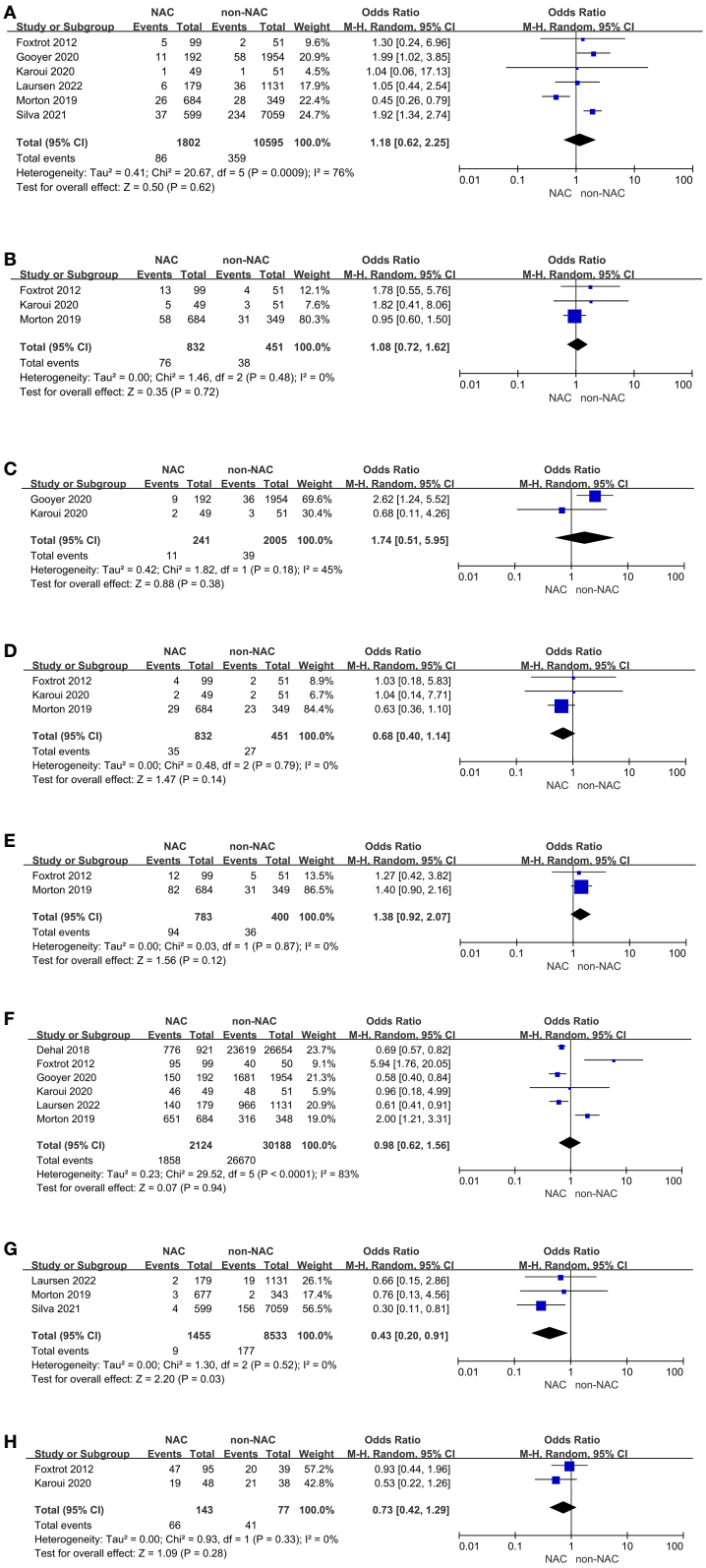


FIGURE 3
Secondary outcomes: **(A)** Anastomotic leak, **(B)** Wound infection, **(C)** Abscess, **(D)** Re-operation, **(E)** Stoma, **(F)** R0 resection, **(G)** 30-day mortality, **(H)** Adverse events.

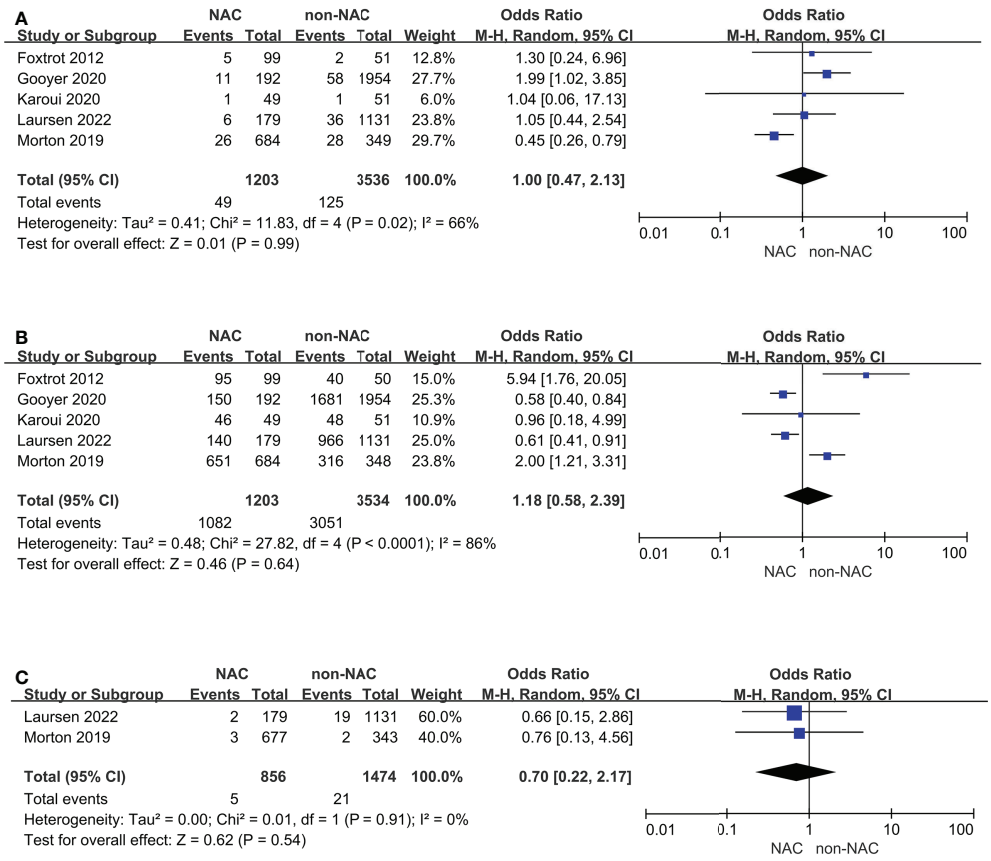


FIGURE 4
Subgroup analyses for cases diagnosed with T3 with extramural depth ≥ 5 mm or T4: **(A)** Anastomotic leak, **(B)** R0 resection, **(C)** 30-day mortality.

chemotherapy in a perioperative strategy. It is also worth noting that looking at survival benefit only misses the entire point of all the other considerations. There is also potential risk of emergency operation during the period of NAC, especially in those patients with tumor progression or poor condition attributed to chemotherapeutic agents. Fortunately, this meta-analysis and the included RCTs have shown that NAC for LACC is feasible, with acceptable toxicity and perioperative incidence rate. Furthermore, one has to consider the wasted finances of potentially unnecessary preoperative treatment. In order to implement NAC more reasonably and effectively, the key point should be to screen the appropriate subgroup and detect markers available to predict neoadjuvant chemosensitivity (24). Further optimization of clinical staging is essential to accurately select patients who may benefit from neoadjuvant therapy and avoid over treatment of low-risk patients (25). Thus, the use of NAC needs to be assessed in multiple aspects, which was the consideration in formulating this systematic review and meta-analysis.

It should also be noted that neoadjuvant therapy may change the biological characteristics of tumors. For instance, the expression of mismatch repair proteins, commonly MSH6, can change after neoadjuvant therapy (26, 27). Moreover, safety and efficacy of the following surgery may also be influenced because of the implementation of neoadjuvant therapy (28). Novel and personalized prognostic markers also need to be developed regarding patients with history of NAC (29, 30). An initial phase II experience indicates that a large proportion of patients with NAC might be converted to a low-risk state before surgery, thereby eliminating the need for following adjuvant chemotherapy (31). However, as the neoadjuvant treatment of colon cancer is still in the exploratory stage, it has not been recognized by the academic community that patients who have benefited from NAC could be exempt from surgery or postoperative chemotherapy.

Several limitations in this study should be mentioned. One was that not enough studies could be searched and included in the pooled analysis, especially for survival data. Additionally,

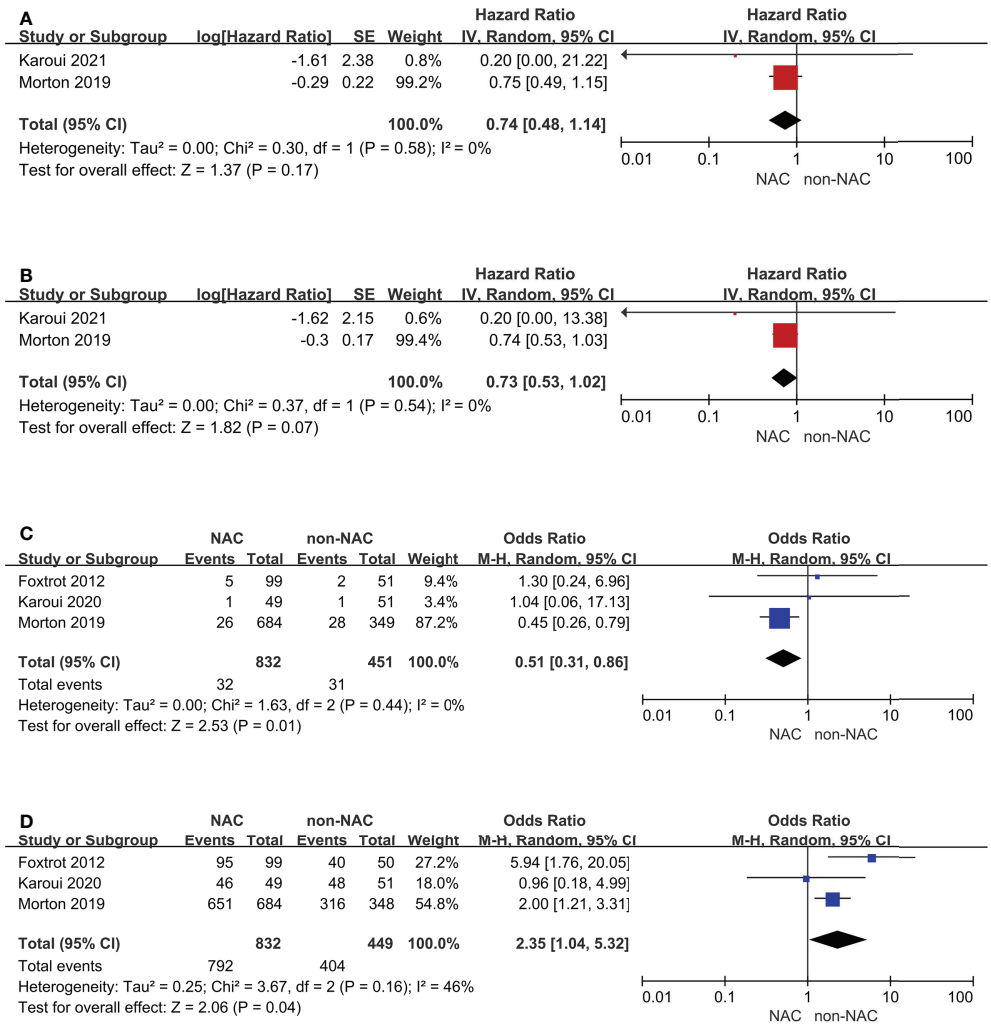


FIGURE 5
Subgroup analyses for randomized controlled trials: **(A)** 3-year overall survival, **(B)** 5-year overall survival, **(C)** Anastomotic leak, **(D)** R0 resection.

there is no consensus on the standard of LACC for now yet. Although a majority of studies defined LACC as T3 with extramural depth ≥ 5 mm or T4, few research have been able to accurately include these cases except RCTs. Recent studies have shown that when planning NAC for LACC, preoperative computed tomography scan with around 60% consistent with pathologic results in T stage or extramural invasion may overestimate the clinical stage and lead to inappropriate treatment (32–34). All of the above limitations have contributed to the heterogeneity among the included studies. Nevertheless, this research is of significance in the field of LACC, timely providing high-level evidence for clinical practice based on the available evidence.

Conclusion

Overall, NAC is safe and feasible for patients with LACC, but no significant survival benefit could be demonstrated. The application of NAC still needs to be prudent in the near future until significant evidence supporting the oncological outcomes is presented.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

ZYL, ZL, QY, and WW contributed to conception and design of the study. JF, DX, and HL performed the paper searching. ZYL and GM performed the statistical analysis. ZYL wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1024345/full#supplementary-material

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Artificial intelligence with magnetic resonance imaging for prediction of pathological complete response to neoadjuvant chemoradiotherapy in rectal cancer: A systematic review and meta-analysis

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Purpose: The purpose of this study was to evaluate the diagnostic accuracy of artificial intelligence (AI) models with magnetic resonance imaging(MRI) in predicting pathological complete response(pCR) to neoadjuvant chemoradiotherapy (nCRT) in patients with rectal cancer. Furthermore, assessed the methodological quality of the models.

Methods: We searched PubMed, Embase, Cochrane Library, and Web of science for studies published before 21 June 2022, without any language restrictions. The Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) and Radiomics Quality Score (RQS) tools were used to assess the methodological quality of the included studies. We calculated pooled sensitivity and specificity using random-effects models, I^2 values were used to measure heterogeneity, and subgroup analyses to explore potential sources of heterogeneity.

Results: We selected 21 papers for inclusion in the meta-analysis from 1562 retrieved publications, with a total of 1873 people in the validation groups. The meta-analysis showed that AI models based on MRI predicted pCR to nCRT in patients with rectal cancer: a pooled area under the curve (AUC) 0.91 (95% CI, 0.88-0.93), sensitivity of 0.82(95% CI,0.71-0.90), pooled specificity 0.86(95% CI,0.80-0.91). In the subgroup analysis, the pooled AUC of the deep learning (DL) model was 0.97, the pooled AUC of the radiomics model was 0.85; the pooled AUC of the combined model with clinical factors was 0.92, and the pooled AUC of the radiomics model alone was 0.87. The mean RQS score of the included studies was 10.95, accounting for 30.4% of the total score.

Conclusions: Radiomics is a promising noninvasive method with high value in predicting pathological response to nCRT in patients with rectal cancer. DL models have higher predictive accuracy than radiomics models, and combined models incorporating clinical factors have higher diagnostic accuracy than radiomics models alone. In the future, prospective, large-scale, multicenter investigations using radiomics approaches will strengthen the diagnostic power of pCR.

Systematic Review Registration: https://www.crd.york.ac.uk/prospero/, identifier CRD42021285630.

KEYWORDS

rectal neoplasms, artificial intelligence, pathological response, radiomics, magnetic resonance imaging

Introduction

More than 700,000 people are diagnosed with rectal cancer each year in the world, 70% of which are locally advanced rectal cancer (LARC) (1). The current standard treatment for LARC is neoadjuvant chemoradiation followed by total mesorectal excision (TME) (2–4). However, individual responses to neoadjuvant chemoradiotherapy (nCRT) are highly heterogeneous, ranging from pathological complete responses(pCR) with no viable cancer cells to small groups of cancer cells or even a small group of patients with tumor progression. Previous studies reported that about 15-27% of patients present pCR after nCRT (5). For those patients, organ preservation methods, such as "wait-and-see" and local excision (6), can achieve a comparable survival rate with pCR as TME, decreasing TME-related morbidity and functional problems (7). However, at present, pathological complete responses can only be confirmed by histopathological examination of surgically resected specimens, so in the personalized medicine of LARC, there is an urgent need to accurately predict pCR in a timely and non-invasive manner before implementing nCRT.

In rectal cancer patients, tumor response to nCRT can be assessed by computed tomography (CT), Positron emission tomography-computed tomography (PET/CT), or rectal ultrasound. However, magnetic resonance imaging (MRI) is the most accurate method to assess and predict pCR after nCRT (8–10). MRI is the imaging modality with the highest soft-tissue contrast. Rectal MRI can accurately evaluate the tumor location, tumor stage, invasion depth, extramural vascular invasion (EMVI), and circumferential resection margin (11). Multiparametric MRI can also reflect the pathophysiological information of rectal cancer, including dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI), diffusion-weighted imaging (DWI), and proton magnetic spectroscopic imaging (12–14). Changes in image morphology and image parameters extracted from contrast-enhanced MRI and DWI can help predict treatment response (15). To that end, mrTRG, a classification system similar to Mandard's tumor regression grade (TRG) system, has been developed, based on hypointensity in T2-weighted sequences of fibrotic tissue in the lesion (16). However, the low predictive value and poor consistency of mrTRG methods for pathological TRG hinder its clinical application (17).

Artificial intelligence(AI) has been frequently and successfully applied in the field of medical image analysis and can automatically identify complex patterns in imaging. Machine learning(ML) is a branch of AI that has been widely used in rectal cancer, including radiomics and deep learning(DL). Radiomics can transform clinical images into mineable data for quantitative analysis through high-throughput extraction (18). Thus, providing non-visual information related to tumor heterogeneity and underlying pathophysiology. Combining AI algorithms and MRI is a promising tool for improving the prediction of diagnosis or prognosis in patients with rectal cancer. In rectal cancer patients, radiomics has been widely used in rectal cancer staging classification (19), rectal cancer liver metastasis (20), distant metastasis (21), colorectal cancer KRAS gene status (22), MSS status (23), aquaporin-1 expression (24) and predicting the early stage of neoadjuvant chemoradiotherapy progress (25).In recent years, several studies based on radiomics have emerged to predict the pathological response to nCRT in patients with rectal cancer, including traditional machine learning models, deep learning models, and delta models. However, no comprehensive review of current research on artificial intelligence (AI) models for predicting pathological responses to nCRT in rectal cancer patients has been conducted, and the overall effectiveness of this prediction model is unknown. Furthermore, because radiomics research is a complicated process with several phases, it is critical to evaluate the method's quality to assure reliable and repeatable models before putting it into clinical applications.

The purpose of this systematic review was to describe available research on radiomics predicting pathological response to nCRT, evaluate the overall effectiveness of prediction models, and evaluate the methodological quality and bias risk in radiomics workflows.

Methods

The Standards for the Reporting of Diagnostic Accuracy Studies (STARD) (26) and Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (27) guidelines were followed. CRD 42021285630 is the registration number.

Search strategy

We searched from the databases of PubMed, Embase, Cochrane Library, and Web of science, for studies conducted before June 20, 2022. Using the technique of blending topic and free words. The key topic terms were "Rectal Neoplasms", "Artificial Intelligence", and "Magnetic Resonance Imaging", as well as related terms. The search strategy and detailed procedures are demonstrated in Table S1.

Inclusion and exclusion criteria

Studies that matched the following criteria were chosen after duplicate literature was eliminated (1): Pathologically proven locally advanced rectal cancer patients (T3/T4 and/or N1+) (2); All patients received neoadjuvant chemoradiation treatment (traditional long course and trial regimens were included) (3); Use of MRI as the examination modality (if other imaging modalities are used, as long as MRI has been studied separately) (4); Predicting pathological responses in patients using artificial intelligence models (5); Provided the information necessary for the reconstruction of 2 × 2 contingency tables (6); Any study design, including retrospective and prospective observational studies (7); the language of the publication was English.

The following criteria were used to exclude our studies (1): each study had at least 10 patients (2); Case reports, review articles, letters, meeting reports, and editorials (3); Studies that included neoadjuvant chemotherapy only (4); Studies that included neoadjuvant radiotherapy only (5); Classification of patients as responsive and non-responsive, rather than pathological complete and non-pathological complete responses (6); No validated studies. The titles and abstracts of all identified studies were examined first, followed by a full-text review of possibly suitable articles.

Data extraction

The following information was extracted from the eligible articles (1): study characteristics: authors (years of publication), country of corresponding author, study type, and study design (2); participants characteristics: neoadjuvant chemoradiotherapy, operation, standard reference, image examination interval, MRI scan parameters (3); model characteristics: image, region of interest (ROI) segmentation, input data, feature selection, modeling methods, verification methods (4); AI model performance: AUC, sensitivity, specificity, pCR population and non-pCR population.

Assessment of study quality

The Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) and Radiomics Quality Score (RQS) were used to evaluate the included studies' methodological quality and study-level risk of bias, respectively. The RQS was proposed by Lambin (28) in 2017 to evaluate radiomics research based on five stages of radiomics research (data selection, medical imaging, feature extraction, exploratory analysis, and modeling). The RQS tool has a total of 16 key items for quantifying the radiomics workflow. Details are in Table S2. The QUADAS-2 standard consists of four parts: patient selection, index test, reference standard, and flow and timing (29), which are detailed in Table S3. To obtain a consensus, two graduate students separately rated the quality and discussed disputes with the evidence-based medicine teacher.

Statistical analysis

We analyzed the raw data with the Midas command in Stata software (30), and we estimated the pooled sensitivity, specificity, positive likelihood ratio (PLR), negative likelihood ratio (NLR), and diagnostic odds ratio (DOR) with 95% CI using a bivariate random-effects model. We created a receiver operating characteristic curve (ROC) with sensitivity on the X-axis and specificity on the Y-axis, as well as the area under the curve (AUC) to demonstrate the diagnostic power of the included research (31).

We used linked forest plots to compare research and discover heterogeneity in confluent sensitivity and specificity (32). We initially visually inspected ROC images and forest plots to examine heterogeneity between study results. The I^2 measure was used to assess heterogeneity in studies. I^2 values >75% are highly heterogeneous (32). Two-sided P<0.05 was considered statistically significant. We plan to perform subgroup analyses to investigate potential sources of heterogeneity. As possible sources of heterogeneity, we considered modeling methods (radiomics and deep learning), sample size (whether greater than 100), radiomics feature extraction software (PyRadiomics and Others), regions of interest (2D and 3D), validation methods (external validation and internal validation) and inclusion of clinical factors (combined models and separate imaging feature models) were performed in subgroup analyses, which also allowed us to assess the impact of various factors on the model's diagnostic performance.

We used a funnel plot visual asymmetry evaluation to identify publication bias (33), which we first published using measurements of effect magnitude plotted against measures of study accuracy. We then officially analyzed test accuracy using Deeks' test and diagnostic odds ratio (DOR).

Results

Literature search

Through searches of PubMed, Embase, Cochrane Library, and Web of Science databases, a total of 1562 articles were retrieved. We browsed the titles and abstracts of 1048 studie, reviewed the full text of 298 studies, and finally reported from 90 articles. The application of AI models in neoadjuvant chemoradiotherapy for rectal cancer was evaluated, and finally, 21 articles were eligible for meta-analysis. The selection process is shown in Figure 1.

Characteristics of included studies

The 21 included studies were published between 2018 and 2022. More than half of the studies (11/21) were based on a population from China (34–44), three from South Korea (45–47), three from Italy (48–50), two from the USA (51, 52), one from Brazil (53), and one from Belgium (54). Two studies were prospective, and all the remaining studies (19/21) had a retrospective design. These 21 studies included a total of 6913 patients with sample sizes ranging from 95 to 1033 (median: 186). The definition of pCR was the same among most of the included studies(17/21), four studies not describing the definition of pCR. Long-course radiotherapy dosesrangeg from 41.8-50.6 Gy with different concurrent chemotherapy (Table 2).

Eleven studies used both 1.5T and 3.0T MRI scan types, seven studies only used 3.0T MRI scan, and two studies used 1.5T MRI scan. Most studies (15/29) used two or more sequences to build their predictive models. Five studies used

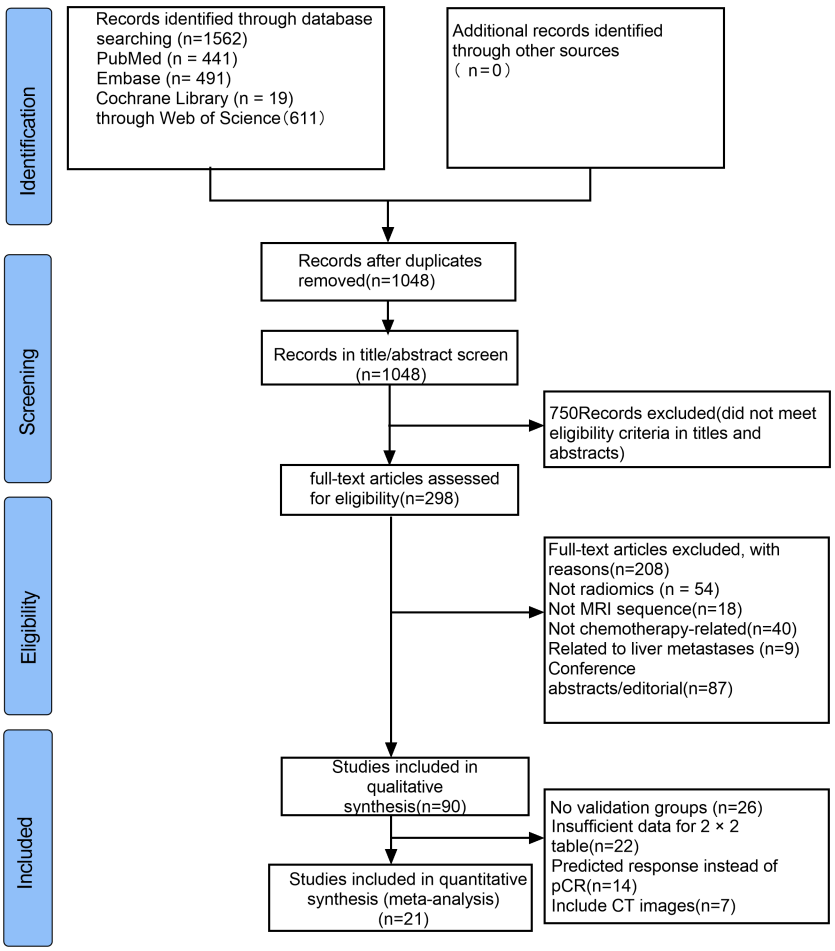


FIGURE 1
Flow diagram of the study selection process for this meta-analysis.

TABLE 1 Summary of general study characteristics.

Study	Country	Study type	No. of patients	MRI field intensity	Sequences	Slice thickness	Image acquisition time	Radiotherapy Dose (Cumulative)	Chemotherapy Regimen	Stand Reference	Definition of pCR	AUC
Antunes 2021	USA	Retrospective	104	1.5 T, 3.0T	T2WI	3.0-8.00mm	Before nCRT	45-50.4 Gy	Capecitabine (825 to 850 mg/m^2/ day)	Surgical pathology	0% viable tumor cells remaining after nCRT	0.712
Boldrini 2022	China	Retrospective	220	1.5 T, 3.0T	T2WI	Not reported	Before nCRT	45 Gy	Oral capecitabine 1650 mg/ m2 * die (d1-7, q7); 5-fluorouracil 225 mg/m2 * die (d1-7, q7) or CapOx 60 mg/m2 of iv oxaliplatin (d1, q7) plus oral capecitabine 1300 mg/m2 * die (d1-7, q7)	Surgical pathology	Absence of tumour disease on surgical specimen	0.75
Bulens 2021	Belgium	Retrospective	125	3.0T	T2WI, DWI	3.0-5.0mm	Before nCRT	45 Gy-50 Gy	Infusion of 5-Fluorouracil 225 mg/m^2/d), capecitabin 825 mg/m^2 bid	Surgical pathology	ypT01N0	0.86
Cheng 2021	China	Retrospective	193	3.0T	T1W,T2WI,T2FS	3.0mm,4.0mm	Before nCRT	45.0-50.4 Gy	mFOLFOX6, CapeOX	Surgical pathology	No tumor regression	0.912
Cui 2019	China	Retrospective	186	3.0T	T2WI, CE-T1WI,ADC	3.0mm,5.0mm	Before nCRT	50 Gy	Capecitabine(800mg/m^2/ day)	Surgical pathology	No viable tumour cells	0.966
Feng 2022	China	Prospective	1033	1.5 T, 3.0T	T2WI, CE-T1WI, DWI	2.0-6.0mm	Before nCRT	50 Gy/45Gy	5-fluorouracil based regimen combined with or without oxaliplatin	Surgical pathology	No remaining viable cancer cells	0·812
Horvat 2018	Brazil	Retrospective	114	1.5 T, 3.0T	T2WI, DWI	3.0mm	Before nCRT	Not reported	Not reported	Surgical pathology	ypT0N0	0.93
Horvat 2022	USA	Retrospective	164	1.5 T, 3.0T	T2WI, DWI	3.0mm,5.0mm	Before nCRT	Not reported	Not reported	Surgical pathology	Not reported	0.83
Jang 2021	Korea	Retrospective	466	1.5 T, 3.0T	T1W,T2WI	3.0mm	Before nCRT	50.4 Gy	Concurrent fluoropyrimidine	Surgical pathology	Not reported	0.76
Jin 2021	China	Retrospective	622	1.5 T, 3.0T	T1WI, CE-T1WI, T2WI, DWI	3.0mm,5.0mm	Before and after nCRT	Not reported	Not reported	Surgical pathology	No viable tumor cells remaining	0.97
Lee 2021	Korea	Retrospective	912	1.5 T, 3.0T	T2WI, CE-T1WI, DWI	3.0mm	Before nCRT	Not reported	Not reported	Surgical pathology	Not reported	0.837
Nardone 2022	Italy	Retrospective	100	1.5 T	T2WI, ADC, DWI	Not reported	Before nCRT	45 Gy	Capecitabine(825 mg/m^2/ day)	Surgical pathology	No viable cancer cells	0.92
Pang 2021	China	Retrospective	275	1.5 T	T2WI	5.0mm	Before nCRT	45 Gy	Oral or intravenous 5-fluorouracil	Surgical pathology	No surviving tumor cells	0.815
Rengo 2022	Italy	Retrospective	95	1.5 T, 3.0T	T2WI	3.0mm,4.0mm	Before nCRT	45 Gy	Oxaliplatin (2-hour infusion 50 mg/m2), 5-FU 200 mg/m2/ die, desamethasone (8 mg) and ondansetron (8 mg)	Surgical pathology	No viable cancer cells	0.833

(Continued)

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TABLE 1 Continued

Study	Country	Study type	No. of patients	MRI field intensity	Sequences	Slice thickness	Image acquisition time	Radiotherapy Dose (Cumulative)	Chemotherapy Regimen	Stand Reference	Definition of pCR	AUC
Shaish 2020	Italy	Retrospective	132	Not reported	T2WI	3.0-8.0mm	Before nCRT	5 Gy	Capecitabine, 5-flourouracil, FOLFOX	Surgical pathology	Not reported	0.80
Shin 2022	Korea	Retrospective	898	1.5 T, 3.0T	T2WI, ADC, DWI	Not reported	Before and after nCRT	Not reported	Not reported	Surgical pathology	No surviving tumor cells	0.82
Wan 2019	China	Retrospective	120	3.0T	T1W,T2WI	3.0mm-5.0mm	Before nCRT	50 Gy	Capecitabine(1650 mg/m2)	Surgical pathology	Absence of residual tumor cells	0.84
Wan 2020	China	Retrospective	165	3.0T	T2WI,T1WI, DWI	3.0mm-5.0mm	Before nCRT	45-50.4 Gy	Capecitabine(825 mg/m^2/ day), oxaliplatin(130 mg/m2)	Surgical pathology	Absence of viable tumor cells	0.91
Yi 2019	China	Retrospective	134	1.5 T, 3.0T	T1WI, CE-T1WI, T2WI	Not reported	Before nCRT	46-50Gy	Capecitabine(825 mg/m2/ day)	Surgical pathology	No viable tumor cells present	0.908
Zhang 2020	China	Prospective	383	3.0T	T2WI,T1WI,DKI	Not reported	Before and after nCRT	Not reported	Capecitabine(825 mg/m2/ day)	Surgical pathology	No viable tumor cells present	0.99
Zhu 2022	China	Retrospective	472	3.0T	DWI	4.0mm	Before nCRT	41.8-50.6 Gy	Capecitabine(825 mg/m2/ day)	Surgical pathology	Absence of any residual cancer cells	0.924

ADC, apparent diffusion coefficient; AUC, Area Under Curve; CE-T1WI, contrastenhanced T1 weighted imaging; DKI, diffusional kurtosis imaging; DWI, Diffusion weighted imaging; nCRT, neoadjuvant chemoradiotherapy; mFOLFOX6, oxaliplatin + fluorouracil; pCR, pathological complete response; T1WI, T1-weighted imaging; T2WI, T2 Weighted Image; T2FS, T2-weighted fat-suppression; USA, United States of America.

only T2WI sequences to construct the models, and the remaining one used DWI sequences (44). All studies included image slice thicknesses between 2.00mm and 8.0mm.

The most used segmentation software is ITK.SNAP (7/21), followed by 3D Slicer (3/21). Most studies performed manual segmentation (15/21), two studies performed semi-automatic segmentation, and one study performed automatic segmentation (39), The segmentation method was not described in the remaining three studies. Ten studies used two-dimensional(2D) segmentation, nine studies used three-dimensional(3D) segmentation, and the other two studies used an unknown segmentation approach.

The most commonly used image feature extraction software is PyRadiomics (6/21), followed by MATLAB (3/21). The number of radiomic features extracted from the images varied from 34 to 8524. To avoid possible overfitting when developing radiomic models, feature selection and dimensionality reduction must be performed because radiomic features often exceed sample size. Each study used a different approach to feature selection and dimensionality reduction, and some studies performed more than one-dimensionality reduction approach. The most commonly used are Pearson correlation and Least Absolute Shrinkage and Selection Operator (LASSO) regression. Repeatability evaluation of imaging features can also be used for feature selection. The thresholds for robust features were set at 0.6-0.915 in seven studies that performed inter-class correlation coefficient (ICC) analysis. Extracted features were described in 12 studies, of which texture features were found in 11 studies, and the features extracted in 9 studies were unknown.

Five studies used deep learning(DL) methods to build models, and the remaining sixteen studies used ML methods to build models. The most common ML classifier is logistic regression. Nine studies used external validation, eleven studies used randomization validation, and the remaining one used cross-validation (53). Fifteen studies used radiomics features alone to construct models, and six studies constructed comprehensive models that combined clinical factors and radiomics features.

The study characteristics and results are summarized in Table 1 and Table 2.

RQS and risk of bias assessment

The included studies' mean RQS score was 10.95, accounting for 30.4% of the overall score. Only one research (37) found the maximum RQS score of 24 (67%). Approximately half of the studies received a score of 10 or above. Because no study took into account the four elements "Phantom study on all scanners", "Imaging at multiple time points", "Cut-off analyses", and "Cost-effectiveness analysis", they obtained a score of zero. Other factors with poor average scores were "biological correlations," "Prospective study", "Potential clinical utility", and "Open science and data" (Figure 2). A detailed description of the RQS scores is provided in Table S4.

TABLE 2 Summary of artificial intelligence-based prediction model characteristics described in included studies.

Study	VOI software	Segmentation	ROI	Feature extraction software	Imaging features	No. of extraced feature	ICC evaluation (threshold)	Algorithm architecture	Validation
Antunes 2021	3D Slicer	Manual	2D	MATLAB	Textural features	764	No	RF	External validation
Boldrini 2022	Not reported	Manual	2D	MODDICOM	Skewness, Entropy	Not reported	No	Linear regression logistic	External validation
Bulens 2021	Not reported	Manual	2D	Not reported	Not reported	8524	No	LASSO regression	External validation
Cheng 2021	ITK.SNAP	Manual	3D	Pyradiomics	First-order, shape-based, texture features	5901	Yes (0.8)	Logistic regression	Split sample
Cui 2019	ITK.SNAP	Manual	2D	AK	Histogram parameters, texture features, factor features	1188	Yes(0.701 -0.915)	LASSO regression	Split sample
Feng 2022	ITK.SNAP	Manual	2D	Pyradiomics	Texture Features, First-order Features, Wavelets Features	2106	Yes (0.6)	SVM	External validation
Horvat 2018	ITK.SNAP	Manual	2D	MATLAB	Texture Features	34	No	RF	FivefoldCV
Horvat 2022	ITK.SNAP	Manual	3D	Not reported	Texture features, Haralick textures, Gabor edges	91	Yes (0.75)	RF	External validation
Jang 2021	Not applicable	Not applicable	3D	MATLAB	Not applicable	Not applicable	Not applicable	DL	Split sample
Jin 2021	Not reported	Not applicable	3D	Not reported	Not applicable	Not applicable	Not applicable	3D RP-Net	External validation
Lee 2021	3D Slicer	Semi-automatic	3D	Pyradiomics	Features on the tumor shape,voxel intensity histogram, texture of tumor areas	3740	No	LR, xgboost, lightgbm,RF, MLP, Ensemble	Split sample
Nardone 2022	Not reported	Manual	3D	LifeX	Texture Features	Not reported	Yes	Logistic regression	External validation
Pang 2021	U-Net	Automatic	2D	PyRadiomics	Not applicable	Not applicable	No	TsraU-Net	External validation
Rengo 2022	Not reported	Not reported	Not reported	WEKA	Not reported	Not reported	No	SVM,RF,J48, Naive bayes,KNN	External validation
Shaish 2020	3D slicer	Manual	3D	Pyradiomics	First-order statistics, 3D shape-based, gray-level cooccurrence matrix, gray-level run length matrix, gray-level size zone matrix, neighboring gray-tone difference matrix, gray-level dependence matrix	3190	No	Logistic regression	Split sample
Shin 2022	3D Slicer	Semi-automatic	3D	PyRadiomics	Not reported	1132	Yes (0.75)	Not reported	Split sample
Wan 2019	Not applicable	Manual	Not applicable	Not applicable	Not applicable	Not applicable	Yes (0.8)	Lasso logistic regression	Split sample

(Continued)

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TABLE 2 Continued

Study	VOI software	Segmentation	ROI	Feature extraction software	Imaging features	No. of extraced feature	ICC evaluation (threshold)	Algorithm architecture	Validation
Wan 2020	Radcloud	Manual	2D	Radcloud	Shape characteristic, first-order statistical characteristics, texture features, high-order statistical characteristics	1049	No	Logistic regression	Split sample
Yi 2019	MaZda	Manual	2D	MaZda	Texture features	340	Yes (0.75)	RF, SVM	Split sample
Zhang 2020	ITK.SNAP	Manual	2D	Python	Not applicable	Not applicable	No	DL	Split sample
Zhu 2022	ITK.SNAP	Manual	3D	Python	Not applicable	Not applicable	No	CNN	Split sample

CNN, Convolutional Neural Networks; CV, cross validation; DL, Deep learning; ICC, Intra-/inter-class Correlation Coefficient; KNN, K-Nearest Neighbor; LASSO, Least Absolute Shrinkage and Selection Operator; LR, Logistic regression; MLP, multi-layer perceptron; ROI, Region Of Interes; RF, Random Forest; SVM, Support Vector Machine; 2D, Two-Dimensional; 3D, Three-Dimensional; VOI, Volume of Interest.

Figure 3 depicts the risk of bias and applicability concerns for twenty-one diagnostic-related studies using QUADAS-2. In each category, the majority of research revealed a low or uncertain risk of bias (Figure S1). In terms of patient selection, eleven studies were deemed to have an uncertain or high risk of bias due to ambiguous methods of participant selection and/or ambiguous detailed exclusion criteria. Concerning the index test, all studies were deemed to have a high or uncertain risk of bias since it was unclear if a threshold was employed or the threshold was not pre-specified. Only one research was deemed to have an unknown risk of bias due to the lack of a description of the reference standard. Concerning the time course, nine studies were deemed to be at high or unclear risk of bias, owing to unclear gaps between indicator tests and reference standards and/or the inability to determine if all subjects got the same reference standards (Supplemental Table S5).

Meta-analysis

A total of 21 studies were included in the meta-analysis, we only evaluated the validation cohorts of those studies, and radiomics assessed the efficacy of pCR after neoadjuvant chemoradiotherapy in rectal cancer: the pooled sensitivity 0.82 (95% CI,0.71-0.90), pooled specificity 0.86(95% CI,0.80- 0.91), pooled PLR 6.0 (95% CI,4.0-8.9), pooled NLR 0.21(95% CI,0.12-0.35)and DOR 29(95% CI,14-61) respectively, and the pooled AUC was 0.91 (95% CI,0.88-0.93).

When we calculated pooled estimates, we discovered significant heterogeneity between studies in terms of sensitivity (I^2 = 78.76%) and specificity (I^2 = 90.92%). Figure 4 shows the forest plot, and Figure 5 shows the noticeable discrepancy between the 95% confidence and 95% prediction areas from the SROC curve, showing a significant probability of variability between studies.

Subgroup analysis

To explore potential sources of study heterogeneity, we performed a subgroup analysis of 21 studies, including six different conditions and twelve subgroups. Radiomics models vary in modeling methods (radiomics and DL), sample size (whether greater than 100), radiomics feature extraction software (PyRadiomics and Others), regions of interest (2D and 3D), and validation methods (external validation and internal validation) and the inclusion of clinical factors (combined models and separate imaging feature models) showed moderate to high diagnostic value in various subgroups. The results are shown in Table 3.

Publication bias

We investigated publication bias for the 21 included papers by first seeing that the funnel plot was symmetric, and then

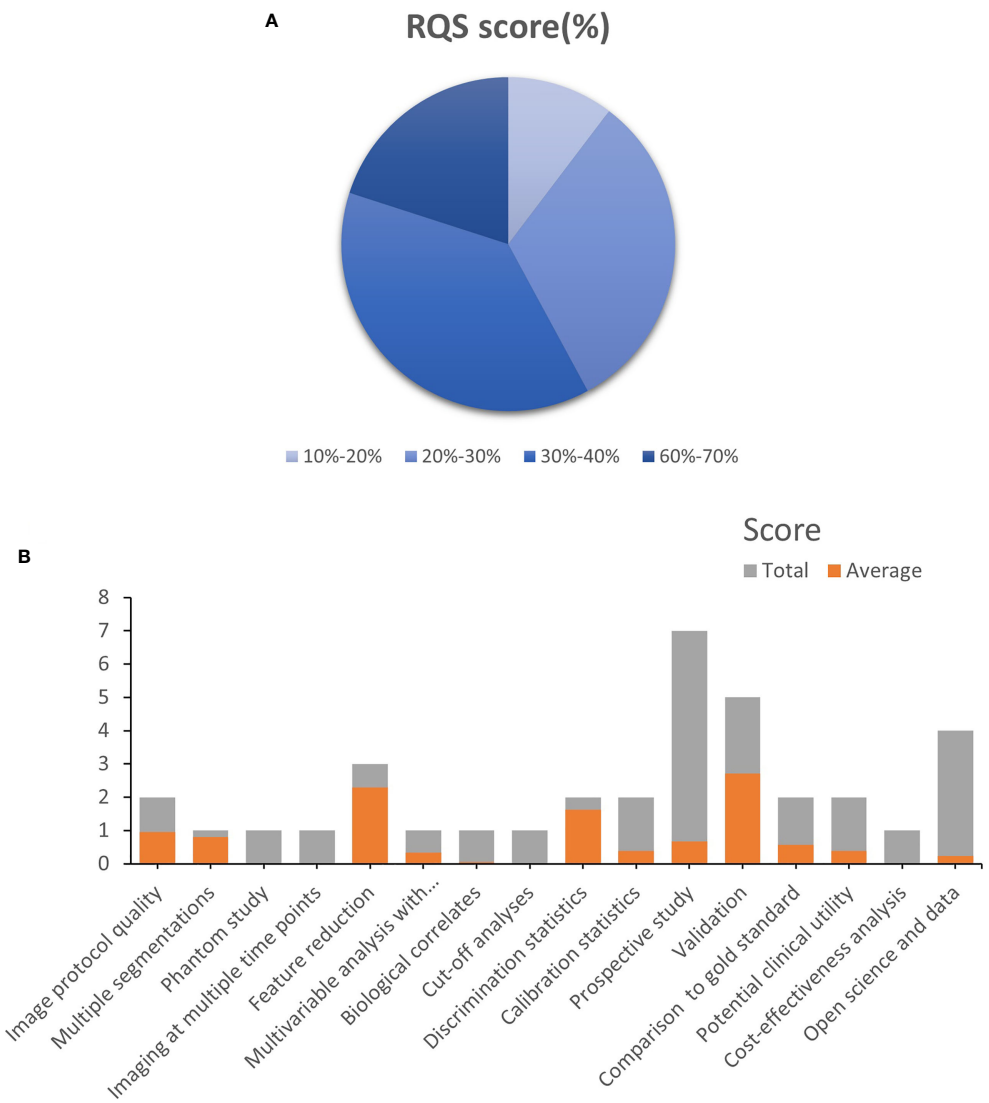


FIGURE 2
Methodological quality was evaluated by using the Radiomics Quality Score (RQS) tool. **(A)**. The proportion of studies with a different RQS percentage scores. **(B)**. Average scores of each RQS item (gray bars stand for the full points of each item, and red bars show actual points).

formally assessing it with the Deek test (P=0.20) (Figure 6), demonstrating that there was no publication bias.

Discussion

This systematic review and meta-analysis explored whether radiomics can be accurate in predicting pathological response to neoadjuvant chemoradiotherapy in patients with rectal cancer, using the QUADAS-2 and RQS tools to assess the quality of included studies. The results showed that the radiomic models had high diagnostic value in predicting pCR, with sensitivity, specificity, and AUC of 0.82(95% CI,0.71-0.90), 0.86(95% CI,0.80- 0.91), and 0.91(95% CI,0.88-0.93), respectively. Confirmation of this information will aid in the development of effective therapeutic regimens for rectal cancer patients. For example, If a patient with rectal cancer shows a pCR after neoadjuvant chemoradiotherapy, TME surgery is not required but waiting and observation.

In several studies, first-order features including skewness, kurtosis, entropy, and energy were found to distinguish pathological complete responses from non-pathological complete responses (34, 35, 37, 51–53). Lower kurtosis was found in pCR patients in one study (53), however, this has not been validated in other studies. Second- and higher-order features also have some predictive power. Texture features are

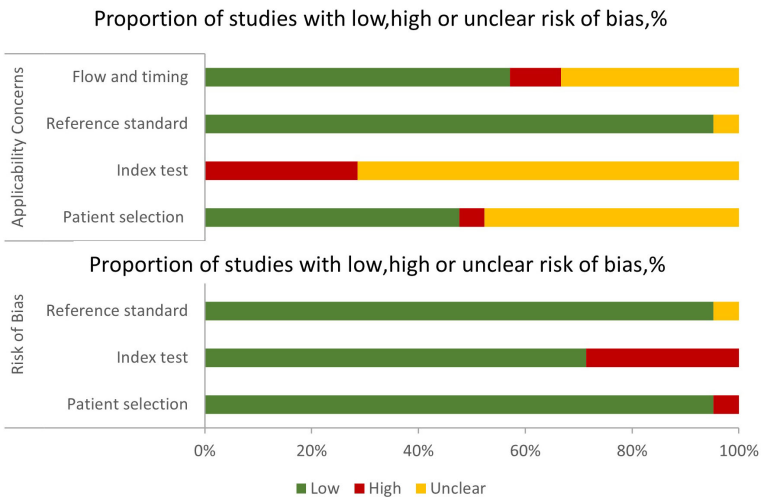


FIGURE 3
Grouped bar charts of the risk of bias and applicability concerns of the included studies were assessed by using a revised tool for the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2).

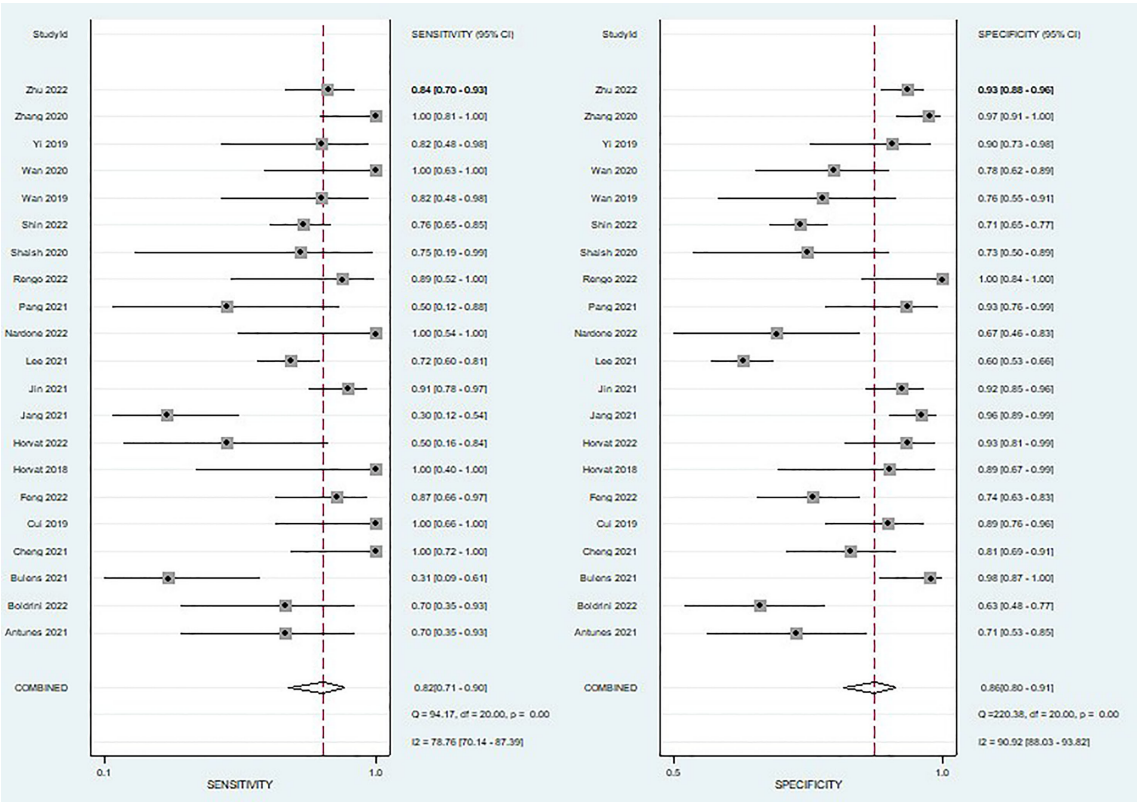


FIGURE 4
Coupled forest plots of pooled sensitivity and specificity of diagnostic performance of predicting pathological complete response to neoadjuvant chemoradiotherapy in rectal cancer. The numbers are pooled estimates with 95% CIs in parentheses; horizontal lines indicate 95% CIs.

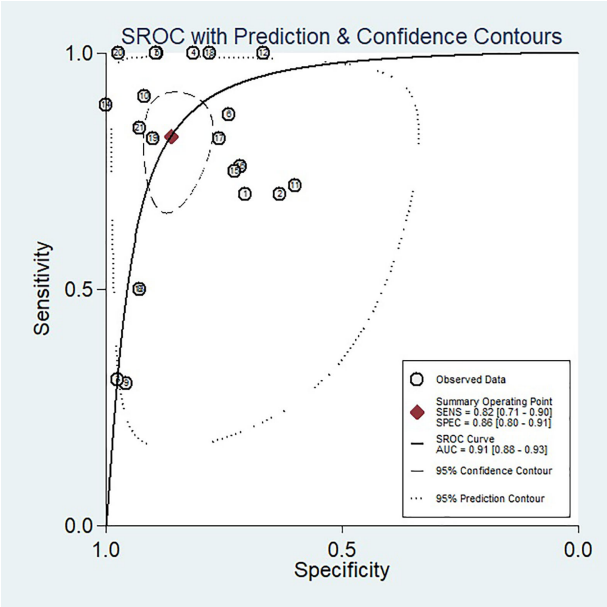


FIGURE 5
SROC curve of the diagnostic performance of artificial intelligence for the prediction of pathological complete response to neoadjuvant chemoradiotherapy in rectal cancer patients. An obvious difference was detected between the 95% CI and 95% prediction regions, indicating a high possibility of heterogeneity across the studies.

changes in image intensity in an image. Texture Analysis (TA) enables researchers to attempt to quantify heterogeneity within the target tumor site, thereby determining the unobservable with more valuable parameters detected (55, 56). Many scholars (35–37, 41, 42, 48, 50–53) have demonstrated that texture features can predict pathological complete response to nCRT. In general, tumors that ultimately failed to achieve pCR after nCRT exhibited elevated or more image heterogeneity, similar to previous findings in breast and lung cancer (57, 58), which demonstrated higher intratumor heterogeneity in patients with a poorer prognosis, including poorer treatment response. Our review also found that combining radiomics signatures across various radiomics categories was more likely to be accurate in predicting nCRT response. This is similar to the literature review by Horvat et al. (59), who found that studies using advanced predictive models had AUCs ranging from 0.72 to 0.93.

The mean RQS score of the 21 included articles was 10.95 (30.4% of the total score). Four items of the RQS in which all included studies performed zero are “Phantom study on all scanners”, “Imaging at multiple time points”, “Cut-off analyses”, and “Cost-effectiveness analysis”. The purpose of a phantom study is to detect different potential feature differences between scanners and suppliers. Many studies included image data from different MRI types (3.0T, 1.5T), vendors (Siemens, Philips), and different medical centers, and phantom studies are a suitable means to gauge these uncertainties and identify features that rely on the vendor. Imaging at multiple time points is based on organ motion or expansion or contraction of the target volume resulting in changes in radiomics characteristics, using remeasurement data (two or more image data sets of a patient acquired in a short period) to obtain stable radiomics features are necessary, especially for the peristaltic hollow organ of the colorectum, however, considering the usual clinical practice work, it is difficult to do this for retrospective studies. Cut-off analyses identified risk groups by medians, previously published cutoff values, or reporting continuous risk variables. Reduce the risk of models with overly optimistic results. Cost-effectiveness analysis is a health economic consideration that argues that cost-quality-adjusted life-year comparisons should be performed with or without radiomics to more accurately determine the economic potential of such studies. The five items where all studies underperformed were “biological correlates”, “Prospective study”, “Potential clinical utility” and “Open science and data”. Only one study combined pathological factors with radiomic features to build predictive models and discussed their biological relevance. Prospective studies are critical enough to link radiomics data to clinical outcomes in appropriate patient populations, however, only two studies were prospective. Three studies considered current and potential applications of models in clinical settings, using decision curves to show the clinical utility of specific models. The openness of data and code contributes to the reproducibility and replicability of radiomics. Radiomics includes multiple complex processes, each one influenced by a variety of factors, including the use of nonstandard nomenclature, the definition of parameters, and the

TABLE 3 The results of subgroup analysis.

Subgroup	Number of study	Sensitivity (95% CI)	I^2 (%)	Specificity	I^2 (%)	PLR	I^2 (%)	NLR	I^2 (%)	AUC
Modeling methods										
Radiomic algorithm	16	0.77(0.71,0.81)	61.9	0.74(0.71,0.76)	82.1	3.47(2.66,4.54)	68.5	0.34(0.24,0.48)	54.3	0.8538
Deep learning	5	0.79(0.71,0.85)	89.5	0.94(0.91,0.96)	0.0	11.66 (7.98,17.02)	0.0	0.22(0.06,0.86)	94.1	0.9724
Sample size										
<100	15	0.80(0.72,0.86)	70.6	0.85(0.81,0.87)	77.6	4.82(3.30,7.05)	64.2	0.27(0.15,0.49)	70.7	0.9009
>100	6	0.76(0.71,0.81)	82.9	0.77(0.74,0.80)	95.4	4.59(2.54,8.30)	92.6	0.29(0.16,0.54)	89.1	0.8771
Radiomic software										
PyRadiomics	6	0.76(0.70,0.82)	51.9	0.69(0.66,0.73)	79.6	2.92(2.07,4.12)	74.4	0.37(0.26,0.53)	30.3	0.8146
others	11	0.80(0.73,0.86)	78.0	0.88(0.85,0.90)	84.3	5.91(3.40,10.27)	80.4	0.20(0.08,0.48)	84.5	0.9227
Segmentation										
2D	10	0.79(0.71,0.87)	75.5	0.84(0.80,0.87)	81.7	5.11(3.16,8.28)	71.1	0.26(0.12,0.56)	78.4	0.9030
3D	9	0.76(0.70,0.81)	79.7	0.78(0.75,0.80)	93.2	4.45(2.72,7.26)	89.1	0.31(0.18,0.54)	83.6	0.8829
Validation										
External validation	9	0.77(0.68,0.84)	72.2	0.83(0.79,0.86)	84.6	4.36(2.55,7.47)	74.5	0.33(0.18,0.62)	78.0	0.8775
Split sample	11	0.77(0.72,0.82)	78.6	0.78(0.76,0.81)	92.1	5.03(3.19,7.93)	87.6	0.26(0.15,0.46)	80.9	0.9025
Models										
Radiomics model	15	0.73(0.68,0.78)	75.6	0.79(0.77,0.82)	91.1	4.66(3.12,6.97)	82.5	0.36(0.25,0.54)	75.3	0.8749
Combined model	6	0.89(0.81,0.94)	37.8	0.82(0.78,0.86)	78.9	5.06(2.88,8.91)	79.5	0.18(0.09,0.34)	35.4	0.9187

AUC, Area Under Curve; NLR, negative likelihood ratio; PLR, positive likelihood ratio; 2D, Two-Dimensional; 3D, Three-Dimensional.

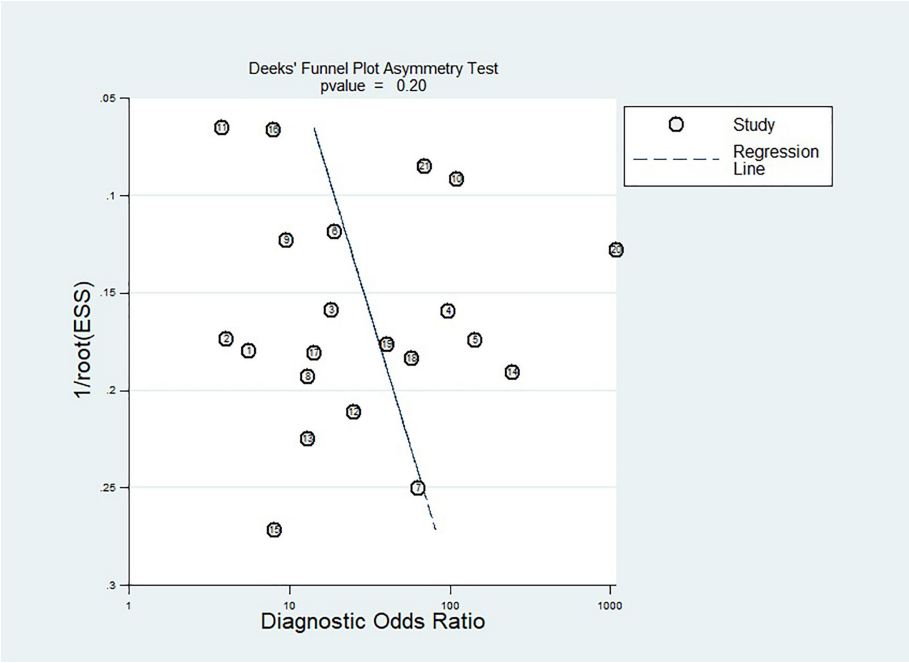


FIGURE 6
Effective sample size (ESS) funnel plots and the associated regression test of asymmetry, as reported by Deeks et al. A p-value < 0.10 was considered evidence of asymmetry and potential publication bias.

selection of software. If researchers do not reveal these complexities, reproducibility, and replicability in radiomics are impossible. As a result, it is expected that various practical concerns, such as radiomics model repeatability, imaging protocol standardization, model overfitting, and external validation of prediction models, should be thoroughly addressed before transferring these models into routine clinical use.

The QUADAS-2 quality assessment revealed some problems with the 21 studies included in the systematic review. Some studies did not state whether the patients were included in continuous or random sampling, which may lead to selection bias. All studies were at risk of bias on the index test, and it was unclear whether thresholds were used or not pre-specified, which may have led us to overestimate the diagnostic performance of our models. Nine studies did not indicate the time interval between imaging and pathological evaluation of resected tissue after rectal cancer surgery. Future studies should avoid patient selection bias and clarify the time interval between imaging and pathological evaluation of resected tissue after surgery.

Our study was highly heterogeneous, with the heterogeneity of 78.76% and 90.92% for sensitivity and specificity, respectively. We, therefore, performed subgroup analyses using six key factors to explore sources of heterogeneity. In the subgroup analysis, we compared the diagnostic performance of DL and radiomics models, and the diagnostic performance of the DL subgroup was higher than that of the radiomics model, (AUC: 0.97 > 0.85), which may be because DL is trained in the capabilities of multi-layer deep neural networks (60). Compared with ML feature extraction methods, DL is more computationally intensive and can extract more image features (61). ML models are traditionally trained to perform useful tasks using manually specified features retrieved from raw data or features learned by other simple machine learning models (62). DL allows computers to acquire meaningful representations and characteristics automatically, directly from raw data, avoiding this time-consuming and challenging process (63). DL models are dominated by various versions of artificial neural networks, although there are others. The major trait that DL approaches have in common is their emphasis on feature learning: autonomously learning data representations (64). This is the key distinction between DL and more "traditional" ML methodologies. Discovering features and accomplishing a task are combined into one challenge and so improved concurrently during the training phase. However, there are only five DL studies in this meta-analysis. More DL studies are needed to confirm this conclusion. Another subgroup analysis showed that the combined model with clinical factors and radiomics features was more powerful than the radiomics feature alone. Because of the constraints of univariate prediction, its prediction performance is less outstanding, however, the multivariate prediction model can overcome these restrictions. A multifactorial pCR prediction model was established based on this approach, which is also the path for future study, and additional imaging and non-imaging data need to be retrieved to construct stronger prediction models (28).

Two of the twenty-one studies we included used the delta model, which is a new radiomics approach that has been developed that accounts for feature variations at different acquisition times (65). With this method, it is possible to study the impact of changes in characteristics after a specific step in a patient's workflow (ie, after specific treatment, time, or biological event). Wan and Nardone et al. (41, 48)used delta models to study changes in radiomics parameters throughout the treatment process and showed that the delta model was a good predictor of patient response. Available data suggest that a delta radiomics approach can also successfully predict tumor behavior in terms of synchronous or metachronous distant metastasis (DM), disease-free and overall survival (66, 67).

Our research has several limitations. First, the heterogeneity of research is obvious. We investigated the causes of heterogeneity using subgroup analyses and discovered that heterogeneity was model-related (DL and radiomics), but because heterogeneity was observed in diagnostic test accuracy reviews features (68), we cannot know the source of all the heterogeneity. Second, because the model was not verified, many large-sample studies were excluded from the meta-analysis. Unvalidated models have low relevance, and validation is an essential aspect of a thorough radiomics study (28). Finally, we only evaluated pCR studies and did not include studies on tumor regression grading (TRG) and T downstaging, it is known that pathologic evaluation of TRG and T downstaging is more subjective than pCR evaluation (69, 70). Precise and objective pathological criteria are lacking for TRG and T downstaging.

Conclusions

In conclusion, our meta-analysis suggests that radiomics is a promising noninvasive approach with a high value for pCR prediction in patients with rectal cancer to neoadjuvant chemoradiotherapy. This has important guiding significance for the individualized treatment of rectal cancer patients in clinical practice. The prediction performance of the DL models for pCR was superior to the radiomics models, and the combined models incorporating clinical factors were superior to the radiomics model alone. Furthermore, more prospective, large-scale, multicenter studies employing radiomics approaches are required in the future to increase pCR preoperative prediction ability.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

L-LJ designed, developed, and refined the study protocol with contributions from Q-YZ, J-HT, GH, L-PZ, and J-XZ. L-LJ,

Q-YZ, and J-HT developed the search strategy and designed the literature search. L-LJ and J-XZ screened titles and abstracts and undertook the data extraction. L-LJ, GH, L-PZ, and J-XZ interpreted the data for the work; L-LJ, Q-YZ drafted the manuscript. All authors were involved in critically revising the draft. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.1026216/full#supplementary-material

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Neutrophils in pancreatic cancer: Potential therapeutic targets

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Pancreatic cancer is a digestive system malignancy and poses a high mortality worldwide. Traditionally, neutrophils have been thought to play a role in acute inflammation. In contrast, their importance during tumor diseases has been less well studied. Generally, neutrophils are recruited into the tumor microenvironment and exert inflammation and tumor-promoting effects. As an essential part of the tumor microenvironment, neutrophils play diverse roles in pancreatic cancer, such as angiogenesis, progression, metastasis and immunosuppression. Additionally, neutrophils can be a new potential therapeutic target in cancer. Inhibitors of cytokines, chemokines and neutrophil extracellular traps can exert antitumor effects. In this review, we describe the role of neutrophils in the development and progression of pancreatic cancer, discuss their potential as therapeutic targets, and aim to provide ideas for improving the prognosis of patients with this malignant tumor disease.

KEYWORDS

anticancer therapy, neutrophil, neutrophil extracellular trap, pancreatic cancer, tumor-associated neutrophil

Introduction

The tumor microenvironment (TME) is a vital part of tumor formation, and TME homeostasis is regulated by signal transduction pathways and metabolism among tumor cells, endothelial cells, stromal cells and immune cells (1). Tumor growth can be modulated by the secretion of signaling molecules by immune cells, so in some cases tumor growth, invasion and metastasis can be regulated by the interactions between cancer cells and immune cells in the TME (2). Therefore, it is important to understand the relationships between cancer cells and the TME for the development of effective therapies. Traditionally, neutrophils have been thought to play a role in acute inflammation. Increased numbers of neutrophils enter tissues and kill microorganisms

by phagocytosis or the release of active substances from granules. Moreover, these cells can also cause severe damage to normal tissues (Figure 1). With increased knowledge of neutrophils, it has been found that neutrophils also participate in chronic inflammation, adaptive immune responses and tumor diseases (3, 4). Tumor-associated macrophages and fibroblast cells are involved in inflammation, which can support cancer progression (5). However, some evidence also suggests that neutrophils can be a new example of cancer-related inflammation and immunity (6, 7).

Pancreatic cancer is a malignancy and poses a serious medical trouble. In 2017, the number of pancreatic cancer cases worldwide was more than twice as high as in 1990. There was a 2.3-fold increase in the number of deaths from 196,000 in 1990 to 441,000 in 2017 (8). Despite progress in the available treatment methods and efficacy, pancreatic cancer patients have poor prognosis. Studies on the carcinogenic mechanism and the search for immune targets based on the TME have become directions in pancreatic cancer research, and tumor-associated neutrophils (TANs) provide new ideas.

In this review, we summarize the functions of neutrophils in pancreatic cancer development processes, such as angiogenesis, progression, metastasis, and immunosuppression. Next, we discuss the potential of neutrophils as anticancer therapeutic targets. We also propose future directions and how neutrophils may affect the therapeutic outcomes of pancreatic cancer patients, which may contribute to a new generation of anticancer therapies for pancreatic cancer patients.

Neutrophil life activity

Production, differentiation and death

Neutrophils account for 50-70% of circulating leukocytes in the human body (9). More than 10^{11} neutrophils may be produced each day (3). Human neutrophils have a half-life of approximately 8 hours in the circulation and are generally considered short-lived cells, but some studies have shown that the average circulating life of human neutrophils is 5.4 days (3, 10, 11). As hematopoietic stem cells differentiate in bone marrow, they first give rise to common myeloid progenitors, followed by granulocytes and monocytes. Granulocyte-colony stimulating factor (G-CSF), produced by bone marrow stromal cells, is a key cytokine that stimulates the production and mobilization of neutrophils in bone marrow (12). G-CSF regulates the differentiation of granulocyte-monocyte progenitor cells into neutrophils and the formation of myeloblasts. The subsequent stages of differentiation are promyelocytic, myelocyte, metamyelocyte, band cell and polymorphonuclear granulocyte (3). Mature neutrophils are released into the bloodstream and play roles in inflammation, infection, and chronic diseases in the body.

There are protective mechanisms to balance the number of neutrophils so that these cells do not become overactive in blood vessels and cause severe damage to normal tissues. The bone marrow is also a site at which circulating neutrophils are recycled. Neutrophil release is negatively regulated by CXC-

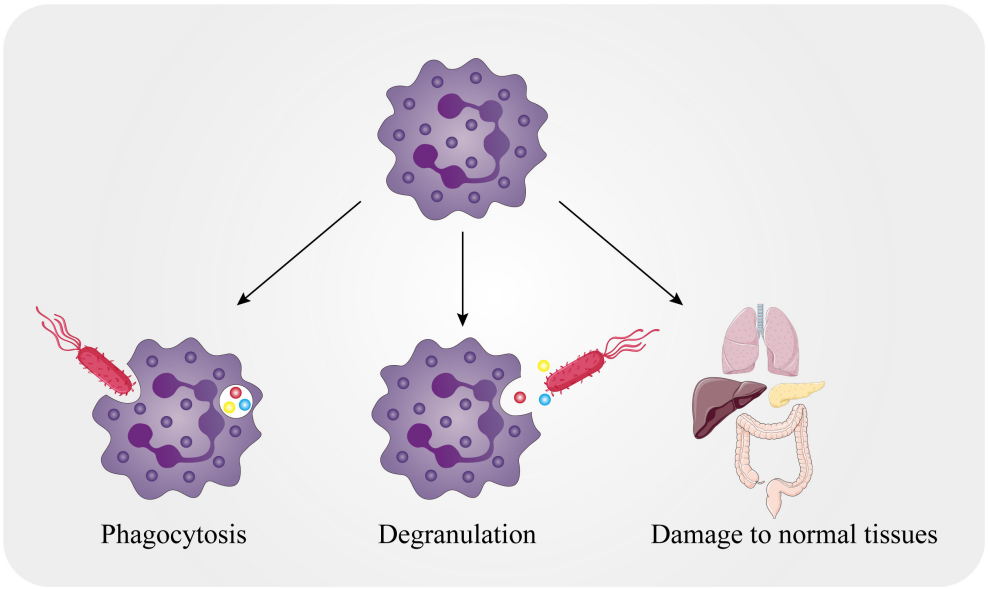


FIGURE 1
Killing mechanisms of neutrophils. The killing mechanisms of neutrophils include both intracellular and extracellular mechanisms. Neutrophils can encase pathogens in the phagocytic body through phagocytosis and can also release particles into the extracellular environment that act on external pathogens. Meanwhile, normal tissues in the body can be attacked by neutrophils.

chemokine receptor 4 (CXCR4) signaling in a cellular autonomous manner (13). In the bone marrow, senescent neutrophils are removed *via* a CXCR4-dependent process. Neutrophils are reassigned from the bone marrow to the blood when CXCR4 signaling is lost (14). In addition, liver Kupffer phagocytosis functions and regulation of the microbiome also limit over the numbers of neutrophils (15, 16). Neutrophils are often activated by a two-step process of priming followed by activation, which avoids non-specific triggering of their cytotoxic mechanisms, and undergo rapid apoptosis which blocks their ability to respond to extracellular ligands (17, 18). Thus, these mechanisms by which both the number and activation of neutrophils are tightly controlled in the circulation ensures that the human body is protected against microbial pathogens and reduces damage to its own tissues.

Neutrophil recruitment

Neutrophil recruitment begins with changes in endothelial cells and processing including tethering, rolling, adhesion, crawling and, finally, transmigration (4). After neutrophils reach the vascular edge, their rolling process is dependent on selectin, and the adhesion process is dependent on integrin, which results in the tight adherence of neutrophils to endothelial cells (19, 20). Platelet endothelial cell adhesion molecule is located on the neutrophil surface and endothelial cell surface, promoting neutrophil migration out of the vascular endothelium by mediating the binding of these two cells (21). Then neutrophils secrete molecules such as collagenase to degrade the vascular basement membrane but preferentially they move through membrane regions with low expression of extracellular matrix components, and then enter the surrounding tissues (4). Then, neutrophils migrate between pericytes, crawl along the cells through intercellular signals and search for gaps through which they can finally leave the vasculature. After extravasation, neutrophils make directional movements along the chemical concentration gradient and accumulate in inflammatory sites.

Chemokines, such as CXCR1, CXCR2 and CXCR4, are critical in neutrophil recruitment (4, 22, 23). In addition, cytokines such as interleukins (ILs) and tumor necrosis factors (TNFs), and intracellular proteins such as poly ADP-ribose polymerase 1, cathepsin C and S100 calcium-binding protein A9 can also increase neutrophil activation and recruitment (24–27). Prolongation of the neutrophil lifespan can further enhance their functional roles. Although the normal lifespan of neutrophils is short, certain cytokines and bacterial products can prolong neutrophil survival by interfering with apoptosis. For instance, G-CSF can delay neutrophil apoptosis by inhibiting the activation of calpain, a calcium-dependent cysteine protease that is upstream of caspase-3, resulting in a delay in apoptosis of approximately 12 hours (28, 29).

Neutrophils and cancer

Circulating neutrophils

Serological indicators are widely used to predict the overall survival (OS, a term that denotes the time of staying alive for individuals that suffer from a specific disease) of tumor diseases due to their advantages of simplicity, economy and noninvasiveness (30). As a marker of systemic inflammation, the neutrophil/lymphocyte ratio (NLR) is currently an attractive biomarker for risk stratification and guiding treatment decisions in cancer patients (31).

Especially in early-stage pancreatic ductal adenocarcinoma (PDAC), finding biomarkers to predict recurrence can lead to a better prognosis. The NLR is associated with the tumor stage, and patients with PDAC whose tumor stages were less than IIA had longer OS and recurrence-free survival (RFS, time from surgery to the date of first recurrence) when the NLR > 2.2 (hazard ratio =3.310, 95% confidence interval: 1.259-8.745). However, the NLR was not associated with OS or RFS in patients with tumor stage greater than IIB (32). Since only a few pancreatic cancer patients have surgical indications at the time of diagnosis, it is important to construct accurate prognostic models in patients with unresectable pancreatic cancer. The NLR is better than other serological indicators (such as the platelet/lymphocyte ratio and prognostic nutritional index) among prognostic factors in nonsurgically resectable pancreatic cancer patients after 6 months of follow-up. Multivariate analysis showed that a high NLR (HR=2.430, 95% CI: 1.484 to 3.977) is an independent predictor of OS (33).

The NLR can also predict how pancreatic cancer will respond to drug treatment. In retrospective studies of pancreatic cancer patients treated with FOLFIRINOX (oxaliplatin, irinotecan, leucovorin, 5-fluorouracil) and immune checkpoint inhibitors, a high NLR was associated with poor prognosis (34, 35).

Neutrophil extracellular traps (NETs)

NETs are formed by the release of cellular contents by activated neutrophils into surrounding tissues or circulation (36). Neutrophils can be stimulated to produce NETs by a variety of substances, including bacteria, viruses and some chemical or biological factors (37). Chemokines in the TME, such as IL-8, can promote the formation of NETs and help recruit more neutrophils (38). Conversely, NETs can also promote tumor growth and have a positive effect on the TME, including enhancing mitochondrial function in tumor cells, blocking the function of immune cells and exhibiting angiogenic activity by increasing capillary length, loop number, and tubule area (39, 40). Thus, NETs can accelerate tumor growth and cause tumor immune escape.

Based on the putative role of NETs in the TME, NETs may have potential as a biomarker for prognosis of some cancer patients. For example, the level of NETs is increased in cancer patients and is significantly higher in patients with advanced stage disease than in patients with early disease (41). In patients with pancreatic cancer, NET was an independent prognostic factor for OS (HR=2.366, 95% CI: 1.408–3.978) and RFS (HR=3.037, 95% CI: 1.809–5.098) and could predict the survival of patients who received gemcitabine-based chemotherapy (42). At present, NETs were confirmed to be involved in the biological process of pancreatic cancer in some preclinical studies. NETs-mediated metastasis and drug resistance of cancer cells will provide new insights into anticancer therapies.

TANs and cancer diseases

TAN can be involved in the progression of tumor disease. A meta-analysis showed that the level of intratumoral neutrophils was independently associated with OS and RFS in cancer patients (43). In the TME, TANs can take an antitumorigenic and pro-tumorigenic phenotype (44). Cytokines in the TME impacts on the balance of these two subpopulations. For instance, TAN can become the "promoting the tumor" type in response to transforming growth factor-β (TGF-β) (45). Neutrophil with this phenotype can produce pro-tumor factors (6). By contrast, low doses of interferon -β induced neutrophil to polarized to the "antitumor" phenotype in C57BL/6 and BALB/C mice, and similar changes were also observed in melanoma patients treated with type I interferon (46). "Antitumor" phenotype TANs produce chemokines, such as CCL3, CXCL9, CXCL10, to recruit CD8+ T cells to the TME (47). There is also evidence that they can increased cytotoxicity and reduced immunosuppression by the production of TNF-α, ROS and CD95, thus providing anticancer effect (48, 49).

The diversity of neutrophils leads to their dual potential in the TME. As a part of tumor-associated inflammation, TANs are involved in tumor growth and metastasis. Additionally, neutrophils can interact with other immune cells and stromal cells, resulting in extracellular matrix accumulation and immune function changes (50). In mouse models, TANs mediate the infiltration of regulatory T (Treg) cells and macrophages in the TME by secreting the chemokines CCL2 and CCL17, leading to the growth of hepatocellular carcinoma cells and increasing the resistance of hepatocellular carcinoma patients to sorafenib (51). In colorectal cancer, tumor growth is related to the gut microbiome because these tumor cells produce IL-17 and mediate the inflammatory response (e.g., driving B-cell infiltration). Neutrophils can limit the number of microorganisms and the expression of IL-17 to reduce inflammation related to tumor progression (52). Conversely, neutrophils can also mediate antitumor responses (50). IL-1 and IL-1β signaling in neutrophils enhances the antimicrobial activities in colorectal cancer, which inhibits bacterial-driven inflammation and alleviated tumorigenesis (53). Hepatocyte growth factor (HGF) acts on the HGF receptor expressed on neutrophils and promotes the production of inducible nitric oxide synthase (iNOS); iNOS releases nitric oxide and promotes apoptosis of tumor cells (54).

Carcinogenic mechanisms of neutrophils in pancreatic cancer

As we mentioned earlier, TANs participate in various cancer-related processes in the TME and are associated with poor prognosis for most cancers. In this section, we will introduce how TANs promote the progression of pancreatic cancer, such as through angiogenesis, pancreatic cancer cell metastasis and immune suppression. A deep understanding of these mechanisms will not only enable us to understand the promoting effect of the TME on pancreatic cancer, but also provide us with new therapeutic targets.

TANs and angiogenesis

Angiogenesis is a critical link in tumor growth and metastasis, and is jointly regulated by tumor cells, stromal cells and their bioactive products, such as various growth factors and extracellular matrix (55). Activated neutrophils release multiple angiogenic factors, including vascular endothelial growth factors (VEGFs), CXCLs and matrix metalloproteinases (MMPs), and form NETs (56).

Histones, which are major components of NETs, significantly increased vascular endothelial tubule formation in a dose-dependent manner (57). After treatment with 100 IU/mL heparin and 62.5 μg/mL polysialic acid for 1 hour, the histone-induced production of tubules in vascular endothelial cells was inhibited. This effect occurred because heparin and polysialic acid are anionic substances that bind to positively charged histones and neutralize their activity (57).

MMP plays an important role in angiogenesis, and MMP-9 promotes the release of VEGF from the extracellular matrix and participates in the interaction between VEGF and VEGF receptors (58). Neutrophils can be a source of MMP-9 in tumor angiogenesis (59). The addition of neutrophils to pancreatic cancer cells can increase the budding rate by more than 2.5 times because MMP-9 may promote endothelial cell migration. After 14 days of treatment with bevacizumab (a VEGF inhibitor) and doxycycline (a drug which could inhibit angiogenesis as effectively as MMP-9 inhibitors), the tumor volume in pancreatic cancer mice was significantly reduced. Furthermore, the average vascular density of pancreatic cancer mice was also significantly reduced (60). Therefore, MMP-9

produced by neutrophils may be a therapeutic target in pancreatic cancer treatment and provide a feasible alternative treatment for pancreatic cancer patients.

Neutrophil gelatinase-associated lipocalin (NGAL) is secreted by neutrophils and is upregulated in a variety of tumor diseases (61). NGAL can potentially inhibit angiogenesis by reducing VEGF production in pancreatic cancer cells. Compared with that in the control groups, adding NGAL reduced the tube formation of human umbilical vein endothelial cells (HUVECs) in MIA PaCa-2 (RRID: CVCL_0428) and PANC-1 (RRID: CVCL_0480) (two pancreatic cancer cell lines) cells by 69.5% ± 5% and 68% ± 7.5%, respectively (62). Moreover, CXCL5 mediates pancreatic cancer angiogenesis in mouse model by activating multiple signaling pathways, including signal transducer and activator of transcription pathways and extracellular signal–regulated kinase pathways in human endothelial cells (63). Due to the important role of neutrophils in tumor angiogenesis, neutrophil suppression may be an effective anticancer strategy.

Progression and metastasis

TANs are involved in tumor progression and migration. As early as 1989, neutrophils were shown to promote lung metastasis of breast cancer (64). TNF, leukotriene B4 and IL in the TME play roles in tumor progression and metastasis influenced by neutrophils (6).

In normal pancreas tissue, obesity promotes the inflammatory response and fibrosis; in pancreatic cancer, cytokines produced by dysfunctional fat cells, such as IL-1β, increase pancreatic stellate cells (PSC) activation and recruit TANs (65). PSCs are the main cell type in the pancreatic cancer stroma, and their large presence suggests that they may contribute to the metabolism of cancer cells (66). TANs also secrete IL-1β, which is involved in PSC activation, immunosuppression and PDAC progression. Moreover, adjuvant chemotherapy showed no significant survival advantage in overweight and obese patients with PDAC; Thus, the cross interaction between adipocytes, TANs and PSCs promotes the progression of PDAC, with IL-1β playing a major role in this process (65).

The purinergic receptor P2RX1, an ATP-gated ion channel, is associated with the inflammatory activation of immune cells (67). A large number of P2RX1-deleted neutrophils were found in the hepatic metastasis model of PDAC. The immune response of P2RX1 negative neutrophils in the PDAC TME is characterized by elevated MMP-9. The metabolic characteristics were a significant increase in the oxygen consumption rate and a nonsignificant increase in the extracellular acid rate (decreased glycolysis in neutrophils and enhanced oxidative phosphorylation in mitochondria). This effect occurs because the deletion of P2RX1 can increase the activity of the neutrophil transcription factor NF-E2 p45-related factor 2 (NRF2) (68). NRF2 is critical in regulating redox, metabolic, protein homeostasis, and inflammation (69). Increased NRF2 activity contributes to the metabolic reprogramming of neutrophils during polarization. Second, NRF2 directly regulates PD-L1 transcription and has a direct impact on CD8+ T-cell failure (68). Because Nrf2 is critical for immunosuppressive microenvironment formation in pancreatic cancer liver metastases *via* shaping the immunosuppressive phenotypes of P2RX1-negative neutrophil, future therapy, such as inhibiting the specific gene to reduce the particular phenotype of neutrophil subpopulation, may help treat pancreatic cancer.

NET formation is dependent on receptor for advanced glycation end products (RAGE) and autophagy pathways and is mediated by citrullination of histones to allow DNA expulsion from cells. Inhibition of autophagy by chloroquine or ablation of RAGE resulted in decreased NET formation (70). NETs can enhance tumor migration and invasion by inducing epithelial cells to transform into mesenchymal cells. Moreover, neutrophils can degrade E-cadherin on pancreatic cancer cells by secreting elastase, leading to increased tumor cell migration and invasion, and resulting in PDAC progression and metastasis (71). The effects of neutrophils on the progression and metastasis of pancreatic cancer are summarized in Figure 2.

TANs and immunosuppression

The TME has the ability to regulate immunosuppression, and understanding the mechanisms by which pancreatic cancer cells evade tumor immunity is crucial for developing more effective therapies. NETs can promote tumor growth and metastasis through a variety of mechanisms: trapping circulating tumor cells and protecting them, thereby preventing T cell and natural killer cell-mediated cytotoxicity (56).

CXCL5, a CXCR2 ligand, are significantly elevated in pancreatic cancer and can recruit TANs. This process is regulated by the activity of the NF-κB signaling pathway in mouse models, suggesting that neutrophils are involved in pancreatic cancer inflammation. Reducing CXCR2 significantly inhibited the number of TANs in pancreatic cancer, leading to spontaneous, T-cell-dependent tumor growth inhibition (72). Because the CXCR2 ligand axis is involved in the recruitment of TANs and the regulation of T-cell immunity in pancreatic cancer, it is expected to be a potential therapeutic target for pancreatic cancer.

Programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are both signaling molecules commonly seen on activated T cells, and have been found to be effective immunotherapeutic targets in cancer (73). IL-17 is highly expressed in tumor tissues. After treatment with IL-17, several chemokines capable of recruiting neutrophils were significantly induced. Researchers found that IL-17 signaling favors CD8+ T-cell inactivation and significantly affects immune

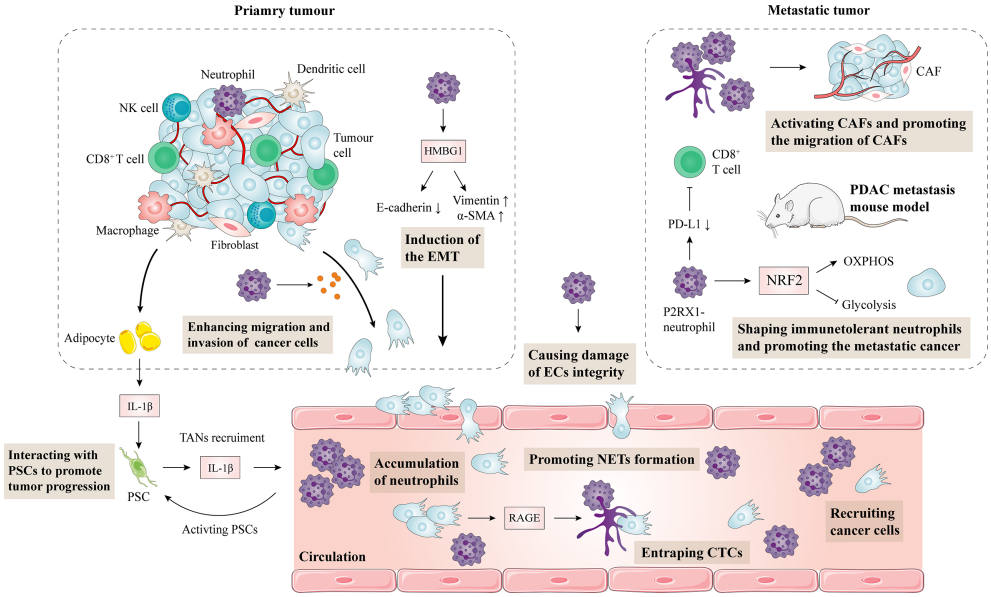


FIGURE 2
Impact of neutrophils on the progression and metastasis of pancreatic cancer. Obesity-induced inflammation and TAN infiltration activate PSCs, leading to connective tissue proliferation in the TME and promoting tumor growth. Conversely, PSCs can also recruit TANs. Neutrophils produce HMGB1 in pancreatic cancer, which induces the epithelial-mesenchymal transformation of pancreatic cancer. Moreover, neutrophils can also secrete elastase to degrade E-cadherin on pancreatic cancer cells, resulting in the enhanced migration and invasion of pancreatic cancer cells. In a mouse metastatic tumor model, NRF2 activity in P2RX1 negative neutrophils is elevated, leading to metabolic reprogramming during polarization. As a result, CD8+ T cells are inhibited, and tumor immune escape is mediated. NETs are upregulated in pancreatic cancer through a RAGE dependent and autophagy mediated pathway. NETs enhance the migration of hepatic stellate cells, activate cancer-associated fibroblasts, and promote hepatic metastasis of pancreatic cancer. Neutrophils are also involved in pancreatic cancer vascular endothelial cell integrity damage and promote metastasis of pancreatic cancer cells. CAF, cancer-associated fibroblasts; EMT, epithelial-mesenchymal transformation; PDAC, pancreatic ductal adenocarcinoma; PD-L1, programmed cell death-ligand 1; PSC, pancreatic stellate cell; IL, interleukin; EC, endothelial cell; NET, neutrophil extracellular trap.

checkpoint blockade (PD-1, CTLA-4) sensitivity. However, inhibition of neutrophils neutralizes IL-17. Thus, IL-17 promotes immunosuppression and resistance to immune checkpoint blockade by inducing neutrophil infiltration in pancreatic cancer (74).

Heterogeneous myeloid-derived suppressor cells (MDSCs) are suppressors of antitumor immunity, making tumor immunotherapy difficult. MDSC can inhibit T cell and NK cell proliferation and promote the function of Treg cells by secreting TGF-β and IL-10; moreover, MDSC can release reactive oxygen species and cause damage to the infiltrating lymphocytes in the TME (75). Neutrophil-like MDSCs (nMDSCs) are significantly increased in PDAC. The expression of high levels of CD13 on nMDSCs more effectively suppresses antitumor immunity through an arginase-1-related mechanism and PDAC patients with higher CD13 expression have a shorter OS (76).

Currently, there have been few studies on the specific mechanism by which TAN indirectly promotes immunosuppression. TANs can promote the recruitment of Tregs to the TME through the release of the chemokine CCL17, leading to the formation of an immunosuppressive microenvironment (77). A link between TANs and other immunosuppressive cell types acting together to impair antitumor immunity in pancreatic cancer needs to be further studied in the future. The interactions between neutrophils and other cells in the pancreatic cancer TME are summarized in Table 1.

Neutrophils as therapeutic targets

Gemcitabine, which produces anticancer activity by interfering with DNA synthesis in cancer cells, has been the most important chemotherapy drug for patients with pancreatic cancer in the past two decades (78). Due to the poor efficacy of chemotherapeutic drugs in some patients, new clinical treatment strategies are increasingly accepted in the treatment of pancreatic cancer. At present, targeted therapy and immunotherapy is representative of a new generation of cancer therapies, and is also the focus of pancreatic cancer research (79). The exploration of the biological function of immune cells in the tumor microenvironment will lead to more effective therapies to suppress the inflammatory response of the TME using cytokine inhibitors, chemokine inhibitors and immune checkpoint inhibitors to enhance anticancer immunity. TAN targeted therapies have been validated in human cancers (80). The

TABLE 1 Interaction of neutrophils with other cells in the pancreatic cancer TME.

Factors	Source	Responder	Effects	Ref.
MMP-9	Neutrophil	EC	Promoting EC migration	(60)
NGAL	Neutrophil	EC	Reducing VEGF secretion	(62)
NRF2	Neutrophil	CD8+ T cell	Regulating immune checkpoint transcription	(68)
CXCL5	Most cells	EC	Mediating angiogenesis in pancreatic cancer	(63)
IL-17	CD4+ T cell	• Neutrophil • T cell	Suppression of antitumor immunity	(74)
IL-1β	• Neutrophil • PSC	• PSC • Neutrophil	Promoting inflammation in PDAC and enhancing chemotherapeutic resistance	(65)
TGF-β	Most cells	Neutrophil	Promoting neutrophils to become the "pro-tumor" phenotype	(45)

TME, tumor microenvironment; Ref., reference; MMP-9, matrix metalloproteinase-9; EC, endothelial cells; NGAL, neutrophil gelatinase-associated lipocalin; VEGF, vascular endothelial growth factor; NRF2, neutrophil transcription factor NF-E2 p45-related factor 2; PD-L1, programmed cell death-ligand 1; CXCL, CXC-chemokine ligand; IL, interleukin; PSC, pancreatic stellate cell; PDAC, pancreatic ductal adenocarcinoma; TGF-β, transforming growth factor-β.

importance of neutrophils in mediating the effects of cancer therapies and the changes in neutrophils during these treatment processes within the TME is an emerging area of research. Several neutrophil-modulating therapies were originally developed for other indications and have effects beyond neutrophils. The current neutrophil modulatory effects of the treatments are summarized in Figure 3.

Chemokine inhibitors

Chemokine systems have been widely considered as potential new drug targets for cancer treatment due to their biological roles in the TME (81). The recruitment and activation of neutrophils is dependent on CXCR2, so CXCR2 is one of the most studied sites of action for neutrophil-targeted therapy. The ligands of CXCR2 mainly include CXCL1, CXCL2, CXCL3, CXCL5, and CXCL8 (82). The receptor-ligand axis of these chemokines can drive the mobilization and recruitment of neutrophils. Therefore, targeting CXCR2 is beneficial for reducing neutrophils in the TME. *In vivo*, blocking CXCR2 inhibits neutrophil mobilization, and the combination of CCR inhibitors and CXCR2 inhibitors enhances the pancreatic cancer response to FOLFIRINOX chemotherapy (83).

Currently, many CXCR4 or CXCR4 ligand inhibitors are in clinical development. For example, the CXCR4 inhibitors LY2510924 and AMD3100 have been evaluated for antitumor activity in combination with other drugs in patients with colorectal and pancreatic cancer (NCT02737072) (84, 85). In a phase II A clinical trial (NCT02826486), investigators evaluated the safety and efficacy of the CXCR4 antagonist BL-8040 in combination with pembrolizumab in metastatic PDAC and found that BL-8040 increased the tumor infiltration of CD8+ T cells. When BL-8040 was combined with pembrolizumab in chemotherapy-resistant patients, the media OS was 3.3 months and the disease control rate was 34.5% in the evaluable population. Additionally, objective response rate, disease control rate, and median response duration were 32%, 77%, and 7.8 months in the cohort that 22 patients received BL-8040 and pembrolizumab with chemotherapy, respectively. These results suggesting that CXCR4 and PD-1 co-inhibition may amplify the benefits of chemotherapy for patients with PDAC (86).

The loss or inhibition of CXCR2 enables the entry of T cells into the pancreatic cancer TME and enhances the antitumor immune function of the TME. In xenograft tumor models, the combination of CXCR2 and PD-1 inhibitors significantly prolonged the survival of mice (87). AZD5069 is a small CXCR2 antagonist that attenuates TGF-β -mediated drug resistance in cancer cells (88). AZD5069 is also evaluated in phase I B and II clinical studies (NCT02583477) for safety and antitumor activity in metastatic PDAC (89). CXCL5, a ligand of CXCR2, induces angiogenesis in pancreatic cancer. Inhibition of CXCL5 with small interfering RNA and neutralizing antibodies reduced tumor growth in a mouse model of pancreatic cancer (63). Although CXCL5 inhibitors have not yet been tested in cancer patients, their blockade of neutrophil recruitment and anti-angiogenesis actions provide a direction for the future treatment of pancreatic cancer patients.

Cytokine inhibitors

Cytokines are key mediators of cell signaling in the TME (90). Recently, cytokines and cytokine receptors have received extensive attention as targeted therapies for cancer, mainly by inhibiting pro-inflammatory cytokines and pro-tumor cytokines (91). As mentioned above, cytokines in the TME can induce neutrophil differentiation and prolong the lifespan of neutrophils. Therefore, inhibition of these cytokines to prevent neutrophils from differentiating into pro-tumor phenotypes is also one of the current targeted therapies.

TGF-β promoted the differentiation of neutrophils into a pro-tumor phenotype. TGF-β inhibitors mainly target the serine/threonine kinase domain of TGF-β receptor 1; for

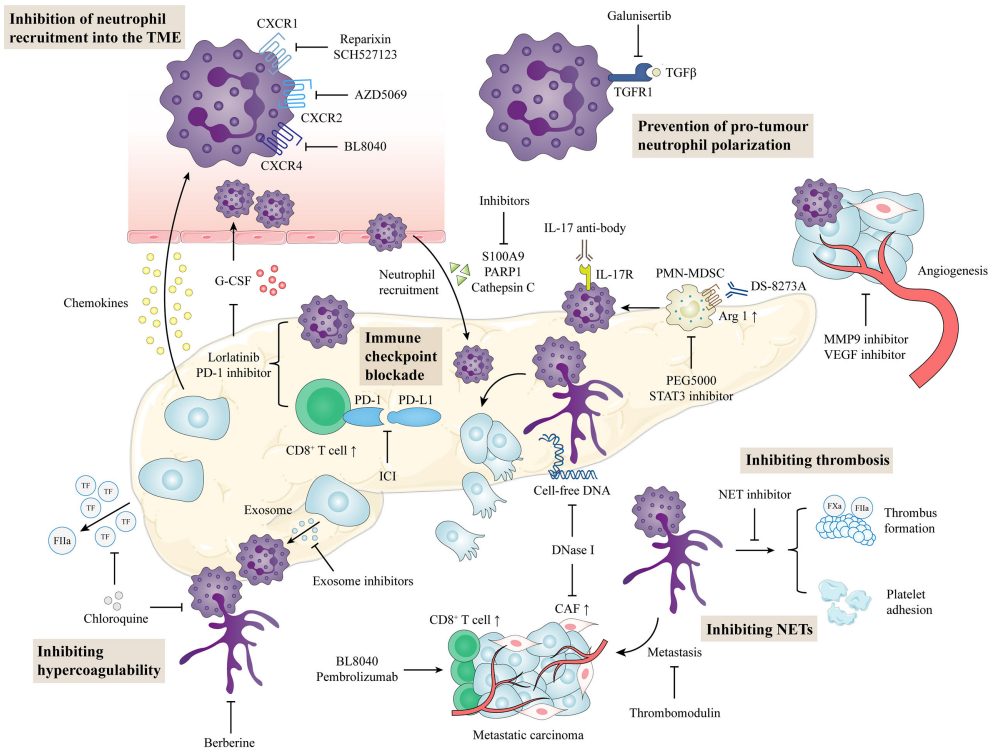


FIGURE 3
Potential neutrophil-directed therapeutic targets in pancreatic cancer. Inhibition of chemokines and cytokines prevents neutrophil activation and recruitment, thereby reducing neutrophils in the TME. TGF-β inhibitors can reduce the tumor-promoting phenotype of neutrophils. In the TME, targeting neutrophil combined with immune checkpoint blockade can enhance the antitumor function in pancreatic cancer. NET inhibitors prevent cancer cell metastasis, circulating hypercoagulable states, and venous thrombosis formation. TME, tumor microenvironment; CAF, cancer-associated fibroblasts; PD-L1, programmed cell death-ligand 1; PD-1, programmed cell death 1; PMN-MDSC, polymorphonuclear-myeloid derived suppressor cell; NET, neutrophil extracellular trap.

instance, galunisertib *in vivo* in combination with immune checkpoint inhibitors can significantly inhibit the growth of pancreatic cancer and enhance the antitumor M1 macrophage infiltration in the TME (92, 93). In current phase I B and phase II trials, galunisertib has also shown good tolerability, safety and antitumor activity in unresectable pancreatic cancer (NCT02734160) (94, 95). Selecting predictive biomarkers of TGF-β inhibition in pancreatic cancer patients may be more effective in predicting treatment effect and patient prognosis.

IL-17 can recruit neutrophils and form NETs that reduce cytotoxic CD8+ T cells in the pancreatic cancer TME (74). In mice with IL-17 overexpression, antibodies to IL-17 and IL-17 receptors reduce pancreatic intraepithelial neoplasia and neutrophil infiltration, and antibodies to IL-17 and IL-17 receptors are currently in clinical trials (96, 97).

G-CSF plays an important role in the activation and mobilization of neutrophils. Lorlatinib is a novel, oral tyrosine kinase inhibitor with anticancer activity in ALK- or ROS1-positive cancer patients (98). Lorlatinib prevents G-CSF and GM-CSF from inducing neutrophil migration. In PDAC, lorlatinib specifically targets neutrophils to inhibit cancer cells by regulating the development of neutrophils in bone marrow cells, reducing the accumulation of neutrophils in the TME, and inhibiting tumor tissue fibrosis. When lorlatinib is combined with anti-PD-1, the number of CD8+ T cells increases and CD44 +, CD69+, CD8+ T cells are activated, suggesting that lorlatinib improves the response of PDAC to immunotherapy (99). Similarly, a combination of suppressing cytokines that promote neutrophil recruitment and blocking immune checkpoints has been demonstrated in several preclinical trials. For instance, combination of anti-CSF1 receptor, anti-PD-1 and gemcitabine decreased the infiltration of myeloid cells and improved the antitumor effect (100). Some clinical trials that evaluating the safety and activity of cytokine inhibitors combined with immune checkpoint inhibitors are ongoing (NCT02947165, NCT04581343, NCT02777710).

Inhibition of NETs

NETs are now considered a promising cancer treatment target. Because NETs are involved in angiogenesis,

immunosuppression and metastasis of cancer, inhibiting their formation or promoting their elimination has been proposed as a novel therapeutic strategy for cancer (46, 56). There are two main methods to inhibit NET: inhibiting the formation of NET and destroying the structure of NET. NET formation is mediated by peptidyl arginine deiminase 4 (PADI4) and elastase, in which PADI4 promotes the expulsion of chromosomes *via* histone citrullination, but berberine can inhibit PADI4 expression *in vitro* (101, 102). In PADI4-deficient mice, pancreatic cancer growth was shown to be restricted (103). Cancer cells can also release exosomes to stimulate NET formation. Exosomes play a role in intercellular communication by transferring intracellular substances such as proteins, metabolites and nucleic acids to recipient cells (104). Cancer-derived exosomes can transfer factors related to cancer progression and promote tumorigenesis by regulating proliferation, metastasis, immune escape and increasing drug resistance processes (105). Studies have shown that cancer-derived exosomes transfer mutant KRAS to neutrophils, thereby promoting NET formation by upregulating IL-8 (106). Therefore, inhibition of exosome release in the TME is also a potential antitumor strategy.

Suppressing the components of NETs is also one of the strategies for targeting NETs. Serum DNA and citrullinated histone H3 are markers of NET formation. DNase is considered a promising cancer treatment for its ability to degrade circulating free DNA, thereby destroying the structure and function of NET (107). In a preclinical model of pancreatic cancer, the use of DNase I significantly reduced the number of fibroblasts accumulated in liver metastases, thereby attenuating NET-induced cancer invasion and metastasis (108). Thrombomodulin protein can degrade NET-derived high mobility group box 1 through thrombin, thereby inhibiting NET-induced epithelial-mesenchymal transformation and preventing the invasion and metastasis of pancreatic cancer cells (71).

Chloroquine is also a candidate to inhibit NETs. Chloroquine destroys the structure of NETs by inhibiting autophagy of glycosylated end-product receptors in pancreatic cancer (70). A meta-analysis evaluating the clinical value of using chloroquine as an autophagy inhibitor in the treatment of cancers showed that autophagy inhibitor therapy significantly improved the objective response rates, OS and progression-free survival of cancer patients, suggesting that the role of chloroquine in the treatment of pancreatic cancer should also be explored (109).

Additionally, gentamicin inhibits NETs release from human neutrophils and reactive oxygen species inhibitor (diphenyleneiodonium chloride) also reduces NET formation in a concentration-dependent manner (110, 111). These drugs are expected to be further validated the ability to inhibit NETs.

Treatment of complications and comorbidities for pancreatic cancer

Pancreatic cancer patients often have different diseases or complications in which neutrophils play different roles. Pancreatic cancer patients are in hypercoagulable state, which is directly related to poor prognosis and venous thrombosis (112–114). Citrullinated histone H3 is one of the markers of NETs, and increased expression of citrullinated histone H3 was observed in the thrombi of pancreatic cancer mice. The thrombus weight decreased after using 1A8, an anti-LY6G antibody, to deplete neutrophils and DNase I to deplete NETs (115). Chloroquine inhibits NET formation and reverses NET-mediated platelet activation and aggregation, as well as tissue factor release. Researchers further found that the rate of venous thromboembolism in patients treated with hydroxychloroquine was 9.1%, while that in the control group was 30% (70, 116).

Neutrophil infiltration was increased in pancreatic cancer specimens from patients with type 2 diabetes mellitus (T2DM). Patients with elevated neutrophils had reduced OS (HR=5.44, 95% CI 1.12 to 26.34) (117). Anorexia and muscle breakdown induced by PDAC are also associated with inflammatory stimulation of neutrophils mediated by the CCR2/CCL2 axis (118). Inflammatory processes and immune system contribute to the metabolic diseases (119, 120). Therefore, based on the role of neutrophils in pancreatic cancer and metabolic disease, such as T2DM, inhibition of neutrophils can simultaneously alleviate the progression of both diseases. Combination therapeutic strategy involving multiple immunomodulatory therapies may prove to be more effective.

Other potential therapeutic targets

Tumor genotypes can affect the TME and play a key role in treatment resistance. In gain-of-function Trp53 mutant mice, intratumoral neutrophil infiltration increased and the numbers of CD3+ T cells, CD4+ T cells and CD8+ T cells decreased. After neutrophil removal, the sensitivity of CD40 agonists combined with chemotherapy and immunotherapy was enhanced (121).

Several bioinformatics analyses have shown a significant correlation between oncogene expression and the infiltration of various immune cells, including neutrophils, suggesting that targeting these genes may also be a future therapy (122).

Chemotherapy induces the invasion of cytotoxic T cells into the liver metastases of pancreatic cancer, but only briefly. Neutrophils lead to tumor cell regeneration in metastases, and reducing neutrophil infiltration or inhibiting the Gas6/AXL signaling axis combined with chemotherapy can inhibit metastatic growth (123).

MDSCs are sensitive to TRAIL receptor 2 agonists. The antitumor efficacy of DS-8273A (an anti- TRAILR2 antibody) was evaluated in a phase I clinical trial (including one pancreatic cancer patient). The results showed that DS-8273A eliminated polymorphonuclear MDSCs (PMN-MDSCs, immature neutrophils) and prolonged progression-free survival (124). STAT3 is involved in the regulation of arginase 1 activity in PMN-MDSCs, leading to immunosuppression in the TME. Inhibition of STAT3 or using human recombinant arginase 1 (PEG5000) may be beneficial to ameliorate this immunosuppressive microenvironment (125, 126).

Areas of future development

Finding novel therapies is currently a hot spot and future research direction for cancer research. As an indispensable part of tumor development, the TME has become a new therapeutic target. Based on the important role of neutrophils in the TME of pancreatic cancer, it is feasible to target neutrophils in the treatment of pancreatic cancer.

Limitation

There are still some challenges in the treatment of pancreatic cancer patients based on neutrophil related oncogenic mechanisms. First, specific reductions of neutrophils in peripheral blood and the TME can inhibit tumor growth and metastasis but can expose patients to opportunistic infections. Since cancer patients often suffer from malnutrition, cachexia, and reduced resistance, such treatments are impractical. Next, blocking chemokines and cytokines also affects the recruitment and function of "antitumor phenotype" neutrophils and other leukocytes, resulting in the limited specificity of currently conducted approaches. Thus, only a few studies have focused on the effects of drugs on specific TAN phenotypes. Moreover, most of the drugs that regulate the TME and target TANs have been studied in animal experiments, but clinical evidence in solid tumor patients is insufficient. Although there have been pilot studies showing a substantial anticancer effect of neutrophil-targeting inhibitors, these need to be followed by more clinical trials so that targeting neutrophil-associated sites or specific phenotypes can be a new treatment for patients with pancreatic cancer. There is also a need for studies that investigate the adverse effects of targeting neutrophils in pancreatic cancer.

Improved future direction

The pancreatic cancer TME is a complex and dynamic structure that directly affects the biological behavior of pancreatic cancer cells at the molecular and clinical levels. Current work focuses on interactions among tumor cells, neutrophils and inflammatory factors. We should study the following aspects in the future.

First, extracellular vesicle-mediated signal transduction between pancreatic cancer cells and immune cells should be intensively studied. Extracellular vesicle can help facilitate an exchange of information within various cells in the TME (127). It has been found that extracellular vesicle-RNA and proteins are involved in the metastasis and chemotherapeutic resistance of pancreatic cancer (128). Inhibition of extracellular vesicle release and uptake in the TME may be another therapeutic option. Second, although immune checkpoint inhibitors have made progress in cancer treatment during the past 10 years, they are only effective in a subset of patients. Since neutrophils can induce the TME to form an immunosuppressive microenvironment, this may be one of the reasons for the poor efficacy of immune checkpoint inhibitors in pancreatic cancer. Therefore, the combination of neutrophil inhibitor therapy and immune checkpoint inhibitor therapy also needs more preclinical studies and clinical trials in the future. Furthermore, cellular metabolism has also emerged as a critical determinant of the function of immune cells in the TME. The metabolism of substances in the TME is not only the result of tumor development, but also the promoting factor of tumor progression (129). A subpopulation of TANs with high glycolytic activity has been found to enhance immunosuppressive and tumor-promoting functions (130). Understanding the metabolic requirements of neutrophils in pancreatic cancer and their effect on the growth, metastasis and immunosuppression of pancreatic cancer will also be a novel research direction upon which to intervene for enhanced immunotherapy.

Neutrophils in pancreatic inflammation and fibrosis

Since pancreatic cancer are closely related to pancreatic chronic inflammation and fibrosis, it is necessary to explore the role of neutrophils in the process of chronic pancreatic fibrosis. Immune cells, especially myeloid cells, play an important role in the pathogenesis of pancreatitis. GM-CSF-mediated increased neutrophil infiltration is the main reason that STAT5 promotes pancreatic fibrosis and chronic pancreatitis (131). However, CXCR2 inhibitors reversed pancreatic inflammation *in vivo* models (132). It is also one of the future directions to conduct joint research with related chronic diseases in cancer mechanistic studies (89). Therefore, it is important to combine pancreatic cancer and chronic pancreatitis in animal models when doing preclinical studies. Including patients with chronic pancreatitis as a

comparison group is also necessary when conducting clinical trials.

Conclusion

Neutrophils play a role in angiogenesis, metastasis and immunosuppression in pancreatic cancer through interactions with other cells in the TME. Various neutrophil modulation therapies are entering preclinical studies and clinical trials for pancreatic cancer. Precision medicine aims to provide patients with more effective personalized medical services (133). The treatment mode of pancreatic cancer is gradually developing toward targeted therapy and precision medicine. The research and development of neutrophil-based therapeutics and targeting neutrophils in combination with other therapies will benefit more patients with pancreatic cancer. Specifically targeting neutrophil-associated sites will be part of therapies for the next generation of cancer patients.

Author contributions

WJ, XL, and WZ contributed to the study conception and design. The original draft of the manuscript was written by WJ and XL. WJ and XL contributed to visualization. Review and editing was performed by CX and WZ. WZ provided language help. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Risk factors associated with peritoneal carcinomatosis of gastric cancer in staging laparoscopy: A systematic review and meta-analysis

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Background: The optimal indications of staging laparoscopy in gastric cancer to detect peritoneal carcinomatosis are still controversial. We performed this systematic review and meta-analysis to quantify the relevance of the preoperative factors with peritoneal carcinomatosis to explore the indications of staging laparoscopy.

Materials and methods: Systematic searches were conducted using Medline, Embase, and the Cochrane Library in December 2021. On the basis of calculating the odds ratio (OR) of each factor, we quantified the association between the risk factors and peritoneal carcinomatosis such as clinical T/N stage, Borrmann type, and tumor markers, using meta-analysis with a random-effects model.

Results: A total of 21 case-control studies and one cohort study were obtained. T stage, N stage, and differentiation degree were most widely studied, with OR values of 2.96 (95% CI: 1.87–4.69), 1.22 (95% CI: 0.86–1.73), and 1.91 (95% CI: 1.42–2.56), respectively. Among all the factors, elevated CA125 (OR = 19.45, 95% CI: 4.71–80.30), Borrmann type IV (OR = 7.68, 95% CI: 3.62–16.27), and large tumor diameter (OR = 5.12, 95% CI: 2.55–10.31) had the highest OR. In particular, CA125 had the best predictability for peritoneal carcinomatosis but was only mentioned by three articles.

Conclusions: There was a cognitive gap between the awareness and importance of risk factors for peritoneal carcinomatosis. In addition to T4 stage, patients with factors with high OR, such as Borrmann type IV, large tumor diameter, and elevated CA125, should undergo staging laparoscopy.

KEYWORDS

gastric cancer, peritoneal carcinomatosis, risk factors, staging laparoscopy, indications

Introduction

There were nearly 1.07 million new cases of gastric cancer in 2020, with incidence ranking fifth and mortality ranking fourth among malignant tumors (1). Peritoneal carcinomatosis (PC), including macroscopic carcinomatosis (P1) and positive cytology (CY1), is the most common metastasis of gastric cancer (2, 3). Current examination methods, such as CT and MRI, are not effective in detecting PC. It was reported that the sensitivity of CT in diagnosing PC changed from 25% to 50.9% and often at a relatively advanced stage (4–6). There is no evidence that PC could be diagnosed effectively by MRI.

The guidelines for gastric cancer recommend staging laparoscopy (SL) combined with peritoneal cytology as the best method for detecting PC (7–9). Its sensitivity changes from 85% to 98% and its specificity is close to 100% (10, 11). However, SL needs to be carried out under general anesthesia, which will increase the cost to patients, and SL could be more cost-effective if used selectively (12). However, the indications for SL of the guidelines are inconsistent. Japanese institutions chose patients with a more advanced stage like bulky lymph nodes or large Borrmann type III to undergo SL (7), whereas Western countries tend to apply SL to all patients with resectable tumors (13). This study aims to summarize the preoperative risk factors of PC to screen patients that are suitable for SL.

Materials and methods

Literature search strategy

The study was performed following the PRISMA statements for systematic reviews and meta-analyses including observational studies. We searched the databases of PubMed, Embase, MEDLINE, and the Cochrane Library for studies that were published before December 2021 using the search strategy "((gastric [Title/Abstract]) OR (stomach [Title/Abstract]) OR (gastroesophageal [Title/Abstract])) AND ((staging laparoscopy [Title/Abstract]) OR (diagnostic laparoscopy [Title/Abstract]))". In addition, the references cited in the publications were manually searched to identify additional relevant studies. Only studies published in English were included. No institutional review board approval was required for this literature review. This review was not registered.

Inclusion and exclusion criteria

Studies included in this systematic review and meta-analysis were required to meet the following criteria: 1. patients were pathologically diagnosed with gastric cancer; 2. PC was diagnosed by SL or open surgery; and 3. PC was diagnosed before any anti-tumor treatment. Studies were excluded on the basis of the following criteria: 1. patients were diagnosed with recurrent gastric cancer; 2. patients were diagnosed clearly as stage IV or with distant metastasis by CT or other non-invasive methods; 3. patients suffered from other malignant tumors at the same time; and 4. patients had a history of malignant tumors.

Data extraction and quality assessment

Two investigators (GMG and ZML) independently screened titles, abstracts, and full texts of the studies for eligibility. Disagreement was resolved between the two reviewers through discussion or, if needed, adjudication by a third reviewer (ZYL). The following information from the included studies were collected in the same way: first author, year of publication, country of patients, duration of study, inclusion and exclusion criteria, sample size, median or mean age, gender, the definition of PC, diagnostic methods of PC, and OR values of risk factors. We would extract the original data to calculate it when feasible, if needed. More specifically, in the latest Japanese guideline for gastric cancer, both P1 and CY1 were defined as M1 stage, so we did the same (7).

Statistical analysis

We quantified the association between risk factors and PC such as age and gender, using meta-analysis with a random-effects model, and presented the results with forest plots. Heterogeneity was tested with Cochran's Q-test, with P-value < 0.1 indicating heterogeneity, and quantified by the I^2 statistic with values of <25%, 25%–50%, and >50% corresponding to low, moderate, and high degrees of heterogeneity, respectively. Then, we examined publication bias with the funnel plot and Egger's test. All analyses were conducted in the statistical software Review Manager (RevMan version 5.3; The Nordic Cochrane Center, Copenhagen, Denmark). P-value < 0.05 was considered to be statistically significant.

Results

Search results and study characteristics

PRISMA flow chart is shown in Figure 1. There were 520 potentially relevant studies initially identified through the database according to the predefined search strategy. In addition, five studies were obtained manually. Five studies were excluded by duplicate checking. A total of 443 studies

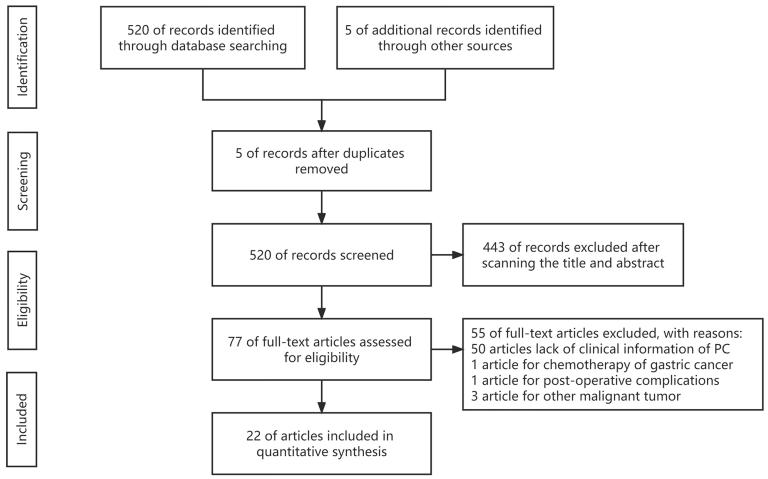


FIGURE 1
Flow diagram.

were excluded after scanning the title and abstract, and the remaining 77 studies were further reviewed by the full-text view. Of these 77 studies, 50 studies were removed because of the lack of clinical information of patients. Five studies were removed because the objects were not PC (one study about chemotherapy, one study about complications, and three studies focusing on other malignant tumors). Finally, 22 studies were included in the meta-analysis.

The characteristics of all studies are summarized in Table 1. The risk factors mentioned in the studies that may be related to PC and the number of studies for each factor are shown in Table 2. PC was defined as P1 and/or CY1 in nine studies, as only P1 in nine and only CY1 in four. The funnel charts of all results can be found in the supplementary materials.

The correlation between T stage and PC

Eleven studies, with 3,877 patients, from 2008 to 2020 were included (10, 14–23). The sample size of the studies varied from 49 to 879.

The pooled results revealed that patients with T4 stage had a higher proportion in the PC group compared with the ones in the non-PC group; the difference was statistically significant; OR = 2.96, 95% CI: 1.87–4.69, P < 0.0001; I^2 = 73% (Figure 2).

The correlation between N stage and PC

Two ways of classification were applied for the N stage. First, patients were divided into positive regional lymph node (N+) and negative (N−) groups, separately. In addition, 15 studies, with 4,587 patients, were included (10, 14–22, 24–28). Then, we divided patients into N0/1 and N2/3 groups, separately. According to this criteria, five studies, with 1,321 patients, were included (14, 17, 20, 22, 29). The sample size varied from 35 to 589.

There was no statistical correlation between N+ and PC, with OR = 1.22, 95% CI: 0.86–1.73, P < 0.0001; I^2 = 66%. On contrary, patients with N2/3 stage had a higher proportion in the PC group, with OR = 2.38, 95% CI: 1.22–4.65, P = 0.01; I^2 = 53% (Figure 3).

The correlation between differentiation degrees and PC

Fourteen studies, with 4,424 patients, from 2006 to 2020 were included (15–23, 26, 27, 29–31). The sample size varied from 32 to 879.

The proportion of patients with poorly differentiated carcinoma was higher in the PC group, and the difference was statistically significant; OR = 1.91, 95% CI: 1.42–2.56, P < 0.0001; I^2 = 49% (Figure 4).

The correlation between Borrmann type and PC

Eight studies, with 2,924 patients, from 2000 to 2018 were included (10, 16–18, 24, 26, 29, 32). The sample size of the studies varied from 32 to 879.

After summing up all the results, we could find that patients with Borrmann type IV had a higher proportion in the PC group, with OR = 6.67, 95% CI: 3.33–13.36, P < 0.0001; I^2 = 85% (Figure 5).

TABLE 1 Baseline characteristics of the included studies.

Author and Year	Type of study	Duration of the study	Total patients	Positive cases	Definition of PC	Statistically significant risk factors in univariate analysis
Fujimura, 2002	Case-control study	1992.4–2000.4	39	21	P1 or CY1	CA125
Yano, 2000	Case-control study	1994.7–1998.5	32	13	P1 and CY uk	No factor
Hwang, 2004	Case-control study	199.7–2003.12	768	88	P1 and CY uk	CA125 CA199
Bentrem, 2005	Case-control study	1993.9–2002.12	371	24	CY1 and P0	No factor
Sarela, 2006	Case-control study	1993.4–2002.5	65	21	P1 and CY uk	N+ PD
Badgwell, 2008	Case-control study	1995.1–2005.12	381	39	CY1 and P0	No factor
La Torre, 2010	Case-control study	2003.7–2008.5	64	7	CY1 and P0	T4 N+ PD
Hur, 2010	Case-control study	2001.1–2005.12	589	72	P1 and CY uk	T4 N+ PD size
Kurita, 2010	Case-control study	2001.1–2008.3	236	41	P1 and CY uk	N2/3 PD Borrmann type IV size
Tsuchida, 2011	Case-control study	1999.6–2003.12	231	86	P1 or CY1	N+ Borrmann type IV
Strandby, 2015	Case-control study	2010–2012	219	21	P1 and CY uk	No factor
Lisiecki, 2015	Case-control study	2014.4–2015.7	51	12	CY1 and P uk	No factor
Ikoma, 2016	Case-control study	1995.1–2012.12	711	228	P1 or CY1	PD Borrmann type IV Signet-ring
Hu, 2016	Case-control study	2004.6–2014.5	582	138	P1 or CY1	T4 size Borrmann type IV
Li, 2017	Case-control study	2011.9–2013.9	249	39	P1 or CY1	Borrmann type IV
Hosogi, 2017	Case-control study	2006.5–2015.9	287	116	P1 or CY1	Borrmann type IV size LD
Huang, 2018	Case-control study	2008.12–2012.12	879	110	P1 or CY1	T4 N+ PD Borrmann type IV size
Rawicz Pruszyński, 2019	Case-control study	2016.8–2018.9	173	39	P1 and CY uk	T4 LD
Nakamura, 2019	Case-control study	2009.1–2017.12	35	28	P1 or CY1	No factor
Harris, 2019	Case-control study	2013.12–2016.10	363	75	P1 and CY uk	PD
Yang, 2020	Case-control study	2014.1–2019.4	672	89	P1 or CY1	T4 PD size
Zhao, 2020	cohort study	2015.4–2015.11	129	43	P1 and CY uk	PD CA125 LD CA199

uk, unknown; N+, positive lymph node metastasis; N2/3, N2 or N3 stage; PD, poorly differentiated carcinoma; Signet-ring, Signet-ring cell carcinoma; Size, tumor diameter ≥ 4 or 5 cm; LD, Lauren diffuse type.

The correlation between tumor diameter tumor and PC

Six studies recorded the diameters of the primary tumors, of which three studies used 4 cm as the cutoff value, and others used 5 cm. We put patients with tumor diameters ≥4 or ≥5 cm together as the large tumor size group. Finally, the number of the included patients was 3,117 (10, 16, 20, 23, 24, 29), with the sample size changing from 231 to 879.

The pooled results showed that patients with large tumor size had a higher proportion in the PC group; the difference was statistically significant; OR = 5.12, 95% CI: 2.55–10.31, $P < 0.0001$; $I^2 = 83\%$ (Figure 6).

TABLE 2 Risk factors and OR value of the pooled results.

Risk factor	OR value	Included studies	Sample size
T4	OR = 2.96, 95% CI: 1.87–4.69	11	3,877
N+	OR = 1.22, 95% CI: 0.86–1.73,	15	4,587
Poorly differentiated carcinoma	OR = 1.91, 95% CI: 1.42–2.56	14	4,424
Borrmann type IV	OR = 6.67, 95% CI: 3.33–13.36	8	2,924
Large tumor	OR = 5.12, 95% CI: 2.55–10.31	6	3,117
N2/3	OR = 2.38, 95% CI: 1.22–4.65	5	1,321
CA125 ≥ 37	OR = 19.45, 95% CI: 4.71–80.30	3	925
Lauren diffusion type	OR = 2.11, 95% CI: 1.60–2.79,	5	1,427
Signet-ring cell carcinoma	OR = 1.71, 95% CI: 1.30–2.26	4	1,328
CA199	OR = 4.22, 95% CI: 1.44–12.34	4	1,110
Gender	OR = 0.90, 95% CI: 0.74–1.09	9	4,054
Age	OR = 1.06,95% CI: 0.89–1.25	6	3,235

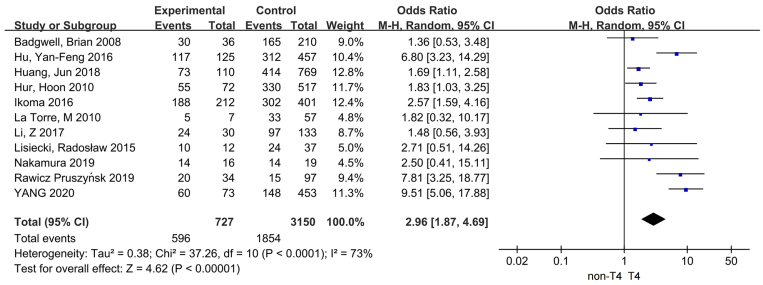


FIGURE 2
The correlation between T4 stage and PC.

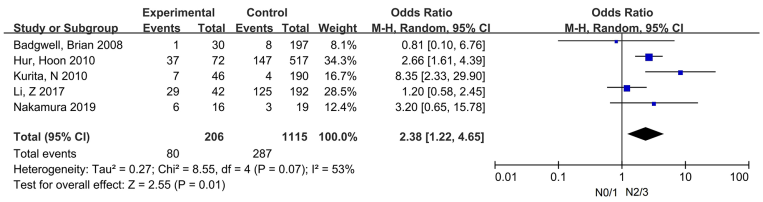


FIGURE 3
The correlation between N2/3 and PC.

The correlation between serum CA125 and PC

Three studies, with 925 patients, from 2002 to 2020 were included (11, 30, 33). All of the studies used 35 ng/ml as cutoff value. The sample size varied from 37 to 728.

The pooled results showed that patients with serum CA125 ≥ 35 ng/ml had a higher proportion in the PC group, with OR = 19.45, 95% CI: 4.71–80.30, P < 0.0001; I^2 = 73% (Figure 7).

The correlation between Lauren diffusion type and PC

Five studies, with 1,427 patients, from 2015 to 2020 were included (15, 16, 19, 24, 30). The sample size of the studies changed from 51 to 804.

The proportion of patients with Lauren diffusion type was higher in the PC group, with OR = 2.11, 95% CI: 1.60–2.79, P < 0.0001; I^2 = 71% (Supplementary Figure 8).

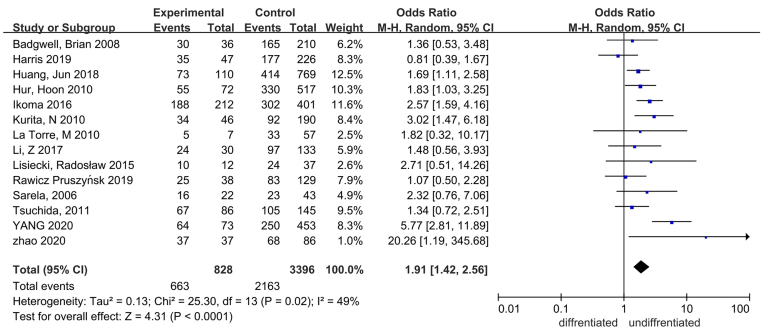


FIGURE 4
The correlation between differentiation degrees and PC.

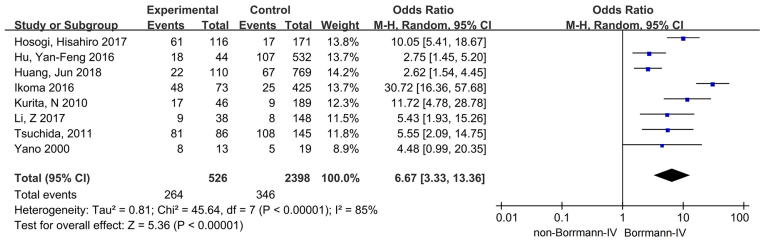


FIGURE 5
The correlation between Borrmann type IV and PC.

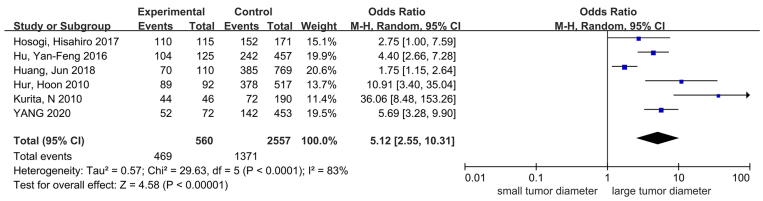


FIGURE 6
The correlation between tumor diameter and PC.

The correlation between Signet-ring cell carcinoma and PC

Four studies, with 1,328 patients, from 2008 to 2017 were included (17, 18, 22, 25). The sample size changed from 185 to 662.

The pooled results showed that patients with Signet-ring cell carcinoma had a higher proportion in the PC group, with OR = 1.71, 95% CI: 1.30–2.26, P = 0.0001; I^2 = 0% (Supplementary Figure 9).

The correlation between serum CA199 and PC

Four studies, with 1,110 patients, from 2002 to 2020, which used 37 ng/ml as cutoff value, were included (11, 17, 30, 33). The sample size of the studies varied from 37 to 768.

The pooled results showed that patients with the serum CA199 ≥ 37 ng/ml had a higher proportion in the PC group, with OR = 4.22, 95% CI: 1.44–12.34, P = 0.001; I^2 = 81% (Supplementary Figure 10).

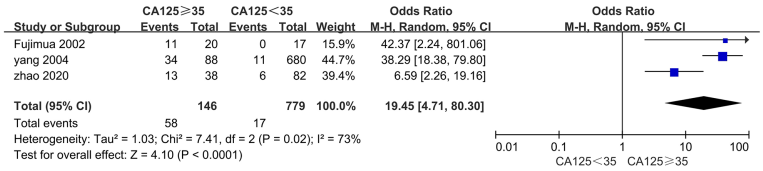


FIGURE 7
The correlation between serum CA125 and PC.

The correlation between gender and PC

Nine studies, with 4,054 patients, from 2005 to 2019 were included (10, 15–18, 20, 26, 28, 31). The sample size varied from 173 to 889.

The pooled results showed that the gender of patients was not statistically associated with PC, OR = 1.06, 95% CI: 0.89–1.25, P = 0.51; I^2 = 63% (Supplementary Figure 11).

The correlation between age and PC

Five studies used 65 years old as the cutoff value and one for 60 years old. We put patients elder than 60 or 65 together as one group. Finally, six studies, with 3,235 patients, from 2005 to 2019 were included (10, 16, 18, 20, 28). The sample size varied from 153 to 878.

The pooled results showed that the age of patients was not statistically associated with PC, OR = 0.90, 95% CI: 0.74–1.09, P = 0.26; I^2 = 0% (Supplementary Figure 12).

Discussion

The guidelines provide different indications of SL for patients with advanced gastric cancer. The NCCN (National Comprehensive Cancer Network) guideline recommends that all locoregional patients undergo SL with cytology (8). However, the JGCA (Japan Gastric Cancer Association) guideline recommends that only patients with large Borrmann type III or IV or bulky lymph nodes require SL (7). In between, the CSCO (Chinese Society of Clinical Oncology) guideline sets the indication as CT suspected PC or T3–4/N+ patients ready for neoadjuvant therapy (9). The conflict between the guidelines raised the need for further research on the risk factors of PC to select patients suitable for SL.

In this meta-analysis, we found that there was a cognitive gap between awareness and importance of risk factors of PC, which could partly explain the reasons for the current divergence. In addition to the clinical stage mentioned by the guidelines, there were also factors significantly statistically related to PC but with low concern, such as tumor size and CA125.

T stage is one of the most discussed factors, and our data support its relevance with PC. The majority of the included studies used T4 as a criterion, and T4's relevance with PC is also consistent with the "seed and soil" hypothesis, which states that more free cancer cells exfoliated from the tumor penetrating serosa could lead to PC (34). However, if all three studies with CY1 only were included in the analysis, then OR (95% CI) was 1.65 (0.79–3.44), which means that there is no statistical difference (19, 21, 22). Yoshida et al. found the occurrence of CY1 or P1 in five of the 1,509 patients with early gastric cancer, and pathological analysis revealed that the primary tumor invaded the submucosa and metastasized to the regional lymph nodes through the lymph vessels; they speculated that tumor cells may metastasize to the peritoneal cavity through the lymphatic system rather than by breaking through the plasma (35). This could be the reason for this subgroup analysis. The analysis of the remaining eight studies involving P1 showed a stronger statistical correlation between T4 and P1. We prefer to recommend SL to patients with T4 stage to avoid missing PC, although the relationship between T4 and CY1 still needs to be explored.

N stage is another most mentioned factor in the studies and guidelines, but the statistical relevance is not obvious. An alternative approach is for N0/1 and N2/3 groups. Although with fewer studies and increased publication bias, there is a significant increase in OR (95% CI) of 2.38 (1.22–4.65). Yoshida et al. proposed that PC can occur through the lymphatic system (35). According to this hypothesis, the more regional lymph nodes that metastasize, the greater likelihood that tumor cells initiate PC by lymph vessels. We cannot propose a definitive SL strategy based on the N stage with insufficient evidence. It may be appropriate to analyze the relationship between the N1–3 stage and PC, separately.

Tumor size is often omitted in the guidelines, with only the Japanese guideline recommending SL for patients with Borrmann type III tumor with diameter ≥ 8 cm (7). However, multiple studies have confirmed the dependency between tumor diameter and PC. Although different lengths were used as the standard of classification, such as 4 or 5 cm, the results indicated that larger tumor diameter was independent risk factor of PC. However, there was no detailed description of how to measure tumor size in the studies. We suggest that patients with large tumor size undergo SL, but the method of measuring the

diameter and the cutoff value of tumor size needs to be further clarified.

Several studies have concluded that Borrmann type IV is risk factor for PC, similarly to the JGCA guideline (10, 16–18, 24, 26, 29). In addition, the results of studies indicated that Borrmann type III is also risk factor for PC (10, 16, 26). This may be because Bormann type IV and large Borrmann type III tumors are usually accompanied by larger tumor size and more advanced stage. As discussed previously, patients with risk factors of PC, such as T4 and N+, are more likely to develop PC. Therefore, we recommend that patients with Bormann type IV and large Borrmann type III gastric cancer receive SL.

Although differentiation degrees have been widely studied, no definite conclusion has been made about its relationship with PC. The pooled results suggested that poorly differentiated carcinoma is a risk factor for PC, but the OR was only 1.91 and eight of the 14 studies had opposite results, with a weight of 45.2%. These diminish the confidence of the conclusion. Furthermore, the result of subgroup analysis showed that poorly differentiated carcinoma was not risk factor for CY1 but a risk factor for P1. This suggests that the relationship between poorly differentiated carcinoma and P1 and CY1 may be investigated, separately.

Regarding CA125, CA199, Signet-ring cell carcinoma, and Lauren's diffuse type, the insufficient studies in the meta-analysis and heterogeneity between results reduce the confidence of the conclusion. We believe that only a system review of their relationship with PC can be made on the base of the current finding. In particular, the OR of elevated CA125 was 19.45, significantly higher than other factors. Moreover, all three studies indicated that elevated CA125 was a risk factor for PC (11, 30, 33). Tumor cells can cause CA125 elevation, and the mesothelial cells in the abdomen and pelvis stimulated by tumors can also increase CA125 secretion. Therefore, we suggest that patients with elevated CA125 should undergo SL and more attention should be paid to its relationship with PC.

There are still limitations in this study. The included studies used different definitions of PC. Four studies defined CY1 as PC and the remaining studies involved P1, of which the conclusions were generally consistent with the overall results. However, no similar relationship was observed in the subgroup analysis of CY1. For example, the relationship between T4 and CY1 was different from between T4 and PC or P1. Moreover, part of the conclusions was based on insufficient evidence, for instance, the relationship between CA125 and PC. In addition, there is heterogeneity and publication bias among the results of the included studies. Meanwhile, the absence of randomized controlled trials has led to the inclusion of only retrospective studies in this article.

In conclusion, this meta-analysis raised the potential conflict between current indications of SL and their actual relevance with PC. We think that patients with T4 stage, Borrmann type IV, large tumor size, and elevated CA125 are more likely to have PC and should undergo SL. In particular, the relationship between CA125 and PC deserves further investigation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

Conception and design of the research: ZYL, GG, and ZML. Analysis and interpretation of the data: GG, ZML, XY, and QW. Writing of the manuscript: GG and ZML. Critical revision of the manuscript for intellectual content: ZYL, GG, and ZML. Final review: ZYL, GG, ZML, and FS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.955181/full#supplementary-material

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Blood-based biomarkers for immune-based therapy in advanced HCC: Promising but a long way to go

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The introduction of immune checkpoint inhibitors (ICIs) represents a key shift in the management strategy for patients with hepatocellular carcinoma (HCC). However, there is a paucity of predictive biomarkers that facilitate the identification of patients that would respond to ICI therapy. Although several researchers have attempted to resolve the issue, the data is insufficient to alter daily clinical practice. The use of minimally invasive procedures to obtain patient-derived specimen, such as using blood-based samples, is increasingly preferred. Circulating tumor DNA (ctDNA) can be isolated from the blood of cancer patients, and liquid biopsies can provide sufficient material to enable ongoing monitoring of HCC. This is particularly significant for patients for whom surgery is not indicated, including those with advanced HCC. In this review, we summarize the current state of understanding of blood-based biomarkers for ICI-based therapy in advanced HCC, which is promising despite there is still a long way to go.

KEYWORDS

hepatocellular carcinoma, immune checkpoint inhibitors, biomarker, circulating tumor DNA, patient outcomes

Introduction

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and is the sixth most common cancer and the third leading cause of malignancy-associated deaths worldwide (1, 2). HCC primarily occurs after liver cirrhosis, and the most common associated risk factor is chronic hepatitis B virus (HBV) infection; other causes include chronic hepatitis C virus (HCV) infection, alcoholic liver disease, and non-alcoholic fatty

liver disease (NAFLD) (3). Liver cancer staging is usually determined by using the 20-year-old Barcelona Clinic Liver Cancer (BCLC) method or its updated versions, and the analysis outcome is used to stratify and allocate HCC patients into appropriate treatment streams (4). According to the BCLC staging system, a considerable number of HCC patients have BCLC stage C HCC, indicating an advanced stage cancer for which curative or locoregional therapies are unsuitable (5, 6). As more than 50% of patients with HCC have a tumor that is too-advanced for curative therapy, HCC represents the second leading cause of death from cancer; with a 3% survival rate at 5 years globally (7). Sorafenib has been used for over ten years as the primary treatment and was the first tyrosine kinase inhibitor (TKI) for patients with advanced HCC. Recently, treatment options for patients with advanced HCC have increased as several novel therapies have gained approval. These include the use of TKIs such as lenvatinib, regorafenib, and cabozantinib and immune-based therapies such as the immune checkpoint inhibitors (ICIs) atezolizumab, nivolumab, and pembrolizumab (8, 9). According to the recent European Association for the Study of the Liver (EASL) guideline, atezolizumab plus bevacizumab or durvalumab plus tremelimumab administration are the preferred first-line treatment in patients with advanced HCC (4, 10, 11).

The liver is characterized by immune tolerance controlled by antigen-presenting cells, which are responsible for the active control of immunogenicity (8). Moreover, during hepatocarcinogenesis, the gradual dysfunction of innate and adaptive immune cells and an increase in the number of immune-regulatory cells contribute to the generation of immunosuppressive tumor microenvironment (TME) (8, 12). M2-polarized tumor-associated macrophages (TAMs), which act as immune suppressors, critically contribute to the immunosuppressive character of the HCC TME (13, 14). Exhausted T cells show upregulated expression of several inhibitory receptors, including programmed cell death protein-1 (PD-1), T cell immunoreceptor with Ig and ITIM domains (TIGIT), T cell immunoglobulin, mucin-domain containing-3 (TIM-3), and lymphocyte activation gene 3 (LAG3), and the effector function of the cells is impaired by transcriptional changes mediated by thymocyte selection-associated HMG BOX (TOX) (15). Blocking such inhibitory receptors with ICIs reinvigorates exhausted T cells and enhances effector function. Over the past ten years, several clinical trials have demonstrated the contribution of ICIs to the improvement of overall survival (OS) of patients with various tumors. Notably, immunotherapies are generally safe and well-tolerated by patients (16); however, under some circumstances, ICI therapy can cause serious adverse events (AEs), resulting in discontinuation and disease hyper-progression in some cases (17). It is critical to identify reliable biomarkers that would facilitate the selection of patients who will be responsive to ICI treatment as well as those likely to suffer serious AEs.

Molecular biomarkers are prevalent in the blood or tissues (18). Several putative biomarkers are being evaluated in clinical trials for ICI therapy against HCC, including programmed death ligand-1 (PD-L1) expression, microsatellite instability (MSI), tumor mutational burden (TMB), DNA damage repair gene alterations, gut microbiome, and various blood biomarkers (16, 19). Among these, markers in blood samples are easily measured and can serve as tools for clinical management, diagnosis, staging, and evaluation of therapeutic response (18). Here, we comprehensively review blood-based biomarkers for the prediction of ICI responses in patients with HCC.

Currently used immune-based treatments for HCC

ICI treatment (nivolumab, pembrolizumab, ipilimumab, tremelimumab, and durvalumab)

Nivolumab, the initial humanized IgG4 monoclonal antibody directed against PD-1, reinvigorates the immune response of the host towards cancer cells through the competitive inhibition of PD-1-dependent signaling (20). In patients with advanced HCC recruited to the phase I/II CheckMate-040 study, nivolumab showed an objective response rate (ORR) and disease control rate (DCR) of 15%–20% and 58%–64%, respectively, with an acceptable safety profile (20). Subsequently, the international phase III randomized controlled trial, CheckMate 495, was conducted to determine the efficacy of nivolumab versus sorafenib as first-line monotherapy in patients with advanced HCC (21). The mean OS for patients treated with nivolumab was similar to that for a cohort administered with sorafenib, i.e., 16.4 vs. 14.7 months (HR, 0.85; $p = 0.0752$), although the predetermined criterion for significance was not met (HR, 0.84; $p = 0.0419$). When compared with the sorafenib group, the cohort that received nivolumab had a superior ORR, i.e., 7% vs. 15%. A more favorable toxicity profile was obtained using nivolumab, and grade 3–4 AEs associated with therapy were also fewer, i.e., 22% vs. 49% (21).

After the successful demonstration of the safety and anti-tumor activity of pembrolizumab in patients with advanced HCC in the KEYNOTE-224 study (22), a randomized placebo-controlled phase III trial (KEYNOTE-240) assessed the efficacy and safety of pembrolizumab versus best supportive care compared to the placebo in patients with advanced HCC. However, the enhanced OS and progression-free survival (PFS) did not reach the required significance, i.e., 13.9 vs. 10.6 months ($p = 0.0238$) and 3.0 vs. 2.0 months ($p = 0.0022$), respectively, when compared with patients administered placebo in sorafenib progressors (23). Another anti-PD-1

antibody, camrelizumab, was also shown to be effective in patients with unresectable HCC. In a phase 2 study with advanced HCC with prior treatment, camrelizumab administration resulted in a 6-month survival rate and response rate of 74.4% and 14.7%, respectively (24).

The checkpoint molecule CTLA-4 also constrains the immune response by restricting the excessive stimulation of effector T cells (25). The human IgG2 monoclonal antibody tremelimumab binds to CTLA-4, which overrides the B7-CTLA-4-mediated downregulation of the T-cell response in the tumor microenvironment. A phase III trial for tremelimumab in patients with advanced HCC reported an ORR of 17.6%, time to progression (TTP) of 6.48 months, and acceptable AEs (26). Ipilimumab is another anti-CTLA-4 antibody that shows superior anti-cancer activity in combination with other drugs (27). In March 2020, a combination of nivolumab and ipilimumab was approved by the FDA for the treatment of patients with advanced HCC who had earlier received sorafenib, which was based on the results of phase I/II CheckMate-040 cohort 4 data (27).

A phase I/II open-label randomized trial investigating combinatorial treatment using durvalumab (anti-PD-L1 monoclonal antibody) plus single-dose tremelimumab in patients with advanced HCC reported positive results (28). In the study, combined therapy with tremelimumab (high priming, 300 mg) plus durvalumab had the longest median OS of 18.7 months, tremlimumab administration alone had a median OS of 15.1 months, durvalumab alone had a median OS of 13.57 months, and the combination of tremelimumab (75 mg) plus durvalumab had the lowest median OS of 11.30 months (28). In a recent open-label phase 3 trial of tremelimumab plus durvalumab, a regimen termed STRIDE (Single 300mg Tremelimumab Regular Interval Durvalumab) was compared with durvalumab alone or sorafenib, for first-line treatment for unresectable HCC patients, STRIDE resulted in the significant improvement of OS when compared with sorafenib (11). Moreover, in the study, durvalumab monotherapy was not inferior to sorafenib as a first-line treatment for unresectable HCC (11). Overall, a very recently published BCLC treatment guideline for unresectable HCC recommends STRIDE regimen as a first line treatment option (4).

ICI + targeted therapy

Atezolizumab is a humanized IgG1 mAb targeting PD-L1. Bevacizumab, commercially known as Avastin, was one of the first approved angiogenesis inhibitors that were effective in treating breast cancer, non-small-cell lung cancer, cervical cancer, and glioblastoma (29). The pivotal open-label phase III IMbrave150 trial assessed the efficacy of the combined treatment with atezolizumab plus bevacizumab compared to sorafenib therapy as the first-line of treatment in unresectable HCC patients (10). The combination therapy resulted in a significant improvement in the twelve-month OS compared to sorafenib monotherapy (67.2% vs. 54.6%, respectively). Additionally, the median PFS was greater (6.8 months) for the combination therapy relative to sorafenib (4.3 months). The hazard ratio (HR) for disease progression or death was 0.59 (95% CI 0.47–0.76; $p < 0.001$) (10).

Lenvatinib, a multikinase inhibitor used in the first-line treatment for advanced HCC, depletes immunosuppressive TAMs and reverses T cell exhaustion within the tumor microenvironment (30, 31). These actions may maximize the clinical efficacy of PD-1 antibodies in reinstating antitumor responses. Based on this hypothesis, a multicenter, open-label study including 104 patients with advanced HCC treated with a combination of lenvatinib and pembrolizumab was conducted. The study reported a median OS and PFS of 22 and 9.3 months, respectively, and grade 3 or higher AEs were reported in 67% of study participants (32). However, phase 3 LEAP-002 trial investigating pembrolizumab plus lenvatinib versus lenvatinib monotherapy did not meet its dual primary endpoints of OS and PFS as a first-line treatment for patients with unresectable HCC (33).

The phase III COSMIC-312 study reached the primary endpoint, demonstrating a significant improvement in PFS with cabozantinib plus atezolizumab compared to sorafenib in unresectable treatment-naive HCC patients, although a statistically significant benefit was not proved for OS (34). Median PFS was 6·8 months (99% CI 5.6–8.3) in the combination treatment group versus 4·2 months (2.8–7.0) in the sorafenib group (99% CI 0.44–0.91, $p = 0.0012$) (34). The improvement in PFS with cabozantinib plus atezolizumab in this study demonstrates that the combination may benefit patients with unresectable HCC (34). Recently, a trial was designed to assess the efficacy of nivolumab in combination with cabozantinib (group A), or nivolumab plus cabozantinib plus ipilimumab (group B), in 71 patients with advanced HCC randomized into two groups for patients that were treated or untreated with sorafenib (35). The disease control rate for groups A and B were 81% and 83%, respectively, and the median PFS was 5.5 and 6.8 months, respectively. Neither arm achieved the median OS. Grade 3/4 AEs were 42% and 71% in groups A and B, respectively (35).

Blood-based biomarkers for immune-based treatment in advanced HCC

In individuals with HCC, no biomarker has shown reliable accuracy in the prediction of response to ICIs. The extensive variability of HCC lesions in terms of genomic profile and TME raises concerns over the utility of analyses performed on lone

tissue specimens. A key merit of circulating biomarkers is that they can be collected easily and measured following immune-based treatment. Recently, the improved survival and response to therapy with nivolumab has been attributed to a small number of biomarkers due to the facilitation of the evaluation of the expression of PD-L1, favorable alpha-fetoprotein (AFP) responses, inflammatory cytokines, and phenotypes of the peripheral serum mononuclear cells. For ICI + targeted therapy, a recent study also showed that pre-existing immunity (high expression of CD274, T-effector signature, and intratumoral CD8+ T cell density) was associated with better clinical outcomes with atezolizumab + bevacizumab therapy (36). In contrast, reduced responses to the therapy were associated with high regulatory T cell to effector T cell ratio and high GPC3 and AFP expression (36). Currently, efforts to identify blood-based biomarkers are on-going for immune-based combination therapies against advanced HCC. Table 1 shows the currently available blood-based biomarkers in immune-based therapies in HCC.

AFP and PIVKA-II

In general, elevated AFP and protein induced by vitamin K absence or antagonist II (PIVKA-II) titers are negative prognostic factors in individuals with HCC (37). AFP concentrations of <400 μg/L before ICI therapy have been associated with increased frequencies of complete or partial responses as the optimal outcomes (52). Early decreases in serum AFP concentrations have been associated with superior responses to ICI therapy in patients with advanced HCC (38). Another report demonstrated a positive correlation between an AFP reduction > 50% or a PIVKA-II reduction > 50%, and ORR of ICI (anti-PD-1), in HCC (48). In addition, AFP and PIVKA-II

TABLE 1 Blood-based biomarkers for immune-based treatment in advanced HCC.

Marker	Characteristic	Evidence	Ref.
AFP PIVKA-II	Traditional blood tumor markers in HCC	AFP concentrations of < 400 μg/L before ICI therapy is associated with increased frequencies of complete or partial responses	36, 37
		PIVKA-II reductions of > 50% were positively correlated with increased OS	38
sPD-L1	Soluble form of PD-L1 protein	High sPD-L1 levels may serve as a negative independent prognostic factor for DFS and OS	39
		High sPD-L1 levels may serve as a prognostic indicator for poor OS outcomes	40
IL-6	A key player of inflammation and cancer cell survival	Pre-treatment elevation of serum IL-6 is a significant predictor of non-response to atezolizumab plus bevacizumab therapy	41
TGF-β	A key player in TME-related immunosuppression	Pre-treatment serum TGF-β titers of < 200 pg/mL were predictive of prolonged OS and PFS for pembrolizumab	8, 42
CD137 (4-1BB)	Activation-induced costimulatory molecule in CD8+ T cells	Longer PFS was observed in patients with high serum CD137 concentrations when compared with those with low concentrations (median, 14.2 vs 4.1 months, p = 0.001) in anti-PD-1 (sintilimab) + bevacizumab biosimilar therapy	43
ADAM9 mRNA	ADAM9: membrane protein associated with cancer invasion	Responders to nivolumab showed a significant decrease in serum ADAM9 mRNA levels	44
KI-67-positive CD4/8+ T cell	Markers for activated T cells	The percentages of Ki-67-positive CD4+ and CD8+ T cells in peripheral blood were higher in responders than in non-responders after nivolumab treatment	45
CD4+ PD-1+ cell	Markers for exhausted T cells	The frequency of peripheral CD4+PD-1+ cells before therapy with tremelimumab was increased in the responders	46
CXCR3+CD8+ effector memory T cells	Markers for functionally preserved CD8 T cells	Associated with objective responses (p = 0.0004 and 0.0255, respectively), PFS (p = 0.00079 and 0.0015, respectively), and irAEs (p = 0.0034 and 0.0125, respectively) in anti-PD-1-treated patients with HCC	47
NLR/PLR	Negative prognostic factor in individuals with HCC	OS was increased in patients with NLR in the lower tertile compared to patients with medium or high NLR tertiles (p = 0.015) An elevated NLR > 4.125 was associated with hyper-progressive disease after nivolumab therapy and reduced survival rate	17, 48
		PLR > or = 300 had reduced OS (6.4 vs. 16.5 months, p < 0.0001) and PFS (1.8 vs. 3.7 months, p = 0.0006).	49
ctDNA	Provides an effective cancer 'fingerprint' by liquid biopsy	Significant correlation between ctDNA levels and tumor burden in anti-PD1-treated patients with various cancers Significant correlation between ctDNA and tumor burden in HCC patients treated with atezolizumab plus bevacizumab	50, 51

ADAM9, A disintegrin and a metalloprotease 9; AFP, alpha-fetoprotein; ctDNA, circulating tumor DNA; DFS, disease-free survival; HCC, hepatocellular carcinoma; HPD, hyperprogressive disease; HR, hazard ratio; ICI, immune checkpoint inhibitors; irAEs, immune-related adverse events; MMP-9, matrix metalloproteinase 9; NLR, neutrophils to lymphocytes ratio; OS, overall survival; PFS, progression-free survival; PIVKA, protein induced by vitamin K absence or antagonist; PLR, platelet to lymphocyte ratio; sPD-L1, soluble programmed cell death - ligand 1; sPD-1, soluble programmed cell death protein-1; TGF-β, transforming growth factor – beta

reductions of > 50% were positively correlated with increased OS ($p = 0.003$ and 0.006) (48). Nevertheless, data from the CheckMate040 study indicated that even though baseline AFP titers of <400 μg/L reflect prolonged OS compared when compared patients with AFP levels ≥ 400 μg/L, the ORR and DCR for nivolumab therapy remained comparable, irrespective of the APF results (53).

A recent study demonstrated that AFP response at 6 weeks after start of atezolizumab + bevacizumab treatment for advanced HCC is a potential blood biomarker for responses to the therapy (45). The authors derived AFP cutoffs of 75% decrease from baseline at 6 weeks to identify responders (45).

Soluble PD-L1/PD-1

In the CheckMate 040 trial, investigators assessed multiple biomarkers present in the TME to identify putative associations with the higher response rate for nivolumab in patients with advanced HCC (53). Multiple gene expression signatures for inflammation were assessed in fresh and stored tumor samples from both the dose-escalation and -expansion arms of the trial. The results showed that patients who expressed PD-L1 in their tumor samples had survival benefits, and the median OS for patients with detectable (> 1%) expression of PD-L1 was 28.1 months (95% CI 18.2–N/A), whereas that for patients who did not express PD-L1 (< 1% expression) was 16.6 months (95% CI 14.2–20.2, $p = 0.032$) (53). Notably, although PD-L1 appears to be a reliable indicator of tumor response, the study was limited by several factors. Specifically, the use of an unstandardized cut-off of 1% for PD-L1 expression as a marker of positivity, the temporal and spatial heterogeneity of PD-L1 expression, and the highly complex analytic methods required for analysis may prevent the wide application of the results (14, 39). Therefore, as a single biomarker in HCC, the use of PD-L1 expression as a prognostic marker for response to ICI therapy appears restricted and remains unvalidated. Given the absence of a notable heterogeneity of HCC between tumor samples and between individuals, the appraisal of immunogenicity within a tumor may require a range of biomarkers instead of using PD-L1 expression as a single marker.

Recently, Chinese researchers have evaluated the prognostic impact of sPD-L1, which was a negative independent prognostic factor (disease-free survival (DFS), HR = 2.58, 95% CI 1.14–5.84, $p = 0.023$; OS, HR 1.77, 95% CI 1.01–3.12, $p = 0.048$), whereas sPD-1 was a favorable independent prognostic factor (DFS, HR 0.32, 95% CI 0.14–0.74, $p = 0.007$; OS, HR 0.54, 95% CI 0.30–0.98, $p = 0.044$) in patients with HCC (40). Another report showed that high sPD-L1 levels may serve as a potential prognostic indicator for poor OS outcomes in patients with HCC (54). In a recent Korean study with an analysis of 72 patient samples, the median sPD-L1 and sPD-1 levels were 25.72 and 341.44 pg/mL, respectively. Further, the sPD-1 levels in patients treated with nivolumab as a second-line therapy changed serially, and a reduction of >50% in sPD-1 levels was observed immediately after nivolumab administration. However, in that study, sPD-1 levels were not associated directly with prognosis in patients with advanced HCC (55).

Cytokines and other serum markers

Interleukin (IL)-6 is produced by various cell types such as tumor cells, stromal cells, and various immune cells in TME. It usually contributes to tumor progression by causing intra- and peritumoral inflammation and promoting angiogenesis (41). A recent study measured 34 plasma proteins in sera from HCC patients treated with atezolizumab plus bevacizumab and identified that elevated plasma IL-6 was a significant predictor of non-response to atezolizumab plus bevacizumab therapy (42). IL8 is a proinflammatory CXC chemokine for neutrophil chemotaxis (41). IL-8 is elevated in various types of malignancies and contributes to tumor angiogenesis and metastasis. A recent study demonstrated that HBV-induced IL-8 promotes HCC metastasis and intrahepatic regulatory T cell accumulation, suggesting the potential negative role of IL-8 in immune-based therapy in HCC (56).

Transforming growth factor-β (TGF-β) is a key player in TME-related immunosuppression and cancer cell circumvention of immune responses (8). The combined delivery of antibodies inhibiting TGF-β and PD-L1 enabled the permeation of T cells into the tumor center and induced potent anti-cancer immune activity (43). In advanced HCC, a robust link exists between TGF-β and exhausted immune signatures (57). Several pertinent serum indicators were assessed in a phase II study which included 29 individuals with unresectable HCC who had undergone treatment with pembrolizumab. Among the serum biomarkers, pre-treatment serum TGF-β titers of <200 pg/mL were predictive of greater OS and PFS (58). Galunisertib, a TGF-β receptor 1 inhibitor, has been evaluated in clinical studies and the results showed an OS of 16.8 months in individuals with advanced HCC in whom pre-therapy AFP titers were < 1.5 × upper normal limit (44).

A recent study further identified soluble levels of CD137 (4-1BB) as one of the blood-based biomarkers for anti-PD-1 (sintilimab) + bevacizumab biosimilar (46). CD137 is an activation-induced costimulatory molecule and its expression on CD8+ tumor-infiltrating lymphocytes reportedly represented a distinct activation state among highly exhausted CD8+ T cells in HCC (59). In addition, a markedly longer PFS was observed in patients with high CD137 concentrations when compared with those with low concentrations (median, 14.2 vs 4.1 months, P = 0.001) (46).

Matrix metalloproteinase 9 (MMP-9) secreted by TAMs has been recently reported to be a potential predictor of immune characteristics and immunotherapeutic responses in HCC (60).

ADAMs (a disintegrin and metalloprotease) are membrane proteins containing both protease and adhesion domains and thus may be potentially important in cancer invasion and metastasis (61). The ADAM9 mRNA levels in blood samples derived from patients with advanced HCC revealed that among four patients treated with nivolumab therapy, two exhibited a clinical response and showed a significant decrease in serum ADAM9 mRNA levels, whereas the other two patients showed no response to nivolumab and no change in ADAM9 mRNA levels. Although the sample size was small, the results of the present study suggested that ADAM9 mRNA might serve as a predictive biomarker for clinical responses to immunotherapy (61).

Circulating immune cells

Recently, our group reported that the percentages of Ki-67-positive CD4+ and CD8+ T cells in peripheral blood were higher in responders than in non-responders after nivolumab treatment (39). The frequency of CD4+PD-1+ cells within serum mononuclear cells before therapy with tremelimumab was increased in the responders (62). Decreased serum B cell PD-1 expression at baseline and PD-L1 expression in monocytes following therapy was linked with the disease control in 16 individuals with HCC, who received nivolumab (63).

A recently identified single-cell peripheral immune signature provides promising non-invasive biomarkers for the early detection of HCC and early assessment of anti-PD-1 immunotherapy efficacy in patients with advanced HCC (64). Single-cell analyses using cytometry by time of flight (CyTOF) identified CXCR3+CD8+ effector memory T cells and CD11c+ antigen-presenting cells as being associated with objective responses ($p = 0.0004$ and 0.0255, respectively), PFS ($p = 0.00079$ and 0.0015, respectively), and immune-related adverse events (irAEs) ($p = 0.0034$ and 0.0125, respectively) in anti-PD-1-treated patients with HCC. Type-1 conventional dendritic cells were also identified as the specific antigen-presenting cells associated with the immunotherapy response, whereas two immunosuppressive CD14+ myeloid clusters were linked to reduced irAEs. Another recent report analyzed CXCR3+CD8+ effector memory T cells and showed cell–cell interactions specific to the response to immunotherapy vs. irAEs (65). The anti-PD-1 and anti-TNFR2 combination led to uncouple the efficacy of ICI and irAEs of it, resulting in enhanced response without increased irAEs, in a murine HCC model (65).

NLR/PLR

Regardless of the underlying causes associated with HCC, inflammation remains a major factor, especially because the evolution of hepatic fibrosis to neoplasia may rely on several intra-hepatic proinflammatory cascades (14, 66). In general, adverse survival statistics and the cancer progression is frequently associated with systemic inflammation (67). Neutrophils may exhibit phenotypic plasticity and can exist both under tumor-promoting and tumor-suppressing states. Tumor-associated neutrophils (TANs) contributes to tumor progression by mitigating antitumor immunity (49). The proinflammatory cytokine IL-17 recruits TANs to the TME of HCC (49). TANs also have additional immunosuppressive functions by the recruitment of regulatory T cells and TAMs to the HCC TME (68). TANs and peripheral blood neutrophils produce CCL2 and CCL17 chemokines, and these recruit macrophages into the TME of HCC (68). A recent report suggested that neutrophils hamper the efficacy of ICIs, especially in non-alcoholic steatohepatitis (NASH)-induced HCC (69). The neutrophils to lymphocytes ratio (NLR) may serve as indicators of systemic inflammation, and the ratio is increased in individuals with HCC, owing to relative increases and decreases in neutrophils and lymphocytes, respectively (70). The association between an elevated NLR and adverse clinical outcomes in individuals with HCC has been demonstrated consistently for several treatment options for HCC, e.g. resection, transplantation, radiotherapy, and TACE (47, 71–73).

NLR has shown promise as a prognostic factor in individuals with advanced HCC treated with nivolumab. In contrast to patients with an NLR ≥ 5, an NLR < 5 before and after therapy was associated with enhanced OS of 23 vs. 10 months; ($p = 0.004$), and 35 vs. 9 months ($p < 0.0001$), respectively (74). Another study using patient data from the Checkmate 040 trial demonstrated that OS was increased in patients with NLR in the lower tertile compared to patients with medium or high NLR tertiles ($p = 0.015$) (53). Furthermore, a multi-center study conducted in Korea demonstrated that an elevated NLR > 4.125 was associated with HPD after nivolumab therapy and reduced survival rate (17). Additionally, the baseline and treatment kinetics for the NLR are effective prognostic indicators in nivolumab-treated patients with HCC (75). During treatment, the NLR increased rapidly in patients with hyperprogressive disease (HPD) (75). In addition, a recent study showed that patients treated with various ICIs and ICI-based combination therapies with an NLR ≥ 5 had reduced OS (7.7 vs. 17.6 months, $p < 0.0001$), reduced PFS (2.1 vs. 3.8 months, $p = 0.025$), and decreased ORR (12% vs. 22%, $p = 0.034$), suggesting that systemic inflammation indicated by NLR is an independent negative prognostic factor in patients with HCC undergoing ICI therapy (76).

The platelet to lymphocyte ratio (PLR) is an additional potential prognostic ranking method, with an increased value indicating a relative rise in the platelet count and a fall in lymphocyte numbers. The former occurs frequently in individuals with HCC and is indicative of portal hypertension, as observed in a study of individuals with late-stage HCC

receiving ICI therapy (74). Another study showed that patients with PLR > or = 300 had reduced OS (6.4 vs. 16.5 months, $p < 0.0001$) and PFS (1.8 vs. 3.7 months, $p = 0.0006$). In that study, NLR emerged as an independent prognostic factor for OS in univariate and multivariate analysis (HR 1.95, $p < 0.001$; HR 1.73, $p = 0.002$, respectively) and the PLR remained an independent prognostic factor for both OS and PFS in multivariate analysis (HR 1.60, $p = 0.020$; HR 1.99, $p = 0.021$) (76).

The prognostic nutritional index (PNI) is an immune-nutritional indicator and is calculated from the serum albumin (ALB) level and the peripheral blood and lymphocyte (LYM) count (77). A recent study defined the inflammation-immunity-nutrition score (IINS), which was simply based on highly sensitive C-reactive protein (hsCRP), LYM, and ALB (50). HCC Patients with low IINS had longer OS and PFS in response to anti-PD-1, suggesting that IINS may serve as an independent prognostic factor for HCC patients treated with anti-PD-1 therapy (51).

Although these preliminary observations hold promise, the inflammatory condition inexorably oscillates during pathological evolution and advance, and therefore the NLR and PLR will change based on the timing of specimen acquisition (67). Generally, these relationships require verification before their implementation in clinical practice through additional analysis in individuals with HCC.

Circulating tumor DNAs

Recently, liquid biopsy potentially offers a non-invasive tool for monitoring and diagnosis for cancer patients. The application of the method has been encouraging for early diagnosis, determination of residual disease, and decision-making to facilitate systemic treatment for HCC (78). Regarding the choice of liquid biopsy analytes that may be used in HCC, circulating tumor DNA (ctDNA) has made an invaluable contribution to the prognosis and monitoring of disease (79). Cell-free DNA (cfDNA) contains only a small proportion of ctDNA, which demands the application of highly sensitive and reliable methods of detection. Point mutations are determined by either droplet digital PCR (ddPCR) or by NGS-based sequencing (79). Subsequent to tumor cell apoptosis and necrosis, ctDNA can be found in the blood stream of cancer patients (80) and provides an effective cancer 'fingerprint' as it has the tumor's molecular characteristics. ctDNA can be differentiated from normal circulating DNA using various markers, including mutation signatures (80, 81) and epigenetic alterations (82). The variation in the concentration of ctDNA in the blood provides a quantitative measure, whereas assessment of gene mutations, changes in DNA copy number, and methylation profiles provide a qualitative assessment of the disease condition. In some situations, ctDNA analysis may help identify mutations that were not obtained from a single tumor biopsy (83). Both genomic and epigenetic biomarker modifications in HCC are important factors to consider in recurrence monitoring and precision oncology (84). The results highlight the value of incorporating ctDNA analysis for the diagnosis and prognosis of patients with HCC. Our recent study using targeted NGS technique identified at least one pathogenic variant of two major HCC driver genes (TP53 and CTNNB1), including 16 variants of TP53 and nine variants of CTNNB1 in 65% HCC patients (13/20) (81).

To relate ctDNA to immunotherapy for cancers, a recent study demonstrated that significant correlation between ctDNA levels and tumor burden in anti-PD1-treated patients with various cancers (85). From the patients enrolled in the phase 1b clinical trial of atezolizumab plus bevacizumab, researchers were able to identify a statistically significant correlation between ctDNA and tumor burden ($p < 0.03$). Of this sub-cohort, patients who achieved a complete response, partial response, stable disease state, and disease progression, ctDNA was undetectable in 70%, 27%, 9% and 0% of these groups, respectively. Furthermore, in cases where ctDNA was not detectable during their treatment period, the patients thereafter experienced a longer PFS (86). Another very recent study using patient cohort treated with camrelizumab plus apatinib demonstrated that patients who were ctDNA positive after adjuvant therapy presented a trend of shorter RFS than those who were ctDNA negative (87). The role of methylated ctDNA in predicting immunotherapy responses is studied in other cancer types such as colorectal cancer (CRC). For example, in four case studies of methylated SEPTIN9 gene (mSEPT9) as a marker of response to immunotherapy in metastatic CRC on four patients, the marker shows that a decrease in ctDNA levels is indicative of a tumor response to immunotherapy while an increase in ctDNA levels corresponds to tumor progression in response to immunotherapy (88, 89). A similar study that focuses on HCC, however, has yet to be found. Another study that looked at predicting immunotherapy responses in HCC but with a methylated RNA molecule (SNRPC) instead, showed a similar pattern in that high-SNRPC groups showed no response to anti-PD1 therapy while low-SNRPC groups showed more patients responding to immune checkpoint inhibitor therapy (90).

TMB, an emergent determinant of immunotherapy responses, is a measure of the total number of somatic non-synonymous mutations per mega-base in the tumor, and its levels between ctDNA and tumor tissues were found to be consistent. Consequently, analysis of blood samples for TMB prior to immunotherapy in advanced primary liver cancer can be beneficial in predicting the responses (91). However, a recent

study using 121 HCC blood samples found that mutational analysis ctDNA was associated with a response to systemic TKI treatment, not with ICI treatment (80).

Limitations and future perspectives

Currently, blood-based biomarkers are newly identified and validated in immune-based treatments for the various cancer types, although the researches have been mostly focusing on immunogenic tumors such as malignant melanoma or lung cancer for which ICIs are being most actively used. Hopefully, soon, blood-based biomarkers including ctDNA may be approved for predicting of responses to ICIs in these types of immunogenic cancers. However, because each type of cancer has its own immune TME and cytokine milieu, a more complex blood-based biomarker discovery may be needed for other types of heterogeneous tumors. Although several studies have been conducted to identify predictive biomarkers to enable the stratification of patients who could benefit from ICI treatment in HCC, few have been prospectively validated and none have resulted in the rewriting of current clinical guidelines or entered clinical practice. Here, we have summarized the progress of immunotherapies for HCC in recent years, with a particular emphasis on predictive biomarkers. However, as immunotherapy development for HCC is still in its infancy, basic research and clinical trials exploring the predictive efficacy of immunotherapeutic biomarkers are still limited, and it is not yet possible to determine which biomarker(s) can effectively predict the efficacy of immunotherapy. Although evaluation of human tissues using various technologies is now routine for the derivation of biomarkers, the utility of straightforward instruments to obtain prognostic data, and which are easily accessible from general blood samples should not be discounted, as they may represent a simpler and more widely available option in daily clinical settings. To address the issue of low concentrations of biomarkers in blood, platforms such as the SomaScan, are able to measure ~7000 proteins in a drop of serum simultaneously, leading the way for novel protein biomarker discoveries (92). One more thing to consider is irAEs. A recent report demonstrated that development of low-grade irAEs was associated with favorable responses for HCC patients treated with ICIs (93). Because there are no reliable biomarkers for irAEs in HCC either, clinicians should be vigilant for detection of irAEs when treating HCC patients with ICIs or combination therapy.

Regardless of the potential value of using ctDNA as a biomarker for diagnosis and treatment responses, there are several limitations associated with its current use. Specifically, in the early stages of tumorigenesis, the levels of ctDNA in the blood are extremely low, which can hinder the early diagnosis of HCC. Further, there is currently no standardized methodology associated with sample collection, preparation, and data analysis. The current method cannot adequately capture spatial tumor heterogeneity, which is indicative of clonal differences within or across tumor metastases (94–96). To resolve these issues, a combinatorial and/or multiparametric process is required to enhance the sensitivity and specificity of using ctDNA as a putative HCC biomarker. The use of blood-based biomarkers including ctDNA to determine the efficacy of immunotherapy against HCC necessitates additional, well-controlled clinical trials, so that the value and clinical relevance of such research endeavors can be realized.

Conclusion

Recent advances in research techniques, such as NGS, scRNA sequencing, and artificial intelligence, should facilitate a more comprehensive understanding of the various components of the TME and their interactions in HCC. Moreover, recent unsatisfactory out-comes in immune-based treatment in advanced HCC urges clinicians to identify blood-based biomarkers for favorable responses to such treatments. Future research should focus on the identification of blood-based protein and cell-free nucleic acid biomarkers for immune-based therapy in HCC.

Author contributions

Conceptualization, PS and JA; methodology, PS; data curation, PS; writing—original draft preparation, IL, PR, and MK; writing—review and editing, PS and JA; supervision, JA and SY; project administration, PS, JA and SY; funding acquisition, PS and JA; All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review of the endocrine organ–like tumor hypothesis of cancer cachexia in pancreatic ductal adenocarcinoma

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Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal types of solid tumors, associated with a high prevalence of cachexia (~80%). PDAC-derived cachexia (PDAC-CC) is a systemic disease involving the complex interplay between the tumor and multiple organs. The endocrine organ–like tumor (EOLT) hypothesis may explain the systemic crosstalk underlying the deleterious homeostatic shifts that occur in PDAC-CC. Several studies have reported a markedly heterogeneous collection of cachectic mediators, signaling mechanisms, and metabolic pathways, including exocrine pancreatic insufficiency, hormonal disturbance, pro-inflammatory cytokine storm, digestive and tumor-derived factors, and PDAC progression. The complexities of PDAC-CC necessitate a careful review of recent literature summarizing cachectic mediators, corresponding metabolic functions, and the collateral impacts on wasting organs. The EOLT hypothesis suggests that metabolites, genetic instability, and epigenetic changes (microRNAs) are involved in cachexia development. Both tumors and host tissues can secrete multiple cachectic factors (beyond only inflammatory mediators). Some regulatory molecules, metabolites, and microRNAs are tissue-specific, resulting in insufficient energy production to support tumor/cachexia development. Due to these complexities, changes in a single factor can trigger bi-directional feedback circuits that exacerbate PDAC and result in the development of irreversible cachexia. We provide an integrated review based on 267 papers and 20 clinical trials from PubMed and ClinicalTrials.gov database proposed under the EOLT hypothesis that may provide a fundamental understanding of cachexia development and response to current treatments.

KEYWORDS

pancreatic ductal adenocarcinoma (PDAC), cachexia, muscle wasting, tissue wasting, endocrine organ-like tumour (EOLT)

1 Introduction

For Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth most common cause of cancer-related deaths worldwide and is projected to become the second most common cause of cancer-related deaths by 2030 (1). Due to its aggressiveness and poor prognosis, mortality remains alarmingly high among patients diagnosed with PDAC. Approximately 80%–85% of PDAC patients are diagnosed at advanced stages with unresectable or metastatic tumors, resulting in a 5-year survival rate below 10% (2). During early-stage PDAC, surgical resection is currently the only curative option, although chemotherapy and radiation therapy are also used as primary treatment options, with or without surgery. However, single-agent chemotherapies are rarely effective in PDAC (3). In general, chemotherapy regimens are not universally effective in PDAC and are associated with significant adverse effects, including the development of PDAC-derived cachexia (PDAC-CC), and cachexia occurs in 32%~71% of patients within 12 to 48 weeks of chemotherapy initiation (4).

Patients with PDAC experience a high prevalence (up to 80%) of cachexia, often with early onset (45% of PDAC patients present with cachexia at the time of diagnosis (5)), which may account for up to 30% of mortality (6). Cachexia is defined as the progressive loss of muscle mass and function (6, 7) and is a catabolic multi-organ syndrome characterized by non-volitional weight loss (muscle or adipocyte loss), adipopenia, fatigue, weakness, loss of appetite, and early satiety (8, 9). When muscle mass loss, it enhances chemo-toxicities and insensitivities, contributing to poor overall survival (10).

In general, tumors demand a high energy supply and can promote the wasting of peripheral tissues *via* hyper-catabolism. Tumors compete with other organs/tissues for energy and nutrients, resulting in elevated resting energy expenditure and inducing a negative energy balance. Energy utilization in tumors also results in increased proteolysis and lipolysis combined with decreased lipogenesis and protein synthesis (8, 9, 11, 12). These metabolic reprogramming effects, combined with poor appetite, lead to rapid weight loss among PDAC patients and can contribute to deterioration in the overall quality of life (QoL) and overall survival (OS) (7, 13–15). The complex, multifactorial nature of the metabolic disruptions in cachexia makes effective treatment challenging. The current lack of consensus regarding how to define cachexia and a scarcity of strong evidence produced by robust, rigorous, and mechanistic studies have limited the development of effective treatments (16). In addition, most cachexia studies focus on symptoms associated with individual organs (such as tumor, muscle, or adipocyte tissues) without considering consider systemic interactions. In this review, we provide an up-to-date overview of current cachexia research in PDAC to provide insight regarding the cachexia mediators that act in different organs and explore whether the endocrine organ–like tumor (EOLT) hypothesis of PDAC can explain the development of systemic complications.

2 Cachexia criteria and stages

Cancer-derived cachexia (CC) is a multifactorial syndrome involving various metabolic changes in several tissues and organs (8, 9, 12, 17–19). Although patients with pancreatic cancer show a wide range of nutritional alterations, the primary symptom is progressive weight loss due to the loss of skeletal muscle mass, with or without the accompanying depletion of adipose tissue (6, 19–23). Other PDAC-CC-related clinical manifestations include inflammation (24–26), anorexia (27, 28) and metabolic reprogramming (9, 29, 30) etc.,

Numerous studies also focus on exploring new PDAC-CC cachectic mediators, corresponding metabolic functions, and the collateral impacts on wasting organs. A systematic review also suggested a network of cytokines (interleukin [IL]-6, tumor necrosis factor-alpha [TNFα], and IL-8) that may be associated with cachexia development (31). Sah et al. (19) suggested that PDAC-CC can be categorized by three distinct metabolic phases: Phase 1 represents the earliest metabolic change, characterized by new-onset hyperglycemia; Phase 2 is associated with a greater than 5% reduction in body weight with pre-cachectic symptoms (appetite loss and impaired glucose metabolism), suggesting the initiation of cachexia; and Phase 3 is associated with dramatic reductions in all monitored metabolites, lipids, subcutaneous fat, and muscle, except fasting glucose.

Traditionally, a Body Mass Index (BMI) < 18.5 kg/m^2 was accepted as a marker of being cachectic. However, sarcopenic obesity can be observed in CC, suggesting that weight loss might not be a defining factor (32). According to the most common consensus, published by Fearon et al. (33), the current standard diagnostic criterion for cachexia is represented by percentage of weight loss, BMI values and metabolic changes (29, 33, 34). Simply, CC were classified into three stages: pre-cachexia, cachexia, and refractory cachexia (Table 1). This classification currently did not fully applicable in clinics but is rather to be considered as a proposal under evaluation. Additional parameters (Table 2) have been developed to improve diagnosis, such as food intake measures, albumin levels, anorexia assessment, markers of systemic inflammation (CRP >10 mg/L), muscle mass measurements, the Skeletal Muscle Index (SMI), bioelectrical impedance analysis (BIA), the Fat-Free Mass Index and cachexia index (CXI). Although these diagnostic measurements did not include in the latest consensus, they suggested that several effective parameters could more accurately identify cachexia. A recent systematic review by Paval et al. described the between-study inconsistencies in grouping criteria as a major hindrance to the conduct of meta-analyses for cachexia (31). Refined CC-

TABLE 1 Cachexia criteria/definition.

Score system	Criteria	Ref
BMI	BMI <18.5 kg/m^2	(35)
Body Weight	-Weight loss ≥10%; -Presence of at least 1 symptom: anorexia, fatigue, or early satiation	(36)
EPCRC	• **Pre-cachexia**: Weight loss ≤5%, anorexia, metabolic changes • **Cachexia:** Weight loss >5% over past 6 months , or BMI < 20kg/m^2 and weight loss >2% , or sarcopenia and weight loss >2% (Skeletal muscle index: males <7.26 kg/m^2; females <5.45 kg/m^2) • **Refractory Cachexia:** Variable degree of cachexia cause poor survival and not responsive to anticancer treatment.	(37, 38)
Glasgow Prognostic Score	CRP >10 mg/L	(39)
Cancer Cachexia Study Group (CCSG)	Multifactorial syndrome: Weight loss, reduce food intake, systematic inflammation CRP >10 mg/L, weight loss >10%, energy intake <1500 kcal/day	(37)
Cachexia Score (CASCO)	Body weight and lean body mass loss; anorexia; inflammatory, immunological and metabolic disturbances; physical performance and QoL.	(40)
Cachexia definition	A complex metabolic syndrome associated with underlying illness and characterized by loss of muscle with or without loss of fat mass, including weight loss (>5%), decreased muscle strength, reduced muscle mass, anorexia, symptoms of fatigue, or biochemical abnormalities (anemia, inflammation CRP >5mg/L, or low albumin).	(41)
Cachexia staging score (CSS)	Defined by 5 components: Weight loss in 6 months, appetite loss, SARC-F questionnaire assessing muscle function and sarcopenia, ECOG performance status, abnormal biochemistry	(42)
Cachexia index (CXI)	Defined by reduced muscle mass (SMI: skeletal muscle index), poor nutritional status (Alb: serum albumin g/dL), and systemic inflammation (NLR: neutrophil-to-lymphocyte ratio). $\text{CXI} = \frac{\text{SMI} \times \text{Alb}}{\text{NLR}}$	(43, 44)

BMI, body mass index; CRP, C-reactive protein; EPCRC, European Palliative Care Research collaborative; CASCO, Cachexia Score.

criteria is critical for evaluating the response to cachexia/antitumor therapy. Because early-onset PDAC-CC can present concomitant with the detection of the primary tumor burden, but cachexia can continue even after the tumors have been surgically removed or effectively treated (15). Patients received either preoperative surgery or chemotherapy/chemoradiation; unintended weight loss coupled with muscle wasting can often be observed, contributing to poor outcomes in PDAC (10, 13, 15, 29). There is no effective strategy to mitigate refractory PDAC-CC. Therefore, the early and precise identification of PDAC-CC is needed to estimate prognosis and prevent progression to the refractory cachexia. More practical, longitudinal definitions of cachexia remain necessary that consider all aspects of the cachexia phenotype.

3 EOLT hypothesis in PDAC-CC

The EOLT hypothesis was proposed to explain how tumor tissues drive disease progression, including CC (31). The EOLT hypothesis states that the tumor acts as an endocrine organ, resulting in dynamic bi-directional communications between the tumor microenvironment (TME) and various organs, leading to the regulation of macroenvironmental changes.

PDAC-CC results in systemic wasting and involves multiple organ dysfunction (Figure 1), accompanied by symptoms including poor appetite, fatigue, depression, muscle wasting, fat wasting, malabsorption, and constipation (Table 3). Tumors secrete cachexia-inducing factors and stimulate host–tumor interactions involve cancer-organ metabolic reprogramming and interorgan signal crosstalk in tumor progression and cachexia development (21, 32). For example, tumor-derived cytokines induce systemic inflammation, stimulating the release of neuropeptides that lead to poor appetite, and the resulting anorexia exacerbates tissue wasting (6, 14, 26). With cachectic environment, adipose and muscle tissues can act like paracrine/endocrine organs in response to cachectic factors, providing reciprocal regulation of energy expenditure and cachexia process (8, 9, 17, 33). Cachexia is a wasting disease that represents metabolic disruptions, mainly catabolisms, driven by systemic inflammation and is characterized by skeletal-muscle proteolysis, adipose tissue lipolysis and hepatic

TABLE 2 Cachexia assessment.

Assessment	Method	Ref
Food intake	PG-SGA-SF: Patient-Generated Subjective Global Assessment Short-Form Ingesta score MNA-SF: Mini Nutritional Assessment Short-Form NIS: Nutritional impact symptoms EORTC QLQ-CAX24 Questionnaire	(35, 45–48)
Anorexia	FAACT: Functional assessment of anorexia/cachexia treatment	(49)
Inflammation	modified Glasgow prognostic score	(50)
Body Weight	-Weight loss ≥10% -Presence of at least 1 symptom: anorexia, fatigue, or early satiation Weight Loss Grading System (WLGS 0, 1, 2, 3, or 4)	(36, 51)
Muscle mass	Muscle mass: mid-upper arm muscle area (men <32 cm^2; women <18 cm^2)	(52, 53)
Skeletal Muscle Index	Computed tomography (men <36.54–45.40 cm^2/m^2; women <30.21–36.05 cm^2/m^2)	(54)
Body composition (body fat and muscle mass)	Dual-energy X-ray absorptiometry (men <5.86–7.40 kg/m^2; women <4.42–5.67 kg/m^2) Bioelectrical impedance analysis (men <6.75–7.40 kg/m^2; women <5.07–5.80 kg/m^2) Fat-Free Mass Index	(53, 55, 56)
Fatigue	Single Item Fatigue (SIF)	(57)
Malnutrition assessment	PINI: Prognostic Inflammation Nutrition Index CRP (mg/L) × α1-acid glycoprotein]/[albumin (g/L) × transthyretin (g/L)] NRI: Nutritional Risk Index NRI = 1.519× albumin (g/L) + 0.417× (current weight/usual weight ×100). Criteria: >100: no malnourishment 97.5-100: mild malnourishment 83.5-97.4: modern malnourishment < 83.5: severe malnourishment. low albumin (<35 g/L); CRP (>5 or >10 mg/L) transthyretin (prealbumin): low transthyretin (variously <110 or <180mg/L)	(58, 59)
Energy expenditure	Harris Benedict formula: Men: BMR = 66.5 + (13.76 × weight in kg) + (5.003 × height in cm) – (6.755 × age) Women: BMR = 655.1 + (9.563 × weight in kg) + (1.850 × height in cm) – (4.676 × age)	(60)

gluconeogenesis (Figure 1) (20, 22, 24, 34). These inter-organ interactions affect metabolisms in the formation of feedback loops. Thus, PDAC-CC can be characterized by two interacting dimensions:

1. Systemic metabolic changes, often associated with *KRAS* mutations (genetic instability).
2. Pro-cachectic mediators and microRNAs (miRNAs) exacerbated in metabolic disruptions.

The high prevalence of cachexia in PDAC is associated with distinct metabolic effects mediated by tumor created environments, including *KRAS* mutations, pro-cachexia mediators, and alteration in pancreas and liver. The present review summarizes the current understanding of PDAC-CC according to the EOLT hypothesis.

3.1 Metabolic alterations and high energy demands in tumors

PDACs are characterized by high energy demands within a nutrient-deprived microenvironment. Aggressive PDAC is characterized by increased glycolysis and glutamine metabolism, closely associated with downstream anabolic pathways in the tumor's hypoxic desmoplastic environment (8, 9, 27, 29, 63). The deprivation of glucose and glutamine and lactic acidosis promote glycolytic and glutaminolysis activity (61, 82, 83). Metabolic alterations are hallmarks of PDAC and PDAC-CC, particularly the dysregulation of glucose and glutamine metabolism (8, 9, 14, 19, 29, 62). However, PDACs under different oxygen and nutrition conditions show distinct and heterogeneous metabolite profiles associated with aerobic glycolysis (the Warburg effect), OXPHOS (oxidative phosphorylation; also known as the reverse Warburg

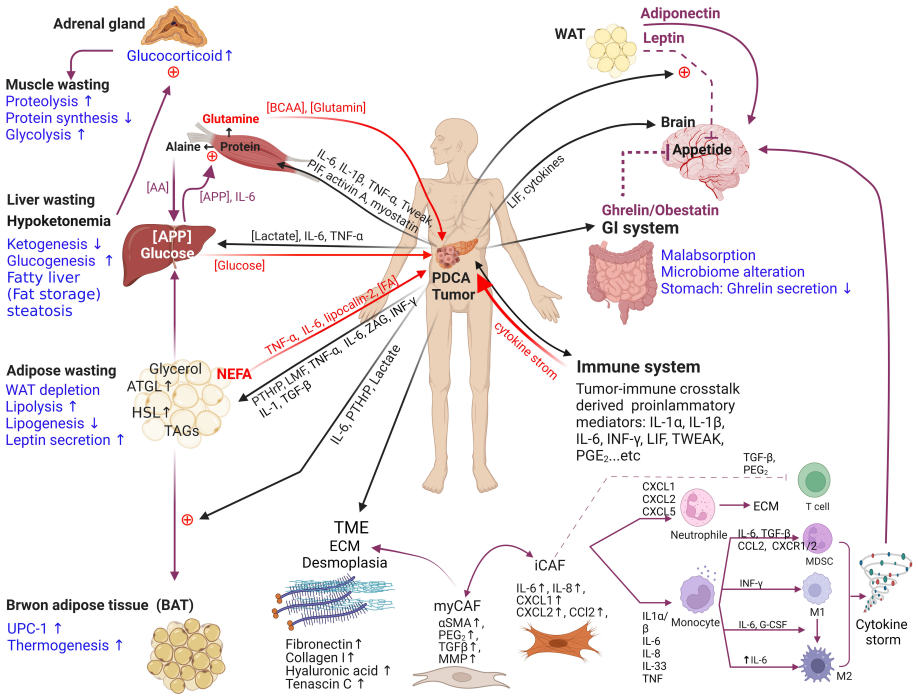


FIGURE 1
The endocrine organ–like tumor (EOLT) hypothesis for multifactorial cachexia syndrome. EOLT states that solid tumors create multiple endocrine/paracrine organs which differs from the "seed and soil" hypothesis. Tumor-organ crosstalk and interorgan signal crosstalk did not rely on reshaped tumors only. Mostly influenced by different cachectic regulators, such as tumor-derived factors, pro-inflammatory immune mediators (ie. IL-6, IL-1α, IL-1β, TNFα, IFN-γ, ZAG, PIF, activin A, LIF, TWEAK, PGE_2), and hormones (including glucocorticoids and PTHrP). These cachectic mediators act as paracrine/autocrine manners, trigger positive feedback to other organs and form a bidirectional circuit (black arrow means mediators derived from tumor; red arrow means mediators derived from peripheral tissues/organs; purple arrow means influence between peripheral tissues/organs). When the communication between tumor and organs exists, metabolic reprogramming (mark in blue: glycolysis↑, proteolysis↑, lipolysis↑ and gluconeogenesis↑) produces bidirectional positive feedback to other organs in cachexia. For example, inflammatory cytokines increase lipolysis in white adipose tissue (WAT), releasing free fatty acids (FAs) that further fuel tumor growth and promote muscle wasting (18–21, 61, 62). Adipocyte also can secrete adipokines (e.g., leptin, adiponectin, and lipocalin-2), IL-6, and TNFα which release *via* extracellular vesicles (EVs) into the circulation to influence the TME or mediate the appetite (61, 63, 64). Similarly, muscle wasting regulates by hormones, adipocyte-derived mediators and tumor-derived factors (65). Cachexia is a wasting disease that represents metabolic disruptions driven by systemic inflammation and is characterized by the depletion of adipose tissue and skeletal muscle Interleukin, IL; tumor necrosis factor-alpha, TNFα; interferon-gamma, IFN-γ; zinc alpha 2-glycoprotein, ZAG; proteolysis-inducing factor, PIF; leukemia inhibitory factor, LIF; TNF-related weak inducer of apoptosis, TWEAK; prostaglandin E_2, PGE_2; tumor-derived parathyroid hormone–related protein, PTHrP; amino acid, AA; acute phase protein, APP; triglycerides, TAG; brown adipose tissue, BAT; white adipose tissue, WAT; uncoupling protein 1, UCP1; extracellular matrix, ECM; branched-chain amino acids, BCAAs; chemokine (C-X-C motif) ligand, CXCL; matrix metalloproteinases, MMPs; α-smooth muscle, α-SMA; tumor microenvironment, TME.

effect), lipid dependence, autophagy, and glutaminolysis (Figure 2). Metabolic alterations are positively correlated with high-grade pancreatic intraepithelial neoplasia (PanIN-3). However, early-onset cachexia also develops independent of PDAC, occasionally presenting in the pre-diagnostic PDAC stage (4). Cachexia is a metabolic disorder involving several nutrient scavenging pathways, including autophagy, micropinocytosis, glycolysis, lipid oxidation, and micropinocytosis (Figure 2**: upper panel**).

PDAC survives and thrives in relatively hypoxic and nutrient-poor niches, driven by [1] reprogramming intracellular nutrient metabolism, including glucose, amino acids, and lipids; [2] scavenging and recycling nutrients; and [3] promoting metabolic crosstalk (Figures 2**: lower panel** and Figure 3) (8, 9, 62). PDAC-CC exacerbates metabolic reprogramming, promoting the deterioration of muscle and adipose tissue (Figure 3), further supporting the energy and nutrient needs of the tumor tissue.

3.1.1 Glucose

Glycolytic flux can result in changes in the pentose phosphate pathway (PPP), the hexosamine biosynthesis pathway (HBP), serine biosynthesis, and the tricarboxylic acid (TCA) cycle, promoting CC development (64). Rate-limiting glycolytic enzymes, such as hexokinase 1/2 (HK1/2), phosphofructokinase 1, and lactate dehydrogenase A (LDHA), are upregulated to facilitate the Warburg effect, resulting in glycolytic flux and the production of lactate from glucose in PDAC-CC (63, 64). The upregulation and translocation of

TABLE 3 The multi-organ response in PDAC-derived cachexia.

Organ	Tissue alterations	Main implications
Brain	• Alterations in appetite • Alterations in taste and smell	• Anorexia • Negative energy balance
Gut	• Changes in microbiota • Altered ghrelin production • Gut barrier dysfunction	• Malabsorption
Liver	• Production of acute phase proteins • Decreased albumin production • Increase gluconeogenesis (increase Cori cycle)	• Acute phase response •Negative energy balance
Skeletal muscle	• Increased proteolysis • Increased glycolysis • Decreased protein synthesis	• Wasting • Atrophy, sarcopenia • Fatigue • Decreased physical performance
White adipose tissue (WAT)	• Activation of thermogenesis • Increased lipolysis • Increased leptin secretion • Release of fatty acids • 'Browning'	• WAT depletion • Decreased food intake and body weight
Brown adipose tissue (BAT)	• Activation of thermogenesis	• Energy expenditure
Pancreatic insufficiency	• Endocrine dysfunction • Pancreatic exocrine insufficiency (PEI)	• Low insulin production • Malabsorption

PDAC, pancreatic ductal adenocarcinoma.
Citation reference (6, 8, 9, 14, 25–27, 66–81).

glucose transporters (GLUT1, encoded by *SLC21A*) in tumor tissues facilitate glucose uptake for aerobic glycolysis. Increased glycolytic flux in response to host–tumor interactions in cachexia results in the production of high lactate levels, leading to lactic acidosis. To address lactic acidosis, PDACs robustly express monocarboxylate transporters (MCT1 and MCT4, encoded by *SLC16A1* and *SLC16A3*, respectively) to coordinate glucose utilization and lactate mobilization (65, 84). Other glucose metabolism pathways are also altered in PDAC, such as the upregulation of the rate-limiting enzyme of the HBP, glutamine: fructose-6-phosphate amidotransferase-1 (GFPT1) (64). Many other mediators regulate glucose metabolism in pancreatic cancer cells. Under hypoxic conditions, hypoxia-inducible factor-1 (HIF-1) can promote glycolysis and upregulate the expression of HBP-related enzymes, such as GFPT2, an isoform of the HBP rate-limiting enzyme GFPT1 (63).

In a model of PDAC-CC, athymic mice injected with high-glycolytic MiaPaCa2 cells showed evidence of cachexia, such as weight loss, fat depletion, and muscle proteolysis (66), suggesting that glycolysis may be involved in PDAC-CC development. Glycolysis was associated with inefficient inter-organ substrate shuttles, as assessed by the lactate-to-pyruvate utilization ratio, LDH activity, and MCT1 expression, which was correlated with cachexia-related weight loss (63). The upregulation of GLUT1 and MCT1/4 promotes glucose utilization and improves the lactate-to-pyruvate utilization ratio in tumor tissue (63, 65). Paradoxically, OXPHOS, also known as the reverse Warburg effect, occurs in muscle, resulting in an increased lactate-to-pyruvate production ratio, providing a potential lactate supply for tumor use and supporting tumor progression and consequent atrophy (85). Additionally, the tumor secretes interferon-gamma (IFN-γ), which mediates the development of insulin resistance *via* reduced glucose and fatty acid [FA] uptake, leading to enhanced lipolysis in WAT (86). Inefficient inter-organ substrate shuttles are regarded as hallmarks of EOLT (Figure 3).

Aerobic glycolysis occurs more commonly in tumor tissues than OXPHOS, which requires a sufficient oxygen supply, although OXPHOS is more efficient for ATP generation (30, 87). Nutrient depletion forces tumors to adapt by inducing nutrient scavenging mechanisms to support cancer progression, which can lead to CC (8, 9, 88). OXPHOS occurs in the mitochondria and is sensitive to stress conditions, as the respiratory complexes in stressed mitochondria produce high levels of reactive oxygen species (ROS) (62). Autophagy is a stress response induced by ROS to remove damaged mitochondria that overproduce ROS, promoting mitochondrial metabolism (89) (Figure 2**: lower panel**). In cachectic patients, increased OXPHOS and dysfunctional autophagy are associated with increased muscle wasting (Figure 3) (90). Autophagy is an important proteolysis pathway activated during PDAC-CC and muscle wasting (91, 92).

Tumors supported by an adequate blood supply can perform aerobic metabolism and tend to exhibit the reverse Warburg effect. In tumors, aerobic metabolisms may utilize intermediates, such as lactate, to fuel the TCA cycle (Figure 2**: lower panel),** decreasing their dependence on glucose. The low uptake of

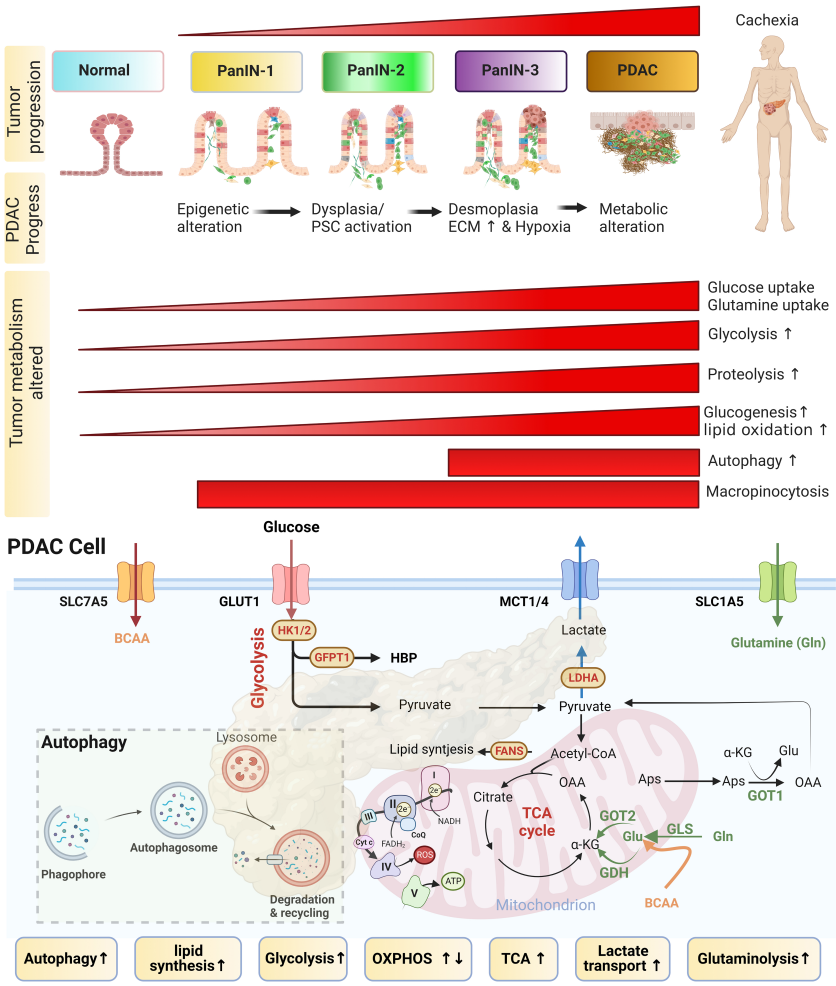


FIGURE 2
Metabolism alterations in PDAC-CC. The upper panel shows that pancreatic ductal adenocarcinoma (PDAC)-derived cachexia (PDAC-CC) arises from the multi-stage progression of precursor lesions, known as pancreatic intraepithelial neoplasia (PanIN). PanINs are characterized by a continuum of increasingly stroma features (from low-grade dysplasia developing to high-grade desmoplasia). A desmoplastic response induced a fibro-inflammatory microenvironment, stimulating an aberrant metabolic response that is associated with cachexia. During early-stage PDAC, histology can be used to identify several distinct types of precursor lesions. The most common types are microscopic PanIN, low-grade (PanIN-1 and PanIN-2), and high-grade (PanIN-3). The progression to PanIN and to PDAC is associated with cachexia development. Multiple metabolic alterations follow the progression of cachexia, resulting in the reprogramming of glucose, amino acid, and lipid pathways. Metabolic alterations include nutrient scavenging pathway), such as glycolysis glutaminolysis, autophagy, proteolysis, lipid oxidation, and micropinocytosis (Most of them were upregulated during the development of cachexia). However, early-onset cachexia can arise independent of the PDAC stage, occurring in the pre-diagnostic PDAC stage. More than one-third of cancer patients were malnourished before chemotherapy, implying that the cachexia occurred early and followed a poor response to chemotherapy. Interestingly, some of cachexia occurs after the chemotherapy. **The lower panel:** The metabolic alterations, including increase glycolysis, glutaminolysis, lactate transport and autophagy ... etc, in PDAC cell associated with PDAC-CC, primarily due to promote the expression in key enzymes (HK1/2, GFPT1, and LDHA) and transporters (GLUT1, MCT1/4, SLC7A5, and SLC1A5). The metabolic shift from the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) to aerobic glycolysis is tightly regulated. HK1/2, hexokinase; GFPT1, glutamine fructose 6-phosphate amidotransferase 1; LDHA, lactate dehydrogenase A; GLUT1, Glucose transporter 1; MCT1/4, monocarboxylate transporter 1/4; SLC7A5 (LAT1), neutral amino acid antiporter; SLC1A5, glutamine transporter.

glucose and the enhanced uptake of intermediate metabolites by tumors under aerobic conditions could protect these tumors from competing with hypoxic tumor regions (such as desmoplastic tumors) for glucose. In addition to OXPHOS, tumors able to perform aerobic metabolism can also utilize glutaminolysis as an alternative energy production pathway requiring activated mitochondrial metabolism (93). Glutamine is the most abundant and versatile nonessential amino acid (NEAA), found in both the blood and the cell cytoplasm, and can be used by both the glutamine-dependent pyruvate cycle and the TCA cycle (30, 94). In contrast, hypovascularization and desmoplasia often occur in PDAC; studies also found that

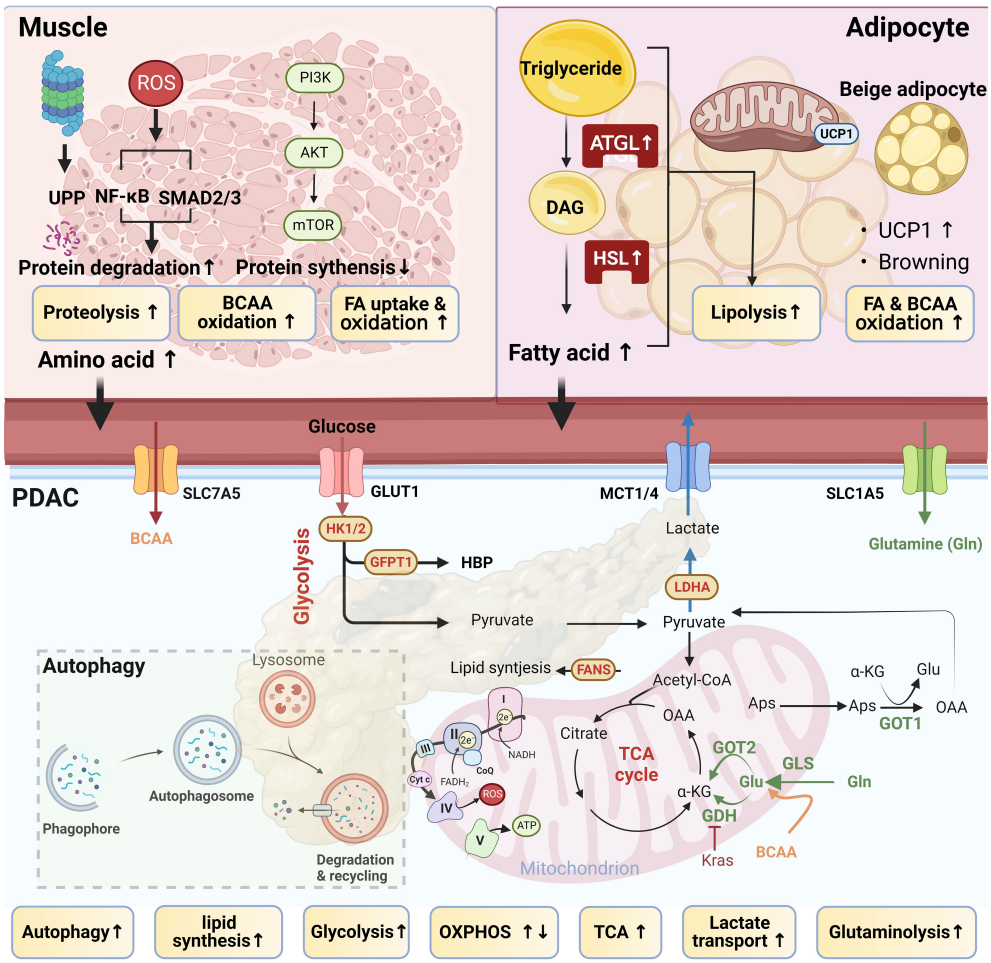


FIGURE 3
Tumor–muscle–adipocyte crosstalk. Pancreatic ductal adenocarcinoma (PDAC)-derived cachexia occurs due to a feedback circuit among tumor, adipocyte, and muscle tissues. In PDAC, tumor-derived factors, including interleukin (IL)-1, IL-6, IL-8, proteolysis-inducing factor (PIF), lipid mobilization factor (LMF), and tumor necrosis factor-α (TNFα), enhance proteolysis, lipolysis, and the catabolic state of muscle and adipocytes, leading to adipose and muscle wasting. Tumor cell–triggered metabolic reprogramming in muscle and adipocytes releases metabolic products, such as branched-chain amino acids (BCAA) and free fatty acids (FFAs), to fuel tumor growth. Lipolysis and proteolysis are the two main processes causing adipose and muscle wasting in cachexia.

HIF-1α (hypoxia-inducible factor-1α) stabilization promotes glycolytic enzymes to shift the metabolism by repressing OXPHOS (95, 96). Supposing that if the function of glycolysis is weakened, OXPHOS and glutamine-based processes will serve as alternative energy generation mechanisms in glucose-limited tumors (87) (Figure 2**: lower panel**).

3.1.2 Amino acids

Altered amino acid (AA) metabolism is a frequent feature in CC. Branched-chain amino acids (BCAA: leucine, isoleucine, and valine) act as important carbon sources and are useful for FA biosynthesis. High BCAA levels in plasma are associated with early PDAC and are often derived from increased protein breakdown in muscle and other body tissues (Figure 3) (97, 98). The utilization of BCAAs by PDAC can result in plasma BCAA depletion during late-stage PDAC. Similar observations have been reported for glutamate, in which the plasma levels of glutamate and the glutamine/glutamate (Q/E) ratio are significantly reduced in cachectic patients and animal models compared with their healthy counterparts (83, 99). Glutamine metabolism is a primary source of nitrogen and carbon, contributing to macromolecular synthesis and redox balance (83). Glutaminase 1 (GLS1) converts glutamine to glutamate, after which glutamate dehydrogenase (GDH) catalyzes the conversion from glutamate to α-ketoglutarate (α-KG). However, GDH is repressed in PDAC, and cytoplasmic aspartate transaminase (GOT1) is upregulated (83) (Figure 2**: lower panel**). Cachexia is associated with more aggressive forms

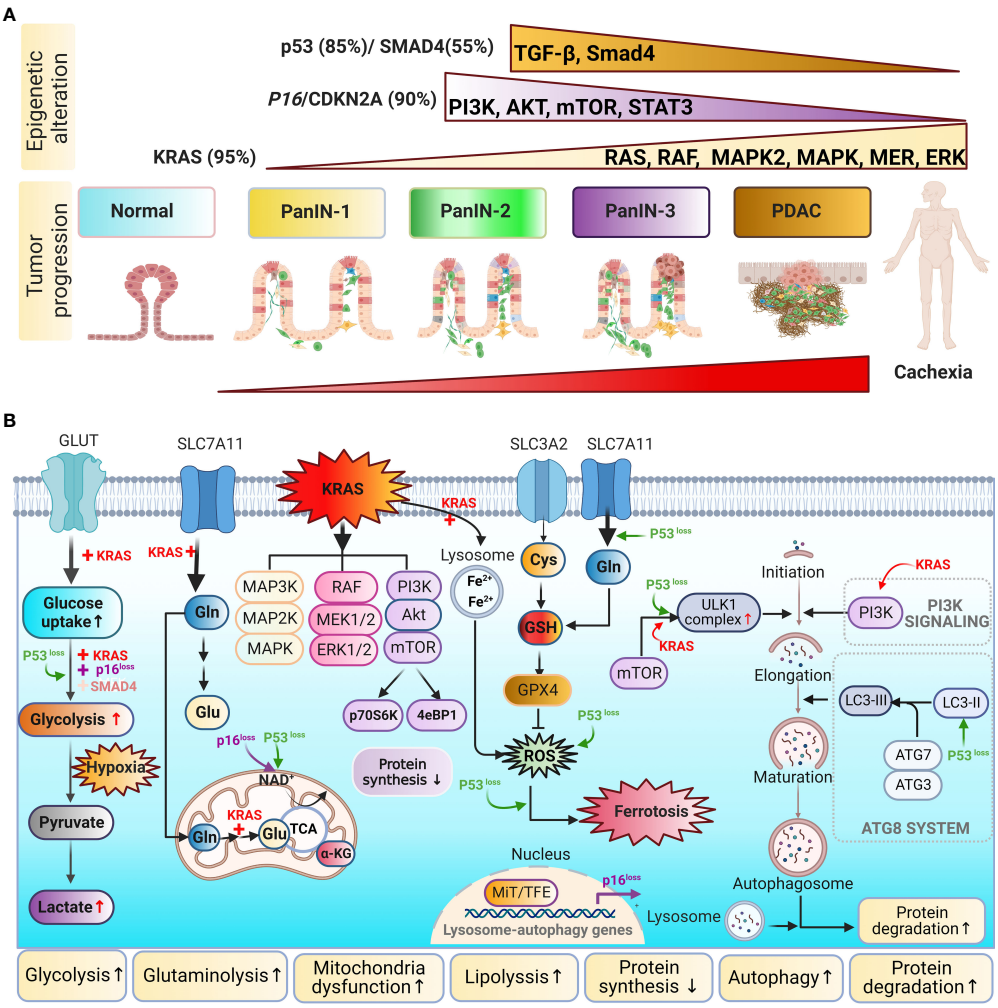


FIGURE 4
Metabolic remolding is influenced by genetic instability in PDAC. **(A)** Genetic mutations: PDAC is affected by high frequencies of aberrations and mutations in *KRAS*, *P16/CDKN2A*, *TP53*, and *SMAD4*. *KRAS* is involved in the RAF/mitogen-associated protein kinase pathway and the phosphoinositol 3-kinase (PI3K) pathway. *P16/CDKN2* mediates the PI3K/AKT/mTOR pathway. *TP53* influences the transforming growth factor-beta (TGF-β)/Smad4 pathway. **(B)** Mutated forms of *KRAS*, *TP53*, *P16/CDKN2*, and *SMAD4* promote glucose (Glc) uptake and enhance glycolytic flux, including the production of lactate (Lac). *KRAS* and *TP53* can both reprogram glutamine (Gln) metabolism to balance cellular redox homeostasis. Pancreatic cancer induces metabolic shifts, including increased glycolysis, lipogenesis, glutaminolysis, and autophagy, which are related to cachexia.

of PDAC, which may reflect the increased access of tumor cells to nutrients derived from protein breakdown and systemic changes in glucose metabolism (97, 100). Higher circulating BCAA levels may arise from the impaired catabolism of AAs that are commonly found in muscle (Figure 3). Muscle wasting is characterized by decreased muscle mass, increased proteolysis, and reduced protein synthesis, changes which are mediated by the proteasome, nuclear factor kappa B (NF-κB), and the mammalian target of rapamycin (mTOR) pathways. The phosphoinositol 3-kinase (PI3K)/AKT/mTOR pathway is a nutrient-sensing mechanism stimulated by decreased glucose availability in the muscle. A higher mTOR activity induced by *KRAS* mutation in PDAC which is positively correlated to higher circulating BCAA levels (12). mTOR activation is responsible for the uptake of BCAA in tumor tissue. Circulating BCAA also can affect subcutaneous adipocyte AA dysmetabolism. Both NF-κB and AKT/mTOR signaling are involved in proteolysis. NF-κB regulates the ATP-dependent ubiquitin–proteasome proteolytic pathway, including muscle-specific E3 ubiquitin ligases (such as muscle atrophy F box protein [MAFbx/atrogin-1] and muscle RING finger–containing protein 1 [MuRF1]), which promote proteolysis and contribute to muscle atrophy (22). Cachexia is the end result of convergent metabolic adaptations induced by tumors to satisfy their metabolic requirements.

TABLE 4 Genetically engineered mouse models (GEMMs) of pancreatic cancer-derived cachexia phenotypes.

	Orthotropic xenograft							Chemically induced	Genetically engineered	
Model	PANC-1	L3.6pl	S2-013 *(SUIT-2)*	COLO-357	MIA PaCa-2	Pan02	PDX	Gemcitabine-induced SW1990	KPC	KPP
Method	Injection: 1×10^6 cells	Injection: 1×10^6 cells	Injection: 5×10^5 cells	1 mm^3 sutured	5×10^6 cells	(IP) 1×10^7 cells	2 mm^3 patient-derived	50 mg/kg, (IP) gemcitabine	$KRAS^{G12D}$ $P53^{R172H}$ PDX-$Cre^{+/+}$	$KRAS^{+/G12D}$ $Ptf1a^{+/ER_Cre}$ $Pten^{f/f}$
Mouse strain	NSG mice NOD-SCID	NSG mice	Athymic nude mice	Athymic nude mice	Athymic nude mice	C57BL/6	NSG mice NOD-SCID	BALB/*c nu/nu* mice	C57BL/6	C57BL/6J Tamoxifen
Age	8-wk-old female	8-wk-old female	6–8-wk-old	6–8-wk-old	6-wk-old	6–8-wk-old male	8-wk-old female	4–6-wk-old	7–12-wk-old	4–5-wk-old
Duration	10 weeks	4–6 weeks	4 weeks	60 d	4 weeks	45 d	8–16 weeks	4 weeks	13–200 d	158 d
Weight loss	–	–	+	+	+	+	+	+	+	+
Muscle wasting	+	+	+*	+	+	+*	+*	–	+*/–	+*
Muscle gene profiles	FoxO1 Atrogin-1 MuRF1 SOCS3	FoxO1, Atrogin-1 MuRF1	MuRF1 Atrogin ZAG HSL	(+) INHBA	(+) SMAD2/3	MuRF1 Atrogin-1 ZAG myostatin	MuRF1 Atrogin-1 FoxO-1	N.A.	MuRF1 FoxO-1 pSTAT3 Atrogin-1	MuRF1 Atrogin-1 Atg5 Bnip3
Note	Chemokine ↑ IP10, MCP1, MIP2, RANTES and MIP1B (spleen)		Metabolic alteration: ROS↑ Glutamine uptake↑	N.A.	Activin A ↑ via (+)PI3K/AKT (-)AKT/TORC	Metabolic alteration: proteolysis↑, lipolysis↑, via TGF-β/NF-κB	(+) JAK/STAT (+) FoxO (+)PI3K/AKT	(+) Anoxia Chemotherapy-induced mild cachexia	(+) Anoxia, Autophagy↑ *Orm1*↑, *Apcs*↑ (+) *Jak2/Stat3*	Clinical muscle wasting phenotype
Inflammation evaluation	TNFα↑, IL1β↑, IL6↑, and KC↑ (murine IL8 homolog)		Not tested	TNFα↑	Not test	MCP-1, IL-6, TGF-β1	IL-1β, IL-1α IL22, TNF, oncostatin M	Not tested	IL-1β, IL-6 *Selp, Arg-1*	N.A.
Metastasis	–	–	N.A.	+	+	Not test	+	N.A.	–/+	+
Ref	(22, 123, 124)	(123)	(125, 126)	(127)	(128)	(129)	(130–132)	(133)	(21, 130, 134–136)	(130)

IP, intraperitoneal; N.A., not available; wk, week; d, day. *fat loss.

3.1.3 Lipids

In addition to glucose and amino acid metabolism, metabolic alterations in cachexia can include lipid metabolism. Approximately 93% of triacylglycerol FAs used by tumors are synthesized *de novo* by the mitochondria and cytosolic acetyl coenzyme A (CoA). Enzymes that participate in *de novo* FA and cholesterol synthesis are upregulated in PDAC, such as FA synthase (FASN) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR). Under pancreatic inflammatory conditions, wasting adipocytes release FAs into the plasma, increasing plasma concentrations of saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated FAs (PUFAs) (101). SFAs and MUFAs promote PDAC progression (102). However, a study performing transcriptomics and metabolomics suggested that lipase and a panel of FAs were significantly decreased in PDAC, and the presence of two SFAs (palmitate and stearate) inhibited tumor cell proliferation (103). Therefore, the roles played by FAs in PDAC appear complicated and remain unclear. PDAC patients present with distinct phenotypes associated with cachexia development, such as adipose tissue loss prior to skeletal muscle wasting or the loss of adipose tissue alone (104). A recent report indicated that soft tissue changes are initiated in PDAC before skeletal muscle loss (19), and the significant loss of visceral adipose tissue has been observed in PDAC-CC (18). In a retrospective cohort study, PDAC-CC was associated with the accumulation of oleic acid in plasma, resulting from malnutritional compensatory mechanisms triggered by the lack of oleic acid uptake into tissue (105). In a pre-cachexia model, increased FA oxidation occurs before muscle mass reduction, suggesting that FA may serve as a dominant energy source in PDAC-CC (18, 106). Adipose tissue lipolysis contributes to circulating FAs and subsequent FA uptake and lipid accumulation in the muscle and tumor tissue, leading to eventual metabolic derangement and muscle wasting after a period of metabolic adaptation. Several lipolytic enzymes are elevated, such as adipose

TABLE 5 Pro-cachectic mediators in PDAC-derived cachexia.

Cachectic mediator	Source	Action	Model of the study	Function	Ref
Pro-inflammatory					
IL-1 α	Tumor	Autocrine	AsPC-1, PANC-1, Capan-1, CFPAC-1, MDAPanc-3, and MDAPanc-28	● IL-1α activates AP-1 and nuclear factor-KB (NF-κB) pathways driving carcinogenesis.	(142)
	Tumor Macrophage Spleen	Autocrine Paracrine	C57BL/6J-congenic KPC model, Orthotopic L3.6pl xenografts (NSG), Orthotopic PANC-1 xenografts	● IL-1α, a catabolic mediator, activates the STAT3 signaling pathway and contributes to myofiber atrophy.	(123, 134)
	Tumor	Paracrine	KPC and IL-1R1 knockout C57BL/6J	● Acting in a paracrine manner, activates NF-κB signaling and expression of LIF in iCAFs.	(143)
	Tumor	Paracrine	PANC-1 and MIA-PaCa2 Orthotopic patient-derived xenograft BALB/c bearing MiaPaCa-2 KCP model, PDAC specimens, n=100	● IL-1α induces inflammatory factors (IL-6 and CXCL8) that lead to JAK/STAT activation.	(144, 145)
IL-1β	Tumor, CAFs Serum	Autocrine Paracrine	Orthotopic PANC-1 xenografts, Capan-1 and PANC-1, Capan-1 xenograft, MIA-PaCa2/CAF xenograft, KCP model PDAC patients: 27 PDAC-CC, total=89	● Increased IL-1β levels are a poor prognosis marker. ● Activates IRAK4 and NF-κB, supports cancer progression and chemoresistance.	(146, 147)
IL-6	Tumor Spleen Serum	Paracrine	Orthotopic L3.6pl xenografts (NSG), Orthotopic PANC-1 xenografts, PANC-1 and T3M4 PDAC specimens (19 PDAC-CC, total=100) PDAC patients (85 PDAC-CC, total 126) PDAC patients (25 PDAC-CC, total 55)	● Increases IL-6 in tumor and spleen, associated with muscle wasting and systemic inflammation. ● IL-6 acts as a poor prognosis marker and a prominent cachexia-associated factor.	(68, 123, 148–151)
	Tumor	Paracrine	KPC and KPC $IL6^{KO}$	● IL-6 causes adipocyte lipolysis and muscle steatosis, dysmetabolism, and wasting.	(21)
	Serum	Paracrine	PDAC serum, n=136 (a retrospectively studied)	● Higher IL-6 levels in tumor and serum mediate muscle wasting and cancer progression.	(152)
	Macrophages Fibroblasts Mast cells T cells	Paracrine	Review article	● Acute-phase response (inflammation). ● Suppresses food intake.	(153)
IL-8	Tumor	Paracrine	PDAC sample n=8 (organoid culture)	● IL-8 is associated with worse survival and muscle wasting.	(154)
	Serum Tumor	Paracrine	PDAC plasma (55 PDAC, total= 127) L3.6pl and COLO-357 PDX model C57BL/6J intraperitoneal injection IL-8	● Elevated serum IL-8 level significantly correlates with cachexia and sarcopenia. ● IL-8 is released from human PC cells in initiating atrophy of muscle cells *via* CXCR2-ERK1/2.	(81, 155)
TNFα (Cachetin) (Cachexin)	Tumor	Paracrine	CHO/TNF-20 cells implanted on nude mice.	● TNFα induces muscle wasting. ● TNFα inhibits both adipocyte and skeletal myocyte differentiation.	(70)
	Serum	Paracrine	PDAC patients (n=63)	● Increased TNFα levels in plasma correlated with poor nutritional status in advanced PDAC.	(156)
	Tumor, Spleen	Autocrine Paracrine	L3.6pl subcutaneous xenograft Orthotopic PANC-1 xenografts	● Elevated TNFα is associated with PDAC cachexia.	(123)
	Macrophage Lymphocyte	Paracrine	Review article	● Mediates muscle wasting, liver lipogenesis, insulin resistance, anorexia, and inflammation	(153)

(Continued)

TABLE 5 Continued

Cachectic mediator	Source	Action	Model of the study	Function	Ref
TGF-β	Serum	Paracrine	KPC mice bearing Panc02 KPC mice bearing FC1242 tumor (Intra-cardiac injection)	● TGF-β is a potent inducer of muscle atrophy, weight loss, and fat loss (increasing catabolism: proteolysis and lipolysis).	(129)
	Tumor	Paracrine	AsPC-1, MIA-PaCa2, BxPC-3, PANC-1, and CFPAC-1 Orthotopic xenograft mouse bearing AsPC	● TGF-β downregulation suppresses tumor growth and muscle wasting.	(157)
INF-γ	Serum	Paracrine	PDAC serum samples, n=90	● Increased serum INF-γ is associated with cachexia.	(158, 159)
	Lymphocytes Natural killer	Paracrine	Review article	● Catabolic effects	(153)
MyD88	Serum	Paracrine	KPC mice	● MyD88 trigger inflammation that influences cachexia development.	(140)
Other factors					
ZIP4	Tumor	Autocrine Paracrine	Orthotopic nude mice bearing AsPC-1 Orthotopic nude mice bearing BxPC-3	● ZIP4 promotes PDAC progression and muscle wasting by activating CREB- RAB27B.	(148)
	Tumor	Paracrine	Orthotopic xenograft mouse bearing AsPC (stable cell line: AsPC-shZIP4-Pre373)	● ZIP4 causes muscle wasting *via* PHLPP2-AKT-TGFβ Signaling Axis.	(157)
ZIP14	Muscle	Paracrine	C57BL/6 mice bearing Panc02 C57BL/6 mice bearing FC1242 tumor	● High levels of ZIP14 in muscles correlated with muscle wasting in PDAC.	(160)
Activin/ Activin A	Serum	Paracrine	PDAC specimens, N=34 KPC model	● Activin A mediated triglyceride degradation and accelerated visceral adipose wasting.	(18)
	Stroma Tumor	Paracrine	PDAC tissue microarrays n=63 Tumor samples *vs.* adjacent-normal KPC mouse model	● High activin A expression in stroma correlated to a worse prognosis.	(161)
	Tumor	Paracrine	KPC model	● PDAC tumors choreograph a systemic activin A response that correlated with muscle wasting.	(162)
	Tumor Serum	Paracrine	PDAC specimens, N=124 GEMM KPC mouse model	● Activin with hormone regulation shows a preferential driver of muscle wasting in males.	(163)
ZAG	Adipocyte	Paracrine	Review article	● Muscle wasting, insulin resistance, inflammation ● Anorexia	(153)
UCP-1	BAT, WAT	Paracrine	PDAC samples, N=8	● Uncouples oxidation of mitochondrial fatty acids. ● Thermogenesis and WAT browning.	(18, 164, 165)
LMF	Tumor	Paracrine	MAC16-murine model	● Lipid mobilizing factor (LMF) secreted from the tumor acts directly on adipose tissue with the release of FFA and glycerol.	(166)
Caveolin-1	Tumor	Paracrine	Athymic mice bearing MIA-PaCa2	● Cav-1 in PDAC stimulated IGF1R/IR, and glycolysis triggered cachectic states.	(167)
MCP-1	Serum	Paracrine	PANC-1 cells PDAC patients (n=70)	● MCP-1 led to inflammation and induced lipolysis by activating hormone-sensitive lipase (HSL). ● Suggesting MCP-1 acts as a biomarker of cancer cachexia.	(75, 168)
DAMPs	Serum tumor	Paracrine		● Extracellular HSP70 and HSP90 function as DAMPs and provoke an innate immune response through activation of TLR7/9 and TLRs. ● Muscle wasting.	(162, 169)
PAMPS	gut microbiota	Paracrine		● Inflammation and muscle wasting.	(170, 171)
Hormone					
Glucocorticoids	Serum	Paracrine	KCP murine model	● Hight GC content mediated skeletal muscle catabolism and hepatic metabolism during cancer cachexia.	(172)

(Continued)

TABLE 5 Continued

Cachectic mediator	Source	Action	Model of the study	Function	Ref
PTH	Tumor	Paracrine	Review article	● Hypercalcemia	(153)
PTHrP	Tumor	Paracrine		● Pro-cachectic factor, Pro-inflammatory stimulant	
	Serum	Paracrine	624 patients	● Regulating UCP1 expression reverse muscle and adipose tissue loss	(173)
Leptin	Adipocyte	Paracrine	Review article	● Leptin reduces appetite and increases energy expenditure.	(88)

TNF-α, tumor necrosis factor-alpha; TGF-β, transforming growth factor-beta; IFN-γ, interferon-γ; IL, interleukin; PTH, parathyroid; PTHrP, parathyroid hormone-related protein; UCP-1, uncoupling protein-1; WAT, white adipose tissue; ZAG, zinc-a2 glycoprotein; DAMPs, danger-associated molecular patterns; PAMPS, pathogen-associated molecular patterns; LMF, lipase maturation factor; MCP-1, monocyte chemoattractant protein-1; ZIP, zinc-regulated, Iron-regulated transporter-like protein; IL, interleukin.

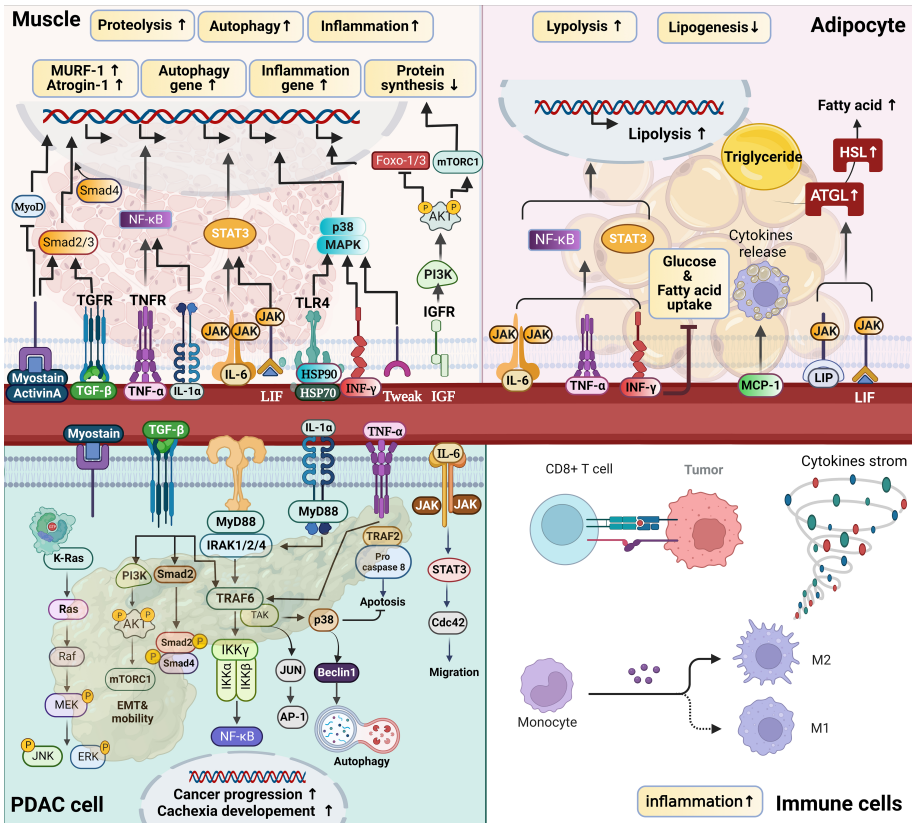


FIGURE 5
Pro-cachectic mediators of catabolism in PDAC-CC. Cachexia signals induce tissue catabolism by modulating gene expression profiles related to protein synthesis and degradation in muscle, lipid depletion, and tumor progression, primarily *via* the nuclear factor kappa B (NF-κB) and Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathways. In tumors, multiple receptors, including the Toll-like/IL1 receptors (TIRs), tumor necrosis factor (TNF), transforming growth factor-beta (TGF-β), and interleukin receptors (IL-6R being the best-studied), utilize overlapping and distinct signal transduction mechanisms to affect cellular outcomes, including increased cytokine production, proliferation, survival, migration, autophagy, and resistance to chemotherapy and immune surveillance. In addition to cytokines in tumors, circulating cytokines affect muscle and adipocytes, resulting in various metabolic alterations. For example, myostatin/activin A binds to type II receptors (ActRIIB), leading to Smad2/3 phosphorylation and the recruitment of Smad4, which results in muscle wasting. Simultaneously, myostatin/activin A signaling inhibits AKT activity and suppresses FoxOs phosphorylation, activating the ubiquitin–proteasome and autophagy–lysosome systems. IL6 binds to receptors to activate JAK/STAT3 signaling, increasing protein degradation. TNFα and IL1 signaling activates the IκB kinase (IKK)–NF-κB axis to initiate proteasome-mediated protein degradation. Higher levels of tumoral and stromal IL-1β expression result in a feedback circuit that attributes to cancer progression and cachexia development.

TABLE 6 MicroRNA (miR) expression levels and functions in pancreatic ductal adenocarcinoma (PDAC) and PDAC cachexia.

miRNA	Pathway	Target genes	Type of study	Location	Biological significance	Ref
miRNA-21 ↑	PI3K–AKT KRAS EGFR Cell cycle Apoptosis TGF-β	G12D, p27, p57, FOXO1, Bcl-2, FasL, PI3K, AKT, PTEN, RECK, SPRY2, P85, VHL, PDCD4, c-Jun	*In vitro*: PC1, Panc-2, and MIA-PaCa2, PANC-1, HS766T, HPAF-II, BxPC-3, Mpanc-96, PL45, Panc03.27, Panc10.05 *In vivo*: PDAC *vs.* healthy pancreatic duct tissue	In MV In tumor In blood	Promotes cell growth, invasion, migration and chemoresistance. Upregulation of miR-21 may inhibit myogenesis *via* regulation of IL6R, PTEN, and FOXO3 signaling. miRNA-21 promote muscle proteolysis *via* TLR7-JUN pathway.	(182–197)
miR-155↑	JAK–STAT TP53 MAPK-p38	TP53INP1, SOCS1, SOCS3, FOXO3a, TP53-induced nuclear protein 1 gene, RHOA, SMAD1/5, ZNF652	*In vitro*: BxPC-3, PANC-1, Capan-2, HS766T, HPAF-II, BxPC-3, Mpanc-96, PL45, Panc03.27, Panc10.05 *In vivo*: nude mice bearing MIA-PaCa2 PDAC *vs.* healthy pancreatic duct tissue	In MV In tumor In blood	Promotes tumor progression, invasion, and migration and mediates apoptosis. Higher miR-155 contributes to cachexia through the inhibition of negative feedback loops of SOCS1. miR-155 mediates TNF-A showing a pro-inflammatory effect.	(182, 190, 198–204)
miR-221/222 ↑ (Tumor)	PI3K–AKT TP53 P16/P27 Cell cycle	MMP-2, MMP-9, TIMP-2, PTEN, P27kip1, P57kip2, PUMA, Cdk4, p16, E2F, CDKN1B, MDM2, ICAM-1, BIM, SOD2, STAT5A	*In vitro*: BxPC-3, SW-1990, PANC-1, MIA-PaCa2, HS766T *In vivo*: PDAC *vs.* healthy pancreatic duct tissue Advanced pancreatic cancer with lymph node metastasis	In tumor	Promotes tumor progression, proliferation, and invasion. Inhibits apoptosis and induces chemoresistance. In C2C12 cell models, downregulated miR221/222 is observed which is associated with cachectic and sarcopenic condition vis MyoD-myomiRs regulatory pathway.	(188, 202, 205–208) (209)
Let-7 ↓	JAK–STAT KRAS	STAT3, SOCS3, N-cadherin, ZEB1	(PDAC) Biopsy specimens	In tumor In serum	Tumor growth and migration. Lower serum levels of let-7d correlated with poor overall survival in PDAC.	(210) (211)
Let-7d↑	KRAS mTOR	KRAS PGR, RPS6KA6, SFRP4	*In vivo*: Pancreatic tissues (PDAC) Biopsy specimens Skeletal muscle biopsies	In tumor In muscle	Cell proliferation, migration, invasion, and apoptosis. Upregulation of let-7d affects muscle cell proliferation and myogenic differentiation which leads to skeletal muscle wasting.	(212–214) (215)
circANAPC7/ miR-373	PHLPP2–AKT–TGF-β	ZIP4 promoter	*In vivo*: Orthotopic xenograft mouse bearing MIA-ZIP4-EV/circANAPC7	N.A.	Suppresses tumor growth and muscle wasting in PDAC.	(157)

MV, microvesicles; EMT, epithelial–mesenchymal transition; N.A., not available.

triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), further suggesting the occurrence of enhanced lipolysis. Increased ATGL and HSL activity correlate with tumor growth and WAT loss in cachexia (107) (Figure 3). Tumor progression is also associated with the shift from WAT to BAT, known as adipose tissue browning. BAT is a metabolic hallmark mediated by uncoupling protein 1 (UCP-1). In KPC and $Kras^{LSLG12D/+}Trp53^{f/f}$ mice, adipose tissue browning is associated with increased UCP-1 expression and occurs prior to the onset of fat wasting, consistent with clinical observations (108). A few studies have suggested that fat loss is an early and precipitating event prior to muscle loss in PDAC-CC, even in the absence of muscle wasting (18, 19, 21, 33, 104, 105, 107). Clinical studies suggested that fat loss may serve as a driving force for cachexia mortality, emphasizing the important roles of adipocytes in PDAC-CC and supporting the need to monitor adipose in patients with CC (20, 21).

Tumors hijack organ and tissue function, causing muscle and adipocyte wasting. Enhanced glycolysis in tumors and the upregulation of lipolysis and proteolysis in wasting tissue counterbalance the reductions in muscle and fat under cachectic conditions (Figure 3). Wasting muscle and adipocytes are among the convergent metabolic adaptations induced by tumors to satisfy their metabolic requirements. Patients with PDAC and PDAC-CC exhibit distinct and heterogeneous metabolic changes. Tumor, muscle, and adipocyte tissues all act as endocrine organs involved in the regulation of metabolic homeostasis, consistent with the EOLT hypothesis. In addition to metabolic alterations, bi-directional feedback occurs between tumor tissues and other organs, driven by the oncogenes and mediators (8, 12, 21, 32, 62).

3.2 Genetic instability–driven cachexic phenotypes and experimental models

In PDAC, tumors become genetically unstable (Figure 4), and mutations in four oncogenes are common in PDAC: *KRAS*

TABLE 7 Therapy for PDAC cachexia.

Target route	Drug	Target/mechanism	Cachexia/ PDAC-CC	Biological significance	Status	Ref
Cachexia mediator/ pathway	NSAID agents thalidomide	Cytokine	Cachexia	Altered cytokine production. Stabilized lean body mass.	Experimental Therapy	(220) (221)
	Infliximab etanercept	TNFα inhibition/ Mediating MuRF1 and Atrogin-1 expression in muscle	Cachexia PDAC-CC	No significant improvements in cachectic patients. TNFA blockade failed to improve muscle wasting.	Phase II Phase I/II	(221–225) (225, 226)
	Landogrozumab LY2495655 monoclonal antibodies (MoAbs)	Myostatin antibody/ Alk4/5/7/Smad and PI3K/AKT/mTOR pathways	PDAC-CC	Increased lean body mass. No benefits on overall survival. Myostatin antibody (LY2495655) with standard-of-care chemotherapy failed to confer additional clinical benefits (overall survival).	Phase II NCT03207724	(227, 228)
	Tocilizumab	anti-IL-6R mAb/ JAK/STAT3 pathway	PDAC-CC	Improved appetite and body weight	Experimental	(229, 230)
	Clazakizumab ALD518 BMS-945429		PDAC-CC Cachexia	Improved anorexia. Failed to reverse muscle atrophy. Improved lean muscle mass, lung symptoms, and fatigue score.	Phase I/II	(231)
	AG490/ Ruxolitinib	JAK/STAT3/ Reduce proteolysis in muscle cells	PDAC-CC	Alleviated cancer cachexia and skeletal muscle wasting. Ruxolitinib plus capecitabine was well tolerated, but no improvement in survival.	Phase II/III NCT00952289 NCT01423604	(232) (233)
	Trabederson AP 12009	TGF-β2 antagonist	Cachexia	Tumor suppression. Effect on anorexia.	Phase II Experimental	(139, 234, 235) (236)
	Bimagrumab (BYM338)	Anti-ACVR2 antibody	PDAC-CC	Increased lean body mass. Improved in thigh muscle volume (TMV), inter-muscular adipose tissue (IMAT) and subcutaneous adipose tissue (SCAT)	Phase II NCT01433263	(139, 237, 238)
	Anamorelin ONO-7643 ANAM	Ghrelin receptor agonist	PDAC-CC	Improved food intake, appetite, adiposity, and lean body mass. Adverse events (hyperglycemia, nausea, and dizziness) exist.	Phase III NCT01395914 NCT04844970 NCT03035409 NCT03637816 NCT01387269 NCT01387282	(239–243)
	Omeprazole	Hsp70/90	Cachexia	Prevented loss of muscle function.	Experimental	(244)
	MicrSoy-20	Gut Microbiota	PDAC-CC	Improved fatigue and appetite loss.	NCT04600154	(245)
	Espindolol MT-102	5-HT1aR/β2 agonist	Cachexia	Improved weight loss and fat-free mass. Acts as a pro-anabolic, anti-catabolic, and appetite-stimulator.	Phase II NCT01238107	(246)
	IMO-8503	TLR7/8/9 antagonist	Cachexia	Suggested a potential therapy for cancer cachexia.	Animal model	(247)
	R848	TLR7/8 antagonist	PDAC PDAC-CC	R848 induces anti-tumor responses and attenuates cachexia, improving the survival.	KPC model	(248)
Hormonal	RU38486	Glucocorticoid antagonist	Cachexia	U38486 was ineffective in muscle wasting.	Tumor-induced animal model	(249, 250)
Nutritional interventions	Ketogenic diet	Metabolism	PDAC-CC	Reversed metabolic alterations and reduced glycolytic flux and glutamine catabolism.	Experimental	(132)
	Glutamine Arginine β-hydroxy-β-methylbutyrate	Metabolism	Cachexia	Shifted away from proteolysis and increased fat-free mass. Failed to improve lean body mass.	Experimental Phase III	(251) (252)
	BCAA	Metabolism	Cachexia	Stimulated muscle protein synthesis Inhibited proteolysis	Clinical trial NCT03253029	(253–255)
	BCAA β-hydroxy-β-methylbutyrate	Metabolism	Cachexia	Fat mass content increased with no change in fat-free mass.	NCT03285217	(256, 257)

(Continued)

TABLE 7 Continued

Target route	Drug	Target/mechanism	Cachexia/ PDAC-CC	Biological significance	Status	Ref
	n-3 polyunsaturated fatty acids		PDAC-CC	Stabilized weight and appetite in pancreatic cancer patients.	NCT03751384	(258–264)
			Cachexia	Resist muscle wasting and improve the survival.	N.A.	(265)
PERT	Pancreatic enzymes	EPI	PDAC-CC	Weight gain, limited weight loss.	NCT02127021	(266, 267)

EPI, exocrine pancreatic insufficiency; CC, cancer-derived cachexia; TNFα, tumor necrosis factor-alpha; BCAA, branched-chain amino acids; TLR, Toll-like receptor; 5-HT, 5-hydroxytryptamine; TGF, transforming growth factor, NSAID, non-steroidal anti-inflammatory drugs; Pancreatic enzyme replacement therapy, PERT.

(>95%), *p16/CDNK2A* (> 90%), *TP53* (~70%), and *SMAD4* (55%) (8, 9, 62, 100). The hyperactivation of oncogenes (e.g., *KRAS*) and the downregulation of tumor suppressor genes (e.g., *TP53* and *CDKN2A*) promote tumor progression through the activation of various signal transduction pathways, including Wnt/Notch, c-Jun N-terminal kinase (JNK), PI3K, KRAS, and transforming growth factor (TGF)-β. A series of genetic and molecular events initiated by early oncogenic mutations in PanINs and later mutations in PDAC have been associated with metabolic alterations (109, 110). PDAC-CC is initiated by a metabolic shift in fuel utilization, in which glycolysis, proteolysis, and lipolysis increase and lipogenesis and protein synthesis decrease (Figure 4). During the pre-cachectic stage, patients experience these metabolic alterations as loss of appetite and impaired glucose metabolism before PDAC diagnosis or significant weight loss is apparent (Figure 2) (19, 67, 105). Early inflammatory signals may trigger the initial muscle and adipocyte wasting signaling cascades (9, 18, 21, 24).

KRAS is the most prevalently mutated oncogene, and *KRAS* mutations are considered to be dominant driver mutations in PDAC. Mutant *KRAS* regulates components of the mitogen-activated protein kinase (MAPK) and PI3K pathways to reprogram intracellular metabolism, including increasing glycolysis, by altering the levels of GLUT1 (111), HBP, and PPP (64, 112). Direct downstream effector cascades affected by *KRAS* mutations include the RAF–MAPK kinase (MEK)–extracellular signal–regulated kinase (ERK) and PI3K–AKT–mTOR pathways. The RAF–MEK–ERK pathway is considered among the most critical. The $KRAS^{G12D}$ mutation is frequently observed in pancreatic cells and promotes glycolysis *via* the upregulation of the MEK–ERK–HIF-1α axis. Elevated HIF-1α results in a feedforward loop between the insulin growth factor (IGF)-1 receptor, HIF-1α, and caveolin-1 to facilitate tumor progression and glycolysis (113).

Besides promoting high levels of glycolysis, *KRAS* upregulates glutaminolysis, allowing glutamine to be used as an additional carbon source for the TCA cycle (114). NEAAs, such as alanine, are alternative carbon sources that can support altered energy metabolism in PDAC (115). *KRAS* mutations increase glycolysis and the metabolism of amino acids, such as alanine and glutamine, activating downstream catabolic pathways, including proteolysis and lipolysis. Genetic mutations promote the recycling of wasting tissues to fuel cancer growth.

KRAS mutations also promote the generation of inflammatory cytokines, which shape the PDAC TME, including IL-6, IL-8, C–X–C motif ligand (CXCL)1, CXCL2, and CXCL5 (116, 117). Some cytokines/chemokines act in both autocrine and paracrine manners to support tumorigenesis and tumor angiogenesis (116).

KRAS activation leads to the loss of p16, accelerating NADH oxidation and supporting increased glycolysis through the production of NAD^+ to support tumor growth (118). However, PDAC cells lack nutrient sensors, and mTOR complex 1 (mTORC1), a nutrient-sensing mechanism, is bypassed in PDAC. Bardeesy et al. proposed that autophagy is driven by the elevated expression of the microphthalmia transcription factor (MiTF) family members MiTF, TFE3, and TFEB in PDAC (119). The loss of *SMAD4* is another frequent event associated with PDAC progression, identified in approximately 50% of PDAC cases. *SMAD4* is a central component in the transforming growth factor (TGF-β) signaling cascade, and *SMAD4* loss enhances glycolysis by altering the expression of the glycolysis enzyme phosphoglycerate kinase (PGK) (120). The loss of *TP53* alters metabolism in PDAC by inhibiting mitochondrial respiration and simultaneously stimulating glycolysis. A recent study demonstrated that *TP53* rewires glucose and glutamine metabolism in malignant PDAC by preventing the nuclear translocation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and stabilizing its function (121). Loss of *TP53* can reduce the expression of fructose-2,6-bisphosphate to promote the glycolysis cycle (122). Therefore, *KRAS*, *P16*, *P53*, and *SMAD4* have counterintuitive effects that promote tumorigenesis, further highlighting the complexity of interactions between genes and metabolisms in cancer progression and cachexia development.

These metabolic changes are consistently observed in numerous preclinical animal models of PDAC cachexia (Table 4). Commonly used animal models of PDAC-CC include [1] intraperitoneal (IP) injections of PDAC cells, which localize to the pancreas; [2] orthotopic models of PDAC, in which cancer cells are injected directly into the pancreas; [3] patient-derived xenograft (PDX) models, in

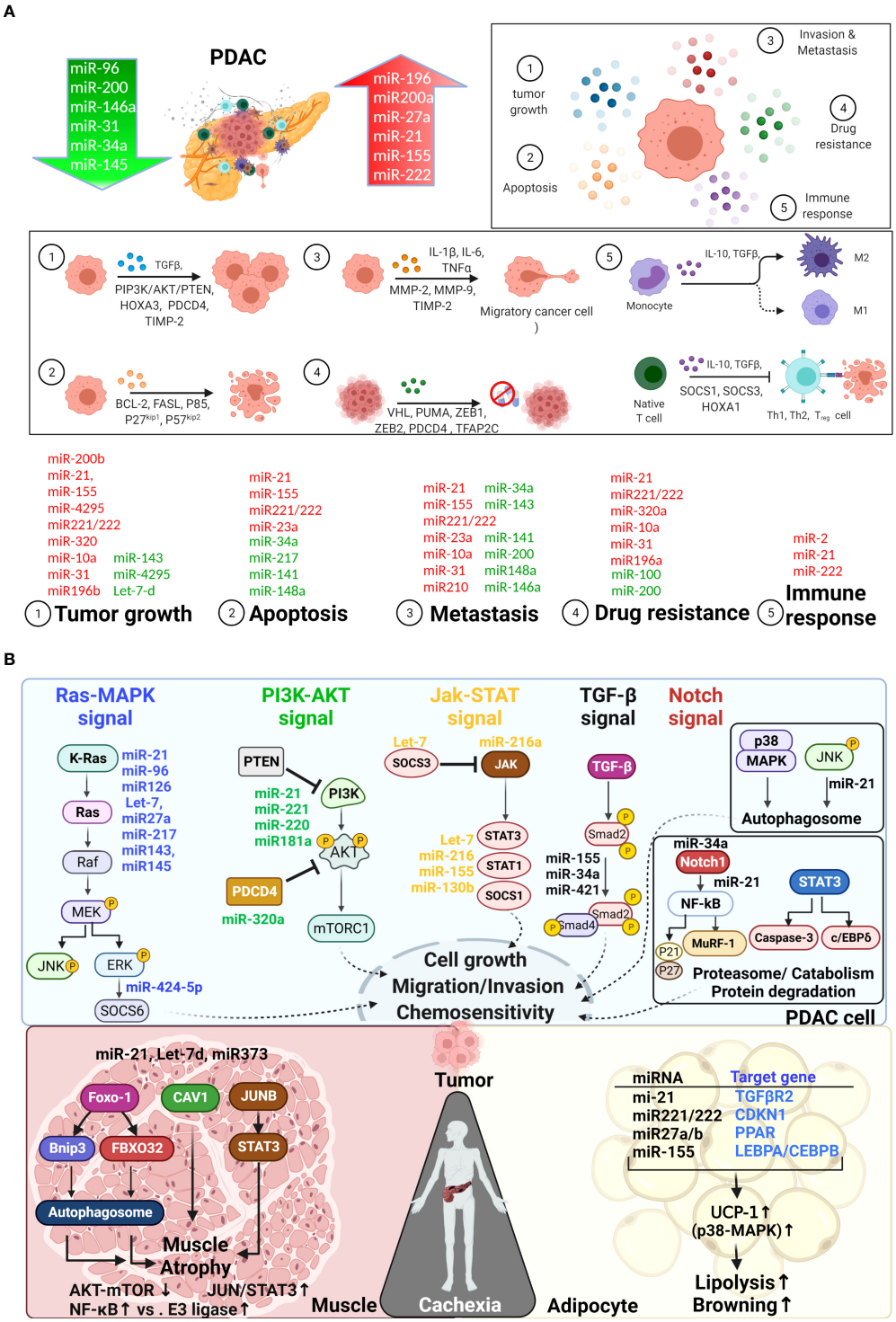


FIGURE 6
Mechanistic role of miRNAs in PDAC cachexia. **(A)** Downregulated microRNAs (miRNAs) are indicated in green, and upregulated miRNAs are indicated in red. Based on the literature, miRNAs participate in the regulation of PDAC progression and metastasis, overcoming host immune responses, and the development of chemoresistance. **(B)**. miRNAs associated with PDAC-CC are primarily involved in KRAS-MAPK, PI3K-AKT, JAK-STAT, and TGF-β, NF-κB and p38-MAPK signaling pathway ... etc. miRNAs can be detected in tumor and serum and mediate crosstalk in the tumor microenvironment between tumor, muscle, and adipocytes, which are associated with the development of PDAC-CC. Some microRNAs are tissue specific. For example, miR-21 (TLR7-JUN), miR-155, let-7d and miR373 are specific contributed to muscle wasting in PDAC-CC (see Table 6 in detail). The most common genes, such as IL-6R, FOXO1, PDK4, and ZIP14, had been associated with muscle wasting in cachexia. In adipocytes, specific microRNAs may mediate the transcription factors C/EBPβ/δ, C/EBPα, and PPARγ, resulting in adipogenesis.

which a portion of a resected human pancreatic tumors are surgically attached to the mouse pancreas; and [4] genetically engineered mouse models (GEMMs) of PDAC. Up to 85% of PDAC patients suffer from CC, and approximately 30% of PDAC patients succumb to cachexia rather than tumor burden (137, 138). PDX and murine allograft models have been applied to study cachexia, resulting in the identification of Toll-like receptors 7/9 (TLR7/9), MyD88, and TGF-β as mediators of cachexia in PDAC (129, 137, 139, 140). Most PDAC-CC studies focus primarily on weight loss, muscle wasting, and the analysis of mRNA markers. A comparison analysis of subcutaneous, IP, and orthotopic PDAC animal models indicated that the implantation site is crucial when attempting to study PDAC-CC (137). Both IP and orthotopic implantation models develop more severe cachexia symptoms (such as muscle wasting, anorexia, and a decrease in locomotive activity) than the subcutaneous implantation model. The orthotopic animal model is histologically similar to PDAC patients, mimicking the TME associated with intact tumors, suggesting that the TME may be involved in cachexia induction. Studies in PDAC animal models have demonstrated that tumor-associated macrophages mediate muscle wasting *via* the activation of signal transducer and activator of transcription (STAT)3 signaling (134, 141).

Most preclinical studies of CC use C-26 (colon cancer) and LLC (lung cancer) mouse models. However, these two models are associated with limitations (1): a limited interval between the onset of CC symptoms and animal death leaves only a small therapeutic window, and (2) translatability to humans may be limited, as the gene expression profiles in these mice did not correlate with those in human cancer tissue biopsies (130). However, GEMMs offer slower cachexia progression and early development than other cancer models, and PDAC-CC animal models are more translatable to humans than models using other cancer types. Therefore, animal models of PDAC-CC are clinically relevant. Preclinical PDAC murine models may be useful for understanding cachexia progression and evaluating therapeutic options for mitigating PDAC-CC. Establishing a model able to fully mimic the human condition remains necessary. Animal models can contribute to improving our understanding of the mechanisms driving tissue wasting for translation into new anti-cachexia therapies.

3.3 Pro-cachectic mediators and microRNAs

3.3.1 Pro-cachectic mediators

Endocrine organs and cells synthesize biologically active compounds that are released directly into the circulation and interact with other cells. Cachexia-associated inflammation is influenced by numerous bioactive molecules, such as TNFα, IL-1, IL-6, and IL-8 (Table 5 and Figure 5). Cachexia-affected organs can act as autocrine or paracrine organs, releasing factors into the bloodstream to promote systemic crosstalk. These cytokines have multifactorial effects, triggering a hypercatabolic feedforward loop between the tumor, adipose tissue, and muscle mediated by the NF-κB and Janus kinase (JAK)/STAT pathways (29, 174, 175) (Figure 5). NF-κB and JAK/STAT activation enhance lipolysis, downregulate lipogenesis, and stimulate the catabolism of lean body mass (12).

A salient feature that distinguishes PDAC from other *KRAS*-mutant cancers is an extensive fibro-inflammatory stroma, which accounts for 80%–85% of the tumor bulk. These stromal cells are recruited and reprogrammed by PDAC cells during cancer progression and cachexia development. Secreted factors (Table 5) enable these cells to communicate with PDAC, creating a dynamic feedback circuit associated with intrinsic *KRAS* signaling in PDAC cells (Figure 5).

Various circulating pro-inflammatory cytokines have been implicated in PDAC-CC, including IL-6, IL-1, IL-8, TGF-β, and TNFα …etc (detailed in Table 5) (12, 21, 29, 123). These cytokines likely derive from various sources and result in systemic effects (123, 176). Oncogenic *RAS* drives the expression of multiple inflammatory cytokines, including IL-1 and IL-6 (21, 146). Inflammatory cytokines released by tumor cells may be prominent cachexia-associated factors that regulate autocrine and paracrine function, promoting tumor progression and cachexia development. For example, IL-6 plays autocrine roles in supporting tumorigenesis *in vivo* and induces weight loss and inflammation in cachexia *via* a paracrine manner (21, 68, 152). Other cytokines, such as IL-1, IL-8, TNFα, and INF-γ, have also been associated with weight loss and poor survival in PDAC (148, 177, 178). TNF-α and IL-1 can induce anorexia, producing both hypercatabolic and anorexigenic effects (Figures 1 and 5). Circulating IL-1β promotes NF-κB activation in the hypothalamus, enhancing glucocorticoid production and resulting in catabolic effects in both muscle and adipose tissue. TGF-β1 can induce proteolysis through the E3 ligase atrogin-1 in animal models. TGF-β inhibition improved muscle wasting in the KPC model (129). Activin A and myostatin belong to the TGF-β superfamily and are associated with muscle wasting through the activation of the Smad2/3 pathway, which decreases AKT–mTOR-mediated protein synthesis and enhances ubiquitin ligase–mediated proteolysis (18, 128). These cytokines drive diverse catabolic processes across multiple cells and organs, forming a catabolic feedforward loop (Figure 1).

In muscle tissue, the JAK–STAT and NF-κB pathways are the dominant catabolic pathways activated by circulating IL-6 and TNFα in muscle wasting (174). IL-6 induces NF-κB activation, which can also upregulate ubiquitin-mediated proteasomal degradation in wasting (179). Proteolysis is a prerequisite for muscle wasting, and both lipolysis and adipopenia may occur prior to muscle loss. Lipolysis results in increased circulating FFAs, triggering the secretion of Atrogin-1 and MuRF1, which induce muscle atrophy.

In adipocytes, lipolysis plays a substantial role in increasing the catabolism of stored fat. ATGL and HSL act to reduce fat to its

component FAs, leading to the loss of body mass. Secreted IL-6 can trigger browning by inducing UCP-1 expression in adipocytes (18, 180). The catabolic effects of IL-6 on WAT *in vitro* are mediated through the JAK/STAT3 and NF-κB pathways (21, 181).

3.3.2 Epigenetics modulation (miRNAs)

Genetic instability and epigenetic changes are both involved in pancreatic oncogenesis and cachexia development (Table 6). Recently, miRNAs, small non-coding RNAs 19–25 nucleotides in length, have been identified in an increasing number of biological processes, including *KRAS* signaling and the JAK–STAT, PI3K–AKT, notch, and TGF-β signaling pathways (Figure 6A). These influences contribute to the control of several cancer-related processes in PDAC, such as tumor growth, apoptosis, metastasis, drug resistance, and the immune response. In addition to roles in oncogenesis and tumorigenesis, aberrant miRNA expression may affect cytokine production or directly alter metabolic processes, resulting in a metabolism remodeling that facilitates PDAC progression and cachexia development (see Figure 6 and Table 6).

Recent studies have detected miRNAs in serum, plasma, tissue, and tumors (Table 6). Studies indicates that miRNAs are commonly found in various EVs, such as exosomes, apoptotic bodies, microvesicles (MV), and lipoproteins, allowing them to target cells and contribute to intercellular signaling through endocrine, paracrine, and autocrine pathways (182, 183, 198, 199, 215–217). Losses in muscle proteins and fat mass are the most important signatures of cachexia and can result in the generation of microvesicles containing miRNAs (see Figure 6B: bottom panel). Most studies have identified miRNAs expressed in tumor cells; however, some miRNAs are tissue-specific or tissue-enriched, involved in either the active or passive stimulation of metabolic changes and inflammatory responses (Table 6). In PDAC-CC, miR-21, miR221/222, miR27a and miR155 are commonly correlated with muscle and adipocyte wasting through the transcription E3 ubiquitin ligases (mediated by FoxO3 or FoxO1 in muscle), JUN–STAT3, or TGF-β; (Figure 6B: bottom panel). Given the roles of miRNAs in gene expression and the regulation of inflammatory responses and metabolic reprogramming, additional study of miRNAs remains necessary. An ongoing observational trial (NCT05275075) aims to analyze the miRNA profiles in patients with PDAC-CC. The causal roles of miRNAs and molecular mechanisms in cachexia remain under debate and require further discussion and study; however, therapeutic approaches for modifying multiple targets have been suggested (218, 219). The study of miRNAs could also contribute to the development of diagnostic or prognostic biomarkers and new targets for cachexia prevention or treatment.

4 Conclusion

The high prevalence of PDAC-CC may be associated with the unique genetic background (*KRAS* mutations) and modulators in PDAC, which mainly exacerbate metabolic disruptions, leading to cachexia development. Systemic metabolic alterations mediated by pro-cachectic factors, systemic inflammation, and epigenetic changes, highlighting that PDAC is a systemic disease rather than a single-organ defect. PDAC can induce metabolic disruptions in organs beyond the pancreas. We applied the EOLT hypothesis (33) to emphasize the systemic effects of PDAC, leading to tissue wasting in PDAC-CC. Currently, no FDA-approved agent is able to treat cachexia, although potential treatments are listed in Table 7. However, further studies remain necessary to generate foundational knowledge for the development of additional therapies or understanding the molecular mechanisms of PDAC-CC.

Author contributions

Y-CY and AA drafted manuscript. L-MC, W-CCe, J-CY, H-CL and W-CCa participated in discussion, literature review, supported, and edited manuscript and W-LM initiated and supported the concept. Y-SS and W-LM edited, and final approved the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

PDAC	Pancreatic ductal adenocarcinoma
PDAC-CC	PDAC-derived cachexia
EOLT	endocrine organ–like tumor
QoL	quality of life
OS	overall survival
CC	cachexia
IL	interleukin
TNF-A	tumor necrosis factor-alpha
BMI	Body Mass Index
CRP	C-reactive protein
SMI	Skeletal Muscle Index
BIA	bioelectrical impedance analysis
CXI	cachexia index
TME	tumor microenvironment
PI	pancreatic insufficiency
OXPHOS	oxidative phosphorylation
PanIN	pancreatic intraepithelial neoplasia
PPP	pentose phosphate pathway
TCA	tricarboxylic acid
HBP	hexosamine biosynthesis pathway
HK1/2	hexokinase ½
LDHA	lactate dehydrogenase A
LDH	lactate dehydrogenase
GLUT1	glucose transporters 1
MCT	monocarboxylate transporters
GFPT	fructose-6-phosphate amidotransferase
HIF-1	hypoxia-inducible factor-1
FA	fatty acid
WAT	white adipocyte tissue
BAT	bBrown adipose tissue
ROS	reactive oxygen species
NEAA	nonessential amino acid
AA	amino acid
BCAA	branched-chain amino acid
Q/E	glutamine/glutamate
GLS1	Glutaminase 1
GDH	glutamate dehydrogenase
A-KG	A-ketoglutarate
GOT1	aspartate transaminase 1
NF-Kb	nuclear factor kappa B
mTOR	mammalian target of rapamycin
MuRF-1	muscle RING-finger protein-1
MAFbx	muscle atrophy F-box
CoA	coenzyme A
FASN	fatty acid synthase
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
SFAs	Saturated fatty acids
MUFAs	monounsaturated fatty acids
PUFAs	polyunsaturated fatty acids
ATGL	adipose triglyceride lipase
HSL	hormone-sensitive lipase
UCP-1	uncoupling protein 1
MAPK	mitogen-activated protein kinase
IGF	insulin growth factor
CXCL	C–X–C motif ligand
TGF-B	transforming growth factor
GEMMs	genetically engineered mouse models
STAT3	signal transducer and activator of transcription 3
JAK	Janus kinase
INF-γ	interferon gamma
MV	microvesicles
EV	extracellular vesicles
FoxO	Forkhead box O
PIF	proteolysis-inducing factor
ZAG	zinc alpha 2-glycoprotein
LIF	leukemia inhibitory factor
TWEAK	TNF-related weak inducer of apoptosis

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Progression of hepatocellular carcinoma after radiofrequency ablation: Current status of research

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Hepatocellular carcinoma (HCC) remains an important disease for health care systems in view of its high morbidity, mortality, and increasing incidence worldwide. Radiofrequency ablation (RFA) is preferred to surgery as a local treatment for HCC because it is safer, less traumatic, less painful, better tolerated, causes fewer adverse reactions, and allows more rapid postoperative recovery. The biggest shortcoming of RFA when used to treat HCC is the high incidence of residual tumor, which is often attributed to the vascular thermal deposition effect, the wide infiltration zone of peripheral venules, and the distance between satellite foci and the main focus of the cancer. Recurrence and progression of the residual tumor is the most important determinant of the prognosis. Therefore, it is important to be aware of the risk of recurrence and to improve the efficacy of RFA. This review summarizes the relevant literature and the possible mechanisms involved in progression of HCC after RFA. Current studies have demonstrated that multimodal treatments which RFA combined with other anti-cancer approaches can prevent progression of HCC after RFA.

KEYWORDS

hepatocellular carcinoma, radiofrequency ablation, residual tumor, tumor progression, prevention

1 Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and accounts for 90% of cases. It is associated with a poor prognosis because of a high recurrence rate and a lack of effective therapeutic options (1). Patients with early-stage HCC can be treated by liver transplantation, surgical resection, or radiofrequency

ablation (RFA) as first-line therapies (2, 3). Although surgical resection and liver transplantation have been regarded as the optimum therapeutic strategies, their application is limited in many cases because of lack of a donor, decompensated liver function, and failure to meet specific (Milan) selection criteria (4).

RFA is a minimally invasive and repeatable treatment that is associated with limited procedure-related morbidity, which results in better cost-effectiveness and quality of life. RFA is also considered as an alternative for patients not eligible for surgical resection or liver transplantation and those awaiting liver transplantation. Many studies have demonstrated that the efficacy of RFA and surgical resection in terms of survival outcomes are similar for a single small HCC of ≤3 cm (5, 6). However, RFA appears to be inferior to surgical resection in terms of local control and disease-free survival (7). Rapid intrahepatic neoplastic progression and metastasis of HCC after RFA, indicating more aggressive biological behavior, has also been found in some clinical centers (8). The mechanisms underlying progression of HCC after RFA remain poorly understood, and it is important to develop targeted therapy that can improve the prognosis of this disease. This review discusses the literature on the potential mechanisms of progression of HCC after RFA in the hope of finding preventative strategies.

2 Tumor progression in HCC after RFA

Since the first experimental hepatic RFA procedure was performed in 1990 (9), there has been extensive research on RFA, and it is now regarded as a curative treatment for HCC. However, many clinical centers have been reporting an increasing number of cases of progression of HCC after RFA. Seki et al. (10) described a patient who underwent transcatheter arterial chemoembolization and RFA for a small HCC measuring 2.5 cm, and enhanced magnetic resonance following treatment showed complete tumor necrosis and did not reveal any tumor around the treated area. However, numerous tumors around the treated area were observed on enhanced computed tomography 50 days after RFA. Koda et al. (11) reported a similar case involving well-differentiated HCC that was treated by RFA and reduced to 2.5 cm in diameter by 6 months after the procedure but rapidly enlarged to 6 cm in the next 2 months and progressed to lymph node metastasis. Autopsy findings showed both sarcomatoid and trabecular HCC cells. This was the first reported case of sarcomatous HCC after RFA. Portolani et al. (12) subsequently reported on three patients with small HCC treated with RFA, in whom imaging confirmed complete ablation. However, tumor regrowth was diagnosed at 3, 4, and 6 months after RFA and was associated with extensive liver and parietal wall involvement. Ruzzenente et al. (13) reported on 87 patients with cirrhosis and 104 HCCs that were treated by RFA. In 4 patients, although complete local necrosis was achieved, rapid intrahepatic neoplastic progression was observed following RFA. After 30 days, there was a rapid increase in alpha fetoprotein in three of these four patients, two of whom died as a result of disease progression after 2–3 months of follow up. Baldan et al. (14) reviewed 401 cases of HCC treated by RFA from 13 centers in Italy and identified tumor seeding in four patients and rapid unexpected disease progression on another 10 patients. Shiozawa et al. (15) investigated 1073 lesions in 538 patients who underwent ultrasound-guided RFA between April 1999 and March 2008 and documented rapid aggressive disease progression in 0.65% of cases. In a study of the perfusion features of local recurrence of HCC after RFA, Wu et al. (16) demonstrated that enhancement was more homogeneous, the border was more poorly defined, washout was more marked, and that there were fewer feeding vessels and areas of inner necrosis in the recurrent HCC than in the initial HCC. Moreover, the tumor stem cell markers CD133 and EpCAM were also both highly expressed in specimens from the patients with recurrent disease. As shown in Figure 1, we reviewed the relevant mechanisms.

3 Cause of residual tumor tissue

RFA of a tumor requires local application of extremely high temperature, which can cause irreversible cell injury and ultimately tumor apoptosis and coagulative necrosis. According to the size and shape of the needle tip, a spherical ablated area is generated in about 10–30 minutes, generally from 2 cm to 5 cm in diameter. With RFA, the zone of active tissue heating is limited to the few millimeters surrounding the active electrode, with the remainder of the ablation zone heated *via* thermal conduction. With an increase in the size of the target area, the efficacy of the treatment is reduced (17). Moreover, certain tissue properties, such as electrical conductivity, thermal conductivity, dielectric permittivity, heat capacity, and blood perfusion rate, have a substantial effect on the growth of ablation zones. Interestingly, a temperature >100°C is less effective because the desiccation that results at these temperatures, which manifests as water vapor and burnt tissue, increases the tissue impedance and therefore limits further electrical conduction through the remaining tissue (18).

Furthermore, a cytotoxic temperature is difficult to maintain if the ablated tumor is close to large blood vessels (19). This heat-sink effect is a commonly described limitation of RFA and occurs when heat that is absorbed by flowing blood or air is carried away from the area of ablation; in these cases, the lower energy

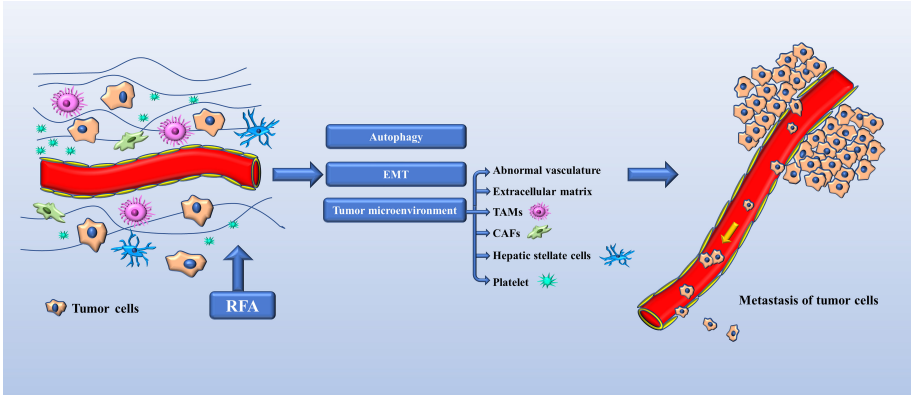


FIGURE 1
Mechanisms of tumor progression in HCC after RFA. EMT (epithelial-mesenchymal transition), TAMs (tumor-associated macrophages), CAFs (carcinoma-associated fibroblasts).

intensity within the passive zone is not able to achieve thermally toxic temperatures in proximity to the cooling vasculature (20). Therefore, tumor tissue that is adjacent to the vasculature is less susceptible to thermal damage.

4 Mechanisms of tumor progression in HCC after RFA

4.1 Changes in biological behavior of HCC cells after RFA

RFA may directly change the proliferation, invasion, and metastasis of HCC cells. Obara et al. (21) assessed the proliferation rate, heat sensitivity, and invasive capacity of several HCC cell lines in response to heat treatment and demonstrated that even a single session of heat treatment could induce further transformation of these cells. Ke et al. (22) established a rabbit model of residual VX2 hepatoma after RFA and identified inadequate RFA caused by temperature that was too low at the target sites to be a potentially important cause of rapid disease progression. Rapid progression of residual hepatic VX2 carcinoma could be facilitated by overexpression of several molecular factors, such as proliferating cell nuclear antigen, matrix metalloproteinase 9, vascular endothelial growth factor (VEGF), hepatocyte growth factor, and interleukin (IL)-6. Zhang et al. (23) also demonstrated that RFA promoted proliferation, migration, and invasion of HepG2 and SMMC7721 cells. Epigenetic regulation also has an important role in maintaining homeostasis when cells are exposed to acute physicochemical stresses. Moreover, in a mechanistic study of the role of m6A machinery in recurrence of HCC after RFA, Su et al. (24) found that sublethal heat treatment increased m6A modification of the epidermal growth factor receptor (EGFR) in the vicinity of the 5' UTR region and promoted its binding with YTHDF1, which enhanced the translation of EGFR mRNA and promoted viability and metastasis of HCC cells after RFA.

4.2 Autophagy

Autophagy is evolutionarily conserved cellular process wherein components of cells are degraded by sequentially formed autophagic vesicles and is also a cellular process used by cancer cells to replicate under various adverse conditions, such as oxidative stress, endoplasmic reticulum stress, mitochondrial stress, and starvation. The possible signaling pathways of autophagy in HCC after RFA are listed in Figure 2. Wang et al. (25) reported that autophagy participated in the enhanced viability and invasion of HCC cells after inadequate RFA. They also found that CD133 became localized to autophagosomes and was suppressed by 3-MA or chloroquine, which could suppress RFA-induced cell viability, invasion, and autophagy. Zhao et al. (26) showed that insufficient RFA induced an anoxic microenvironment, autophagy, and autophagic flux in tumor cells, which have an important role in tumor relapse and proliferation. Furthermore, Xu et al. (27) demonstrated that insufficient RFA increased autophagy in residual HCC cells *via* the hypoxia-inducible factor 1 (HIF)-1α/BNIP3 pathway, which is involved in increased proliferation, migration, and invasion of tumor cells. Chen et al. (28) found that the heat shock protein90 (HSP90)/Akt/mTOR pathway is involved in the signaling between autophagy and HSPs after incomplete thermal ablation. And subsequent studies found that the HSP90 inhibitor 17-AAG, in combination with the autophagy inhibitor 3-mA, promoted

hepatocellular carcinoma apoptosis following incomplete thermal ablation more significantly than monotherapy, suggesting an association between heat-induced heat shock processes and autophagy (29). Jiang et al. (30) showed that sublethal heat stress induced protective autophagy against heat-induced apoptosis in HCC *via* the ATP-AMPK-mTOR axis, and the inhibition of autophagy by CQ or siRNA targeting the autophagy-related genes Beclin-1 and Atg5 enhanced heat-induced apoptosis. Zhang et al. (31) found that activated hepatic stellate cells promote progression of residual HCC cells after sublethal heat treatment from autophagic survival to proliferation *via* HGF/c-Met signaling. In an animal model, inhibiting autophagy in combination with c-Met inhibitor significantly thwarted tumor progression of residual HCC after incomplete thermal ablation *via* the suppressed autophagy, the decreased proliferation and the increased apoptosis.

4.3 Epithelial-mesenchymal transition

Trans-differentiation of epithelial cells into motile mesenchymal cells, a process known as epithelial-mesenchymal transition (EMT), is activated in an aberrant manner under pathological conditions, including organ fibrosis and cancer (32). To acquire an invasive phenotype for metastatic progression in cancer, carcinoma cells exploit EMT to facilitate their dissociation from the primary tumor and dissemination into the circulation. EMT also endows tumor cells with enhanced stemness and increased resistance to immune clearance and various iatrogenic insults (33). The mechanisms of EMT in tumor progression after RFA of HCC are listed in Figure 2.

An increasing body of data suggests that EMT also participates in progression of HCC after RFA. Some studies have shown that P-ERK1/2 plays an important role in heat-induced EMT (34). Four studies (35–38) performed heat treatment on MHCC97H, HepG2, HuH7, and HEP3B cell lines and found that ERK was significantly phosphorylated. EMT was attenuated after inhibition of P-ERK1/2, which was similar to other ways of activating EMT. They found that P13K, P46-Shc, and Periostin were activated as upstream proteins of ERK after heat treatment, causing tumor invasion and metastasis. Flotillin-1 and flotillin-2 were found to be upregulated in HCCLM3 cells following heat treatment and in residual HCCLM3 xenograft cells after RFA, which altered the status of EMT and metastatic potential *via* activation of the Akt/Wnt/β-catenin signaling pathway (39). Zhang et al. (40) also found that incomplete RFA enhanced the invasive and metastatic potential of residual cancer, accompanying with EMT-like phenotype changes by activating β-catenin signaling in HCCLM3 cells. Subsequent study found that a combination of interferon alpha and an herbal compound known as "songyou

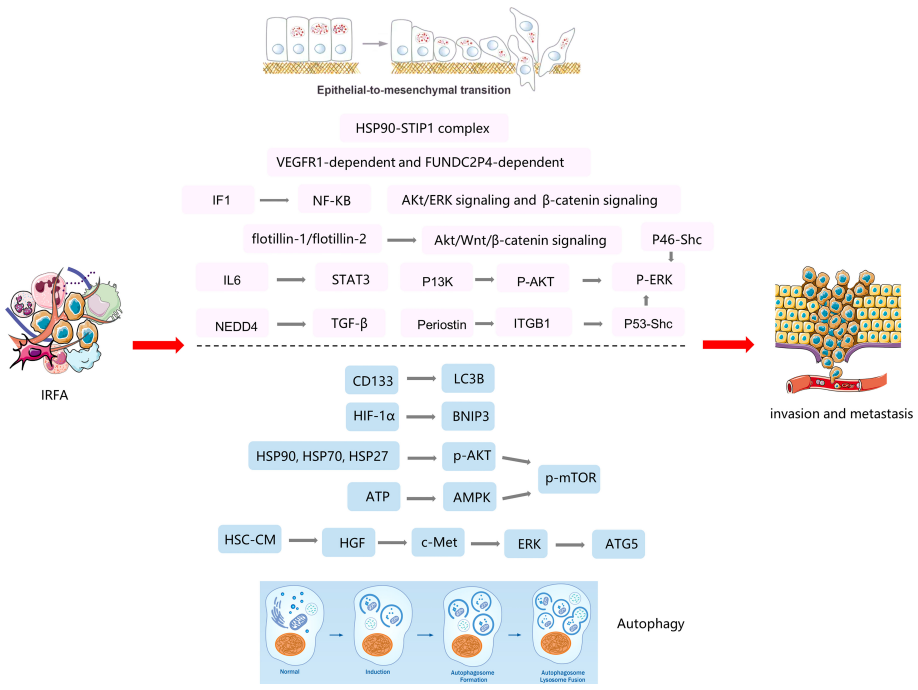


FIGURE 2
The mechanisms of EMT and autophagy in tumor progression after insufficient RFA(IRFA) of HCC.

yin" significantly weakened the enhanced metastatic potential of residual HCC after RFA *via* attenuation of EMT and that this effect was mediated by inhibition of activation of β-catenin (41). Su et al. (42) found that incomplete RFA induced the formation of Stress-induced phosphoprotein 1 (STIP1) - HSP90 complex, which mediated heat-induced EMT and metastasis in HCC cells. Tan et al. (43) showed that sublethal heat treatment increased the expression of cancer stem cell markers and markers of metastasis and promoted the ability of HCC cells to migrate after RFA. They also found that blockade of VEGFR1 could reduce heat-induced enhancement of migration and stemness. Kong et al. (44) found that ATPase inhibitory factor 1 (IF1) promoted EMT and angiogenesis in HCC after inadequate RFA, that this ability was markedly inhibited after IF1 knockdown, and that the sensitivity of HCC cells to sorafenib was attenuated after RFA *via* the nuclear factor kappa-B signaling pathway. Zeng et al. (45) reported that downregulation of lncRNA FUNDC2P4 promoted EMT, leading to proliferation, invasion, and migration of tumor cells by reducing expression of E-cadherin in residual HCC after RFA, which suggests that FUNDC2P4 may have value for prevention and treatment of recurrent HCC. Zhou et al. (46) demonstrated that insufficient ablation at a low temperature induced EMT and promoted tumor aggressiveness that was mediated by the IL-6/STAT3/Snail pathway. Targeting EMT could suppress tumor progression in HCC after RFA. Li et al. (47) showed that the upregulation of Nedd4 in HCC insufficient ablation tissues was induced by METTL14-mediated N6-methyladenosine modification after sublethal heat treatment. Besides, Nedd4 enhanced TGF-β/smad/EMT signal transduction by directly binding to TGFBR1 and forming K27-linked ubiquitin at Lysine 391 mediated HCC progression. Knockdown of Nedd4 inhibited HCC metastasis and growth *in vitro* and *in vivo*.

5 Tumor microenvironment

The tumor microenvironment in HCC is a complex and spatially structured mixture of hepatic non-parenchymal resident cells, tumor cells, immune cells, and tumor-associated fibroblasts. All these cell populations interact in a dynamic manner through cell-cell contact and release or recognition of cytokines and other soluble factors. This complex interplay between cells has a substantial influence on tumor immune evasion (48).

5.1 Abnormal vasculature

Rapid development of new vascular networks is required in tumors in order to support a high cell proliferation rate. These networks are different from those of normal blood vessels and are characterized by distorted and chaotic branches, heterogeneity of the vascular lumen, incomplete pericyte coverage, an abnormal basement membrane, increased vascular permeability, hypoxia, and increased tissue hydraulic pressure (49), which are crucial for metastasis and escape of cancer cells (50). Many studies have shown that RFA promotes angiogenesis in residual liver cancer tissue and worsens its abnormal vasculature.

5.1.1 Tumor-associated endothelial cells

Tumor-associated endothelial cells (TAECs) form the inner layer of tumor blood vessels and are an important part of the tumor microenvironment. Unlike normal endothelial cells, TAECs show morphological and phenotypic abnormalities at the cellular and molecular levels. Furthermore, angiogenic ability and drug resistance have been shown to be significantly higher in TAECs than in normal endothelial cells (51). TAECs are constituents of blood vessels that provide oxygen and nutrients for tumor cells and act as gatekeepers that allow these cells to escape and enter the circulation (52). An imbalance of tumor endothelial cells leads to loss of normal vascular barrier function and provides a channel for metastasis of tumor cells (53). Kong et al. identified significant enhancement of the migration and tube formation ability of TAECs after RFA, which may play a key role in the rapid growth of residual HCC. TAECs could also increase the invasive ability of HCC cells by secreting a variety of cytokines, including IL-8, IL-6, monocyte chemoattractant protein-1, and Gro-α. Furthermore, expression of E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 was found to be upregulated in TAECs after insufficient RFA, suggesting that upregulation of adhesion molecules may be one of the mechanisms of the enhanced adhesion between TAECs and HCC cells. Angiogenic capability and drug resistance was also found to be higher in TAECs than in normal endothelial cells (54).

5.1.2 Angiogenesis

Angiogenesis is the term used to describe the formation of new blood vessels in already existing vasculature. This process is the result of the synergistic action of tumor cells and the tumor stroma and is a prerequisite for metastasis (55). Folkman first proposed that tumor growth and metastasis depend on angiogenesis in 1971 (56). The mechanisms that drive angiogenesis are complex and have an important role in progression of HCC after insufficient RFA. VEGF plays an important part in angiogenesis; its most important member is VEGFA, which can directly stimulate the movement, proliferation, and division of vascular endothelial cells and increase the permeability of the microvasculature. Ke et al.

(22) found that inadequate RFA caused by a low target temperature resulted in a significant increase in expression of VEGF in residual tumor tissue and promoted metastasis of liver cancer. Liu et al. (57) also showed that RFA promoted growth of residual HCC by increasing expression of VEGF *via* activation of CaMKII-induced ERK. Ahmed et al. (58) found that RFA of normal liver tissue stimulated tumor growth in distant subcutaneous tissue, which was mediated *via* the hepatocyte growth factor/c-Met pathway and activation of VEGF and that this process could be suppressed by inhibition of VEGF.

Tumors with a high cell proliferation rate and undergoing active growth have a significantly reduced oxygen supply, especially cells in the core of the tumor, and activation of HIF-1 promotes the release of more pro-angiogenic factors, especially VEGFA, from tumor cells and stromal cells (59). Kong et al. (60) found that RFA could promote angiogenesis in residual HCC *via* HIF-1a/VEGFA and that the HIF-1a inhibitor YC-1 reversed this process. Xu et al. (61) demonstrated that hypoxia and hypoxia-driven angiogenesis have an important role in the recurrence of HCC after RFA and that sorafenib is an effective inhibitor of the HIF-1a/VEGFA pathway.

5.1.3 Vascular permeability

Increased vascular permeability results mainly from the loss of connexin between endothelial cells and causes destruction of the integrity of the vascular barrier, which in turn affects the ability of tumor cells to cross the vascular barrier. Endothelial cells are connected by connective proteins, including adhesion connexins, such as VE-cadherin and catenins, and by tight junctions, such as ZO-1 and claudin-5. Studies have confirmed that loss of endothelial intercellular connexin can promote permeability of the tumor vasculature and metastasis (62). Kong et al. (63) found that ICAM-1 induces aggregation and activation of platelets, increases endothelial permeability *via* Ezrin/VE-cadherin, and promotes tumor migration across endothelial cells in HCC after insufficient RFA.

5.1.4 Vasculogenic mimicry

Vasculogenic mimicry (VM) is different from the classical tumor angiogenesis pathway, independent of endothelial cells, and involves hollow lumens composed of basement membrane and peripheral cancer cells (64). VM has been discovered to be a method of angiogenesis in many malignant tumors and provides a novel strategy for the clinical treatment of angiogenesis in tumors, which is related to the invasion, metastasis, and poor prognosis of HCC. Cancer stem cells and EMT participate in VM (65). Jia et al. (66) found that platelet lysates in patients with HCC after RFA can promote EMT and activation of Akt, ERK1/2 and Smad3 signals, further promoting tumor VM and metastasis of HCC after RFA. Kong et al. (44) also demonstrated that EMT participated in VM and promoted progression of HCC after insufficient RFA.

5.2 Extracellular matrix

The extracellular matrix (ECM) is a non-cellular three-dimensional macromolecular network composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins, and several other glycoproteins. The ECM not only provides a physical scaffold in which cells are embedded but also regulates many cellular processes, including growth, migration, differentiation, survival, homeostasis, and morphogenesis. Cells embedded in the ECM interact with this macromolecular network *via* their surface receptors, which include integrins, discoidin domain receptors, cell surface proteoglycans, and the hyaluronan receptor CD44 (67). During tumorigenesis, marked alterations take place in the ECM, leading to formation of a fibrotic stroma with increased stiffness, excessive deposition of ECM components, and release of proteolytic enzymes that result in abnormal ECM remodeling upon activation. These changes in the ECM further promote tumor progression and metastasis. The ECM has been identified to have an important role in the progression of residual cancer of HCC after RFA (68).

Zhang et al. (38) revealed that an increase in the ECM protein collagen I promotes progression of heat-exposed residual HCC cells, indicating the importance of collagen I in modulating residual HCC after incomplete heat treatment, and proposed that sorafenib could reverse collagen I-induced protumor effects. Zhang et al. (69) also showed that the increased matrix stiffness that occurs after RFA promoted proliferation, motility, and progression of heat-exposed residual HCC cells.

5.3 Tumor-associated macrophages

Macrophages play an important role in tumorigenesis. Regulation of the biological behavior of tumor cells by manipulation of the function of macrophages is a current focus in tumor research (70). Macrophages have strong plasticity, and their activated states and types have different effects on the biological behavior of tumors. Th1-activated macrophages (classical activation/M1-like) have anti-tumor activity and Th2-activated macrophages (bypass pathway/M2-like) are related to tumor growth and metastasis (71).

Tumor-associated macrophages (TAMs) are a major component of the tumor microenvironment and play pivotal

roles in progression of HCC. Many studies have indicated that tumor-associated macrophages promote initiation, angiogenesis, and metastasis of tumors and suppression of adaptive immunity by production of a large number of cytokines, chemokines, growth factors and matrix metalloprotease in the tumor microenvironment (72). Collettini et al. (73) identified a large number of macrophages around the RFA area, which suggested that tumor-associated macrophages participate in progression of HCC after RFA. Rozenblum et al. (74) found that RFA induced large concentrations of macrophages around the necrotic area and that blockade of either IL-6 or c-met significantly reduced the proliferation of hepatocytes, with blockade of IL-6 reducing accumulation of both macrophages and myofibroblasts in the vicinity of the area of coagulation necrosis. Kumar et al. observed an increase in markers of tissue inflammation in the periablational rim and serum after hepatic RFA, including increased production of cytokines and recruitment of inflammatory cells (including macrophages, myofibroblasts, T-cells, and natural killer cells). Increased activation of COX-2 after hepatic RFA contributes to infiltration of periablational macrophages and inflammation-mediated distant tumor growth, which can be successfully suppressed with a COX-2 inhibitor (75).

5.4 Carcinoma-associated fibroblasts

Carcinoma-associated fibroblasts (CAFs) constitute a substantial proportion of the non-neoplastic mesenchymal cell compartment in various human tumors. These fibroblasts are phenotypically converted from their progenitors *via* interactions with nearby cancer cells during the course of tumor progression. The resulting CAFs, in turn, support the growth and progression of carcinoma cells. These fibroblasts have a major influence on the hallmarks of carcinoma and promote malignancy by secretion of tumor-promoting growth factors, cytokines, and exosomes, and by remodeling of the ECM (76).

Kumar et al. (75) observed an increase in alpha-smooth muscle actin (αSMA)-positive activated myofibroblasts after RFA. However, periablational recruitment of activated myofibroblasts was lower after daily exposure to celecoxib following RFA than after RFA alone. Rozenblum et al. (74) found that RFA induced a large accumulation of activated myofibroblasts around the necrotic zone. In addition to the accumulation of myofibroblasts, RFA induced proliferation of hepatocytes in both the ablated lobe and an untreated lobe, and blockade of either IL-6 or c-met significantly reduced global proliferation of hepatocytes. These changes, which were mediated *via* IL-6- and/or c-met, could have accounted for a proportion of the local and distant tumor recurrences observed after treatment. Ahmed et al. (77) demonstrated that the increase in heat shock protein induced by RFA could promote tumor growth and progression. Ma et al. (78) also showed that the gain-of-function p53 protein could bind selectively to the chaperone protein heat shock protein 90 and be packaged into small extracellular vesicles, which could be transferred to fibroblasts.

5.5 Hepatic stellate cells

CAFs in the liver are mainly derived from hepatic stellate cells (HSCs) (79). HSCs are liver sinusoidal resident vitamin A-storing cells and are considered to be the most relevant profibrogenic cell type operating in chronic liver diseases. During the process of liver injury, these cells undergo phenotypic transformation from 'quiescent' cells into 'activated' cells, which are characterized by proliferation, contractility, increased synthesis and secretion of ECM, altered matrix protease activity, and pro-mitogenic cytokines (80, 81). CAFs are composed of both fibroblasts and α-SMA-positive myofibroblasts, which are the hallmark of activated fibroblasts. Expression of α-SMA has long been regarded as the most reliable marker for detection of activated fibroblast populations in CAFs (82).

Zhang et al. (31) found that activated HSCs promoted progression of residual HCC cells after sublethal heat treatment from autophagic survival to proliferation *via* hepatocyte growth factor/c-Met signaling. A combined treatment regimen that included inhibition of autophagy and c-Met signaling could suppress progression of residual HCC after incomplete thermal ablation. Furthermore, Zhang et al. (83) demonstrated that activated HSCs can promote the stemness traits of residual HCC cells after incomplete thermal ablation and that metformin may be able to reverse this process. Zhang et al. (84) also showed that activated HSCs promoted progression of heat–treated residual HCC by release of POSTN, which could be inhibited by calcipotriol. Calcipotriol plus cisplatin could be used to thwart the accelerated progression of residual HCC after suboptimal heat treatment.

5.6 Platelets

Current research suggests that platelets have an important role in tumorigenesis, contributing to inflammation, angiogenesis and metastatic dissemination of tumor cells (85). In HCC, platelet activation is also an important risk factor for a poor postoperative prognosis (86). There is also research indicating that antiplatelet therapy reduces the risk of recurrence after surgical resection and improves overall survival in patients with HCC associated with viral hepatitis B (87).

Platelets can interact with adhesion molecules on the surface of endothelial cells and regulate the barrier function of these cells by releasing vesicles (88), which is an important step in the process of distant metastasis, and can increase vascular

permeability. Kong et al. (63) found that ICAM-1 activates platelets in residual tumor tissue after RFA and promotes vascular permeability in TAECs *via* VE-cadherin and that anti-platelet and anti-ICAM-1 therapy could prevent progression of HCC after RFA. Furthermore, Jia et al. (66) compared the effect of platelet lysates in HCC cell lines before and after RFA and found that lysates obtained from patients after RFA of HCC could promote proliferation, migration, invasion, and VM of HCC cells. They also demonstrated that platelet lysates from patients who had undergone RFA accelerated metastasis of HCC cells to the lung.

In conclusion, as shown in Figure 3, the tumor microenvironment plays an important role in the tumor progression after IRFA of HCC. This provides a theoretical basis for the exploration of combined targeted therapy after RFA of HCC.

6 Strategies to prevent progression of HCC after RFA

Recent efforts have focused on multimodal management of HCC in which RFA is combined with other anti-cancer approaches to prevent progression of HCC after RFA.

6.1 Pathological complete ablation

In terms of pathology, HCC usually consists of the main tumor lesion, peritumoral microvascular invasion (MVI), and satellite lesions. In the late stage, portal vein tumor thrombus and extrahepatic metastasis can be found. Imaging can reveal the main tumor lesion and larger satellite lesions but not MVI and smaller satellite lesions. The extent of HCC that cannot be seen by imaging is usually much larger than that of the main lesion. Pathological complete ablation with no residual viable tumor cells requires complete ablation of all tumor tissue, including the main tumor lesion, peritumoral MVI, and satellite lesions, and is the most effective way of preventing disease progression (89).

In most cases, RFA completely ablates only the main HCC lesion, even if there is an ablation margin, what is obtained is usually the imaging complete ablation. The residual MVI and satellite lesion around the tumor will lead to tumor progression. The most effective way to achieve pathological complete ablation is to increase the ablation margin. Jiang et al. (90) demonstrated that the minimum ablation margin was significantly smaller for a tumor with local progression than for one without local progression. Li et al. (91) and Laimer et al. (92) also found that enlarging the ablation margin could significantly reduce the risk of tumor recurrence and improve the long-term survival rate. In a previous study, we demonstrated that repeated RFA with an ablation margin and transarterial chemoembolization improved the outcome in patients with large solitary HCCs measuring ≥10 cm (93). Moreover, our yet to be published current research demonstrates that long-term overall survival is not significantly different between anatomic resection and RFA with an ablation margin ≥1.0 cm in patients with a solitary HCC measuring ≤3 cm. Therefore, when performing RFA as a local ablation therapy to reduce the risk of local disease progression and improve the overall survival rate, we need to ablate not only the target tumor but also the apparently non-tumorous surrounding liver tissues, which could be harboring micrometastases and contain areas of microvascular invasion.

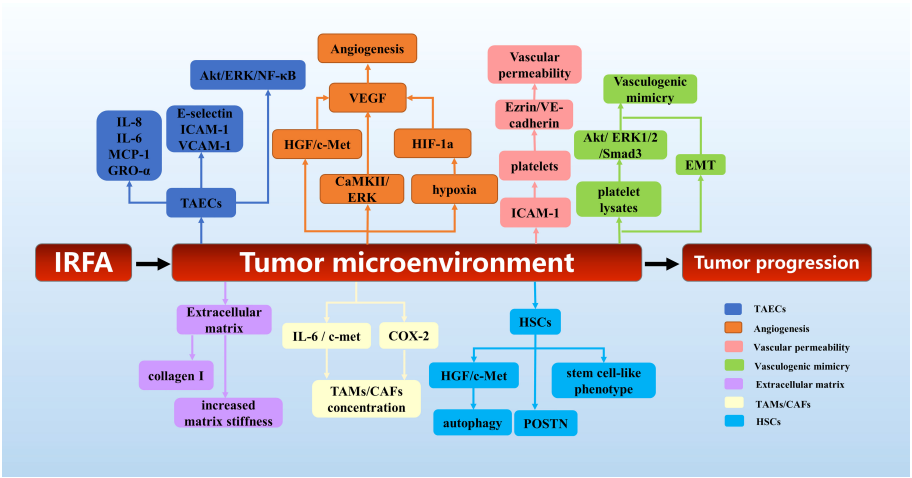


FIGURE 3
The mechanisms of tumor microenvironment in tumor progression after RFA of HCC.

6.2 RFA plus targeted therapy or immunotherapy

In the past few years, a number of promising targeted therapies have emerged for HCC. Sorafenib is the classic molecular targeted agent and is approved by the US Food and Drug Administration (FDA) for the treatment of advanced HCC. When used to treat HCC, sorafenib is reported to activate several signaling pathways, in particular those for Raf/MEK/ERK, the VEGF receptor, and platelet-derived growth factor receptor. Sorafenib can also be used to prevent progression of HCC after RFA (94). Dong et al. (37) demonstrated that sorafenib suppressed EMT of HCC cells after inadequate RFA and could prevent progression of HCC after RFA. Kong et al. (44) showed that sorafenib could inhibit and prevent migration of a colony of HCC cells after RFA, that overexpression of IF1 could attenuate the effect of sorafenib in these cells, and that inhibition of IF1 could improve the therapeutic effect of sorafenib. Xu et al. (61) also found that sorafenib blocked the HIF-1α/VEGFA pathway, inhibiting tumor invasiveness and inducing apoptosis in hepatoma cells after RFA. Furthermore, Mertens et al. (95) demonstrated that sorafenib promoted necrosis after RFA, decreasing tissue repair and preventing disease progression. This reduction in tissue repair is caused by inhibition of neovascularization and reduced cell proliferation. Bevacizumab is the first anti-angiogenesis agent approved by the FDA. It is a humanized monoclonal antibody against VEGFA. One study in a rat model demonstrated that bevacizumab is useful for preventing rapid progression of residual HCC following RFA (60).

More recently, immunotherapy has emerged as the standard first-line treatment for patients with advanced HCC. Combination of RFA with cellular immunotherapy has attracted interest because of its synergistic anticancer effects and is expected to eradicate residual disease after RFA and prevent disease recurrence (96). Ma et al. (97) found that autologous RetroNectin-activated killer cells and suggested that adaptive immunotherapy might help to prevent recurrence of HCC after RFA. Kitahara et al. (98) also demonstrated that intratumoral injection of OK432-stimulated dendritic cells could prevent progression of residual HCC after RFA.

Although some studies have demonstrated that targeted therapy or immunotherapy after RFA provides better outcomes than RFA alone, more multicenter randomized clinical trials in large samples are needed to confirm the benefits of RFA plus targeted therapy or immunotherapy. Moreover, how to develop individualized treatment strategies to obtain the best treatment effect needs to be taken into consideration in clinical research.

6.3 RFA combined with other agents

Metformin is recommended as first-line therapy for all patients with a new diagnosis of type 2 diabetes mellitus. There are some epidemiologic data highlighting the positive effects of metformin on the incidence of cancer and its mortality. Metformin also appears to hold promise as a treatment for HCC. In one study, metformin was found to inhibit cell proliferation, invasion, and angiogenesis and to induce apoptosis in HCC (99). Zhang et al. (23) also found that metformin inhibited the growth of HCC cells after insufficient RFA and suggested that it could be used to prevent progression of HCC after RFA.

Arsenic trioxide (ATO) has been approved by the FDA as first-line treatment for acute promyelocytic leukemia (100). Recent *in vitro* studies have demonstrated that ATO can suppress HCC cells *via* various mechanisms, including suppression of proliferation, slowing invasion and migration, as well as reversing multidrug resistance (101–103). These effects suggest that ATO may be able to eradicate residual tumor cells. Dong et al. (104) demonstrated that ATO blocked the paracrine signaling of Ang-1 and Ang-2 by inhibiting p-Akt/Hif-1a and further suppressed angiogenesis of HCC after insufficient RFA. Chen et al. (105)also found that extensive angiogenesis after RFA could augment the enhanced permeability and retention effect and increase the enrichment of ATO-loaded ZIF-8 nanoparticles, which markedly inhibited residual tumor progression.

7 Conclusion

Various factors contribute to progression of residual HCC after RFA. Current research on the mechanisms of disease progression after RFA for HCC is mainly focused on changes in the biological behavior of tumor cells and remodeling of the tumor microenvironment. A number of studies performed in the clinical practice setting have confirmed that multimodal therapies that include RFA can indeed improve the outlook for patients with HCC. Further efforts are needed to optimize the protocol for each of the combination therapies and to establish the best combination strategy to prevent progression of HCC after RFA.

Author contributions

JK and WS designed the manuscript. SLW, ZL, CYY, SD, JG, SK, RZ, SH, SHW, LX, CY performed literature research. SLW, ZL, CY drafted the article. JK and WS revised the article. All authors have read and approved the final article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Systemic therapies in hepatocellular carcinoma: Existing and emerging biomarkers for treatment response

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy and the third most common cause of cancer-related death worldwide. Due to asymptomatic patients in the early stage, most patients are diagnosed at an advanced stage and lose the opportunity for radical resection. In addition, for patients who underwent procedures with curative intent for early-stage HCC, up to 70% of patients may have disease recurrence within 5 years. With the advent of an increasing number of systemic therapy medications, we now have more options for the treatment of HCC. However, data from clinical studies show that with different combinations of regimens, the objective response rate is approximately 40%, and most patients will not respond to treatment. In this setting, biomarkers for predicting treatment response are of great significance for precise treatment, reducing drug side effects and saving medical resources. In this review, we summarized the existing and emerging biomarkers in the literature, with special emphasis on the pathways and mechanism underlying the prediction value of those biomarkers for systemic treatment response.

KEYWORDS

hepatocellular carcinoma, predictive biomarker, systemic treatment, molecular targeted therapy, immunotherapy

1 Introduction

Liver cancer is the third most common death-related malignant tumor in the world, and China bears the brunt of it with the highest number of deaths annually (1). Hepatocellular carcinoma (HCC) represents the most frequent histologic type of primary liver cancer, accounting for 75%-85% of cases (1). Due to the early

asymptomatic period, most patients are diagnosed at an advanced stage. Even for early-stage HCC patients, the recurrence rate after surgical resection remains high (2, 3).

In recent years, a growing number of studies have focused on the systemic treatment of HCC. Targeted therapy and immunotherapy have played an important role in the combined treatment of HCC. Tyrosine kinase inhibitors (TKIs) targeting pathways involved in the amplification and proliferation of tumors (4). Since sorafenib was approved as the first molecular targeted agents (MTAs) for the treatment of advanced HCC in 2007 (5), molecular targeted therapy has made rapid progress. With a growing number of clinical studies being carried out, sorafenib and lenvatinib have been approved as first-line MTAs, and regorafenib, cabozantinib, anlotinib and ramucirumab have been approved as second-line MTAs. The general mechanism of immunotherapy is to enhance anticancer immunity in the tumor microenvironment by blocking the negative feedback pathway of the immune system (6). Programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) are major molecules involved in suppressing the immune response (6, 7). Although studies have demonstrated the effectiveness of systemic therapy in improving the prognosis of HCC patients (8), its limited response rates are still a bothersome issue to be solved. By monotherapy, the response rate to sorafenib is less than 5% (9), and the response rate to immunotherapy is less than 20% (10, 11). A low response rate prevents the majority of HCC patients from receiving treatment benefits. Therefore, biomarkers that can aid in the selection of HCC patients who respond to systemic therapy are critical. In this review, we summarized potential biomarkers of response to systemic therapy in HCC.

2 Clinical factors

2.1 Aetiological factors

As a multicausal disease, the causes of HCC include hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, chronic alcohol intake, and nonalcoholic steatohepatitis. Different etiologies represent different tumorigenesis mechanisms and may have different susceptibilities to antiangiogenic drugs. A recent retrospective study performed by Tomonari et al. showed that in HCC patients treated with lenvatinib, the objective response rate (ORR) was higher in the nonviral group than in the viral group, although the difference was not significant. While the progression-free survival (PFS) and overall survival (OS) was significantly longer in the nonviral group than the viral group, suggesting that nonviral status might serve as a biomarker for lenvatinib treatment (12). The authors held that fibroblast growth factor 19 - fibroblast growth factor receptor 4 (FGF19-FGFR4) pathway, which was target of lenvatinib, were involved in the tumorigenesis of non-alcoholic steatohepatitis- and alcohol-associated HCC and consequently, these HCCs responded better to lenvatinib. Moreover, in a meta-analysis, Shao et al. reported that among sorafenib-treated HCC patients, both HCV-positive and HCV-negative patients had a significantly lower risk of death than controls (13). In addition, the benefit from sorafenib in HCV-positive patients was significantly greater than that in HCV-negative patients (Hazard ratios were 0.65 versus 0.87). And Kolamunnage-Dona et al. suggested that sorafenib was associated with reducing tumor growth rate and deterioration of liver function of HCV-induced HCC patients (14). The underlying mechanism might be as follow. The upregulation of Raf and downstream signaling could be induced by the transcription regulation of HCV core (15). And Raf signaling pathway represents one of sorafenib targets. Moreover, miRNA-dependent modulation of Mcl-1 by HCV protein enhances sorafenib sensitivity (16). These results suggested that HCV-positive patients may have a better response to sorafenib treatment. These studies indicated that nonviral-associated HCC or HCV-associated HCC responded better to first-line TKIs. However, HBV infection represents the major etiology of HCC patients, and how to improve the response rate of HBV-associated HCC patients to sorafenib and lenvatinib treatment still needs further investigation.

2.2 Lung metastasis

The lung is the predominant location for extrahepatic metastasis of HCC, accounting for more than 40% of HCC (17–19). Studies have been conducted to investigate the response to systemic therapy of HCC with lung metastasis. Yau et al. performed a phase II open-label trial of sorafenib monotherapy treating advanced HCC in an Asian population with prevalent hepatitis B. They found that patients with lung metastases had a worse clinical benefit than those without lung metastases. The authors speculated that this could be due to the distant metastasis of HCC itself being a poor prognostic factor (20). In another retrospective study, sorafenib responders had a higher rate of lung metastases than those who did not respond to sorafenib, although the difference was not statistically significant. However, considering the number of lung metastases, the incidence of multiple lung metastases ($n \geq 5$) in responders was significantly higher than that in non-responders (21). An in-depth study of HCC molecular subtyping has provided clues to this phenomenon, reporting that macrotrabecular-massive HCC, characterized by aggressiveness with both angiopoietin 2 (Ang-2) and vascular endothelial growth factor A (VEGFA) overexpression, with a hallmark feature of angiogenesis, was the most common subtype with a high potential for metastasis (22) (23). And the angiogenesis could be promoted by VEGFA through Raf/mitogen-activated protein kinase pathway, which is one of the major target of sorafenib.

On the other hand, studies on HCC immunotherapy have reported consistently favorable results for lung metastasis compared with the primary tumor. Lu et al. reported that intrahepatic lesions of HCC were less responsive to immune checkpoint inhibitors (ICIs) than extrahepatic lesions, with lung metastases most positively responding to ICIs (24). The distinct tumor microenvironment between the liver and the lung may underlie the difference in treatment response. The lung tumor microenvironment is reported to be richer in immune cells than other organs (25, 26). In contrast, immunosuppressive cells in the liver can contribute to the immunosuppressive microenvironment of the organ (27). Moreover, tumor volume is another factor affecting the response to immunotherapy. Huang et al. reported that, small HCCs had more immune cell infiltration than large HCCs, including CD8+ T cells, M1 macrophages, and monocytes, and small lesions had better sensitivity to ICIs than large lesions (28). As lung metastasis, in most cases, is relatively smaller than primary liver tumors, this may explain the difference in ICI therapy response between the primary and lung metastases of HCC.

2.3 Adverse events

For molecular targeting therapy, AEs mainly manifest as skin toxicity, digestive system reactions, and hypertension. Vincenzi et al. retrospectively analyzed the association between skin toxicity (rash and hand-foot skin reaction (HFSR)), disease control rate (DCR), and time to progression (TTP) in HCC patients receiving sorafenib and found that for patients with skin toxicity during treatment, both DCR and TTP were better than those without (29). A meta-analysis by Wang et al. found that patients who developed HFSR during sorafenib treatment had better TTP than those who did not (30). In addition, studies further investigated the predictive value of different HFSR grades for the efficacy of sorafenib and found that HFSR grade ≥ 2 was the most favorable predictor of response (31–33).

The digestive system AEs mainly manifested as diarrhea. In a retrospective study, Cho et al. found that the presence of HFSR and diarrhea were correlated with a prolonged TTP for HCC patients receiving sorafenib (34). Likewise, combining patients with partial response (PR) and stable disease (SD), HFSR and diarrhea were found to be predictors of sorafenib response (35).

Hypertension is also a common side effect of molecular targeted therapy. Considering the similarity between the mechanism of hypertension and the antitumor mechanism of sorafenib, Van Leeuwen et al. believed that the development of hypertension is a pharmacodynamic marker of treatment efficacy (36). Yang et al. reported the superiority of TTP in HCC patients with hypertension over those without hypertension during apatinib treatment (37). In addition, Lee et al. suggested that HFSR, diarrhea, and hypertension have predictive value in response to sorafenib, and with the increase in the number of AEs, TTP and OS were improved (38).

Immune-related adverse events (irAEs) have also been proposed as a predictive biomarker for immunotherapy response. Lu et al. showed that HCC patients with irAEs (mainly rash) had a significantly higher tumor response rate and DCR than those without irAEs when treated with anti-PD-1 antibodies (39).

The TKI-associated AEs were reported to be probably induced by the inhibition of vascular endothelial growth factor receptor and platelet-derived growth factor receptor (40, 41). As for irAEs, the underlying mechanism is still being explored. The cross-reactivity of immune response between tumors and normal tissue might provide us some clues (42). Besides, Hinrichs et al. proposed that healthy tissue might have molecular mimicry expressed, which are antigens identical to tumor antigens. When treated with ICIs, those healthy tissue may have immune response similar to that of tumor (43). Therefore, both TKI-associated AEs and irAEs could be considered as external manifestation of agents effect. Given that these AEs are potentially predictive biomarkers for systemic therapy response, it is important to encourage patients to adhere to their medication while managing AEs. Table 1 summarized the clinical factors predictive of response to systemic treatment for HCC.

3 Blood biomarkers

3.1 Tumor markers

3.1.1 Alpha-fetoprotein and Des-γ-carboxyprothrombin

Approximately 70% of HCC patients have elevated baseline levels of AFP. At present, it is mainly used as a diagnostic serum marker of primary liver cancer in clinical practice. Shao et al. studied the predictive function of early AFP response (defined as a greater than 20% decrease in AFP from baseline within four weeks of treatment initiation) for the treatment of antiangiogenic agents (44). The results showed that the early AFP responder group had a better ORR and DCR than non-AFP responders. Similar results have also been yielded by other studies (45–47). These studies revealed that early AFP changes during sorafenib treatment have the potential to help select HCC patients who might benefit from sorafenib. And AFP response phenomenon could be due to the killing of HCC tumor cells during treatment. However, AFP responses are only detected during treatment; for those without AFP responses, this may lead to a waste of medical resources and missed opportunities for treatment.

DCP is also a tumor marker for HCC diagnosis and prognosis (48–50). In a retrospective study, Ueshima et al. investigated the predictive function of DCP and found that after 2 weeks of sorafenib treatment, patients with DCP levels ≥2-fold higher than before treatment had significantly longer TTP than those

TABLE 1 Clinical Predictive factors for the systemic treatment of hepatocellular carcinoma.

Clinical factors	Reference	Hazard ratio (95% confidence interval)		Positive or Negative
		PFS, TTP or PD	OS	
Nonviral status	Tomonari et al. (12)	0.324 (0.174–0.602)	0.277 (0.116-0.662)	Positive for lenvatinib
HCV	Shao et al. (13)	0.65 (0.53–0.80) versus 0.87 (0.79–0.96) of HCV+ versus HCV- for OS		Positive for sorafenib
Kolamunnage-Dona et al. (14)		NA	NA	
Lung metastasis	Arao et al. (21)	NA	NA	Positive for sorafenib
	Lu et al. (24)	NA	NA	Positive for ICIs
(immune related) Adverse events				
Skin toxicity	Vincenzi et al. (29)	0.412 (0.176 – 0.820)	NA	Positive for sorafenib
HFSR	Wang et al. (30)	0.41 (0.28-0.6)	0.45 (0.36-0.55)	Positive for sorafenib
Skin toxicity	Shomura et al. (31)	NA	0.267 (0.102-0.701)	Positive for sorafenib
HSFR	Wang et al. (32)	0.74 (0.58-0.96)	0.53 (0.443-0.67)	Positive for sorafenib
	Cho et al. (34)	HSFR:0.4 (0.19-0.82)	0.4 (0.24-0.67)	Positive for sorafenib
		Diarrhea:0.34(0.15-0.74)	0.52(0.31-0.88)	
Hypertension	Yang et al. (37)	0.563 (0.413–0.768)	0.520 (0.349–0.775)	Positive for apatinib
	Lu et al. (39)	0.22 (0.09-0.57)	NA	Positive for PD-1 blockade

PFS, Progression-free survival; TTP, Time to progression; PD, Progressive disease; OS, Overall survival; HCV, Hepatitis C virus; ICIs, Immune checkpoint inhibitors; PD-1, Programmed death-1; NA, Not available.

without DCP elevation (51). This finding was explained by the fact that hypoxia induced by sorafenib contributes to the production of DCP; in this setting, an early increase in DCP could reflect the efficacy of sorafenib (51).

The predictive role of AFP and DCP has also been investigated in immunotherapies (52–56). As with TKI treatment, early AFP reduction after treatment initiation was also found to be correlated with objective response to immunotherapy. In contrast, reductions in DCP were associated with an objective response to immunotherapy treatment (55), indicating that posttreatment changes in DCP may have opposite predictive values when treated with TKIs or immunotherapy. This may limit its predictive power in the situation of combining TKI treatment and immunotherapy. With the predictive role of AFP and DCP being elucidated, these two tumor markers may not only contribute to the diagnosis of HCC but also help to select appropriate remedies for HCC patients.

3.2 Interleukin-6/interleukin-8

IL-6 and IL-8 are key inflammatory response mediators that promote angiogenesis. Preclinical studies have confirmed their promotion of sorafenib resistance through different mechanisms (57–59). The IL-6/signal transducer and activator of transcription 3 (STAT3) signaling pathway is involved in the angiogenesis and proliferation of HCC and STAT3 is one of the targets of sorafenib (Figure 1). Besides, as a cytokine derived from macrophage, IL-8 induces tumor angiogenesis and recruits immunosuppressive cells to tumors. In a nonrandomized phase II study, Boige et al. found that in patients with HCC treated with bevacizumab, low IL-8 levels at any time point were associated with better DCR (60). More recently, Öcal et al. showed that lower levels of IL-6 and IL-8 were associated with objective responses to sorafenib treatment (61). Moreover, there are also studies exploring the relationship between the changes in IL-6/IL-8 and the efficacy during treatment. Shao et al. found that patients with PD after receiving sorafenib plus tegafur had significantly higher posttreatment IL-6 and IL-8 levels than control patients (62).

IL-6 exerts both positive and negative effects on tumor immunity. It induces T-cell infiltration but also suppresses T cells by recruiting myeloid-derived suppressor cells (63–65). Myojin et al. reported that high serum IL-6 and interferon alpha were significantly associated with PD after atezolizumab plus bevacizumab treatment (66). Whether IL-6 can promote disease progression after or under immunotherapy or whether the negative immune effect of IL-6 can override the positive immune effect and appear to be related to the disease progression response after immunotherapy is worthy of further investigation.

3.3 Markers related to angiogenesis and proliferation

Because of the antiproliferative and antiangiogenic effects of TKIs, it is reasonable to assume that angiogenesis or proliferation-related biomarkers may to some extent reflect the therapeutic response of TKIs.

3.3.1 Vascular endothelial growth factors

Sorafenib blocks tumor angiogenesis by targeting vascular endothelial growth factor receptor-2/-3 (VEGFR-2/-3) and

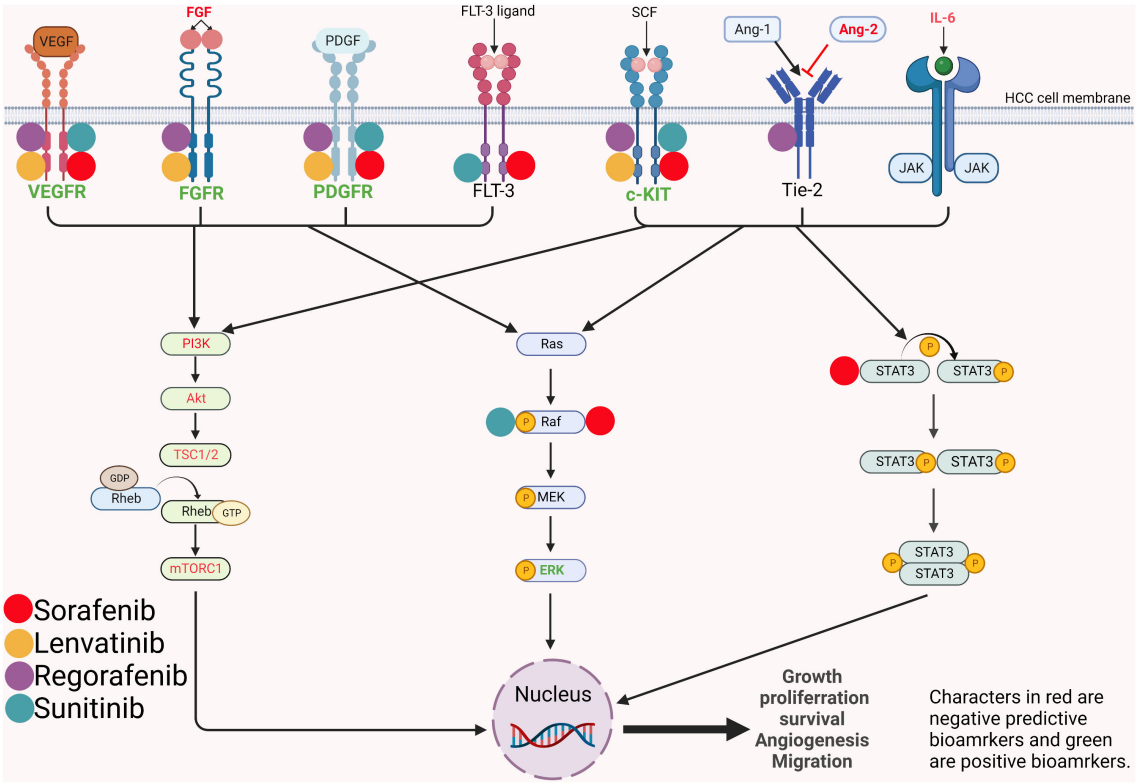


FIGURE 1
Mechanism and pathways involved in the angiogenesis and proliferation of hepatocellular carcinoma and potential predictive biomarkers. VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor; PDFGR, platelet-derived growth factor receptor; FLT-3, FMS-like tyrosine kinase 3; SCF, stem cell factor; Ang, angiopoietin; Tie2, tyrosine-protein kinase receptor; IL-6, interleukin-6; JAK, janus kinase; PI3K, phosphatidylinositol 3-kinase; Akt, protein kinase B; TSC, tuberous sclerosis complex subunit; Rheb, ras homolog enriched in brain; mTORC1, mammalian target of rapamycin complex 1; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal–regulated kinase; STAT, signal transducer and activator of transcription.

platelet-derived growth factor receptor-beta (PDGFR-beta) tyrosine kinases (67) (Figure 1). In a retrospective study of 30 patients (68), Miyahara et al. found that baseline cytokine levels, including VEGFA, were significantly higher in PD patients than in non-PD patients when treated with sorafenib. There was a trend of worse treatment response with more elevated baseline cytokines. Zhu et al. and Faivre et al. demonstrated that high serum VEGFC was associated with longer TTP and higher DCR under sorafenib and sunitinib treatment (69) (70). Different results suggest that VEGFA and VEGFC might have opposite predictive functions for MTAs. The divergent predictive value of VEGFA and VEGFC might be explained by their different receptors. VEGFA promotes the carcinogenesis of HCC through VEGFR-1 and VEGFR-2. While the receptors of VEGFC are VEGFR-2 and VEGFR-3, which are major targets of sorafenib and sunitinib.

3.3.2 Angiopoietin-2 and FGF 19/FGF 23

Normally, Ang-2 binds to its receptor Tie2 and promotes the proliferation of vascular endothelial cells to form new blood vessels in the presence of VEGF (71). In disease states, elevated Ang-2 is associated with tumor cell proliferation and may lead to vascular leakage and metastasis (72) (Figure 1). Ang-2 has been found to be a negative predictive factor for TKI treatment. Miyahara et al (68) described that Ang-2 had a similar predictive function as VEGFA and that Ang-2 levels were significantly elevated in patients with PD prior to sorafenib treatment. Similarly, recently, Yang et al. reported that higher baseline Ang-2 levels were significantly associated with a nonobjective response in HCC patients treated with lenvatinib (73).

FGFs are also involved in the pathogenesis of HCC. Lenvatinib is a multiple kinase inhibitor targeting VEGF receptor 1–3 (VEGFR1-3), PDGFR-α, c-Kit and FGF receptor 1-4 (FGFR1-4) (Figure 1). In a study evaluating multiple biomarkers, Shigesawa et al. found that in lenvatinib-treated patients, FGF 19 levels in the objective response group were significantly lower than those in the nonobjective response group (74).

Based on the REFLECT study, Finn et al. observed that increases in FGF19 and FGF23 and decreases in Ang-2 were associated with tumor response in lenvatinib-treated patients but not in sorafenib-treated patients (75). Considering that FGF19 is a ligand of FGFR4, by inhibiting FGFRs, lenvatinib treatment could lead to an increase in FGF19 through inverse feedback. Moreover, lenvatinib treatment reduced Ang-2 levels through its anti-VEGF function. Therefore, elevated FGF19 and decreased Ang-2 could represent the effect of lenvatinib and may guide therapeutic decisions in the future.

3.4 Circulating tumor cells and circulating tumor DNA/circulating free DNA

Since HCC diagnosis is based on radiological features, HCC tumor specimens are often not available prior to treatment. In recent years, liquid biopsy has been proposed as a new tumor biopsy technique. It is able to obtain CTCs and nucleic acids released by tumor cells.

CTCs are tumor cells isolated into the blood from primary or metastatic tumors. Tumor cells expressing PD-L1 are reported to respond better to PD-1/PD-L1 inhibitors (11). Winograd et al. found that of 10 HCC patients treated with PD-1 inhibitors, 6 had baseline PD-L1+ CTCs, and 5 of these 6 patients responded to treatment (76). The remaining 4 patients without baseline PD-L1+ CTCs showed no response.

By using liquid biopsy, Nakatsuka et al. found that HCC patients who both responded and non-responded to MTAs had an increase in cfDNA after treatment, but those who respond had a significantly greater increase in cfDNA (77). This is mainly because tumor cells killed by the treatment release tumor DNA into the plasma, resulting in an increase in cfDNA. Next-generation sequencing also confirmed tumor gene mutations in cfDNA after treatment, supporting the claim that necrotic tumor cells release DNA into plasma.

Hsu et al. investigated whether ctDNA could monitor the efficacy of atezolizumab combined with bevacizumab in HCC patients (78). They found that patients whose ctDNA level became negative during treatment had a higher response rate than patients whose ctDNA was positive.

Clinically, evaluating tumor response mainly depends on Response Evaluation Criteria in Solid Tumors (RECIST) or modified RECIST (mRECIST) criteria by radiology, but tumor size may not present evident change during treatment. Thus, the changes in ctDNA and cfDNA may better assist in screening patients suitable for systemic treatment. The aforementioned circulating predictive biomarkers were summarized in Table 2.

4 Imaging features of response prediction

As the most important diagnostic tool for HCC, imaging techniques were also studied to potentially have predictive power for treatment response.

4.1 Imaging features of TKIs response prediction

4.1.1 Dynamic contrast-enhanced ultrasound

DCE-US, by using intravenous injection of contrast agent Sonovue microbubble and vascular imaging software, is applied to accurately detect microvessles and tissue perfusion in tumors. Due to the antiangiogenic effects of TKIs, tumor vascularity is altered during MTA treatment. Therefore, DCE-US is an ideal tool to detect these changes.

In a prospective single center study, Frampas et al. found that the area under the time-intensity curve of contrast-enhanced ultrasound was significantly associated with targeted treatment response, a decrease in the area under the time-intensity curve of more than 40% at month 1 correlating to non-progression at month 2 treatment (79). Zocco et al. compared five DCE-US functional parameters (peak intensity, PI; time to PI, T_P; area under the curve, AUC; slope of wash in, P_w; mean transit time, MTT) between sorafenib responders (CR+PR+SD) and non-responders (PD) and found a strong correlation between three parameters (AUC, PI and P_w) on day 15 after treatment with tumor response (80).

4.1.2 Contrast-enhanced computed tomography

Because of the abundance of vascularity within HCC, the tumor could be obviously enhanced on CE-CT imaging. Colagrande et al. investigated the predictive role of the volume of enhancement of disease (VED, defined as volume lesion × arterial enhancement coefficient/volume lesion) on the efficacy of sorafenib and found that clinical benefit patients had a significantly higher rate of VED_{T0} (VED before treatment) > 70% than PD patients (81). Similarly, Nakamura et al. found that patients with pretreatment arterial perfusion (Pre-AP) of HCC on CE-CT higher than 71.7 mL/min/100 mL owned higher OS rate than those without. It made sense because those tumors that had a VED_{T0} > 70% or a Pre-AP > 71.7 mL/min/100 mL possessed a higher level of vascularization, and consequently were more vulnerable to anti-angiogenic therapy.

TABLE 2 Circulating predictive biomarkers for the systemic treatment of hepatocellular carcinoma.

Circulating biomarkers	Reference	Hazard ratio (95% confidence interval)		Cut-off	Positive or Negative
		PFS, TTP or PD	OS		
AFP response	Shao et al. (44)	0.307 (0.140-0.671)	0.356 (0.152-0.833)	AFP decrease by 20% within 4 weeks of treatment	Positive for antiangiogenic therapy
	Yau et al. (45)	0.31 (0.13-0.76)	0.3 (0.09-1.02)	AFP decrease by 20% after 6 weeks of treatment	Positive for sorafenib
	Shao et al. (52)	0.128(0.041-0.399)	0.089 (0.018-0.441)	AFP decrease by 20% within 4 weeks of treatment	Positive for ICIs
	Lee et al. (53)	NA	0.234 (0.096–0.569)	AFP decrease by 20% within 4 weeks of treatment	Positive for ICIs
	Sun et al. (55)	0.38 (0.23-0.61)	0.50 (0.32–0.80)	AFP reduction > 50%	Positive for PD-1 blockade
DCP	Ueshima et al. (51)	NA	NA	≥2-fold higher than pretreatment levels at 2 weeks	Negative for sorafenib
	Sun et al. (55)	0.60 (0.39–0.93)	0.54 (0.35–0.84)	AFP reduction > 50%	Positive for PD-1 blockade
Interleukin-6	Ocal (61)	NA	2.99 (1.22–7.3)	8.58 pg/mL	Negative for sorafenib
	Myojin (66)	2.785(1.216–6.380)	NA	4.77pg/L	Negative for Atezolizumab plus Bevacizumab
Interleukin-8	Boige et al. (61)	NA	2.19 (1.02–4.7)		Negative for sorafenib
VEGF-A	Miyahara et al. (68)	NA	NA	Mean value	Negative for sorafenib
	Zhu et al. (69)	NA	1.386 (1.119-1.715)	1162.4pg/L	Negative for sorafenib
VEGF-C	Zhu et al. (69)	0.633 (0.505-0.793)	0.829 (0.674-1.020)	906.9pg/L	Positive for sorafenib
Ang-2	Miyahara et al. (68)	2.51 (1.01–6.57)	NA	Mean value	Negative for sorafenib

PFS: Progression-free survival; TTP, Time to progression; PD, Progressive disease; OS, Overall survival; AFP, alpha-fetoprotein; DCP, Des-γ-carboxyprothrombin; VEGF, vascular endothelial growth factor; ICIs, Immune checkpoint inhibitors; PD-1, Programmed death-1; Ang-2, angiopoietin-2; NA, Not available.

4.1.3 Dynamic contrast-enhanced magnetic resonance imaging

DCE-MRI is a noninvasive method to measure tumor blood flow, vascular permeability, and interstitial and intravascular volume changes. The volume transfer constant (K^{trans}) on DCE-MRI, reflecting the permeability of vessels, is an endpoint for vascular response evaluation. Hsu et al. discovered that baseline K^{trans} was significantly higher in patients with PR or SD than in patients with PD when treated with sorafenib and metronomic tegafur/uracil (82). Moreover, further analysis showed specifically that patients with vascular response, defined as a 40% or greater decrease in K^{trans} after 14 days of treatment, had significantly higher rates of PR and SD than patients without. Higher baseline K^{trans} reflected richer vascularization within HCC, and greater decrease in K^{trans} after treatment implied better sorafenib effect on tumors. Both were reasonable predictive biomarkers for sorafenib response. In addition, the authors also found a correlation between vascular response and hypertension and HFSR, indicating that hypertension and HFSR may also correspond to the response to TKI treatment, which has been reported in many articles.

4.2 Imaging features of immunotherapy response prediction

Immune suppression plays an essential role in HCC progression. Immune suppression mainly results from the exclusion of infiltrating T cells and functional suppression of T cells, the latter usually caused by PD-1/PD-L1 expression on tumor cells or immune cells. Current studies investigating imaging biomarkers to predict immunotherapy response mainly focus on the prediction of T-cell infiltration and the expression of PD-1/PD-L1.

4.2.1 Conventional imaging

4.2.1.1 Contrast-enhanced MRI

In a retrospective study evaluating gadoxetic acid-enhanced MR imaging features on HCC infiltrating CD8 cells and PD-L1 expression, Sun et al. showed that irregular tumor margin (ITM) and peritumoral low signal intensity (PLSI) on hepatobiliary phase images were predictors for PD-L1 positivity, absence of an enhancing capsule (AEC) and PLSI were predictors for $CD8^+$ high density, and PLSI and ITM were predictors for both (83). In

addition, the combination of PLSI and ITM and the combination of PLSI and AEC were found to be correlated with the response to immunotherapy.

4.2.2 Novel imaging

4.2.2.1 Gd-EOB-DTPA-enhanced MRI

The activated Wnt/β-catenin signaling pathway is characterized by immune cells exclusion, decreased expression of PD-L1 and increased expression of organic anion transporting polypeptide 1B3 (OATP 1B3). Hepatocytes can take up Gd-EOB-DTPA through OATP 1B3 (84). Given that the Wnt/β-catenin signaling pathway was associated with immunotherapy resistance in HCC, Aoki et al. investigated the predictive role of the hepatobiliary phase of Gd-EOB-DTPA-enhanced MRI on HCC response to immunotherapy (85). The authors found that the ORR, DCR and TTP of hypointense nodules tended to be better than those of hyperintense nodules. It was because that hyperintense lesions were tumors with Wnt/β-catenin signaling activated and resistant to immunotherapy (Figure 2). Sasaki et al. had similar results when evaluating HCC patients treated with a combination of atezolizumab plus bevacizumab (86).

4.2.2.2 Radiomics

Radiomics is a new concept proposed in recent years. It refers to the high-throughput extraction of a large number of imaging features that describe tumor and tumor microenvironment characteristics.

Based on the hypothesis that disparate phenotypes of tumors could be detected by high-dimensional imaging data, Liao et al. developed a radiomics-based score (Rad score) using seven imaging features of CE-CT in patients with HCC (87). The score was found to be associated with the percentage of tumor-

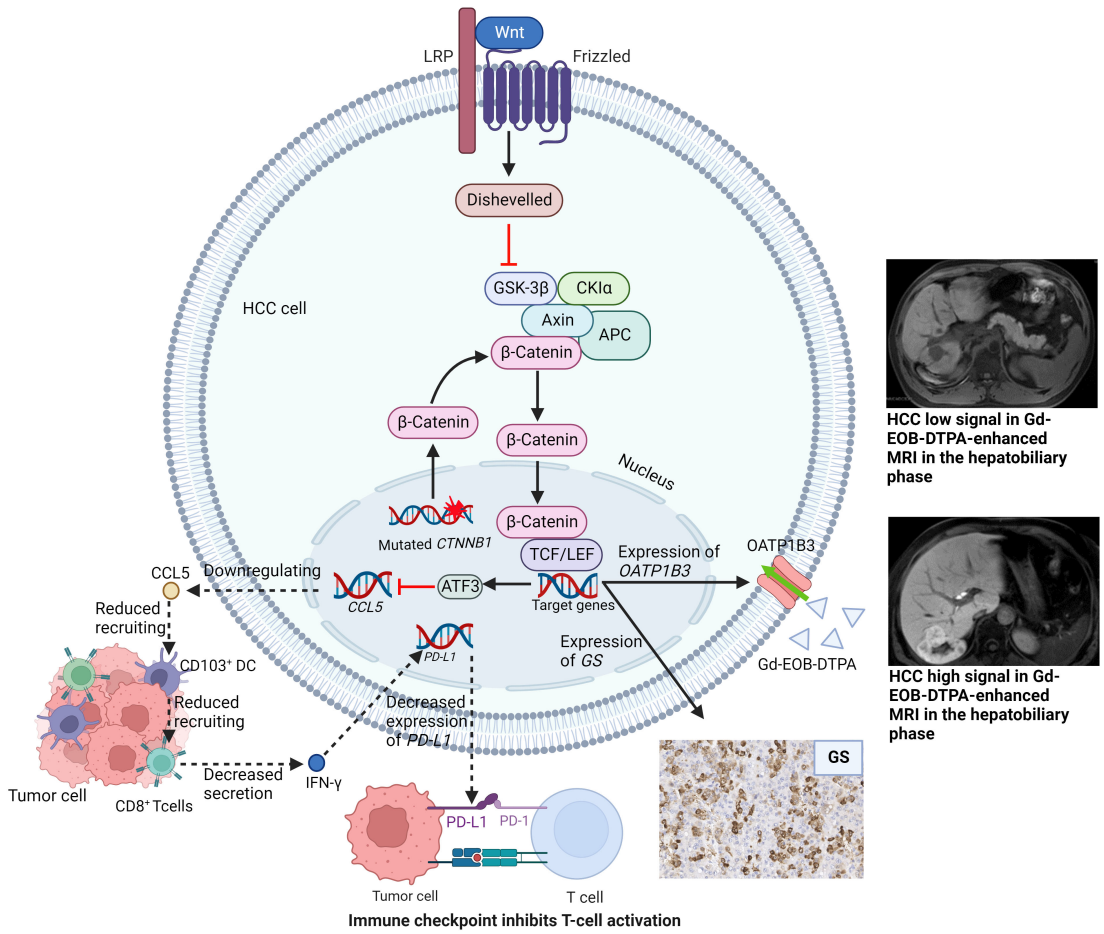


FIGURE 2
Wnt/β-catenin signaling pathway with related target genes activated and subsequent manifestations. LRP, lipoprotein Receptor-Related Proteins; GSK-3β, glycogen synthase kinase-3beta; CKIα, casein kinase I α; TCF/LEF, T-cell factor/lymphoid enhancer factor; ATF3, activating Transcription Factor; CCL5, C-C chemokine ligand 5; DC, dendritic cells; IFN-γ, interferon-γ; PD-1/PD-L1, Programmed death-1/programmed death-ligand 1; GS, glutamine synthetase; OATP1B3, organic anion transporting polypeptide 1B3.

infiltrating lymphocytes and PD-1/PD-L1 expression on tumor/immune cells.

Hectors et al. retrospectively analyzed radiomics features of HCC patients by MRI. The results turned out that radiomics features were distinctly related to immune cell markers(CD3, CD68 and PD-L1). Furthermore, an association between PD-1 mRNA and radiomics was also found (88).

These correlations between radiomics and immune cells/PD-1/PD-L1 indicated its potential role in predicting immunotherapy response of HCC patients. Nevertheless, it needs to be validated in the future. The report by Hectors et al. included patients without treatment before undergoing MRI and Liao et al. excluded patients treated before CT scan. To our knowledge, the radiologic and biological characteristics of HCC may be alterable during treatment. Patients who are deemed to be appropriate candidates for immunotherapy before treatment might not benefit from the cure after receiving a period of remedy. In this setting, the delta radiomics, which is capable of capture the quantitative changes of tumor radiomic features on treatment going (89), may help clinicians better screen out the proportion of patients suitable for treatment continuation. Similar clinical research has been conducted on rectal cancer and high-grade soft-tissue sarcoma (90, 91). However, to our knowledge, report about the predictive value of delta radiomics on HCC treated with TKIs or immunotherapy has not been published.

Imaging biomarkers serve as a noninvasive method to help select an appropriate HCC patient proportion sensitive to systemic therapies, which is convenient to perform and poses nearly no threat to patient safety. However, the remaining problems are that the results might be influenced by the different precisions of imaging devices and different radiologists in clinical practice. These problems may be solved with the development of artificial intelligence.

5 Tumor tissue biomarkers

Biomarkers from tumor tissue at different levels, including DNA, RNA, proteins and cells, from our perspective, are the most accurate predictors of treatment response. These biomarkers are able to directly reflect tumor characteristics and can help to screen liver cancer patients suitable for systemic therapy more accurately.

5.1 Proteins within tumor tissue

The mitogen-activated protein kinase (MAPK) signaling pathway, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38MAPK and ERK5, regulates the inflammatory response and participates in tumorigenesis and angiogenesis of HCC (92, 93).

In human HCC, the abnormal activation of ERK signaling could be frequently observed. The Raf/MAPK ERK kinase (MEK)/ERK pathway is one of the targets of sorafenib (Figure 1). Based on a phase II study of sorafenib in advanced HCC patients, Abou-Alfa et al. found that patients with higher pretreatment expression of phosphorylated ERK (pERK) within tumor cells had significantly higher TTP than patients with lower expression of pERK (94). Chen et al. had a consistent result with Abou-Alfa et al. that higher pERK and VEFGR expression was associated with increased TTP (95).

Yamauchi et al. observed that positive immunohistochemistry for fibroblast growth factor receptor (FGFR4) in biopsy samples before treatment was associated with a longer progression-free survival (2.5 *vs.* 5.5 months, P 5 0.01) and a favorable objective response rate treated with lenvatinib, but this association was not observed with blood soluble FGFR4 (96).

For PD-1/PD-L1 inhibitors, the predictive role of PD-L1 expression level was the most commonly investigated. Logically, patients with higher expression levels of PD-1/PD-L1 respond better to PD-L1 inhibitors, which has been reported in many types of cancers, such as lung cancers (97) and melanoma (98). The predictive role of PD-L1 has also been investigated in HCC.

In the KEYNOTE-224 clinical trial, Zhu et al. found that the objective response to pembrolizumab was associated with PD-L1 expression (11). In a randomized phase 2 trial, Qin et al. also showed that the ORR of camrelizumab was significantly higher in HCC patients with expression of PD-L1 ≥1% than in patients with PD-L1 <1% (99). However, patients with negative PD-L1 expression have also been reported to respond to ICIs (10, 100). Moreover, another remaining problem is whether the expression of PD-L1 on tumor cells or immune cells or on both cells represents the selection of sensitive patients to ICIs needs further investigation because the associations between response and PD-L1 expression on both cells have been reported (11, 99, 101–103).

In addition, combined immunotherapy has been proposed in recent years. The results from IMbrave150 study showed that atezolizumab plus bevacizumab therapy was superior to sorafenib monotherapy (104). And a phase IB study reported that combination of pembrolizumab and lenvatinib provided better efficacy than immunotherapy alone (105). These phenomena might be due to that VEGF and FGF suppressed interferon gamma secretion and T cell cytotoxicity, while upregulating the expression of PD-1 (106). Therefore, by targeting VEGF and FGF, the efficacy of immunotherapy was synergeticly enhanced. Additionally, Zhu et al. suggested that anti-VEGF agents might strengthen the antitumor activity of immunotherapy through targeting myeloid cell inflammation and inhibiting the angiogenesis within tumors (107). Hence, predictive biomarkers for combined immunotherapy are worthy of being investigated. Zhu et al. found that HCC with high expression of PD-L1, VEGFR and infiltrating CD8+ T cell had better benefit from atezolizumab plus bevacizumab therapy than

monotherapy (107), indicating that HCC patients with these features probably appropriate to combined immunotherapy.

5.2 Gene alterations

5.2.1 Molecular targeted therapy

Alterations in genetic levels are essential for the formation of tumor phenotypes and features observed by examinations or in the clinic. The difference in genetic alterations has greatly contributed to the heterogeneity of HCC. Assessment of response prediction for TKIs using genes has been performed by many researchers.

Arao et al. showed that the amplification of *FGF3/FGF4* was observed in three of ten HCC samples from patients who responded to sorafenib, while no amplification of *FGF3/FGF4* was found in 38 patients with SD or PD (21).

With the help of next-generation sequencing, Harding et al. found that HCC patients with activating mutations in the PI3K–mTOR pathway in tumors had significantly lower DCR and shorter PFS and OS after sorafenib treatment (108).

5.2.2 Immunotherapy

HCC can be divided into different molecular subtypes, each with its own genetic mutational signature and distinct phenotype. Calderaro et al. summarized the phenotypic and molecular features of HCC (109). With different mutated genes, HCC subtypes hold different activated signaling pathways and different immune cell infiltration. Thus, HCC subtypes may be capable of helping screen patients suitable for immunotherapy. Of all the subtypes, notably, CTNNB1-mutated HCC with activating WNT/β-catenin pathway has a well-differentiated tumor phenotype and lacks immune cell infiltration (Figure 2).

In a prospective study aimed at matching HCC patients to molecular targeted therapy and immunotherapy, Harding et al. proposed that patients with WNT/β-catenin pathway activation treated with ICIs had a lower DCR than patients without (108). This result might be explained by the lack of immune cell infiltration in *CTNNB1*-mutated HCC. Oversoe et al. showed that the integrated analysis of circulating tumor DNAs and DNAs in tumor tissue could improve the detection rate of *CTNNB1* mutation in HCC patients (110), indicating that the combined analysis of tumor tissue and blood might better select HCC patients suitable for immunotherapy.

5.3 Infiltrating immune cells in the tumor microenvironment

The antitumor effect of ICIs relies on immune cell infiltration. In the tumor microenvironment, the infiltration of immune cells has been shown to be predictive of the response to ICIs, especially CD8+ cytotoxic T cells. The CheckMate 040 trial found that in HCC patients treated with nivolumab, CD3+ T cells were associated with response (10). Kaseb et al. found that in HCC patients receiving combination therapy with nivolumab and ipilimumab, CD8+ cell infiltration was significantly positively associated with clinical response (111). Ng et al. found that patients with higher levels of CD38+ macrophage infiltration had a higher overall response rate when receiving immunotherapy than those with lower levels of CD38+ macrophages, which may be related to the secretion of IFN-γ

TABLE 3 Predictive biomarkers within tumors for the systemic treatment of hepatocellular carcinoma.

Tumor biomarkers	Reference	Hazard ratio (95% confidence interval)		Cut-off	Positive or Negative
		PFS, TTP or PD	OS		
Proteins					
pERK	Chen et al. (95)	1.504 (1.292-1.217)	NA	NA	Positive for sorafenib
VEGFR	Chen et al. (95)	0.284 (0.411-1.109)	NA	NA	Positive for sorafenib
FGFR4	Yamauchi et al. (96)	0.30 (0.13–0.69)	NA	NA	Positive for lenvatinib
PD-L1	Qin et al. (99)	NA	NA	NA	Positive for camrelizumab
Genes					
FGF3/FGF4 amplification	Arao et al. (21)	NA	NA	NA	Positive for sorafenib
CTNNB1	Harding et al. (108)	NA	NA	NA	Negative for immunotherapy
Infiltrating immune cells	Ng et al. (112)	0.384 (0.193 to 0.765)	0.463 (0.232 to 0.926)	5% positivity of total immune infiltrates being CD38+ cell	Positive for immunotherapy

PFS, Progression-free survival; TTP, Time to progression; PD, Progressive disease; OS, Overall survival; pERK, phosphorylated extracellular signal-regulated kinase; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; PD-L1, programmed death-ligand 1. NA, Not available.

by macrophages (112). Table 3 summarized predictive biomarkers within tumors.

6 Conclusion

The advent of systematic therapy offers a new management strategy for HCC. However, the reality is that only a small fraction of patients respond and improve survival with targeted therapy and immunotherapy. Predictive biomarkers for treatment response have been investigated, but until now, no biomarkers have been approved to guide treatment decisions. Clinical traits of patients, such as adverse events, are easily available. These features may represent an extrinsic manifestation of the drug's action and could aid in the selection of patients who respond to treatment at an early stage. Circulating biomarkers, including tumor markers, inflammatory markers, circulating DNAs and tumor cells, serve as noninvasive methods for response prediction. Biomarkers within tumor tissues represent the direct detection of the intrinsic characteristics of tumors. In our opinion, these biomarkers are the most valuable. However, due to the unrequired biopsy for the diagnosis of HCC, it is difficult for physicians and surgeons to obtain tumor tissue before treatment. Here, the necessity of HCC biopsy before treatment is advocated to better understand the intrinsic features of tumors and help guide therapy selection. Moreover, with the emergence of liquid biopsy, the combination of tumor tissue and circulating tumor cells may help us better understand the intrinsic features of tumors and better recognize different HCC subtypes. A single biomarker is insufficient for response prediction. For example, although studies have shown that PD-L1-positive patients respond better to PD-1/PD-L1 blockade, some PD-L1-negative patients respond to the treatment. Future research should also focus on predictive models or scores consisting of multiple biomarkers that are able to combine the predictive values of multiple factors. By this we can maximize the screening of suitable patients for systemic treatment. In clinical practice, patients receive a combination of targeted therapy and immunotherapy; thus, seeking biomarkers predictive of response to the combination therapy would be of more practical significance.

Author contributions

HW and KX contributed to the conception of the study; PH and HW search the database. PH made the figures and tables; PH, HW and JW wrote this paper; PH, HW and KX revised this paper. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Roles of intercellular cell adhesion molecule-1 (ICAM-1) in colorectal cancer: expression, functions, prognosis, tumorigenesis, polymorphisms and therapeutic implications

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Colorectal cancer (CRC) is a major global health problem and one of the major causes of cancer-related death worldwide. It is very important to understand the pathogenesis of CRC for early diagnosis, prevention strategies and identification of new therapeutic targets. Intercellular adhesion molecule-1 (ICAM-1, CD54) displays an important role in the the pathogenesis of CRC. It is a cell surface glycoprotein of the immunoglobulin (Ig) superfamily and plays an essential role in cell-cell, cell-extracellular matrix interaction, cell signaling and immune process. It is also expressed by tumor cells and modulates their functions, including apoptosis, cell motility, invasion and angiogenesis. The interaction between ICAM-1 and its ligand may facilitate adhesion of tumor cells to the vascular endothelium and subsequently in the promotion of metastasis. ICAM-1 expression determines malignant potential of cancer. In this review, we will discuss the expression, function, prognosis, tumorigenesis, polymorphisms and therapeutic implications of ICAM-1 in CRC.

KEYWORDS

ICAM-1, colorectal cancer, polymorphisms, prognosis, tumorigenesis

Introduction

Colorectal cancer (CRC) with a particularly high prevalence in China is a major health problem and one of the major causes of cancer-related death worldwide (1, 2). In recent years, both the morbidity and mortality of CRC have increased (1), thus it is very important to understand the pathogenesis of CRC for early diagnosis, prevention strategies and identification of new therapeutic targets. Some studies have focused on the identification of biomarkers in CRC, taking cell adhesion molecules (CAMs) as their subject.

CAMs refer to those cell surface structures that allow cells to adhere to each other and the extracellular matrix. They are critical regulators of cellular homeostasis and function, and play crucial roles in tumorigenesis, progression and metastasis (3, 4). Intercellular adhesion molecule-1(ICAM-1, CD54) is one of CAMs, which displays an important role in the the pathogenesis of CRC. In this review, we will discuss the expression, function, prognosis, tumorigenesis, polymorphisms and therapeutic implications of ICAM-1 in CRC.

ICAM-1 structure, expression and function

ICAM-1, located on chromosome 19p13, is a cell surface glycoprotein of the immunoglobulin (Ig) superfamily of CAMs, and consists of 5 extracellular Ig-like domains, a transmembrane domain and a short cytoplasmic tail (5). The Ig-like domains mediate ICAM-1 interactions with its two major ligands, macrophage-1 antigen (Mac-1, CD11b/CD18) and lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) (6).

ICAM-1 is expressed in various cell types (epithelial cells, keratinocytes, fibroblasts and immune cells) and plays an essential role in cell-cell, cell-extracellular matrix interaction, cell signaling and immune process (7, 8). It serves as a biosensor to transducer outside-in-signaling *via* association of its cytoplasmic domain with the actin cytoskeleton following ligand engagement of the extracellular domain. Upon ligation, ICAM-1 undergoes dimerization and clustering through homotypic binding between Ig domains (7, 8).

Epithelial cells (ECs) of normal human colon do not express ICAM-1, but it can be expressed subsequent to malignant transformation. It has also been shown that ICAM-1 is related to the mesothelial adhesion, malignant potential, occurrence and progression of CRC (9–12). The interaction between ICAM-1 and its ligand may facilitate adhesion of tumor cells to the vascular endothelium and promote metastasis subsequently. The patients with increased ICAM-1 expression have more advanced stage, as it promotes the tumor growth (11, 13). Its expression was also associated with the cell differentiation of CRC. Higher ICAM-1 expression was found in better differentiated CRC cells compared to lower ICAM-1 expression in poorly differentiated CRC cells, which demonstrated that ICAM-1 promoted CRC differentiation and retarded metastais (14). ICAM-1 may play an important role in the immune response. The increased ICAM-1 expression might reflect the elevated immunity against tumor cells and ICAM-1 renders tumor cells more sensitive to lymphocyte-mediated lysis (15).

The prognostic significance of ICAM-1 expression in CRC

The prognostic significance of ICAM-1 expression remains controversial in CRC. ICAM-1 plays a dual role in CRC, and its impact depends on whether this protein is expressed in a membrane-bound or a soluble form (16–20). In CRC, the increased expression of membrane-bound ICAM-1 was associated with the favorable prognosis (16, 21). Maeda K et al. reported a better prognosis for CRC patients with membrane-bound ICAM1-positive (16). Tachimori A et al. also reported that an increased membrane-bound ICAM-1 expression inhibited the tumour growth and was correlated with a favorable prognosis in CRC (22). Leqi Zhou et al. and Mlecnik B et al. reported that a high expression of ICAM-1 was relevant to a prolonged survival (21, 23).

The favorable prognosis may be attributed to two mechanisms: one is that ICAM-1 may play an important role in the immunosurveillance and enhances lymphocyte-mediated cytotoxicity (15, 24–26). T cells are important for killing tumor cells and the increased ICAM-1 expression on $CD8^+$ T cells activates the antitumor function of $CD8^+$ T cells. ICAM-1 expressed by tumor cells may lead to T cell-specific recognition and enhanced T cell adhesion (27). Tachimori et al. showed that more lymphocytes adhered to CRC cells when ICAM-1 expression was upregulated (22). The other potential mechanism is that ICAM-1 may play an important role in the tumor microenvironment (TME). Upregulation of ICAM-1 in CRC cells could increase cytotoxic lymphocytes (CTLs) infiltration and the expression of cytolytic immune effector molecules in the TME, which is associated with favorable prognosis in CRC (4, 28–30). Increased CTLs was observed in the TME of ICAM-1 positive CRC compared to that of ICAM-1 negative CRC (13). Fisher et al. showed that ICAM-1 blockade decreased CTLs infiltration in the TME (31). The increased ICAM-1 expression on other cells in the TME also enhances the tumor infiltration and function of CTLs (23, 32, 33).

However, in some reports, the ICAM-1 expression was correlated with a worse prognosis (34, 35). For instance, Ionescu C et al. reported overexpression of ICAM-1 was correlated with lower overall survial (OS) (34). There is no clear explanation for the apparently contrary roles of ICAM-1,

suggesting that the function of ICAM-1 is context dependent and modulated by the action of other membrane receptors.

Factors regulating ICAM-1 expression

Several studies have focused on the factors regulating ICAM-1 expression, and the mechanisms seem to be multiple. Figure 1 demonstrates the potential mechanisms of ICAM-1 expression in CRC.

Cytokines

A variety of cytokines may regulate ICAM-1 expression, such as TNFα, IFNγ and IL-1β (36, 37). The regulatory mechanism is mainly involved in activating signal pathways through the binding on the ICAM-1 promoter.

Tumor necrosis factor

TNF-α can regulate ICAM-1 expression, which results in enhanced lymphocytic infiltration and tumor apoptosis (36). TNF-α response element is important in regulating ICAM-1 expression. This response element possesses an NF-κB binding site and NF-κB plays a significant role in TNF-α induced ICAM-1 expression (38).

Interferon-γ

IFNγ can also induce ICAM-1 expression and STAT-1 (signal transduction and activator of transcription) protein is upregulated during IFN-γ response (39). The IFN-γ response element (IRE) plays a important role in ICAM-1 expression (40). Upon IFN-γ stimulation, IRE forms a binding complex (IRE-BC) with nuclear proteins, which is required for induction of ICAM-1 expression.

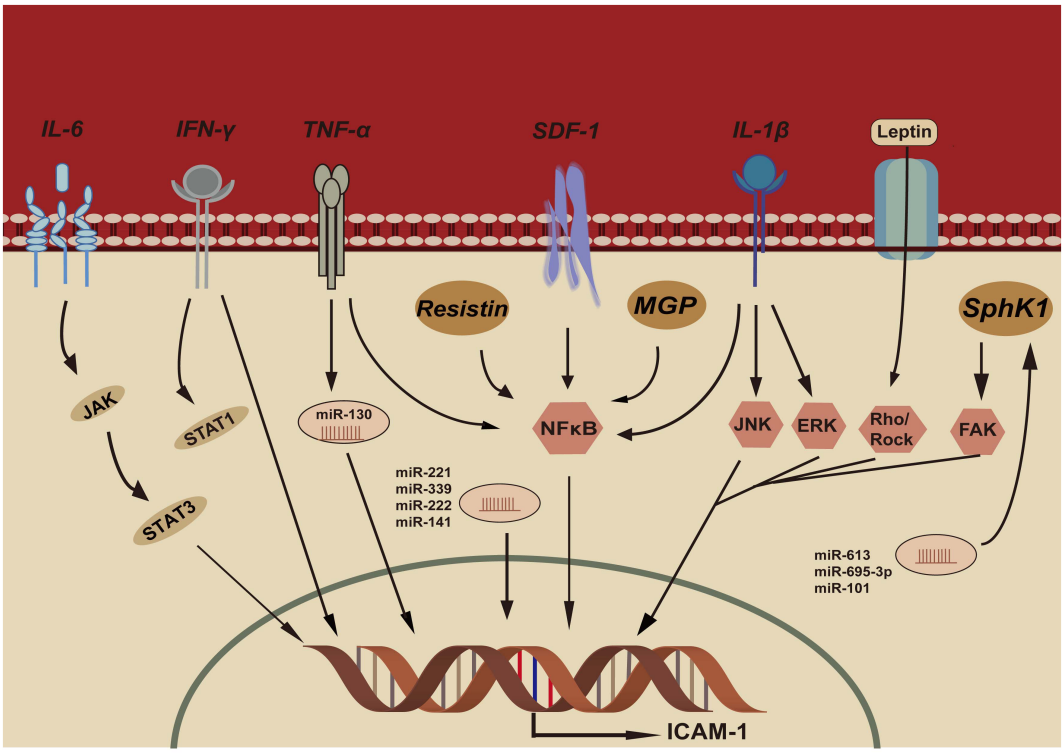


FIGURE 1
IL-6, Interleukin-6; IFN-γ, Interferon- γ; TNF-α, Tumor necrosis factor- α; SDF-1, Stromal cell derived factor-1; IL-β, Interleukin- β; MGP, Matrix gla protein; SphK1, Sphingosine kinase-1; JAK, Janus kinase; STAT3, Signal transducer and activator of transcription 3; STAT1, Signal transducer and activator of transcription 1; NFκB, NFκB pathway; JNK:c-Jun N-terminal kinase pathway; ERK, Extracellular signal-regulated kinase pathway; FAK, Focal adhesion kinase pathway; Rho/Rock, Rho/Rho-associated kinase pathway; ICAM-1, Intercellular adhesion molecule-1.

Interleukin-6

IL-6 is a T-cell-derived cytokine that induces maturation of B cells. IL-6 plays a critical role in metastasis of cancer cells by modulating ICAM-1 expression (40). CRC patients exhibited high level of IL-6 and IL-6 induces ICAM-1 expression *via* IL-6 receptor. JAK-STAT3 pathway and the AP-1 binding site of ICAM-1 are involved in IL-6 mediated ICAM-1 expression (41).

IL-1β

IL-1β has been reported to induce ICAM-1 expression and is involved in multiple immune and inflammatory responses (42). IL-1β also activates NF-κB pathway, ERK pathway and JNK pathway for ICAM-1 expression.

Resistin

Resistin is an adipose tissue-secreted form and could also be expressed in peripheral blood mononuclear cells, macrophages, and bone marrow cells (43). Resistin exerts its biological effects by binding to Toll-like receptor 4 (TLR4) and NF-κB can be activated by TLR4 which leads to ICAM-1 expression (44, 45).

Transcription factors

The expression of ICAM-1 may be regulated by a few transcription factors, such as stromal cell derived factor-1 (SDF-1), Leptin, C/EBPβ (36).

SDF-1

Abnormal expression of SDF-1 has been detected in CRC and ICAM-1 expression was up-regulated by SDF-1 (46). MAPKs pathway may be involved in the SDF-1-mediated expression of ICAM-1 (47). By MAPKs pathways, SDF-1 activates NF-κB and C/EBPβ to bind to the promoter of ICAM-1, thus leading to ICAM-1 up-regulation in CRC cells (46).

Leptin

Leptin can induce ICAM-1 expression and the Rho/ROCK (Rho-associated coiled-coil-forming protein kinase, ROCK) pathway may be involved in the leptin-mediated expression of ICAM-1 (48). Z Dong et al. revealed that leptin can induce ICAM-1 expression by the Rho/ROCK pathway (49).

Sphingosine kinase 1

SphK1 is an oncogene and is associated with angiogenesis, anti-apoptosis and survival of tumor cells (50). The SphK1 expression is enhanced in CRC and enhanced the ICAM-1expression by regulating the FAK pathway in CRC cells (51, 52). The ICAM-1 expression is upregulated with the overexpression of SphK1 and downregulated with the suppression of SphK1 in CRC cells.

Matrix Gla protein

MGP is a secreted, calcium-binding matrix protein. Overexpressed MGP could be found in CRC, and it may be associated with tumor progression and invasion. Li X et al. revealed that MGP expression increased in CRC and MGP promoted the phosphorylation of NF-κB by upregulating intracellular free calcium concentrations, activating the expression of ICAM-1 (53).

MicroRNAs

MicroRNAs (miRNAs) also play an important role in regulating the ICAM-1 expression in CRC. Recent studies indicate that ICAM-1 is a direct target of miRNAs, and these miRNAs bind to the untranslated region (UTR) of ICAM-1 and regulate ICAM-1 expression. Mir-221 binded to 3'UTR of ICAM-1 mRNA which resulted in transcription suppression of IFN-γ induced ICAM-1 expression (54). In addition, miR-222 and miR-339 have also been shown to bind 3'UTR of ICAM-1 promoter to suppress the ICAM-1 expression and promoted resistance of cancer cells to CTLs (55, 56). MiR-130 was induced by TNFα and lead to the increased ICAM-1 expression (57). MiR-141 binded to the 3'UTR of ICAM-1 directly and inhibited TNF-α induced ICAM-1 expression in ECs (58). Some miRNAs modulate ICAM-1 expression through down-regulating SphK1 expression in CRC cells, such as miR-613, miR-659-3p, miR-101. These miRNAs targeted SphK1 and downregulated ICAM-1 expression (59–62). SphK1 was an important target of miR-101, and miR-101 down-regulated SphK1 to inhibit ICAM-1 expression in CRC cells (62).

Genetic variations

Genetic variations in the ICAM-1 gene can regulate the protein expression in various diseases. ICAM-1 rs5498 may affect the expression of ICAM-1 in CRC patients. Wang MY et al. reported that ICAM-1 rs5498 may affect the level of sICAM-1 (63). The level of sICAM-1 at ICAM-1 5498 allele

locus in K individuals was higher than that at non-K allele. Wang QL et al. also reported patients with KK genotype showed an increased ICAM-1 expression in CRC and ICAM-1 expression was higher in patients with KK genotype than that with KE+EE genotypes (64). ICAM-1 5498 is a non-synonymous mutation, which leads to the increased expression of ICAM-1 and affects the function of ICAM-1.

ICAM-1 expression and metastasis in CRC

ICAM-1 expression may decrease CRC metastasis. In CRC, the high expression of membrane-bound ICAM-1 was associated with a lower incidence of liver and lymph node metastases (14, 16, 17). The upregulation of ICAM-1 inhibited tumor metastasis in CRC cell lines (22, 23). Mlecnik B et al. reported upregulation of ICAM-1 in CRC cells lowered the frequency of distant metastasis (23). The transfection of ICAM-1 into CRC cells inhibited tumor metastasis (27). Tachimori A et al. also demonstrated that liver metastases decreased in CRC cells expressing ICAM-1 compared with CRC cells not expressing ICAM-1 (65).

Mechanisms of ICAM-1 in CRC metastasis

Metastasis of CRC is a complex process that is influenced by a variety of factors. Among these factors, ICAM-1 plays a key role, but the mechanisms of how ICAM-1 decreases metastasis of CRC are not completely clear. One potential mechanism is that ICAM-1 can activate the immune system to prevent metastasis of CRC (13). ICAM-1 promotes recognition and destruction of tumor cells by the immune cells (4). ICAM-1 increased lymphocytes recruitment, promoted lymphocytes to attach to CRC cells and lymphocyte-mediated tumor lysis, which may improve the immunosurveillance and restrict tumor metastasis (20, 24). It can also sensitize metastatic tumor cells to CTL-mediated killing and prevent tumor metastasis (66). A second mechanism may be that ICAM-1, as a morphogen, enhances tumor cells attachment to the extracellular matrix by promoting motility in the context of remodeling. Taglia L et al. showed that ICAM-1 mediated tumor cells attachment to the extracellular matrix and prevented tumour cells from detaching from the primary tumor and thus retarded metastasis (16). Thirdly, ST6GAL1 could mediate tumor metastasis by regulating the stability of ICAM-1 (22). It might increase ICAM-1 stability through sialylation and consequently inhibit CRC metastasis (67, 68). Fourthly, the mechanism may be due to GRP's activation of the immune surveillance system (2, 69). FAK phosphorylation mediates GRP's activation of the immune system and ICAM-1 is the downstream proteins of FAK pathway (58–63, 66–72).

Soluble ICAM-1 in CRC

Apart from the membrane-bound ICAM-1 expressed on CRC cells, there exists a soluble form of ICAM-1 (sICAM-1) in serum. SICAM-1 was firstly identified in the serum of healthy volunteers by Seth et al, and its level is elevated in malignancies (73). Although the splice variant of sICAM-1 is truncated at the transmembrane domain, it retains all five extracellular Ig-like domains similarly to full-length ICAM-1 molecule, and its ability is conserved. In agreement with the small size of the transmembrane and cytoplasmic domains, sICAM-1 is only slightly smaller in size than its membrane-bound form.

The mechanism of sICAM-1 production is unclear, but it may be produced by proteolytic cleavage of membrane-bound ICAM-1, and released from the local cancer cell and enter the serum (73). Secondly, sICAM-1 is an inflammation-associated marker and is therefore increased in patients with an inflammatory TME. Thirdly, ICAM-1 rs5498 may have an effect on the levels of sICAM-1. Bielinski SJ et al reported the ICAM1 rs5498 G allele was associated with the level of sICAM-1 (74).

The level of sICAM-1 was elevated in CRC patients and can serve as a biomarker (Table 1) (15, 20, 75, 79, 80). The level of sICAM-1 was positively correlated with the tumor size,

TABLE 1 Comparison of sICAM-1 levels between patients and controls.

CRC patients	median age (years)	Gender (male:female)	sICAM-1 levels in patients (ng/mL)	controls	sICAM-1 levels in controls (ng/mL)	P value	References
40	NR	20:20	366.1±114.1	24	306.4±98.2	0.037	(15)
63	70	33:30	285.0	51	203	NR	(20)
56	57	32: 24	743.7±113.7*	25	345.7±49.8	P<0.001	(75)
297	67	185:112	266.5	40	242.7	NR	(76)
138	64	89:49	160.9 ±109.9	40	76.1 ±15.6	<0.001;	(77)
46	66	20:26	228.0±52.59	40	201.7±24.7	P<0.02	(78)

NR: not reported, *Dukes C and D.

advanced stage and metastasis in CRC patients (9, 14, 15, 20, 75, 79–82). Basouglu et al. found that the serum sICAM-1 level was higher in CRC patients than that in the healthy controls and patients with advanced stage had higher sICAM-1 levels than those with a lower stage (81). Mantur et al. and Kang et al. also observed that CRC patients with higher sICAM-1 level were at a higher advanced stage (15, 75). The sICAM-1 level in patients with distant metastases increased compared with patients without metastases. High levels of sICAM-1 have been shown to be associated with liver metastasis in CRC (14, 15, 18). SICAM-1 levels decreased significantly after curative surgery for CRC (15).

Previous studies demonstrated that patients with higher sICAM-1 level revealed poor prognosis (14, 16, 23), while the patients with lower sICAM-1 level displayed an improved OS (83). Yamamoto Y et al. also reported high sICAM-1 level was associated with shorter OS in CRC patients treated with chemotherapy plus bevacizumab (84). Elevated sICAM-1 level is associated with a decreased OS and serves as independent prognostic biomarker, but the mechanisms are not completely clear. SICAM-1 can bind to circulating CTLs, inhibit the interaction between CTLs and tumour cells, and block immune recognition of tumor cells (85). It can also block NK cell-mediated toxicity and thus allow tumour cells to escape immune destruction (76). Moreover, it can promote angiogenesis and stimulate tumour cells growth (86). These findings are possible explanations for the poor prognosis.

ICAM-1 single nucleotide polymorphisms in CRC

SNPs are the most common type of DNA sequence and analysis of ICAM-1 SNPs is important for studying the genetic features of CRC. Previous studies have suggested that ICAM-1 SNPs are associated with the risk of CRC.

ICAM-1 SNPs and CRC risk

Several studies have assessed the relationship between CRC risk and ICAM-1 SNPs, but these results were controversial (Table 2). Some studies showed that ICAM-1 SNPs were associated with an increased CRC risk (5, 64, 87). George Theodoropoulos et al. firstly reported that ICAM-1 rs5498 was associated with an increasing CRC risk in CRC patients (87). Anbarasan C et al. and Wang QL et al. also reported ICAM-1 rs5498 increased the risk of CRC (5, 64). But in a meta-analysis, the ICAM-1 rs5498 decreased the risk of CRC in Caucasians (88). We found that ICAM-1 rs5498 was not correlated with the risk of CRC in Chinese CRC patients, but ICAM-1 rs5498 decreased the CRC risk in the subgroup of age≥61 (89). Ravindran Ankathil et al. found that ICAM-1 rs5498 did not show significant association for CRC risk in Malaysian CRC patients (90). In our previous study, ICAM-1 rs3093030 polymorphism did not influence CRC risk (89). For ICAM-1 rs179969 polymorphism, the frequencies of homozygous wild type was significantly higher in controls compared to CRC patients. The different findings may be due to different ethnicities, regions, ages or the limited sample sizes. In the future, an analysis of different SNPs may make it possible to describe the exact relations between polymorphisms and CRC risk.

ICAM-1 SNPs and tumor differentiation in CRC

ICAM-1 SNPs are correlated with differentiation of CRC. ICAM-1 rs5498 KK genotype in poorly differentiated patients was significantly higher than that in well- or moderately-differentiated patients, whereas ICAM-1 rs5498 KE+EE genotype in poorly-differentiated patients was lower than that in well- or moderately-differentiated patients. Wang QL also found that ICAM-1 rs5498 is significantly associated with well differentiation of CRC (64). Liu LB et al. reported ICAM-1

TABLE 2 The relationship between CRC risk and ICAM-1 SNPs.

ICAM-1 SNPs	CRC patients	Gender (male:female)	median age (years)	Controls	CRC risk	ethnicities	References
rs5498	222	128:94	NR	200	increased	Greek population	(87)
rs5498	87	49:38	55.0	102	increased	Chinese population	(64)
rs5498	309	NR	NR	302	decreased	Caucasians	(88)
rs3093030	1003	620:383	61.1	1303	no significant difference	Chinese population	(89)
rs5498							
rs5498	280	140:140	53.2	280	no significant difference	Malaysian population	(90)
rs179969					increased		
rs5498	195	102:93	6.1	188	decreased	Chinese population	(91)

NR, not reported.

rs5498 was associated with the degree of tumor differentiation in the population of North China (91). The differentiation of CRC that correlates with ICAM-1 rs5498 may be of different ICAM-1 expression. ICAM-1 SNPs and multidrug resistance in CRC

MDR is one of the important factors leading to the failure of chemotherapy. Topo II and P-gp are MDR-associated protein and expression of them had a vital significance in chemotherapy for CRC. ICAM-1 rs5498 polymorphism was associated with MDR in CRC in a Chinese population (91). The high expression of Topo II and P-gp was observed in ICAM-1 rs5498 KK genotype, indicating that ICAM-1 rs5498 KK genotype might be associated with MDR in CRC (91).

Anti-Tumor Therapy Targeting ICAM-1

Targeting ICAM-1 and its associated pathway might provide a new insight for treatments of CRC. However, ICAM-1 plays diverse roles in anti-tumor responses and immunity, therefore, the targeting treatments of ICAM-1 may be difficult. Blocking of ICAM-1 has been proven useful in rheumatoid arthritis (92), but targeting ICAM-1 in tumors have shown disappointing results.

Chimeric antigen receptor (CAR)-T cell therapy has shown remarkably effective in cancer treatment and ICAM-1 could be a promising target for CAR-T cells. Wei H et al. demonstrated ICAM1-specific CAR-T cells could recognize ICAM-1 expressing breast cancer cells and inhibit tumor growth *in vitro* and *in vivo* (93), which provided a reference for CAR-T cell therapy in CRC. Further, CpG-ODN (oligodeoxynucleotides, ODN) vaccination caused up-regulation of ICAM-1 on tumor-associated blood vessel endothelia leading to tumor-infiltration of T cells and tumor suppression in mouse model of pancreatic carcinoma (94). Administer cytokines is the straight way to increase inflammatory signals, but this could lead to severe adverse events, so delivery of cytokines directly to the tumor site could reduce adverse events (95). Angiogenic factors in the TME can decrease ICAM-1 expression, so targeting angiogenesis could also increase the ICAM-1 expression (96). NF-κB pathway plays a central role in ICAM-1 expression, so blocking NF-κB pathway can inhibit the ICAM-1 expression. The acai polyphenolic extract inhibited the ICAM-1 expression by targeting NF-κB pathway (97). Flubendazole, the benzimidazole derivative used in the treatment of parasitic disease, suppressed the growth of colon cells by down-regulation of NF-κB and ICAM-1 (98). Perhaps, with a better understanding of the various functions of ICAM-1 and how their expression and function are regulated, the clinical value of ICAM-1 could be revisited for the improvement of therapeutic strategies.

Conclusions and future perspectives

In this review, we provide the first comprehensive description of the knowledge regarding ICAM-1 in CRC. The pathogenesis of CRC involves various mechanisms, and ICAM-1 plays different roles. During cancer development, ICAM-1 mediates anti-tumor response including tumor antigen uptake, activation of tumor-specific T cells, leukocyte trafficking into the tumor site and tumor cell killing. ICAM-1 remains the focus of continued investigations and may serve as a promising prognostic biomarker, and a potential target for emerging therapies.

Author contributions

GT and YZ provided direction and guidance throughout the preparation of this manuscript. ZQ and YW wrote and edited the manuscript. WT and YX reviewed and made significant revisions to the manuscript. RQ, ZZ and YP collected and prepared the related papers. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Comparative efficacy and toxicity of immune checkpoint inhibitors in combination with or without chemotherapy treatment for advanced esophageal squamous cell carcinoma: A systematic review and meta-analysis

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Introduction: We did a systematic review and meta-analysis to assess the efficacy and safety of immune checkpoint inhibitors combined with or without chemotherapies in patients with esophageal squamous cell carcinoma.

Methods: Data related to the treatment of esophageal squamous cell carcinoma with immune checkpoint inhibitors therapy were retrieved from the database construction to August 2022. The risk of bias was assessed using the Cochrane Manual standard and RevMan 5.3 software for data synthesis. The outcome measures observed included overall survival, 12-month survival, disease control rate, objective response rate, treatment-related adverse events of grade 3 or higher, and progression-free survival. The adverse reactions included fatigue, diarrhea, hypothyroidism, rash, anemia, and anorexia.

Results: In this meta-analysis, a total of 17 randomized controlled trials were included. In first-line therapy, immune checkpoint inhibitors combined with or without chemotherapy in the treatment of esophageal squamous cell carcinoma was more effective than chemotherapy alone. Overall survival, 12-month survival rate, and objective response rate were statistically significant. Among second-line treatments, immune checkpoint inhibitors combined with or without chemotherapy in the treatment of esophageal squamous cell carcinoma had statistically significant overall survival, 12-month survival, objective response rate, treatment-related adverse events of grade 3 or higher, and progression-free survival compared with chemotherapy alone.

Conclusion: Both first- and second-line immune checkpoint inhibitors are effective for esophageal squamous cell carcinoma, and the adverse reactions are controllable and safe.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/, identifier CRD42021282586.

KEYWORDS

immune checkpoint inhibitors, esophageal squamous cell carcinoma, toxicity, efficacy, meta-analysis

1 Introduction

Esophageal cancer ranks seventh in terms of incidence (604,000 new cases) and sixth in mortality overall (544,000 deaths); the latter means that esophageal cancer is responsible for 1 in every 18 cancer deaths in 2020. Approximately 70% of cases occur in men, and incidence and mortality rates are two- to threefold higher than in women (1). Esophageal cancer is one of the most important contribution of China to the worldwide burden of cancer, with epidemiological hot spots in Asia and Africa. In China, esophageal cancer is ranked as the third most common cancer in men and the fifth most common cancer in women and was the fourth leading cause of death from cancer in 2015 (2, 3). In addition, because of its anatomical location, esophageal cancer is often described as a disease. However, esophageal cancer has histological subtypes due to different etiologies; the most important subtypes include esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (AC), of which esophageal squamous cell carcinoma accounts for 80%–90% (1, 4). Furthermore, the proportion of pathological types of esophageal cancer in China is significantly different from that in developed countries (5). In Western regions, nearly two-thirds of esophageal cancer cases are ACs (1). Therefore, a large number of clinical studies also pay attention to this issue when enrolling patients and often use organizational grade type or geographical location as stratification factors (6). This leads to different therapeutic outcomes based on histological subtypes.

Abbreviations: ICIs, immune checkpoint inhibitors; OS, overall survival; DCR, disease control rate; ORR, objective response rate; PFS, progression-free survival; RCTs, randomized controlled trials; ESCC, esophageal squamous cell carcinoma; AC, esophageal adenocarcinoma; PD-1, programmed cell death protein 1; PD-L1, programmed death ligand 1; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; IL, interleukin; ROS, reactive oxygen species; MDSCs, myeloid-derived suppressor cells; HRs, hazard ratios; CI, confidence intervals

Previous studies have shown that some factors play an important role in the occurrence and development of esophageal cancer. Gastroesophageal reflux disease and obesity are the main pathogenic factors of esophageal adenocarcinoma, while smoking and alcohol consumption are the main causes of esophageal squamous cell carcinoma (7). Gastroesophageal reflux disease and obesity-driven inflammation generate a pro-tumorigenic microenvironment consisting of pro-inflammatory M2-type macrophages, neutrophils, myeloid-derived suppressor cells (MDSCs) and TH2 cells, and proinflammatory mediators that include interleukin (IL)-1β, IL-8, IL-6, reactive oxygen species (ROS), and tumor-promoting TH2 cytokines. Because esophageal cancer TME is rich in immune cells, it is considered to be sensitive to ICIs (8).

One of the major breakthroughs in cancer treatment over the past decade has been the discovery of immune checkpoint proteins, which effectively suppress the immune system through a variety of mechanisms. Immune checkpoint inhibitors (ICIs) have revolutionized cancer treatment, showing higher efficacy in several cancers, such as non-small cell lung cancer (NSCLC), melanoma, malignant mesothelioma, renal cell carcinoma, urothelial carcinoma, gastric cancer, and head and neck carcinoma (9–16). When programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) activates the immune checkpoint cascade, it leads to tumor-specific T-cell inactivation and immune evasion. Therapy with immune checkpoint inhibitors, such as anti-PD-1, anti-PD-L1, and/or anti-CTLA-4 drugs, can rejuvenate T cells and enable the adaptive immune system to target tumor cells (17–19).

As the biology of esophageal cancer might substantially vary in different regions of the world, so might the response to checkpoint inhibition (20). Recent studies concerning immunotherapeutic agents have been to revolutionize therapeutic strategies for esophageal cancer patients. Treatment with anti-PD-(L)1 drugs currently represents the mainstay of ESCC ICIs and can result in impressive response rates and durable disease remission, but only in a subset of patients.

Taking KEYNOTE-590 clinical study as an example, it examined first-line chemotherapy with or without pembrolizumab in patients

with squamous cell carcinoma of the esophagus, adenocarcinoma of the esophagus, or Siewert-type GE junction adenocarcinoma. It demonstrated that pembrolizumab plus chemotherapy improved overall survival (OS) in patients with ESCC with PD-L1 CPS≥10 tumors, all squamous cell carcinomas, and all patients with CPS≥10. Progression-free survival (PFS) was also improved (21).

In this meta-analysis and literature review, we tried to analyze the efficacy and safety of ICIs combined with or without different chemotherapies in patients with ESCC.

2 Materials and methods

2.1 Literature search

We followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines for this systematic review and meta-analysis (22). We searched PubMed, The Cochrane Library, Embase, Web of Science, CNKI, Wanfang, VIP, and Biomedical database (from the establishment of the database to August 2022), by searching for articles on ICIs therapy with or without chemotherapy for ESCC. The method of combining mesh words and free words is used to search literatures. The language limit is Chinese and English. The search terms were "Esophageal Cancers," "Neoplasm, Esophageal," "programmed death receptor 1," "Checkpoint Inhibitors, Immune," "ocrelizumab," "Nivolumab," "pembrolizumab," and "telimomab." We also reviewed the references of articles included in the final selection. The detailed search strategy can be found in Supplementary File 1.

2.2 Study selection

Inclusion criteria were as follows: (1) studies whose subjects were patients with confirmed ESCC by pathological diagnosis; (2) studies that reported directly hazard ratios (HRs) and 95% confidence intervals (95%CIs) for OS or disease-free survival (DFS), or sufficient data were provided to calculate the HR and 95%CIs; (3) studies that included immune checkpoint inhibitor therapy for esophageal cancer; (4) studies whose target population was patients with advanced esophageal squamous cell carcinoma; and (5) abstracts and conference articles meeting the above inclusion criteria.

Excluded criteria were as follows: (1) meta-analyses, reviews, surveys, letters, case reports, and book chapters, and studies based on the National Cancer Database, or the Surveillance, Epidemiology, and End Results database; (2) duplicate publications; (3) single-arm clinical trial; (4) unable to obtain data; and (5) studies that have <10 cases.

2.3 Data extraction

Two authors independently extracted information from eligible articles. The authors resolved differences through discussion. The extracted data were as follows: (1) basic characteristics (first author, year of publication, study type, sample size, the protocol of test group and control group, and treatment line); (2) HRs and 95% CIs extracted for OS or DFS [if the studies did not directly report HRs and 95% CIs, we calculated the data based on the methods described by a previous study (23)]; and (3) the number of patients for each clinicopathological feature. If the relevant information of some items was not provided, these items were marked as "not available (NA)." When the HRs for the survival outcomes were reported by both univariate and multivariate analyses, only the HRs from the multivariate analysis were extracted (24).

2.4 Outcome

2.4.1 The main outcome

The main outcomes were overall survival and objective response rate.

2.4.2 The secondary outcome

Secondary outcomes were 12-month survival, disease control rate, treatment-related adverse events of grade 3 or higher, and progression-free survival.

2.5 Quality assessment of included studies

The quality of the included literatures was evaluated according to the standard Cochrane manual, including ① randomization method, ② distribution scheme hidden, ③ whether blind method is used, ④ integrity of result data, ⑤ selective reporting of research results, and ⑥ other sources of bias. The quality evaluation was carried out by two researchers independently and cross-checked. In case of disagreement, the decision was made by discussion or referring to the opinion of the third researcher.

The quality of the included literatures was evaluated according to the modified Jadad scale, including the following. ① Was the study described as randomized? ② Was the method of randomization appropriate, ③ Was the study described as blinded?④ Was the method of blinding appropriate? ⑤Was there a description of withdrawals and dropouts? ⑥ Was there a clear description of the inclusion/exclusion criteria? ⑦ Was the method used to assess adverse effects described? ⑧ Was the method of statistical analysis described? The quality evaluation was carried out by two researchers independently and cross-checked. In case of disagreement, the decision was made by discussion or referring to the opinion of the third researcher.

2.6 Statistical analysis

Meta-analysis was performed using RevMan 5.3 software provided by the Cochrane Collaboration. The count data were expressed by odds ratio OR and 95%CI. χ^2 test was used for heterogeneity among included studies. The random-effect model was chosen if obvious heterogeneity was present ($I^2 > 50\%$); otherwise, the fixed-effect model was selected. If there was statistical heterogeneity among study results ($p<0.1$, $I^2>50\%$), the source of heterogeneity was analyzed. We used sensitivity analysis to determine the source of heterogeneity, and the factors that might lead to heterogeneity were subgroup analyzed.

3 Results

3.1 Literature retrieval results

A preliminary search was conducted for 5,116 articles, and 1,582 duplicate articles were deleted after review by literature management Endnote 20 software. A total of 3,534 articles inconsistent with the theme of this meta-analysis were excluded by reading the title and abstract. After full text screening, 17 randomized controlled trials were included. For specific screening process and results, see Figure 1.

3.2 Study characteristics

The 17 clinical trials included 8,080 patients (21, 25–37). Among them, 4,034 patients received immune checkpoint inhibitor treatment, and 4,046 patients did not receive the treatment. Eight studies were on first-line therapy; the rest were on second-line therapy. Seven studies reported adverse outcomes. The characteristics of each study are shown in Table 1.

3.3 Quality assessment

For the 17 included literatures, Review Manager's own literature evaluation tool was used for evaluation. Four literatures were conferences or abstracts, and the full text could not be obtained. We obtained the methodological part of relevant trials from the clinical trial registration website (www.clinicaltrials.gov/) for quality evaluation (Figures 2, 3).

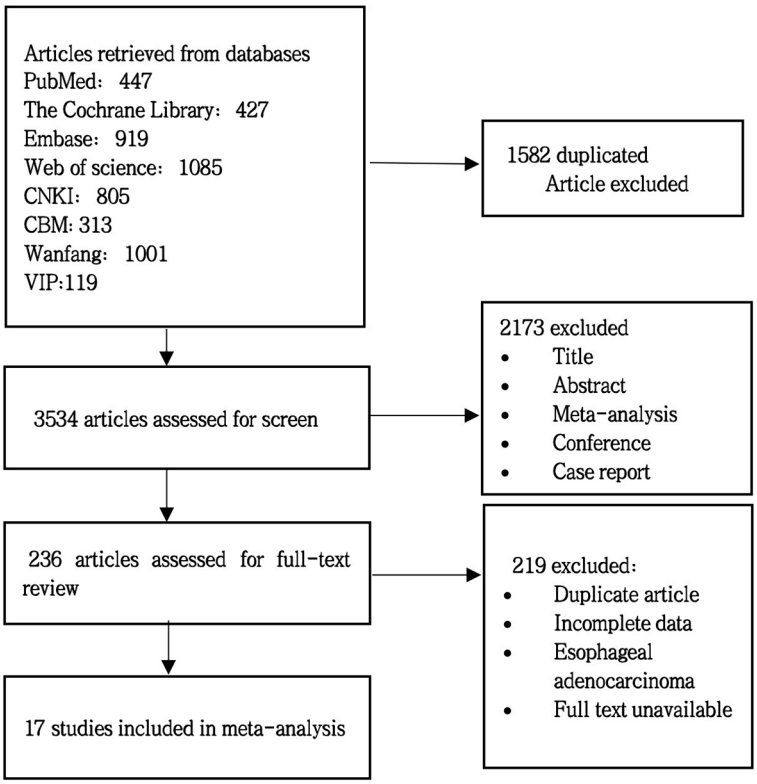


FIGURE 1
Literature screening flowchart.

TABLE 1 Baseline characteristics of studies included in the meta-analysis.

Author	Year	Country	People	Study design	Intervention	Control	pathology	ICIs	Intervention	Control	Treatment line	Outcomes	Adverse reaction
Huiyan Luo	2021	China	China	RCT	298	298	ESCC	PD-1	Camrelizumab+PTX+cis-platinum	Placebo+PTX+Cis-platinum	First line	ABCEFG	②④③⑥
Ian Chau	2021	NA	NA	RCT	321	324	ESCC	PD-1	Nivolumab+Chemotherapy	Fluorouracil+Cis-platinum	First line	ACD	NA
J. Ajani	2021	USA	various countries	RCT	256	256	ESCC	PD-1	Tislelizumab	PTX/Docetaxel/ Irinotecan	Second line	F	NA
Jianming Xu	2020	China	China	RCT	95	95	ESCC	PD-1	Stintilimab	Chemotherapy	Second line	CDF	NA
Jing Huang	2020	China	China	RCT	228	220	ESCC	PD-1	Camrelizumab	Docetaxel/Irinotecan	Second line	ABCDEG	②③④⑥
Jong-Mu Sun	2021	Korea	various countries	RCT	274	274	ESCC	PD-1	Pembrolizumab+5-FU+Cis-platinum	Placebo+5-FU+Cis-platinum	First line	AG	NA
Ken Kato	2019	Japan	various countries	RCT	210	209	ESCC	PD-1	Nivolumab	Docetaxel/PTX	Second line	ABCEFG	①③④⑤⑥
Lin Shen	2021	China	China	RCT	327	332	ESCC	PD-1	Stintilimab+TP/CF	Placebo+TP/CF	First line	AC	NA
Masanobu Takahashi	2020	Japan	Japan	RCT	136	138	ESCC	PD-1	Nivolumab	PTX/Docetaxel	Second line	ACEG	①②③④⑤⑥
R.Xu	2021	China	China	RCT	257	257	ESCC	PD-1	Toripalimab+PTX+cis-platinum	Placebo+PTX+Cis-platinum	First line	BCDG	NA
Takashi Kojima	2020	Japan	various countries	RCT	198	203	ESCC	PD-1	Pembrolizumab	Docetaxel/PTX/ Irinotecan	Second line	BCD	NA
Y.cao	2022	China	various countries	RCT	170	170	ESCC	PD-1	Pembrolizumab	PTX/Docetaxel/ Irinotecan	First line	AEFG	NA
Xiaochuan Liu	2022	China	China	RCT	34	35	ESCC	PD-1	Sintilimab+PTX+5-FU+Cis-platinum	PTX+5-FU+Cis-platinum	Second line	AEFG	NA
Zhihao Lu	2022	China	various countries	RCT	327	332	ESCC	PD-1	Sintilimab+Chemotherapy	Placebo +Chemotherapy	Second line	NA	②③④⑤⑥
Lin Shen	2022	China	various countries	RCT	256	256	ESCC	PD-1	Tislelizumab	Chemotherapy	Second line	ABCDFG	①②③④⑥
H.Yoon	2022	Japan	various countries	RCT	326	323	ESCC	PD-1	Tislelizumab+Chemotherapy	Placebo +Chemotherapy	First line	DF	NA
Doki Y	2022	Japan	various countries	RCT	321	324	ESCC	PD-1	Nivolumab+Chemotherapy	Chemotherapy	First line	CDF	①②③④⑤⑥

Adverse reaction: ① Fatigue; ② Hypothyroidism; ③ Diarrhea; ④ Anemia; ⑤ Rash; ⑥ Decreased appetite; Outcomes: A,Overall survival; B,12-month overall survival; C,Treatment-related adverse reactions; D,Treatment-related adverse reactions of grade 3 or higher; E,Disease control rate; F,Objective response rate; G,progression-free survival; ESCC, esophageal squamous cell carcinoma; ICIs, Immune checkpoint inhibitors; NA,not available; PTX,paclitaxel; 5-FU,5-Fluorouracil.

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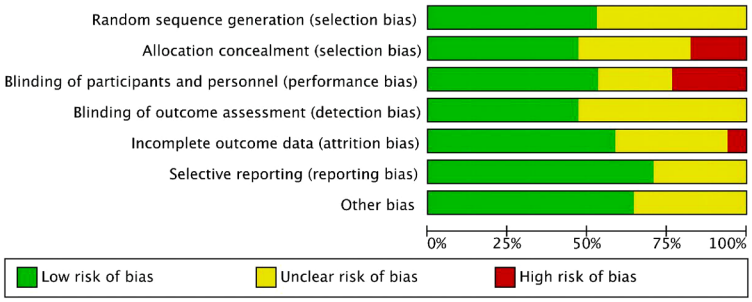


FIGURE 2
Risk of bias and applicability concerns summary.

3.4 Meta-analysis

In first-line therapy, immune checkpoint inhibitors combined with or without chemotherapy in the treatment of esophageal squamous cell carcinoma was more effective than chemotherapy alone. Overall survival, 12-month survival rate, and objective response rate were statistically significant. Among second-line therapy, immune checkpoint inhibitors combined with or without chemotherapy in the treatment of esophageal squamous cell carcinoma had statistically significant overall survival, 12-month survival, objective response rate, treatment-related adverse events of grade 3 or higher, and progression-free survival compared with chemotherapy alone. In the adverse effects, hypothyroidism and decreased appetites have statistical significance.

3.4.1 First-line ICIs

3.4.1.1 Overall survival

OS was reported in six studies, including 1,646 cases in the experimental group and 1,654 cases in the control group. The results of heterogeneity analysis were $p<0.00001$, $I^2 = 100\%$, indicating great heterogeneity. Therefore, the random effect model was adopted. The results of the meta-analysis showed that OR and 95%CI=2.83[2.27, 3.40], $p<0.00001$, $p< 0.05$, suggesting that the difference in overall survival rate between the two groups was statistically significant (Figure 4A).

3.4.1.2 Twelve-month overall survival

The 12-month overall survival rate was reported in three studies, including 811 patients in the experimental group and 811 cases in the control group. The results of heterogeneity analysis were $p=0.19$ and $I^2 = 40\%$, indicating small heterogeneity. Therefore, the fixed effect model was adopted. Meta-analysis results showed that OR and 95%CI=1.96[1.60, 2.40], $p<0.0001$, $p<0.05$, suggesting that the 12-month overall survival rate between the two groups was statistically significant (Figure 4B).

3.4.1.3 Treatment-related adverse reactions

Six studies reported treatment-related adverse reactions, including 1,769 cases in the experimental group and 811 cases in the control group. The results of heterogeneity analysis were $p<0.00001$, $I^2 = 86\%$, indicating great heterogeneity. Therefore, the random effect model was adopted. Meta-analysis results showed that OR and 95%CI=0.01[−0.03, 0.04], $p=0.69$, $p>0.05$, suggesting that there was no significant difference in treatment-related adverse reactions between the two groups (Figure 4C).

3.4.1.4 Treatment-related adverse reactions of grade 3 or higher

Six studies reported treatment-related adverse reactions of more than grade 3, including 1,768 cases in the experimental group and 1,716 cases in the control group. The results of heterogeneity analysis were $p<0.00001$, $I^2 = 94\%$, indicating great heterogeneity. Therefore, the random effect model was adopted. Meta-analysis results showed that OR and 95%CI=0.96 [0.54, 1.70], $p=0.89$, $p>0.05$, suggesting that there was no significant difference between the two treatment-related adverse reactions of grade 3 or higher (Figure 4D).

3.4.1.5 Disease control rate

The disease control rate was reported in three studies, including 725 cases in the experimental group and 725 cases in the control group. The results of heterogeneity analysis were $p=0.32$, $I^2 = 12\%$, indicating small heterogeneity. Therefore, the fixed effect model was adopted. Meta-analysis results showed that OR and 95%CI=1.32[1.00, 1.75], $p=0.05$, $p\geq0.05$, suggesting that there was no significant difference in disease control rates between the two groups (Figure 4E).

3.4.1.6 Objective response rate

Objective response rate was reported in six studies, including 1,628 cases in the experimental group and 1,628 cases in the control group. The results of heterogeneity analysis were $p=0.54$, $I^2 = 0\%$, indicating small heterogeneity. Therefore,



FIGURE 3
Risk of bias and applicability concerns graph.

the fixed effect model was adopted. Meta-analysis results showed that OR and 95%CI=2.11[1.78, 2.49], p<0.00001, p<0.05, suggesting that there was a statistically significant difference in objective response rate between the two groups (Figure 4F).

3.4.1.7 Progression-free survival

PFS was reported in six studies, including 1,582 cases in the experimental group and 1,662 cases in the control group. The results of heterogeneity analysis were p< 0.00001, I^2 = 100%, indicating great heterogeneity. Therefore, the random effect

model was adopted. Meta-analysis results showed that OR and 95%CI=0.00[−0.39, 0.39], p=1, P>0.05, suggesting that there was no significant difference in PFS between the two groups (Figure 4G).

3.4.2 Second-line treatment

3.4.2.1 Overall survival

OS was reported in four studies, including 608 cases in the experimental group and 602 cases in the control group. The results of heterogeneity analysis were p < 0.00001, I^2 = 99%, indicating great heterogeneity. Therefore, the random effect model was adopted. The results of meta-analysis showed that OR and 95%CI=2.62[1.70, 3.54], p < 0.00001, p<0.05, suggesting that the OS between the two groups was statistically significant (Figure 5A).

3.4.2.2 Twelve-month overall survival

Twelve-month overall survival was reported in three studies, including 636 patients in the experimental group and 632 cases in the control group. The results of heterogeneity analysis were as follows: p=0.92, I2 = 0%, indicating small heterogeneity. Therefore, the fixed effect model was adopted. Meta-analysis results showed that OR and 95%CI=1.84[1.45, 2.34], p<0.00001, p < 0.05, suggesting that there was significant difference in 12-month overall survival rate between the two groups (Figure 5B).

3.4.2.3 Treatment-related adverse reactions

Five studies reported treatment-related adverse reactions, including 981 cases in the experimental group; the control group included 975 cases. The results of heterogeneity analysis were as follows: p < 0.00001, I^2 = 92%, indicating great heterogeneity. Therefore, the random effect model was adopted. The results of meta-analysis showed that OR and 95%CI=0.30[0.11, 0.79], p=0.02, p < 0.05, suggesting that there was statistically significant difference in treatment-related adverse reactions between the two groups (Figure 5C).

3.4.2.4 Treatment-related adverse reactions of grade 3 or higher

Five studies reported treatment-related adverse reactions of grade 3 or higher, including 923 cases in the experimental group; the control group included 917 cases. The results of heterogeneity analysis were as follows: p < 0.0001, I^2 = 85%, indicating great heterogeneity. Therefore, the random effect model was adopted. The results of meta-analysis showed that OR and 95%CI=0.19 [0.11, 0.34], p<0.00001, p < 0.05, suggesting that the difference in treatment-related adverse reactions above grade 3 between the two groups was statistically significant (Figure 5D).

3.4.2.5 Disease control rate

Disease control rates were reported in five studies, including 635 patients in the experimental group; the control group included 616 cases. The results of heterogeneity analysis were as follows: p < 0.00001, I^2 = 91%, indicating great heterogeneity. Therefore, the random effect model was adopted. Meta-analysis results showed that OR and 95%CI=−0.03[−0.22,0.16], p=0.76, p > 0.05, indicating no statistically significant difference in disease control rates between the two groups (Figure 5E).

3.4.2.6 Objective response rate

Objective response rates were reported in seven studies, including 1,059 patients in the experimental group and 1,058 cases in the control group. The results of heterogeneity analysis were as follows: p=0.02, I2 = 61%, indicating great heterogeneity. Therefore, the random effect model was adopted. Meta-analysis results showed that OR and 95%CI=1.84[1.21, 2.80], p=0.005, p < 0.05, suggesting that there was statistical significance in the difference in objective response rate between the two groups (Figure 5F).

3.4.2.7 Progression-free survival

PFS was reported in five studies, including 818 cases in the experimental group; the control group included 811 patients. The results of heterogeneity analysis were as follows: p < 0.00001, I^2 = 92%, indicating great heterogeneity. Therefore, the random effect model was adopted. The results of meta-analysis showed that OR and 95%CI=−0.97[−1.05, −0.89], p<0.00001, p<0.05, suggesting that the difference in PFS between the two groups was statistically significant (Figure 5G).

3.4.3 Adverse reactions

3.4.3.1 Weakness

Weakness was reported in five studies, including 953 cases in the experimental group; the control group included 954 cases. The results of heterogeneity analysis were p<0.00001, I^2 = 95%, indicating great heterogeneity. Therefore, the random effect model was adopted. The results of meta-analysis showed that OR and 95%CI=0.57[0.15, 2.22], p=0.42, p>0.05, suggesting no statistically significant difference in weakness between the two groups. (Figure 6, ①)

3.4.3.2 Hypothyroidism

Hypothyroidism was reported in four studies, including 714 cases in the experimental group and 709 cases in the control group. The results of heterogeneity analysis were p=0.76 and I^2 = 0%, indicating small heterogeneity. Therefore, the fixed effect model was adopted. The results of meta-analysis showed that OR and 95%CI=20.83[8.38, 51.78], p<0.00001, p<0.05, suggesting that there was statistically significant difference in hypothyroidism between the two groups. (Figure 6, ②)

3.4.3.3 Diarrhea

Diarrhea was reported in six studies, including 1,181 cases in the experimental group and 1,175 cases in control group.

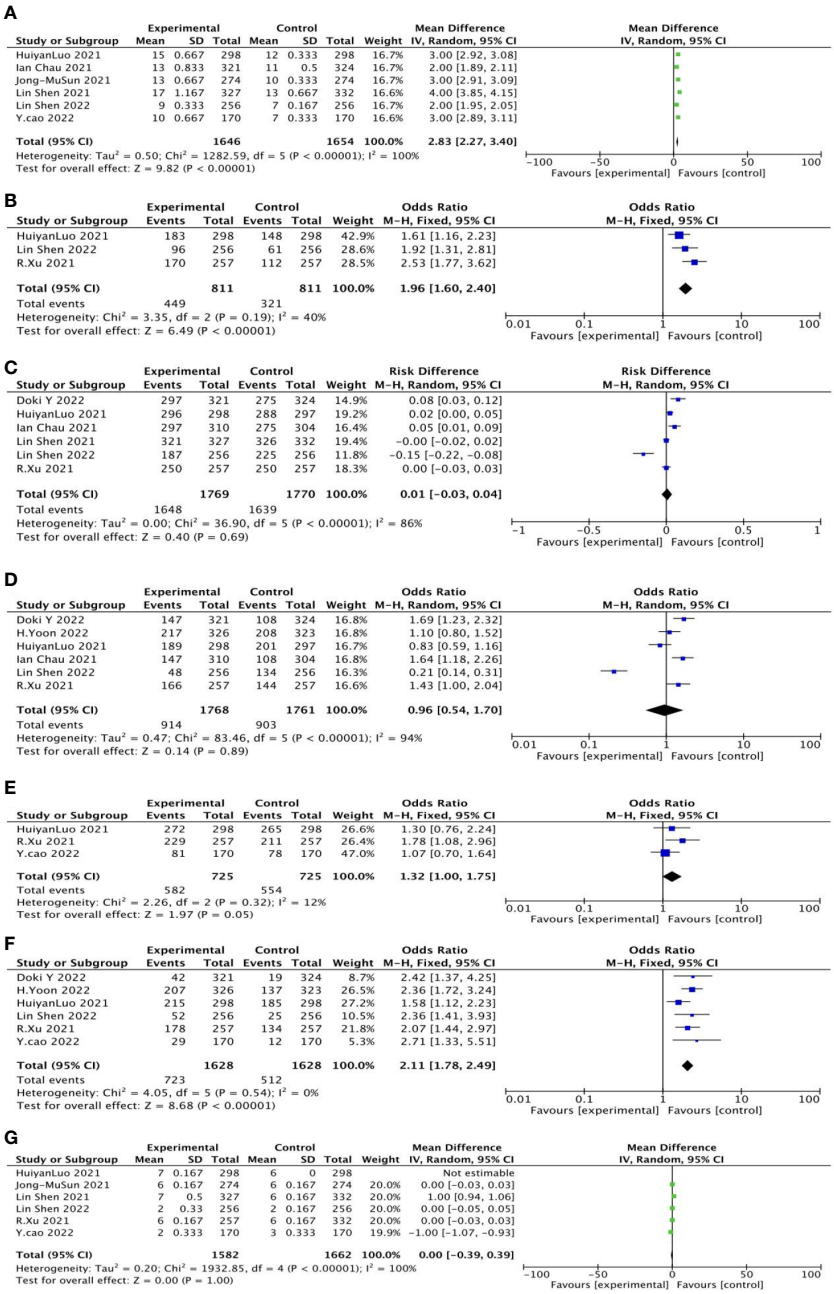


FIGURE 4
General forest plot for each outcome in first-line treatment.

The results of heterogeneity analysis were $p < 0.0001$, $I^2 = 93\%$, indicating great heterogeneity. Therefore, the random effect model was adopted. Meta-analysis results showed that OR and 95%CI=0.39[0.14, 1.11], p=0.08, p>0.05, indicating no statistically significant difference in diarrhea between the two groups (Figure 6, ③).

3.4.3.4 Anemia

Anemia was reported in six studies, including 1,181 cases in the experimental group; the control group included 1,175 cases. The results of heterogeneity analysis were $p<0.00001$, $I^2 = 90\%$, indicating great heterogeneity, so the random effect model was adopted. The results of meta-analysis showed that OR and 95%

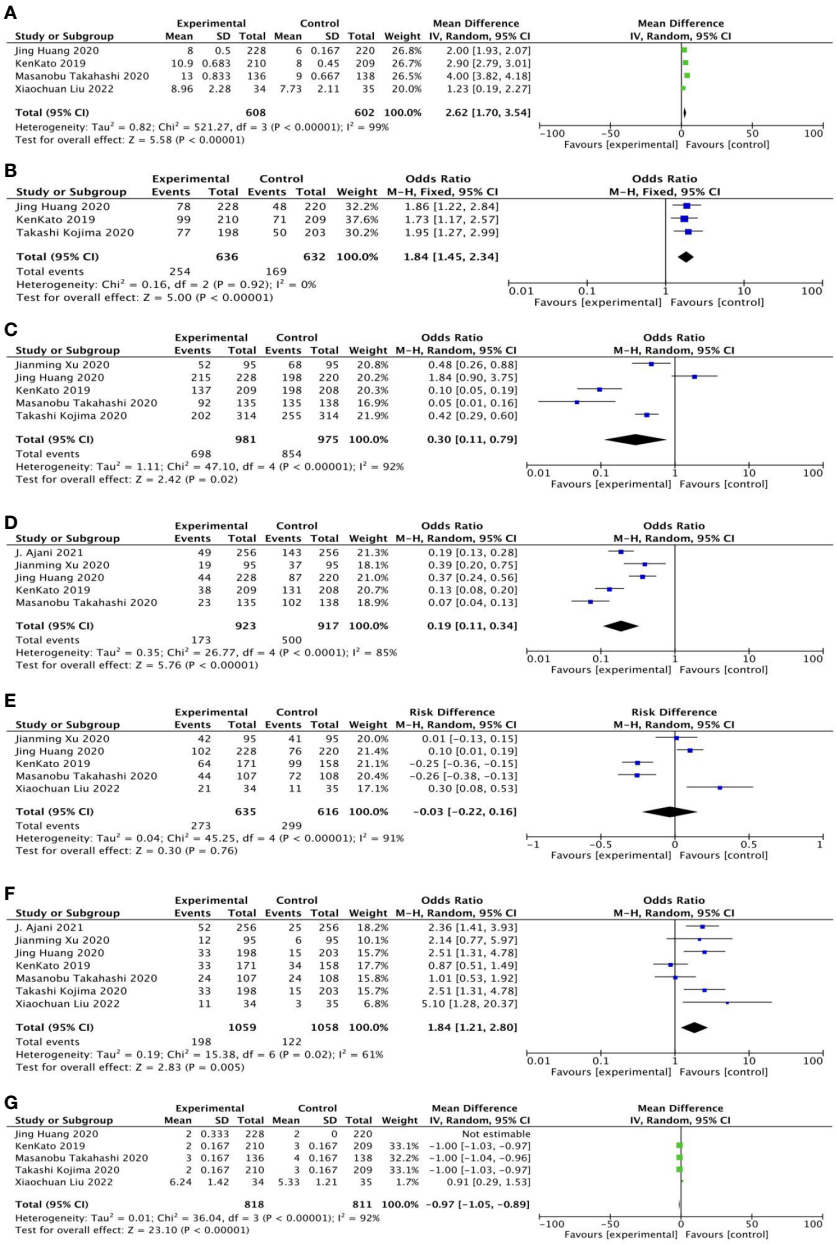


FIGURE 5
General forest plot for each outcome in second-line treatment.

CI=0.26[0.12, 0.57], p=0.0007, p<0.05, suggesting statistically significant difference in anemia between the two groups (Figure 6, ④).

3.4.3.5 Rash

Rashes were reported in three studies, including 440 cases in the experimental group; the control group included 442 cases. The results of heterogeneity analysis were p=0.23 and I^2 = 32%, indicating great heterogeneity. Therefore, the random effect model was adopted. Meta-analysis results showed that OR and 95%CI=0.85[0.55, 1.31], p=0.45, p>0.05, indicating no statistically significant difference in rash between the two groups (Figure 6, ⑤).

3.4.3.6 Decreased appetites

Decreased appetites were reported in five studies, including 924 cases in the experimental group; the control group contained 918 patients. The results of heterogeneity analysis were as

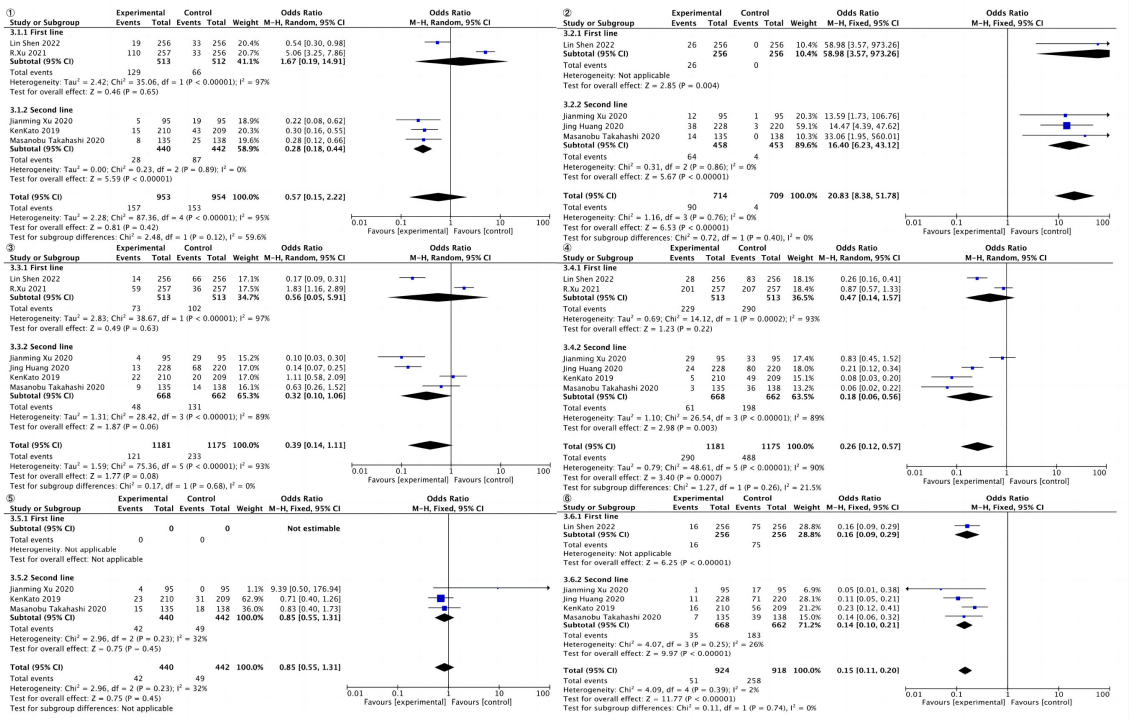


FIGURE 6
Total forest of adverse reactions.

follows: p=0.39, I^2 = 2%, indicating small heterogeneity. Therefore, the fixed effect model was adopted. The results of meta-analysis showed that OR and 95%CI=0.15[0.11, 0.20], p<0.00001, p<0.05, suggesting statistically significant difference in decreased appetites between the two groups (Figure 6, ⑥).

4 Discussion

In recent years, the randomized exploration of ICIs treatments has drawn extensive attention in many patient categories with promising results, and esophageal cancer had entered the era of ICIs.

However, in patients with advanced esophageal squamous cell carcinoma, the efficacy of immune checkpoint inhibitors is not known, so we performed meta-analysis, and we also performed meta-analysis of related adverse effects. In 2019, KEYNOTE-181 showed that second-line treatment with PD-L1-positive advanced/metastatic esophageal cancer or esophagogastric junction adenocarcinoma with PD-L1 monotherapy significantly extended patients' overall survival (OS) compared to standard chemotherapy (25). Subsequently, similar results were observed in the ESCORT study, Attract-03 study, and RATIONALE-302. Since then, ICIs have become the standard second-line treatment for patients with ESCC (26–28). Therefore, in the indication of second-line treatments, an enrichment strategy design led to the approval of ICIs for PD-L1-high patients. Our study shows that among second-line treatments, ICIs combined with chemotherapy in the treatment of esophageal squamous cell carcinoma had statistically significant OS, 12-month overall survival (12 OS), treatment-related adverse reactions, treatment-related grade 3 or more adverse reactions, objective response rate (ORR), and PFS compared with chemotherapy alone.

As the first-line clinical trials of advanced esophageal cancer data published, ICIs have become the standard of treatment of first-line esophageal cancer. ICIs combined with chemotherapy in the first-line treatment of esophageal cancer ameliorate the clinical outcomes and improve survival benefits in patients with ESCC. At the same time, the ORR increased from 27%–45% in the chemotherapy group to 45%–72.1% with the addition of ICIs.

Based on the results of our meta-analysis of esophagogastric cancer chemotherapy regimens with or without ICIs, a total of 17 RCTs were included in this meta-analysis, involving a total of 8,080 patients with esophageal cancer; the major conclusions can be drawn that may directly affect clinical practice. In KEYNOTE-590 study (21) and CHECKMATE 648 study (29), cisplatin +5-FU (FP) regimen was selected in the control group, and the overall ORR rate of patients was <30%. The ORR rate of Taxol + cisplatin (TP) was 45% in the ESCORT-1 (30), Orient-15 (31), and Jupit-06

(32) studies. Although not a head-to-head clinical study, a significant difference in effectiveness between FP and TP regimens was observed. Relevant studies have confirmed that in synchronous chemoradiotherapy for patients with esophageal cancer, there is no significant difference in the efficacy of different chemotherapy schemes combined with radiotherapy. However, the prognosis of ICIs combined with different chemotherapy regimens is different. This may be related to the synergistic effect of ICIs combined with chemotherapy. A previous network meta-analysis showed that the efficacy and safety of different first-line chemotherapy regimens for esophageal cancer were different (38). At the same time, it also reflects that the synergistic effect of different chemotherapy regimens on ICIs is also different. Some studies indicate that 5-Fu/DDP could induce immunogenic cell death in the tumor microenvironment of ESCC. However, the interaction between ICIs and chemotherapy is still unknown. They showed that immunogenic cell death (ICD) was induced in ESCC by proving the maturation of DCs (39). As a consequence, compared with FP regimen, combining with TP regimen is more effective, which provides a basis for the selection of chemotherapy regimen.

Our results show that, in the first-line treatment of esophageal squamous cell carcinoma, ICIs combined with chemotherapy was more effective than chemotherapy alone. OS, 12-month survival rate, and objective response rate were statistically significant. ICIs antitumor mechanisms lead to the particularity of its adverse effects; the effects on the body's immune system will produce the corresponding adverse reaction, In our meta-analysis, in the treatment with ICIs alone compared with chemotherapy alone in the second-line treatment, related adverse reactions and incidence of grade ≥3 TRAEs are statistically significant, but significant statistical differences were not observed in the first-line treatment. Compared to traditional chemotherapy, ICIs have generally fewer side effects.

In addition, six of the most common ICI-related adverse reactions were analyzed. It was found that ICIs often resulted in hypothyroidism, anemia, and loss of appetite. The toxic and side effects of chemotherapy drugs mainly include cytopenia (white blood cells, platelets, hemoglobin, granulocytes, etc.), gastrointestinal reactions (nausea, vomiting, diarrhea, and constipation), liver and kidney dysfunction, cardiotoxicity, and nerve dysfunction (40). Our results indicate that ICIs have more advantages in adverse reactions than chemotherapy, but drug reactions should be carefully monitored and treated in time during drug administration.

Previous studies have demonstrated that there are many risk factors for irAEs, for example, sarcopenia, sex, tumor histology, underlying comorbidities, treatment modality, concurrent medications, preexisting autoantibodies, cytokine assays (IL-6, IL-10, IL-17, CXCL9, CXCL10, CXCL11 etc.), blood cells, gut microbiome, and genetic variability (41).This may explain why adverse effects were not statistically significant in the ICIs group compared with the control group in the first-line treatment of ESCC. However, when esophageal cancer patients are treated with second-line therapy, their nutritional status is often poor, and their skeletal muscle is decreased, which may lead to severe immune-related adverse reactions. Therefore, compared with the chemotherapy alone group, the adverse reactions of ICIs are significantly more serious.

Currently, PD-L1 is a biomarker that can benefit from ICIs. In KEYNOTE-590 study and CHECKMATE 648 study, patients with positive PD-L1 expression had an even greater reduction in risk of death. In the subgroup of patients with negative PD-L1 expression, the risk of death was not significantly reduced, and none of these patients could benefit from combination therapy (21, 29). However, in the ESCORT 1 study, Orient-15 study, and Jupit-06 study led by Chinese experts, the expression of PD-L1 seemed to have little influence on the final benefit of patients after the combination of ICIs and chemotherapy (30–32). The reason may be that TP and FP chemotherapy regimens commonly used in Chinese patients are more effective than those in relevant studies in western countries, resulting in higher benefits for the whole population. In addition, ethnic differences between eastern and western patients lead to different responses to ICIs and different efficacy. Therefore, the detection of PD-L1 expression is still necessary and is the best predictor of efficacy at present. However, it is still necessary to consider the related effects of different race and chemotherapy regimen, combined with the clinical characteristics of patients and other relevant markers, to more accurately identify the beneficiaries of ICIs.

In the field of treatment of esophageal squamous cell carcinoma, the exploration of biomarkers is very important. Biomarkers also need to be combined with clinical features related to esophageal cancer. At the same time, the representativeness of PD-L1 expression results is still limited due to the small size of esophageal cancer biopsy samples. In the future, it is an important research trend to explore more representative biomarkers.

Although ICIs have shown some benefit in ESCC, because of both intrinsic and acquired immune resistance, there are still a large number of patients who do not respond well to ICIs. Research has shown comprehensively characterized tumor-infiltrating immune cells and revealed the landscape of the suppressive immune state for ESCC. In the ESCC TME, there were exhausted T cells, exhausted NK cells, regulatory T (Treg) cells, alternatively activated macrophages (M2), and tolerogenic dendritic cells (tDCs) in these tumors, indicating an inflamed but immune-suppressed TME in ESCC (42).

Some limitations of this work should be acknowledged. Meta-analysis is inherently observational, and, despite our best efforts to investigate inconsistency and to assess the impact of effect modifiers using sensitivity analysis, it is possible that the results are affected by unmeasured confounding. Estimates that rely substantially on indirect evidence should be interpreted with care. In addition, since many large RCTS are currently under way, this paper includes the conferences and abstracts with available data at present, which is highly likely to produce bias in some literatures. Due to the limited literature included for each outcome index, it is

impossible to analyze the source of the variance. The dose of immunosuppressant is different from the regimen, and there is no unified standard. Of course, this paper also has certain advantages, for example, the study subjects were all patients with esophageal squamous cell carcinoma, and more outcome indicators and adverse reactions were collected. The era of ICIs for esophageal squamous cell carcinoma has arrived, and the future is promising. However, ICIs in the field of ESCC has a long way to go.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

YM, YX, and DS wrote the manuscript. YXZ, HL, and HZ performed the literature review. XY, QY, and JC performed the statistical analysis. CW and YQZ revised the text. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.958783/full#supplementary-material

SUPPLEMENT FILE 1
Detailed retrieval strategy and PICOs.

SUPPLEMENT FILE 2
modified Jadad scale.

SUPPLEMENT FILE 3
Sensitivity analysis.

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HCC biomarkers – state of the old and outlook to future promising biomarkers and their potential in everyday clinical practice

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Hepatocellular carcinoma (HCC) is one of the most common and deadly tumors worldwide. Management of HCC depends on reliable biomarkers for screening, diagnosis, and monitoring of the disease, as well as predicting response towards therapy and safety. To date, imaging has been the established standard technique in the diagnosis and follow-up of HCC. However, imaging techniques have their limitations, especially in the early detection of HCC. Therefore, there is an urgent need for reliable, non/minimal invasive biomarkers. To date, alpha-fetoprotein (AFP) is the only serum biomarker used in clinical practice for the management of HCC. However, AFP is of relatively rather low quality in terms of specificity and sensitivity. Liquid biopsies as a source for biomarkers have become the focus of clinical research. Our review highlights alternative biomarkers derived from liquid biopsies, including circulating tumor cells, proteins, circulating nucleic acids, and exosomes, and their potential for clinical application. Using defined combinations of different biomarkers will open new perspectives for diagnosing, treating, and monitoring HCC.

KEYWORDS

hepatocellular carcinoma, biomarker, personalized medicine, alpha-fetoprotein, liquid biopsy, circulating tumor cells, circulating nucleic acids, exosomes

1 Hepatocellular carcinoma – a high disease burden

Hepatocellular carcinoma (HCC) is one of the most frequent tumor types worldwide. Its clinical importance has been rising tremendously in the past two decades: The incidence increased about 75% (1). Concurrently, HCC is ranked 5th most common tumor worldwide (2). In 2020 905,677 new cases of liver cancer worldwide were diagnosed. About 80% of them were HCC (2).

Regarding death caused by cancer, HCC ranks 3rd place (2). The World Health Organization expects more than one million deaths due to liver cancer by 2030 (3). Men are almost three times more likely to be affected than women.

73% of HCC cases occur in Asia (2). The reason for this geographic variance is the endemic occurrence of hepatitis viruses, the most common risk factor for HCC. Despite the decrease in the number of Hepatitis B virus (HBV) infections due to the global introduction of vaccination programs, one-third of all HCC cases worldwide are still based on chronic HBV infection (33%) (1, 4). Hepatitis C (HCV) infection is the second most important risk factor. Even after the introduction of new, highly efficient combination therapies with polymerase inhibitors, protease inhibitors, and non-structural protein 5A (NS5A) inhibitors in 2013, more than one-fifth of HCCs are due to HCV infection (1). Another relevant risk factor with a substantial global disease burden and potential to grow is the metabolic syndrome. Since 1975, overweight and obesity have tripled worldwide (5). Other risk factors are alcohol-related liver cirrhosis (30%), primary biliary cholangitis, primary sclerosing cholangitis, hemochromatosis, and alpha1-antitrypsin deficiency (1). Aflatoxin is a mycotoxin that can also trigger HCC and is mainly found in nuts, dried fruits, and spices.

Physicians should regularly monitor the risk groups depicted above for the occurrence of HCC. Given the high burden of disease, there is a tremendous need for valid and cost-effective biomarkers that can

- identify individual risks for developing HCC,
- detect HCC in an early stage,
- predict therapy response to specific therapies,
- monitor response to therapy and predict adverse side effects of cancer therapies,
- and predict cancer recurrence.

These biomarkers should be easy to obtain and easy to analyze routinely.

2 What characterizes an optimal biomarker?

Modern medicine depends on reliable biomarkers for screening, diagnosis, disease monitoring, prognosis, predicting therapy success, response to therapy, and treatment safety. The BEST (Biomarkers, EndpointS, and other Tools) glossary of the FDA-NIH Biomarker Working Group defines biomarkers as a characteristic that is measured as an indicator of normal biological processes, pathogenic processes or biological responses to an exposure or intervention, including therapeutic interventions." (6). They contain molecular, histologic, radiographic or physiologic characteristics." (6, 7). The FDA-NIH Biomarker Working Group distinguishes between (6):

i. Susceptibility or risk biomarkers are associated with the chance of developing cancer and are essential to define risk populations for surveillance.
ii. Diagnostic biomarkers detect cancer occurrence.
iii. Predictive biomarkers identify patients that might benefit from specific cancer therapies.
iv. Monitoring biomarkers indicate the activity of the disease and response to therapy.
v. Pharmacodynamics or response biomarkers show changes in biological characteristics in response to the dosage of cancer therapy.
vi. Safety biomarkers predict adverse side effects of cancer therapies.
vii. Prognostic biomarkers anticipate cancer recurrence or progression (Figure 1).

For clinical applicability, biomarkers must meet the following criteria:

- The biomarker has to be valid, reliable, and objective.
- The sample collection must only involve a minimal risk for the patient.
- The biomarker has to be easy to collect.
- The sample must be stable under clinical and laboratory conditions.
- Samples should be able to be analyzed on a routine basis.
- Analysis has to be feasible and rapid.

3 State of the old – conventional biomarkers recommended by guidelines

Unfortunately, there are only a few biomarkers for HCC in daily practice so far: alpha-fetoprotein (AFP), HSP70 (HSPA7), glypican 3 (GPC3), and glutamine synthetase (GS). They are not even considered in all major guidelines due to their limited diagnostic quality. In the following, we will briefly review the use of biomarkers for HCC in daily practice:

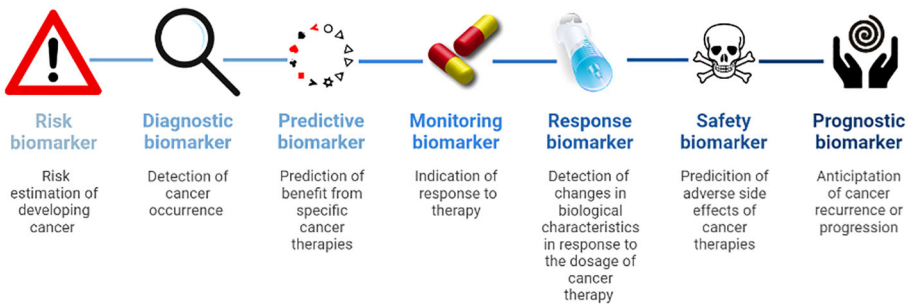


FIGURE 1
Overview of biomarker types.

3.1 Susceptibility biomarkers

Up to date, no susceptibility biomarkers are recommended by HCC guidelines and in daily clinical practice for stratifying the risk of HCC occurrence.

3.2 Diagnostic biomarkers

AFP is the most established clinical biomarker in clinical practice for detecting HCC. It is a transport protein for copper, nickel, fatty acids, and bilirubin expressed during the embryonic phase in human cells. Serum AFP levels diminish rapidly after birth and remain very low during adulthood (8). Of clinical relevance, in adults, AFP is mainly found in tumor cells of the liver, testes, and ovaries. It has been shown that AFP dampens the immune response and can enhance immunological tolerance toward tumors. In detail, secreted AFP interferes with the maturation and function of dendritic cells, leading to a decreased antigen presentation and induction of immune responses (9, 10). Furthermore, AFP interferes with T cell proliferation and shifts T cell response to a more regulatory phenotype. Thus, AFP promotes the immune system's tolerance toward the tumor (11, 12).

Although AFP is commonly used as a biomarker for detection, it imposes enormous limitations: AFP has a very low-test sensitivity and specificity. Several studies showed disappointing or even contradictory results (9). AFP expression is absent in around 80% of early HCC (13). In many cases, liver damage also leads to the upregulation of AFP expression and secretion (14, 15). Especially in patients with high viral load, the determination of AFP displays low reliability. Low cut-off levels above 20 ng/ml show high sensitivity but low specificity, whereas high cut-off levels above 200 ng/ml raise specificity but lower sensitivity (16). Thus, serum AFP levels cannot reliably discriminate between chronic liver damage (e.g., fibrosis and cirrhosis) and HCC. Therefore, the Practice Guidance of the American Association for the Study of Liver Diseases (AASLD) and the Pan-Asian adapted European Society for Medical Oncology (ESMO) Clinical Practice Guidelines recommend biannual ultrasound with or even without determination of AFP levels for patients with liver cirrhosis and hepatitis-virus-infected patients (17, 18). Child Pugh-Stage and co-morbidities should allow curative or palliative therapy. The European Association for the Study of the Liver (EASL) even explicitly opposes the determination of AFP for the reasons given above (19). Nevertheless, according to new meta-analyses, the combination of ultrasound and AFP is a reliable diagnostic approach to detect HCC (7, 20). Zhang et al. recommended 400 ng/ml AFP protein in terms of sensitivity and specificity, whether AFP is used alone or combined with ultrasound (21).

As for AFP, the EASL guidelines assess other conventional diagnostic biomarkers, AFP-L3 and Des-gamma-carboxy-prothrombin (DCP), as unsuitable for cost-efficiency reasons. Nonetheless, studies of last years revealed that a combination of the clinical markers gender and age with the biomarkers AFP-L3, AFP, and DCP, is a good diagnostic marker (22). The combination is named the GALAD score after the initials of the biomarkers.

AFP-L3, an isoform of AFP, binds the lectin lens culinaris agglutinin (LCA) and is produced by malignant hepatocytes (23). A meta-analysis has shown that AFP-L3 has high specificity but low sensitivity for the diagnosis of early-stage HCC, suggesting that AFP-L3 is more valuable for ruling out HCC in conditions with elevated AFP levels than for diagnosing early HCC (24).

DCP is a defective prothrombin and results from a lack of post-translational carboxylation of the prothrombin precursor in HCC cells. Most large-scale studies have been performed in patients mainly of HCV- or HCB-related etiology (25–28).

Johnson et al. developed the score initially (22). In their study, the performance of the GALAD model was significantly better than the simple combination of AFP-3, AFP, and DCP alone. In a prospective phase 3 cohort study by Tayob et al. of 50 HCC patients and 484 controls, the GALAD score was associated with a substantial improvement in sensitivity for

detecting HCC. However, in this study, a limitation of the GALAD score also shows up since the advantage of increased sensitivity was offset by a high number of false-positive results (29). In contrast, the study of Best et al. showed a high specificity of 93.3% (30) and was reliable in the subgroup of HCV and NAFLD patients. In another case-control study from Germany and a pilot cohort study in Japan, the GALAD score has been shown to detect early-stage HCC with high accuracy in patients with NASH, with and without cirrhosis (30–32) and thus could facilitate the monitoring of patients with NASH. In addition, another study shows that the GALAD score is also excellent for distinguishing HCC from chronic liver disease in an HCV subgroup of a cohort of Chinese patients (33). Therefore, despite limitations, the GALAD score seems one of the best options for HCC detection and will likely find its way into the guidelines.

If the diagnosis of HCC cannot be confirmed by typical contrast agent behavior in imaging, the EASL guidelines recommend a biopsy (19), with subsequent staining of HSP70, Glypican-3 (GPC3), and glutamine synthetase (GS).

HSP70 is a chaperone involved in protein folding, protein translocation, and regulation of transcription. In contrast to normal cells, many tumor entities, including HCC, overexpress HSP70 and secrete it into the extracellular matrix. Expression profiling identified HSP70 as a molecular marker for the detection of early HCC (34, 35). In addition, HSP70 serum levels enable discrimination between chronic hepatitis, cirrhosis, and HCC. However, this observation is limited by analyzing a relatively small cohort (86 healthy donors, 50 donors with chronic hepatitis, and 47 HCC patients) (36), and HSP70 is secreted by other tumors as well.

GPC3 belongs to a family of glycosylphosphatidylinositol-anchored cell surface heparin-sulfate proteoglycans. Sung et al. showed that GPC3 is upregulated in HCC tissue and plays an important role in the proliferation of malignant cells (37). Hippo et al. demonstrated that, compared to AFP, GCP3 is a more reliable marker to distinguish between patients with small, well-differentiated HCC and liver cirrhosis (38). However, a meta-analysis by Xu et al. revealed that GCP3 is inferior to AFP in the differential diagnosis between HCC and liver cirrhosis (39). Indeed, GCP3 expression displays no correlation to the expression of AFP.

The enzyme GS catalyzes the synthesis of glutamine, the primary energy source of tumor cells, from glutamate and ammonia in the liver. GS's mRNA, protein, and activity were increasingly upregulated in precancerous lesions to advanced HCC (40, 41). GS displays a 50-59% sensitivity and specificity of 86-90% (42, 43).

A combination of the three markers, GCP3, the chaperone HSP70, and glutamine synthase for early detection of HCC revealed a sensitivity of 72% and a specificity of 100% (42). Thus, the combination of multiple markers results in advanced sensitivity and specificity.

3.3 Predictive biomarkers

Predictive biomarkers play almost no role in the choice of HCC therapy. There are two exceptions:

The curative treatment options for HCC are resection, local ablation procedures, and liver transplantation. Due to the pervasive organ shortage, bridging therapy to transplantation is required, and patients must be carefully selected. According to national guidelines, liver transplantation should not be considered if AFP levels are above 1000 ng/ml due to poor postoperative prognosis (18, 44–46).

Palliative treatment options for advanced-stage HCC include immunotherapies (atezolizumab/bevacizumab, tremelimumab/durvalumab, nivolumab/ipilimumab, pembrolizumab) and protein tyrosine kinase inhibitors (sorafenib, lenvatinib, donafenib, regorafenib, cabozantinib, apatinib). Ramucirumab is a recombinant human monoclonal antibody that inhibits vascular endothelial growth factor receptor (VEGFR) and is approved for second-line therapy. The REACH-2 trial demonstrated that ramucirumab improved overall survival compared to placebo in patients with hepatocellular carcinoma and AFP levels of at least 400 ng/ml who had previously been treated with sorafenib (47). Patients with lower AFP levels did not benefit.

3.4 Monitoring biomarkers

Imaging techniques primarily assess the response of patients with HCC to local or drug therapies. None of the guidelines recommend specific biomarkers for assessing response to therapies. Bruix et al. analyzed two phase 3 studies of prognostic factors and predictors of the benefit of sorafenib in patients with HCC. AFP was not a predictive biomarker of sorafenib benefit (48). However, a recent meta-analysis showed that post-treatment AFP response was significantly associated with overall and recurrence-free survival (49).

3.5 Prognostic biomarkers

Like the assessment of response to treatment, screening for relapse after curative treatment is mainly performed by imaging with MRI, CT, or ultrasound. Imamura et al. showed in a study of 249 patients undergoing hepatectomy that AFP levels above 32 ng/ml indicate relapse (50).

In summary, AFP is the only biomarker used for early detection, prediction, and monitoring of response to treatment and disease recurrence. It has significant limitations in sensitivity and specificity, especially when used alone without combination with other biomarkers. Therefore, there is a great need for other or complementary biomarkers to improve the quality of care for patients with HCC.

4 Promising new biomarkers for clinical application

Conventional tissue biopsies are invasive and associated with risks for patients. Sometimes tissue biopsies are impossible or very difficult to perform due to the location of the tumor or the presence of multiple lesions. In contrast, biomarkers from body fluids are a promising tool for diagnosing and monitoring tumor diseases, especially because they are not or only minimally invasive and can therefore be obtained without or with low risk. The idea of liquid biopsy is based on molecular analysis of circulating cells that have been shed from the tumor and products from malignant tissue that have been released into biological fluids, such as the bloodstream. Thus, liquid biopsies provide access to tumor-derived materials, including circulating nucleic acids, proteins, exosomes, and circulating tumor cells (CTCs). However, the differences between these various groups of markers in terms of accessibility, stability, and detection must be considered.

4.1 Circulating nucleic acids

Nucleic acids are released into the bloodstream after induction of apoptotic or necrotic cell death of tumor cells. These circulating nucleic acids can be divided into two subgroups: (i) circulating tumor desoxyribonucleic acid (ctDNA) and (ii) cell-free ribonucleic acid (RNA).

4.1.1 Circulating tumor DNA

In 1948 Mandel et al. described for the first time that freely circulating DNA is released from dying cells into the peripheral blood (51). Later Leon et al. observed that circulating DNA appeared more frequently in the serum and plasma of cancer patients (52) and reflected the tumor burden (53). Furthermore, ctDNA provides direct access to molecular key information, including genomic (point mutations or copy number variations [CNV]) as well as epigenetic data (changes in DNA methylation) (54).

Apoptotic and necrotic cells release DNA into the extracellular matrix. This DNA can be detected as circulating cell-free DNA in the blood. Solid tumors often show large necrotic areas due to undersupply of oxygen and glucose. Therefore, various tumors, including HCC, can be detected by an increased level of cell-free DNA. Iizuka et al.'s study highlighted the potential diagnostic value of monitoring the amount of cell-free DNA to detect HCC (55). However, elevated cell-free DNA levels have been observed in multiple cancers. Therefore, the amount of cell-free DNA is not HCC-specific. Nevertheless, combining the determination of the amount of cell-free DNA with the detection of HCC-specific protein biomarkers like AFP results in a sensitivity of 87% and specificity of 100% to detect HCC (56). A recent study presents a novel computer-based prediction model that uses comprehensive fragmentomic profiling of cell-free DNA in plasma for early detection of liver tumors. The model showed excellent performance with a sensitivity of 98.8% and 96.8% specificity in detecting primary liver cancer (sensitivity for HCC 96.2% and intrahepatic cholangiocarcinoma 100%). In addition, early-stage primary liver cancer detection was 95.9% (stage I) and 97.9% (stage II). For tumors less than 3 cm in size, the sensitivity was 98.2% (57).

The use of modern technologies such as next-generation sequencing (NGS) allows the detection of mutations in DNA isolated from the blood (58). For example, tumor-specific point mutations of various genes have been detected in the ctDNA of HCC patients. NGS analyses revealed that seven genes and DNA regions derived from ctDNA harbor the most important mutations associated with poor survival in HCC: (i) TERT promoter, (ii) TP53, (iii) CTNNB1, (iv) AXIN1, (v) JAK1, (vi) EPS15 and (vii) CACNA2D4 (59–67). Several studies have shown that analysis of point mutations in these genes is a valuable tool with a clinically relevant impact on prognosis and early detection of HCC (59, 68, 69).

However, the use of somatic mutations previously detected in primary tumor tissue as biomarkers is limited by their variability and low concentration in plasma (70). Besides detection and analysis of point mutations, CNV can be used as early biomarkers and prognostic parameters for HCC (63–66, 71–78).

For example, a characteristic CNV was found in preresection plasma samples of patients with HCC, whereas this CNV was almost absent in post-resection plasma samples. Thus, CNV is an additional marker for detection and treatment surveillance (79, 80).

Furthermore, genomic alterations and epigenetic modifications (e.g., DNA methylation) were detected in ctDNA (81–85). DNA methylation is a mechanism to regulate gene expression and control DNA stability and DNA-protein interactions. DNA methylation is essential in cancer development, especially in HCC formation (54, 86–88). The methylation status of several tumor suppressor genes correlates with HCC occurrence and progression. These genes include p15 and p16, APC, SPINT2, SFRP1, TFP12, GSTP1, and RASSF1A (86, 89, 90). Moreover, specific hypomethylation of the long interspersed nuclear element-1 (LINE-1) (91), methylation of the insulin-like growth factor-binding protein 7 (IGFBP7) gene (92) and hypomethylation of the promotor region of the transcriptional repressor CTCFL were associated with reduced survival in HCC patients (93).

Therefore, combined determination of (i) the amount of circulating cell-free DNA, (ii) additional biomarkers, and (iii) genetic analysis of ctDNA, including detection of tumor-specific point mutations, CNV, and alteration in methylation patterns, are essential tools for detection and prognosis of HCC.

Furthermore, genetic aberrations detected in ctDNA provide molecular information about tumor development and possibly response towards treatment. Recent developments allow the analysis of small amounts of ctDNA. This advance will facilitate analyses of ctDNA in the future.

Conclusion: ctDNA provides a comprehensive representation of genomic alterations of different tumor regions. Isolation procedures are well-established. Improvements in technology allow for higher sensitivity of analytical assays. The short half-life of ctDNA allows real-time monitoring of cancer development with more accurate clinical correlations. The amounts of blood required for isolation are practical from the clinical point of view. The number of patients and the control groups are also large enough in most studies, and international studies are available in addition to many Asian studies. Most studies with ctDNA are in biomarker development phase 1 or 2 (57, 84, 94, 95). The limitations are that it is difficult to distinguish between ctDNA and circulating free DNA (released from non-malignant cells) and the low concentration of ctDNA in blood. In addition, the short half-life, which on the one hand allows real-time monitoring, is on the other hand a challenge for the analysis and storage of the samples. In comparison to CTCs ctDNA is not suitable for functional assays. Furthermore, analysis is time-consuming and costly, and most emerging assays have not yet been clinically validated (Table 1; Figure 2).

4.1.2 Cell-free tumor RNA

In addition to DNA, RNA is also released into the extracellular space. It should be highlighted that RNA is less stable compared to DNA. Therefore, cell-free RNA is associated with proteins, proteolipids, or encapsulated into exosomes preventing its degradation. Three different groups of RNA can be detected in the bloodstream and used as potential biomarkers: circular RNA, micro (mi)RNA, and long-non-coding (lnc)RNA (Figure 3).

4.1.2.1 Circular RNA

Circular RNAs are differentially expressed in various cancer tissues (111–114), including HCC (115), and they are closely associated with the initiation and development of cancer. Circular RNAs arise from aberrant by-products or abnormally spliced transcripts (116). Most up-regulated circular RNAs are positively associated with HCC progression, whereas down-regulation usually displays suppressive effects and prevents

TABLE 1 Overview of relevant biomarker classes for HCC.

Biomarker	Phase of biomarker development	Biomarker type	Sample	Patient risk	Availability	Stability	Analysis on a routine basis	Rapid analysis
ctDNA	Phase 2	Diagnostic (0.57-0.97)[1] (96, 97)	Blood Hepatic tissue	Blood: (+) Hepatic tissue: ++	+/-	++	+	++
	Phase 2	Monitoring						
	Phase 2	Prognostic						
circRNA	Phase 3	Diagnostic (0.81-0.89)[1] (98–101)	Blood Hepatic tissue	Blood: (+) Hepatic tissue: ++	+/-	+/-	+	++
	Phase 2	Monitoring						
	Phase 3	Prognostic						
miRNA	Phase 3	Diagnostic (0.79-0.88)[1] (102–105)	Blood	(+)	+++	+	+	++
	Phase 3	Prognostic						
lncRNA	Phase 2	Diagnostic (0.88-0.92)[1] (106–108)	Blood Hepatic tissue	Blood: (+) Hepatic tissue: ++	++	+	+	++
	Phase 2	Prognostic						
Proteins: AFP	Phase 5	Diagnostic (0.61-0.93)[1] (21, 97)	Blood Hepatic tissue	Blood: (+) Hepatic tissue: ++	+/-	+++	+++	+++
	Phase 5	Monitoring						
	Phase 5	Prognostic						
Exosomes	Phase 2	Diagnostic (n.a.)	Blood	(+)	+++	+++	+	+
	Phase 2	Prognostic						
CTCs	Phase 2	Diagnostic (0.70-0.93)[1] (97, 109, 110)	Blood	(+)	+++	+++	+	+
	Phase 2	Monitoring						
	Phase 2	Prognostic						

[1] pooled AUC in systematic reviews.
Relative properties: - limited; + low; ++ medium; +++ high

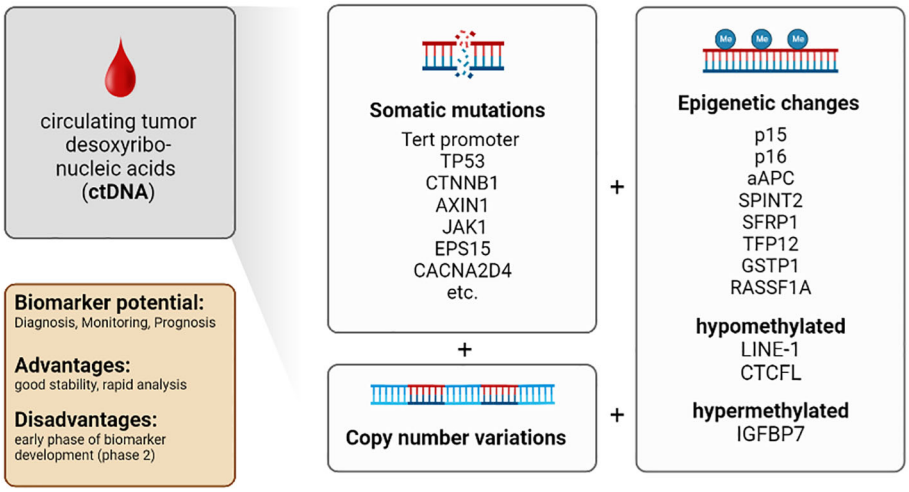


FIGURE 2
Characteristics of the biomarker class circulating tumor desoxyribonucleic acids (ctDNA) in patients with HCC.

HCC development. Most data on circular RNA are based on tissue analysis. Therefore, circular RNAs can also be used as markers in normal biopsies. The particular advantage, however, is that these RNAs can also be found in blood and thus be sampled minimally invasively (117, 118).

Several circular mRNAs showed highly interesting results in context with early detection and diagnosis of HCC (115, 119–123). For example, the circular RNAs hsa_circ_001565 (B4GALT2), hsa_circ_000224 (C17orf107), and hsa_circ_000520 (VIM) display a sensitivity of up to 97% and a specificity of up to 92% in the detection of HCC (124). Very similar results were obtained for hsa_circRNA_104075 (NUP153), hsa_circ_0005075 (EIF4G3), hsa_circ_0028502 (SLC24A6) and hsa_circ_0076251 (ZFAND3). They showed comparable sensitivities of up to 96% and specificities of up to 98% (115, 122, 125).

Besides their potential as biomarkers for the early detection of HCC, circular RNAs are of high prognostic value (124, 126–132). Exemplary is a study showing that hsa_circ_0001727 (circZK - SCAN1) expression was positively correlated with HCC prognosis (129). Moreover, the detection of hsa_circ_001565 (B4GALT2), hsa_circ_000224 (C17orf107), and hsa_circ_000520 (VIM) was associated with prolonged relapse-free survival (124). In contrast, low expression of hsa_circRNA8662-12 (TRIM33-12) was closely correlated with poor prognosis (133).

Conclusion: Circulating free RNA provides an up-to-date snapshot of the transcriptome. It can indicate cancer and trace it back to its site of origin. Limitations are sample instability and high variability of circulating free RNA expression between different individuals. Therefore, studies aiming to define panels of cell-free circulating RNAs that can be used as general biomarkers for HCC are needed. In addition to the small amount and not yet very detailed purification protocols, the high variability and heterogeneity of cell-free circulating RNA are problematic and currently do not allow for clinical application. In addition, there is always a risk that samples will be contaminated with transcellular mRNA. Unfortunately, there are only very few studies from Europe or the USA. Most of the studies originate from East Asia and are in development phase 3 (121, 123, 131, 134) (Table 1). Thus, there is still much development work to be done here.

4.1.2.2 Cell-free micro (mi)RNA

In 2008 Lawrie et al. were the first to describe microRNA (miRNA) as tumor biomarker (135). miRNAs are a member of endogenous non-protein-coding RNA with a size of approximately 20-22 nucleotides. miRNAs are relatively resistant to RNase degradation, boiling, and freeze-thaw cycles (136, 137). miRNAs can be used as markers in tissues and the blood. In terms of patient safety, miRNAs obtained in a liquid biopsy are of particular interest. Especially the stability of these RNAs and their release into the bloodstream makes them attractive as a biomarker. Over the past ten years, miRNAs have become the most intensively studied nucleic acid biomarkers in HCC and have proven valuable in the diagnosis and prognosis of the disease. Nevertheless, many studies have conceptual weaknesses, such as very different non-validated purification methods for miRNAs or unclear sequencing and identification protocols.

miRNAs bind to the corresponding 3´UTR of their target messenger RNA (mRNA) and induce mRNA degradation. Interestingly, there is a correlation between abnormal circulating miRNA levels and pathological characteristics of

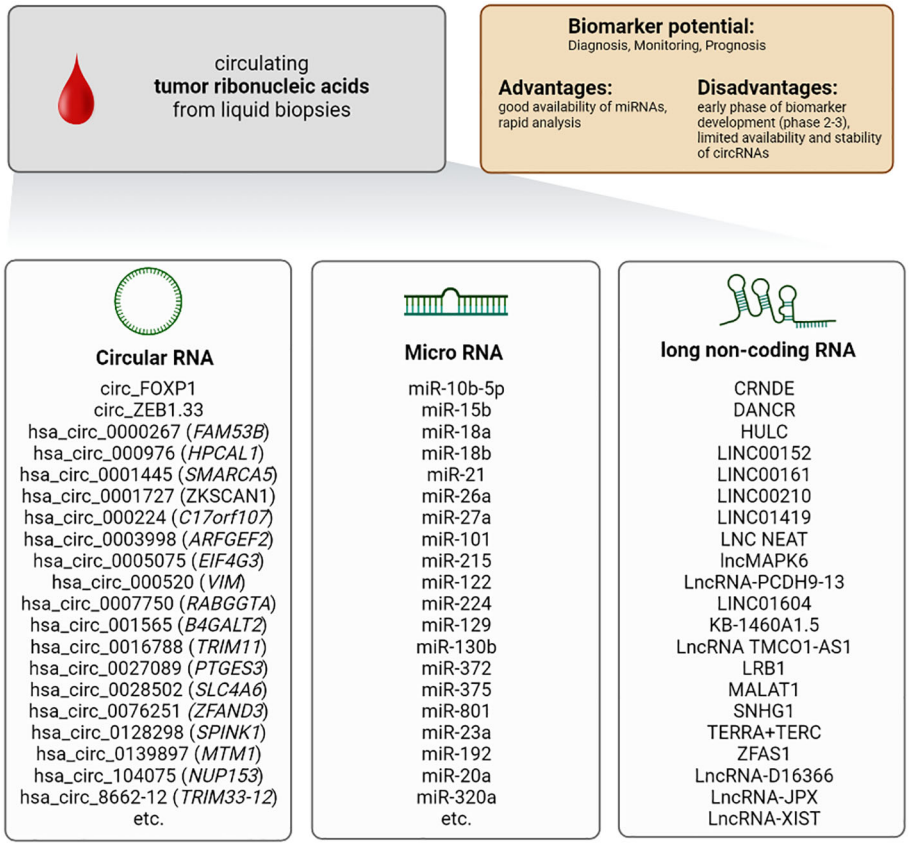


FIGURE 3
Characteristics of the biomarker class circulating tumor ribonucleic acids (cell-free RNA) in patients with HCC.

certain tumors (138–140), including HCC (141). So far, over 70 miRNAs have been proposed as potential biomarkers for HCC (86, 142–159). A meta-analysis evaluated miRNA-21 as a biomarker for early diagnosis of HCC with a sensitivity of up to 88% and a specificity of up to 87% (160). Interestingly, the sensitivity and specificity of the combined miRNA panel miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505 were significantly higher than the sensitivity and specificity of the established biomarker AFP regarding detection of small (AUC: 0.833 vs. 0.727) and early-stage HCC (AUC: 0.824 vs. 0.754). Another panel of miRNAs (miR-192, miRNA-125b, and miR-23a) was suitable for predicting the survival time of HCC patients (161). Huang et al. developed in a phase 3 study an HCC risk score consisting of 5 miRNAs (miR-18a, miR-26a, miR-27a, miR-222, miR-223) that correlates with an increased risk of HCC development in cirrhotic patients (162). Thus, miRNA combinations represent a strategy to develop novel diagnostic tools for HCC and improve treatment surveillance.

Conclusion: The advantage of using miRNA as a biomarker is its wide range of applications, as miRNAs are involved in many pathogenic processes and have high specificity and reproducibility. miRNAs display inherent stability, and the serum concentration is relatively high. The main challenge in establishing miRNAs as biomarkers is that although the available studies have identified a large number of miRNAs as potential markers, the miRNAs identified vary depending on the specific study. Therefore, a “universal marker” is still lacking. This is due to the lack of standardized protocols for the purification of miRNAs and the fact that the expression of these miRNAs is directly linked to the developmental stage and characteristics of the individual tumor. In addition, comorbidities can lead to an increase of unspecific miRNAs, which interfere with detecting cancer-specific miRNAs. Nevertheless, it should be possible to define miRNA panels that could act as universal markers. These panels can then be used for both prognosis and diagnostics purposes. In studies containing information about the required blood volumes, the required volumes were in a range that could be used in the clinic (≤10ml). Unfortunately, some studies lack this information. Most studies include a relatively large number of participants (>100). The control groups are also sufficiently

big in most cases. Interestingly, most recent studies were conducted in East Asia (China) or North Africa (Egypt). However, the number of patients with HCC is increasing in Western countries due to lifestyle and the resulting metabolic syndrome. There is a high heterogeneity of the miRNAs found, and each study finds different miRNAs that can be used as biomarkers. Most studies are in phase 2 (155, 157), and few are in phase 3 (162). We need more studies with clearly defined protocols for the purification of miRNAs and also very precisely characterized cohorts of patients. Therefore, very little can be said about miRNAs as biomarkers for HCC. Generally, they are a potential option that fulfills all the prerequisites for a good marker (Table 1).

4.1.2.3 Cell-free long non-coding RNA (lncRNA)

Circular RNA and miRNA are not the only RNAs that can serve as biomarkers for HCC. Furthermore, long non-coding RNA (lncRNA) shifted into the focus as novel potential biomarkers in HCC. Comparable to miRNAs, lncRNAs belong to the group of non-protein-coding RNA transcripts. They exceed a length of 200 nucleotides. Like other non-protein-coding RNAs, lncRNAs can be detected in the blood. Upon isolation, lncRNAs are stable in the plasma (163, 164). They can interact with proteins, DNA, and other types of RNA. They act as modulators of gene expression by regulating transcriptional and post-transcriptional processes and controlling various cellular processes, e.g., genomic imprinting, cell cycle, cell proliferation, differentiation, and apoptosis (165, 166). Various lncRNAs are involved in cancer pathogenesis, controlling invasion, migration, and metastasis of tumor cells (167–169), and several studies showed that dysregulation of lncRNAs promoted the development of HCC (170).

Therefore, lncRNAs might contribute to the panel of beneficial biomarkers in hepatic tumors. One of the first and most studied lncRNAs in HCC is Highly Upregulated in Liver Cancer (HULC). Several studies demonstrated that circulating HULC could be used as a diagnostic marker, being up-regulated in the blood of patients with HCC (171, 172). Another lncRNA of clinical relevance is LINC00152. The amount of LINC00152 in the blood increases from healthy donors to patients with liver cirrhosis and displays the strongest up-regulation in patients with HCC. This close association with progress from liver cirrhosis to HCC underlines LINC00152 abilities as a potential diagnostic biomarker (172, 173). A further significant increase in sensitivity and specificity was achieved by combining HULC with LINC00152 or combining HULC, LINC00152, and AFP (174). However, a more detailed analysis is needed to prove their value as biomarkers for HCC (175).

Conclusion: The advantage of using lncRNA as a biomarker is its relatively high stability. lncRNAs are involved in multiple pathological processes and can obtain new insights into the progression of the disease. The disadvantages are similar to those of miRNAs. Since lncRNAs are involved in many physiological and pathophysiological processes, detecting tumor-specific lncRNA is difficult. Interference by co-morbidities cannot be excluded. Some studies directly compare tissue samples and blood samples. However, many studies involve small patient and control cohorts. In addition, most studies have been conducted in East Asia. Care should be taken to include European and American cohorts in the future. Most studies are in biomarker development phase 2 (172, 176–179). Therefore, further detailed studies are needed to improve data on individual lncRNAs and establish defined lncRNA panels that can be used as general markers for detecting HCC. The large heterogeneity of lncRNAs identified in the previous studies, which unfortunately overlap only partially, is the major obstacle to clinical application.

Overall, circulating nucleic acids occur in different forms, e.g., circulating tumor DNA (ctDNA) or cell-free RNA. Although circulating nucleic acids are relatively inert towards degradation, it is still more elaborate to purify, store and analyze them compared to proteins. The advantage of nucleic acids as markers is that they contain essential biological information and may be of prognostic value. But these properties provide a large heterogeneity in terms of expression in tumors of different patients, making clinical application difficult. Interestingly, a combination of nucleic acids and marker proteins displays the highest specificity and sensitivity in HCC diagnosis and could thus help develop new identification panels that can be used universally for detecting HCC. Therefore, combining different biomarkers, regardless of nucleic acid, protein or CTC, might be the most promising approach for the future (Table 1).

4.2 Proteins

Tumor cells secret various proteins into the extracellular matrix, e.g., proteases required for invasion and metastasis, proteins dampening the immune system, cytokines, growth factors, and angiogenic factors for proliferation. In addition, tumor cells undergo apoptotic or necrotic cell death due to a lack of oxygen and energy. These dying cells release proteins into the bloodstream.

Proteins are very stable in the blood, and there are several methods for identification by immune-linked and biochemical methods. Detecting tumor-specific proteins is less complex and expensive than identifying and purifying cell-free DNAs and RNAs. As described above, proteins are the most commonly used biomarkers in clinical routine (Figure 4).

4.2.1 Cytokeratin 19

Cytokeratin 19 belongs to the keratin family. It is a filament protein essential for the structural integrity of cells. Cytokeratin 19 has been associated with poor clinical prognosis in HCC patients in several studies. The co-expression of Cytokeratin 19, AFP, and Glypican-3 is an excellent predictive factor for metastasis and adverse treatment outcomes (180). In addition,

concurrent expression of these three proteins was also associated with poor survival (181–183).

4.2.2 Golgi protein 73

Golgi protein 73 is a transmembrane protein localized in the Golgi apparatus. Its function is incompletely understood. Golgi protein 73 expression is linked to patients with liver disease, particularly HCC (184, 185). In the serum of HCC patients, Golgi protein 73 concentration is significantly elevated compared to patients with liver cirrhosis (186). Whether Golgi protein 73 alone is superior to AFP in detecting HCC and discrimination from liver cirrhosis is controversial (186, 187). However, combining both markers has improved the detection of early HCC and discrimination from liver cirrhosis (187).

4.2.3 Annexin 2

Annexin 2 is a calcium-dependent, phospholipid-binding protein linked to cell mobility and protein interaction with the actin cytoskeleton. Recent studies reported that Annexins, including Annexin 2, can interfere with immune functions and induce tolerance (188). Interestingly, Annexin 2 is upregulated in HCC and can indicate tumor malignancy (189). Annexin 2 also showed better sensitivity and specificity than AFP to detect early HCC (190). Thus, Annexin 2 might be a helpful marker for early tumor detection (189), although more detailed studies are needed to estimate the potential of Annexin 2 for clinical application.

4.2.4 Osteopontin

Osteopontin (OPN) is an extracellular matrix (ECM) protein whose elevation is associated with tumor invasion, proliferation, and metastasis in several cancers (191). Using tissue microarrays, Desert et al. analyzed 366 samples from patients with normal liver, cirrhosis, dysplastic nodules, or HCC. They show that OPN increases in expression during hepatocarcinogenesis (192). Wu et al. showed in a recent study that OPN induces JAK2/STAT3/NOX1-mediated ROS production, leading to hepatocellular carcinoma progression (193). Several phase 2 studies show osteopontin has diagnostic (194, 195) and prognostic potential (196, 197) as a biomarker.

4.2.5 Midkine

Midkine, also known as neurite growth-promoting factor 2 (NEGF2), is a secreted protein that functions as a cytokine and growth factor and mediates its signal through proteoglycan and non-proteoglycan receptors on the cell surface (198–201). Midkine enhances the angiogenic and proliferative activities of cancer cells. Expression of midkine (mRNA and protein expression) is increased in several cancers, including HCC. Thus, Midkine can serve as a biomarker in HCC, as shown in phase 3 trials (202–205). Midkine is mainly overexpressed in AFP-negative patients, so it increases detection rates of HCC (205).

4.2.6 Dickkopf-1

The glycoprotein Dickkopf-1 (DKK-1), expressed mainly in the placenta and embryonic tissues, is an antagonist of the Wnt/β-catenin signaling pathway and is elevated in several cancer types. DKK-1 shows higher efficacy for detecting HCC than AFP in phase 4 trials (206–208), but midkine is more precise than DKK-1 in cirrhotic HCV patients (209). A combination of Golgi protein 73, AFP, and Dickkopf-1 increases the sensitivity and specificity of HCC detection (210).

4.2.7 Squamous cell carcinoma antigen 1 and 2

Squamous Cell Carcinoma Antigen (SCCA) consists of two proteins, SCCA-1 and SCCA-2, which are serine protease inhibitors. Several studies investigated the diagnostic value of

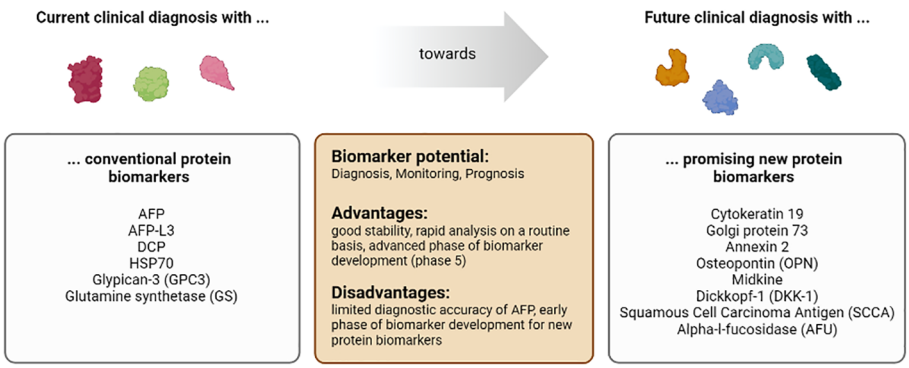


FIGURE 4
Characteristics of the biomarker class proteins in patients with HCC.

SCCA and its immune complex SCCA-IgM in HCC. A recent phase 4 trial with 203 cirrhotic patients revealed that patients with HCC have higher levels of SCCA-IgM than those without it during a five-year follow-up (211). Another phase 4 trial with 91 patients showed that SCCA-IgM also offers predictive value (212). But gender differences have to be considered, as low levels of SCCA-IgM after transarterial embolization indicate more prolonged survival in males and shorter in females (213). SCCA and SCCA-IgM show moderate diagnostic accuracy in several meta-analyses (214–216). Combination with AFP increases prognostic value significantly (214).

4.2.8 Alpha-l-fucosidase

Alpha-l-fucosidase (AFU) is a lysosomal enzyme that is present in low concentrations in human cells, blood, and body fluid and hydrolyzes fucose-containing sugars. Its activity is increased in the serum and tissue of HCC patients. Still, it is not specific to HCC, as high levels are also found in patients with diabetes, pancreatitis, and hypothyroidism (217). A retrospective phase 3 study with 280 HCC patients due to HBV B AFU shows good early detection prosperities of HCC (218), but midkine is a more sensitive predictor than AFU in HCC due to HCV (209). The combination of AFU with AFP raises sensitivity and specificity, especially in hepatitis-negative patients (219), but is worse than AFP alone in patients with HCC due to HBV (220).

Conclusion: The major advantage of protein biomarkers is that the detection is easy to perform, less error-prone, and inexpensive. Results can be obtained quickly and without complex equipment. Therefore, protein biomarkers are optimal for clinical use. The major disadvantage of protein biomarkers is that tumors can escape from detection due to individual differences in the protein expression pattern. However, this can be overcome by using defined combinations of different biomarkers. In the future, defined combinations of protein biomarkers with other types of markers, e.g., circulating nuclear acid and CTCs, may improve detection specificity and sensitivity as well as the prognosis of HCC.

4.3 Exosomes

Exosomes are extracellular vesicles with a diameter of 30-200nm. They are formed in the endosomal compartment of eukaryotic cells. Exosomes are specifically released, facilitating intracellular transport processes, and enabling communication between cells. Their content consists of various components, such as proteins and nucleic acids (Figure 5). Therefore, exosomes give detailed information about the secreting cell or tissue (221). In the liver, mainly hepatocytes, immune cells, and non-parenchymal liver cells release exosomes (222). It should be emphasized that the administration of antibiotics from the subgroup of fluocinolones, especially ciprofloxacin, can increase the secretion of exosomes (223). Furthermore, it must be admitted that studies on exosomes as markers are still in the early stages.

4.3.1 Exosomal lipids and proteins

Exosomal membranes are composed of lipids characteristic for different tissue, including tumors. To date, mainly *in vitro* data are available on the potential role of exosomal lipids as biomarkers for HCC (224, 225). Thus, further analysis must show whether exosomal lipids are suitable biomarkers *in vivo*.

Exosomes contain macromolecules, e.g., proteins protected from extracellular degradation processes. Therefore, the exosomal content is potentially interesting for tumor detection and prognosis. One study identified an HCC-specific exosomal protein profile that included CD44, cell division cycle 42 (CDC42), RAS related protein (RRAS), MET, G protein subunit alpha 13 (GNA13), metalloproteinase domain 1 (ADAM1), GNAS complex locus (GNAS), eukaryotic translation initiation factor 4A3 (EIF4A3) and S100 family proteins (226). An additional study detected HSP70, Hsp90, glypican 3, and the well-established marker AFP specific to HCC (227). However, whether exosome-derived protein panels are more valid biomarkers than freely circulating proteins needs to be analyzed in clinical studies, comparing both parameters according to the purification method, the stability, and thus the robustness of the analysis.

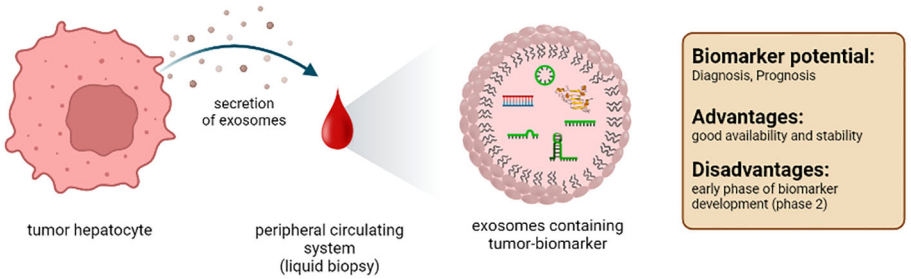


FIGURE 5
Characteristics of the biomarker class exosomes from a liquid biopsy in patients with HCC.

Probably the closest study to a clinical application is the HCC EV ECG score, an extracellular vesicle-based protein assay for detecting early-stage hepatocellular carcinoma (228). Here, exosomes isolated from plasma are analyzed for surface expression of EpCAM, CD63, CD147, CD63, and GPC3. The analysis of this panel of surface markers results in a sensitivity of 91% and a specificity of 90% in the early detection of HCC. Thus, this score could complement current monitoring methods and improve patient outcomes.

4.3.2 Exosomal nucleic acids

In addition to proteins, exosomes also contain nucleic acids, e.g., DNA and RNA. Our knowledge of exosomal DNA as biomarkers for HCC is limited to date. Exosome-derived DNA is protected against degradation; it is of high molecular weight and, therefore, suitable as an HCC biomarker (56, 229). More intensively than DNA, exosome-derived RNA has been explored as a potential biomarker for HCC (230, 231). Several types of RNA can be found in exosomes that are protected from degradation by the exosomal membrane and thus have a significantly longer half-life than free RNAs: mRNA, circular RNA, miRNA, and lncRNA. These RNA species, alone or in combination with other exosomal components, represent interesting biomarkers for HCC. For example, RAB11A mRNA was present in exosomes purified from the serum of patients with HCC. Combined with exosomal lncRNA-RP11-513115.6 and miR-1262, it turned out to be an effective biomarker with high sensitivity and specificity in distinguishing patients with HCC from patients with chronic hepatitis C virus infection (232). In addition, exosomal circular RNAs are particularly interesting for predicting the prognosis of HCC. Many circular RNAs are abundant and stable in exosomes derived from patients with HCC, such as hsa_circ_0088030 (circPTGR1), Cerebellar degeneration-related protein 1 antisense RNA (*Cdr1*), and circDB. These circular RNAs promote cancer cell proliferation and metastasis and are indicators of aggressive tumors with poor prognosis (233–236). Another RNA species detected in exosomes are miRNAs. Detection of exosomal miRNA-210 and miRNA-224 is specific for HCC. Both miRNAs promote angiogenesis and enhance the proliferation and invasion of the tumor (237, 238). Exosomal lncRNAs are also potentially significant for the HCC diagnosis. For example, exosomal-derived lnc-FAM72D-3 and lnc-EPC1-4 levels are significantly increased in the serum of patients with HCC (239, 240).

Overall, exosomes contain similar biomarkers compared to those detected in the blood. The vesicle protects the marker molecules from damage and degradation. This is particularly important for nucleic acids as they are targets for degradation in the blood. Further studies must confirm that exosomes and their contents are suitable biomarkers in HCC.

Conclusion: Exosomes contain a multitude of important information about the cells from which they originate. Their lipid composition, protein, and nucleic acid content are like a fingerprint of the (tumor) cell from which they originate. However, the purification of these small extracellular vesicles and their analysis are complex and require sophisticated equipment. The quality of the isolation of the inter-exosomal proteins or nucleic acids is highly dependent on the methodology, especially since standardized protocols are not yet available.

Several phase 2 trials show promising results for diagnostic and prognostic biomarker usage (82, 241–243). We need more international studies to prove the importance of exosomes as tumor markers, as most studies are from Asia. From a scientific and clinical point of view, exosomes seem to fulfill all the conditions for an ideal marker (Table 1).

4.4 Circulating tumor cells

In 1869, Ashworth detected "cells similar to those in the tumours" in the blood of a patient with a metastatic tumor (244). This was the first description of CTCs and the first detection of tumors by blood analysis. However, at this time, reliable detection and identification of these cells were a nearly unsolvable challenge. Furthermore, in 1895, x-ray imaging by Roentgen was developed as a novel diagnostic tool. Thus, scientists primarily focused on this new imaging method, and the idea of using CTCs to detect tumors was put on hold. Today we know that imaging tools have their limitations, and additional strategies for early detection and prognosis are urgently needed. Therefore, CTCs again became a focus as biomarkers, especially since detection, isolation, and analysis to investigate CTCs have made tremendous progress (Figure 6).

CTCs are cancer cells that circulate in the blood upon being shed off from the tumor. The genetic information of these cells indicates mutations and, therefore, contains hints on sensitivity and resistance towards therapy. In addition, CTCs can be used to form organoids, which serve as personalized tumor models to analyze cell signaling and mimic therapeutic approaches *in vitro* (245–249).

Nevertheless, the isolation and analysis of these cells are still challenging despite novel techniques. Once cells detach from the extracellular matrix, apoptotic cell death, anoikis, is induced in most cells (250). Thus, most tumor cells die within a few hours upon shedding, resulting in a low frequency of CTCs (251, 252). In the blood of patients with metastatic tumors, there is approximately one CTC per $1x10^9$ cells (253). This low frequency makes identification and isolation extraordinarily challenging and asks for strict definitions regarding identification.

The CellSearch™ definition is considered to be the current state-of-the-art standard. This definition states that a CTC is a circulating nucleated cell larger than 4µm, expressing the epithelial cell adhesion molecule (EpCAM) and cytokeratins 8, 18, and 19. To separate CTCs from immune cells, the CTC has to be negative for the leukocyte-specific antigen CD45 (254).

However, only about one-third of CTCs derived from HCC patients are positive for EpCAM and cytokeratins. Thus, the application of CellSearch™ criteria is unsuitable for all subgroups of HCC (255, 256). Another concern is that the level of CTCs correlates with tumor burden. Therefore, sensitivity in the early stage of the disease might be low (252).

Nevertheless, several studies using CellSearch™ showed interesting results for CTCs as diagnostic and prognostic markers. One study analyzed patients with HCC before and one month after liver resection. The number of CTCs was a reliable diagnostic and prognostic marker, indicating the reoccurrence of the tumor (257). Another study using CellSearch™ criteria demonstrated the frequent presence of CTCs in patients with intermediate and advanced HCC (258). Further CellSearch™-based studies showed that the appearance of EpCAM-positive CTCs in HCC could be used to predict recurrence and is associated with poor prognosis (259, 260).

In one study, the nanofiltration technique CanPatrol™ was used to identify clusters of CTCs and white blood cells. Patients exhibiting those clusters show significantly shorter disease-free survival and overall survival (261). Another study using CanPatrol™ shows that a high percentage of mesenchymal CTCs are closely related to the expression of CK19, which is associated with a poor prognosis in HCC patients (262). Qi et al. use CanPatrol™ combined with an RNA-ISH assay to enrich and classify CTCs from patients with HCC. In this study, a slightly increased percentage (≥2%) of mesenchymal CTCs before resection was shown to be a predictive factor for early tumor recurrence (263).

Interestingly, other identification criteria based on less strict definitions also revealed good results for detecting HCC. One study reported that CTCs identified only based on their morphology were associated with shorter survival in HCC (264). In another study, erythrocytes and $CD45^+$ immune cells were depleted from the pool of circulating cells. Afterward, the expression pattern of the leftover cells was monitored by polymerase chain reaction (PCR) using differential expression of EpCAM, CD90, CD133, and CK19 as identifiers for CTCs. Of note, this analysis revealed a sensitivity of 72.5% and a very high specificity of 95% to detect HCC in healthy donors and discriminate HCC from chronic HBV infection and benign hepatic lesions. Using AFP as a biomarker displayed a sensitivity of 57% and a specificity of 90%. Of clinical relevance, this approach also performed well in patients with early-stage HCC and showed a sensitivity of 71.8% and a specificity of 95%. Thus, this method could be a risk prediction and treatment surveillance tool enabling early decision-making to adjust effective antitumor strategies (265). Results of an interesting recent study showed that the detection of CTCs in patients who have undergone LTx allows early prediction of recurrence. Therefore, serial CTC detection may be helpful in the postoperative monitoring of HCC recurrence. However, it has to be considered that the study design is limited due to its small patient cohort, relatively short follow-up of the course, and its single-center design. Therefore, further clinical studies have to follow (243).

Conclusion: The major advantage of CTCs as biomarkers is that these cells contain all the information about the tumor.

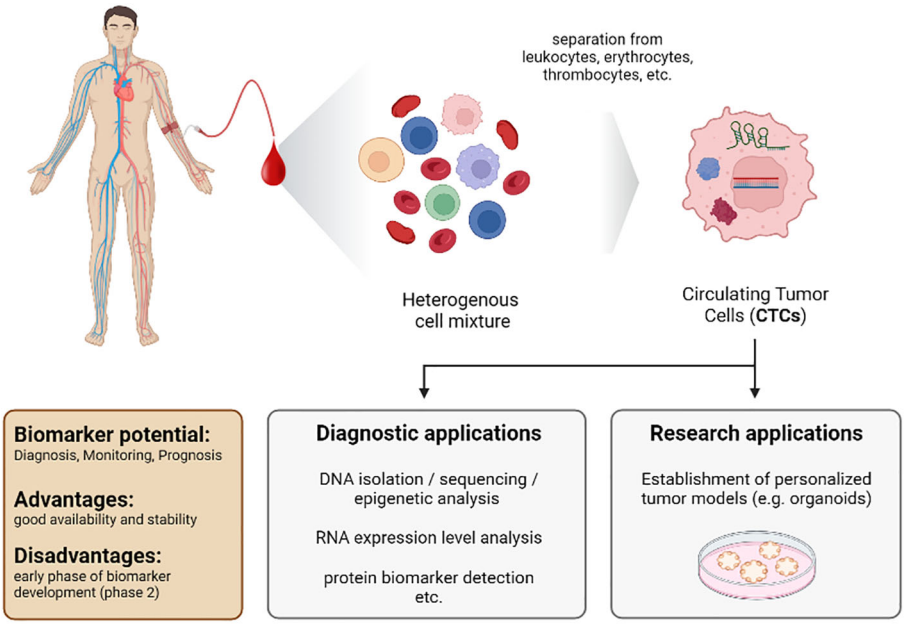


FIGURE 6
Characteristics of the biomarker class circulating tumor cells (CTCs) from a liquid biopsy in patients with HCC.

Furthermore, looking for markers of epithelial-mesenchymal transition determines metastasis formation and aggressiveness of a tumor. Therefore, the analysis of CTCs will advance our understanding of the biology of metastatic diseases and the development of treatment strategies. The required blood volumes are also practical and do not exceed 10ml. The disadvantage of CTCs as biomarkers is that purification and detection are complicated, time-consuming, and costly. The necessary technical equipment may not be available in all clinical settings. Thus, further improvement in the detection and isolation of CTCs is required to use them routinely as biomarkers in the clinic. On the one hand, PCR-based methods might address the challenges in detecting and isolating CTCs and lead to less time-consuming and cost-intensive investigations. On the other hand, such strategies entirely depend on the quality and definition of target transcripts for the detection and staging of the tumor. It is important to note that the control groups in most studies of CTCs were small. In addition, most studies were conducted in East Asia (China, Japan, and Taiwan). Thus, ethnic differences cannot be excluded. Most studies are in phase 2 of biomarker development (257, 261, 263, 265–268) (Table 1). Prospective studies are needed.

5 Final conclusion

HCC is one of the most common tumor diseases with rising incidence and high mortality. Management of HCC especially requires early diagnosis and therapy. Therefore, we need reliable, valid, and objective biomarkers for screening, diagnosis, disease monitoring, prognosis, predicting response to therapy, and treatment safety. Identification of novel non-invasive biomarkers for HCC has become the focus of research. There is an urgent need to define circulating markers that can replace invasive methods like liver biopsies and provide additional information about the tumor. These markers would enable more personalized medicine, including the prediction of therapeutic response. CTCs, ctDNA, circulating RNA, and exosomes are attractive candidates for liquid biopsy since they fulfill many essential characteristics of an ideal biomarker.

A big step towards ideal biomarkers is certainly the analysis of ctDNAs. ctDNA is stable and provides epigenetic and genetic data on the tumor. Detection of mutated DNA and methylation profiling is suitable for early detection of HCC and estimation of prognosis. However, NGS and methylation profiling is complicated and time-consuming. In addition, the different studies often identify completely different markers. It is difficult to understand why there are often no similarities between the markers identified in the studies. One reason is certainly a high heterogeneity between individual tumors. However, this cannot explain the lack of common markers in the studies. The differences must also be due to different study conditions. Here we need standardized and validated protocols for ctDNA purification and analysis. The quality of the samples is the key to a valid statement on the quality of ctDNA as a potential tumor marker.

miRNA and lncRNAs have shifted into the focus of cancer research. So far, more than 70 miRNAs have been proposed as potential biomarkers for HCC, and more and more lncRNA markers have been identified. Most remarkable results for the detection and prognosis of HCC were obtained using well-defined marker panels. These combinations of different RNAs showed very good results in sensitivity and specificity. Other combinations, e.g., with protein markers, are also possible. However, there are often entirely different marker panels, and the studies are not able to confirm the panels from other studies. Again, the quality of the samples plays a crucial role. We need standardized protocols to be used in studies worldwide. This is the only way to identify unique markers that can be used universally. This is true for HCC, but also all other tumors.

Proteins, including AFP, are the best-characterized biomarkers. They do not need elaborate purification and detection methods. Thus, they are optimal to be used in clinical routine. However, they often lack specificity. This disadvantage can be overcome using a combination of different markers or a panel of protein markers and other parameters. A very good example for the combination of several markers is the GALAD score. The score shows that combining three protein markers that can be easily determined in routine clinical practice (AFP, AFP-L3, and DCP) with patient metadata can significantly improve the predictive value. The combination of the three protein markers significantly increases prediction sensitivity but decreases specificity. However, adding simple patient metadata such as age and gender to the protein markers significantly improves both sensitivity and specificity. However, other combinations of tumor markers are also conceivable. The combination of protein biomarkers and nucleic acid markers also shows initial success and leads to improved sensitivity and specificity in prediction. Again, it must be said that more studies from Europe and North America are important to support the findings and reconcile ethnical differences.

Even though fewer data are currently available, analysis of exosomes could provide novel options to detect and understand the development of HCC. Exosomes contain unique functional information, e.g., about interactions between cancer cells and distant cells or the tumor microenvironment. Thus, they are important to gain more insights into tumor physiology. There is certainly a long way to go for exosomes as biomarkers until clinical application. However, they represent a tool with many advantages. Many macromolecules are already degraded in the body or during the purification process. In exosomes, they are protected. Thus, errors can be avoided, and differences due to purification can be prevented. This could help to solve the challenges described above for nucleic acid biomarkers.

CTCs are the carbon copy of the tumor itself. On the one hand, they can provide genetic information about mutations and epigenetic alterations of the tumor. Moreover, CTCs exhibit the transcriptome and proteome of the cancer of origin. Thus, they

fulfill all criteria of an ultimate biomarker. On the other hand, they occur sparsely in blood, and there are no optimal/general surface markers for HCC-derived CTCs. Thus, purification and identification are very challenging and costly. Nevertheless, the blood volumes required for purification are practical (not exceeding 10ml). Regardless, new appropriate purification and analysis methods must be developed to routinely use these biomarkers in the clinic. Additionally, it is unfortunate to note that most studies on CTCs and other tumor biomarkers were conducted in East Asia. We need more studies in the Western industrialized nations since it is precisely here that an increase in HCC is apparent. This is primarily due to lifestyle and associated malnutrition.

We are on the right track in identifying new biomarkers, but we need more and better studies. Since HCC is a worldwide challenge, we need international studies to consider ethnical differences. To establish reliable universal markers, we need standardized and validated purification, storage, and processing protocols for the corresponding macromolecules or CTCs. This is the only way to identify and develop new good tumor markers.

After developing and validating novel biomarkers, the final step has to be their integration into the clinical routine. The novel liquid biopsy-based tools will not replace the established methods but will supplement them to optimize patient care. These markers are non-invasive or minimally invasive and, therefore, easier to implement, as only small volumes of biological material (e.g., blood) are required. As mentioned before, the challenge is certainly the purification and analysis of the samples. However, there are great technological advances that help us to overcome these obstacles. Therefore, we can look optimistically into the future and assume that we will have significantly more and better biomarkers for HCC in the near future.

Author contributions

Conceptualization: MM, SoS and KG. Investigation: SoS and KG. Original draft preparation: MM, KG and SoS. Review and editing: MM, KG, CK, SoS, AK, StS, DT, HT. Table preparation: SoS, KG, BV, KN, NE. Visualization: KG, SoS and DT. Supervision: MM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Meta-analysis of the effect of PGM on survival prognosis of tumor patients

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Objective: A systematic evaluation of the impact of phosphoglucose translocase PGM on the survival prognosis of tumor patients was conducted to understand its impact on tumors so as to improve the quality of survival and to find effective therapeutic targets for tumor patients.

Methods: The following were searched in the databases China National Knowledge Infrastructure (CNKI), Wanfang, Wipu, PubMed, EMBASE, ScienceDirect, Web of Science, and Cochrane Library: "PGM1", "PGM2", "PGM3", "PGM4", and "PGM5" as Chinese keywords and "PGM1", "PGM2", "PGM3", "PGM4", "PGM5", "PGM1 cancer", "PGM2 cancer", "PGM3 cancer", "PGM4 cancer", "PGM5 cancer", and "phosphoglucomutase". Relevant studies published from the database establishment to April 2022 were collected. Studies that met the inclusion criteria were extracted and evaluated for quality with reference to the Cochrane 5.1.0 systematic evaluation method, and quality assessment was performed using RevMan 5.3 software.

Results: The final results of nine articles and 10 studies with a total of 3,806 patients were included, including 272 patients in the PGM1 group, 541 patients in the PGM2 group, 1,775 patients in the PGM3 group, and 1,585 patients in the PGM5 group. *Results of the meta-analysis*: after determining the results of the nine articles, it was found that the difference was statistically significant with a p-value <0.05 (hazard ratio (HR) = 0.89, 95% CI 0.69–1.09, p = 0.000). To find the sources of heterogeneity, the remaining eight papers were tested after removing the highly sensitive literature, and the results showed I^2 = 26.5%, p < 0.001, a statistically significant difference. The HR for high expression of PGM1 and PGM2 and PGM5 was <1, while the HR for high expression of PGM3 was >1.

Conclusion: Although PGM1, PGM2, PGM3, and PGM5 are enzymes of the same family, their effects on tumors are different. High expression of PGM1,

PGM2, and PGM5 can effectively prolong the overall survival of patients. In contrast, high expression of PGM3 reduced the overall survival of patients. This study of PGM family enzymes can assist in subsequent tumor diagnosis, treatment, and prognostic assessment.

KEYWORDS

cancer, PGM, survival prognosis, glycogen metabolism, meta-analysis

Introduction

Phosphoglucomutase (PGM), a key enzyme involved in the synthesis and breakdown of glycogen, is essential in the formation of carbohydrates from G-6-P and the formation of G-6-P from galactose and glycogen (1). At this stage, five enzymes of the PGM family have been identified: PGM1, PGM2, PGM3, PGM4, and PGM5. The coding sequences of these five enzymes are homologous, but due to their different substrates and functions, their effects on tumors are also different (2). This study found that PGM is basically involved in glucose metabolism (3, 4). PGM2 expression levels may affect the reduction of glucose-1,6-bisphosphate expression levels in human erythrocytes (5). PGM3 is targeted to inhibit the hexosamine biosynthesis pathway, inhibit tumor growth, and promote apoptosis (6). PGM4 has been less studied, and no in-depth studies have been found in the literature. Mutations in PGM1 gene cause PGM1 deficiency, which is classified as an inborn metabolic disorder and was once identified as a glycogen accumulation disorder. Existing studies have found that PGM1 deficiency is a congenital glycosylation disorder (7–9). An increasing number of studies have found that PGM1 influences tumor development through its involvement in glycogen metabolic processes (10, 11). PGM5 has been studied mainly in muscle tissue and is highly expressed in cardiac muscle, skeletal muscle, and smooth muscle (12). It is mainly distributed in the periphery of myofibroblasts, localized at intercellular adhesion junctions, and plays an important role in cell adhesion junctions and cytoskeleton maintenance. Available studies have shown that PGM5 is important for the diagnosis and prognosis of a variety of tumors (13, 14). At this stage, there are many ways to treat tumors, such as surgery, chemotherapy, and radiotherapy. However, the efficacy of these treatments is not significant for patients with advanced stages. In order to improve the survival quality of tumor patients and find effective therapeutic targets, it is necessary to study the effect of PGM on the survival prognosis of tumor patients.

In this review, we conducted a meta-analysis of the data related to PGM homologous enzymes affecting the survival prognosis of tumor patients to understand the role of PGM family-related enzymes in tumors and to assess the risk of PGM1, PGM2, PGM3, and PGM5 on survival prognosis so as to provide more evidence-based medical evidence for clinical treatment and prognosis judgment.

Methods

Data sources

The following were searched in the databases China National Knowledge Infrastructure (CNKI), Wanfang, Wipu, PubMed, EMBASE, ScienceDirect, Web of Science, and Cochrane Library: “PGM1”, “PGM2”, “PGM3”, “PGM4”, and “PGM5” as Chinese keywords and “PGM1”, “PGM2”, “PGM3”, “PGM4”, “PGM5”, “PGM1 cancer”, “PGM2 cancer”, “PGM3 cancer”, “PGM4 cancer”, “PGM5 cancer”, and “phosphoglucomutase”. Relevant studies published from the database establishment to April 2022 were collected.

Study selection

Inclusion criteria: 1) PGM homologous enzymes (PGM1, PGM2, PGM3, PGM4, and PGM5), 2) OS data are included in all literature, 3) large data sample size, and 4) clear and complete data sources. Exclusion criteria: 1) missing data for the study, e.g., number of missing pieces; 2) incomplete information and incomplete research; 3) duplicate publication; 4) overviews, case reports, etc.; 5) unclear efficacy evaluation; and 6) excluded articles related to research on anoxic microenvironment.

Data extraction

On the basis of the inclusion and exclusion criteria, two evaluators independently conducted database searches and screened the literature, and differences in opinion, when encountered, were resolved through discussion. Information extraction was performed for the final included literature: 1)

basic information of the literature: title, author, time of publication, and country or region; 2) clinical study information: type of study, number of cases, and treatment protocol; and 3) outcome indicators: if two evaluators could not agree, a third evaluator was asked to participate in the decision.

Quality evaluation

Quality assessment of the included literature was performed using the *Cochrane Handbook for Systematic Reviews of Interventions*.

Statistical analysis

The OS data plots for each group were first analyzed using Engauge Digitizer software, and the final hazard ratio (HR) and 95% CI were obtained using the tool of Jayne F. Tierney et al. The quality of the literature was evaluated using RevMan 5.3 software, and a meta-analysis of all the outcome indicators of the included studies was performed using Stata 12 software, using HR and its 95% CI as effect evaluation criteria. Statistical heterogeneity of the literature was analyzed using I^2, and when $p \geq 0.05$ and $I^2 \leq 50\%$, studies were considered to have no statistical heterogeneity, and fixed-effects models were used. When $p < 0.05$ and $I^2 > 50\%$, studies were considered to have statistical heterogeneity, random-effects models were used, and the sources of heterogeneity were further discussed.

Results

Study selection

According to the search strategy, a total of 3,847 relevant articles were obtained for the initial screening. Duplicates were removed, and reviews, conferences, and case reports were excluded. The titles, abstracts, and full texts were further read; 21 were re-screened by combining the inclusion and exclusion criteria. There was 0 article on PGM4, and nine articles finally met the criteria. The literature screening process and results are shown in Figure 1.

General characteristics

Ten studies from nine included papers had a total of 3,806 patients, including 272 in the PGM1 group, 446 in the PGM2



FIGURE 1
Flow chart of literature screening.

group, 1,503 in the PGM3 group, and 1,585 in the PGM5 group. The basic characteristics of the nine papers were analyzed and are shown in Table 1.

Methodological quality evaluation

The quality evaluation and risk assessment of the included literature are shown in Figure 2.

Meta results

Effect of PGM homologous enzymes on hazard ratio

In this included literature, 10 data sets from nine papers were statistically analyzed. Forest plot analysis revealed a mixed picture of the effect of PGM homologous enzymes on tumor cells. *Evaluation criteria*: the impact of the expression of PGM homologous enzymes in tumor patient tissues on patient survival prognosis was examined. A heterogeneity test was performed on these 10 groups of data, I^2 = 73.4%, p = 0.000, and a meta-analysis was performed using a random-effects model because the I^2 value was >50%. The results showed that the HR for high expression of PGM1 and PGM2 and PGM5 was <1, while the HR for high expression of PGM3 was >1. The difference was statistically significant (HR = 0.89, 95% CI 0.69–1.09, p < 0.001) (Figure 3). Thus, high expression of PGM1, PGM2, and PGM5 inhibited tumor development. High expression of PGM3 promoted tumor development.

Sensitivity analysis

As we can see from Figures 4. 5, almost all studies' combined effect sizes were within the 95% CI. However, by comparing the specific data through sensitivity analysis, we can see that the three reports by authors Hyunmin Lee, Zhongqi Cui, and Bing Chen have the potential to create heterogeneity in the overall data (Figures 4, 5).

Data analysis (after excluding heterogeneity)

We found that the literature by Lee et al. was responsible for the elevated heterogeneity, and removing the literature had the potential to cause elevated heterogeneity separately in order to clarify the source of heterogeneity. After we removed this literature, we performed heterogeneity tests on the remaining eight papers and found that I^2 = 26.5%, p < 0.001, a statistically significant difference. Since the I^2 value was <50%, we made forest plots using a fixed-effects model (Figure 6). The results showed that high expression of PGM1 and PGM2 and PGM5 inhibited tumor development and that high expression of PGM3 promoted tumor development.

Publication bias

Publication bias was evaluated using Stata 12 software, and Egger's test was used for evaluation. To ensure the effectiveness of the funnel plot test, nine groups of data were included, one of which analyzed the survival prognosis of two groups of tumors. Therefore, a total of 10 groups of data were included for analysis. The possibility of bias was determined by funnel plot with p-value = 0.641, p > 0.05, indicating no publication bias (Figure 7).

Discussion

Tumor is one of the major diseases that people need to overcome in the 21st century. Through the meta-analysis of

TABLE 1 Basic characteristics of the included studies.

Author	Year	Country	Related factors	Tumor type	Data source	Survival analysis	n	HR (95% CI)
Guang-Zhi Jin, et al. (10)	2018	China	PGM1	Liver cancer	Non-database	OS/CIR	272/272	0.75 (0.45–1.24)
Zhongqi Cui, et al. (15)	2021	China	PGM2	Colon cancer	TCGA	OS	446	0.54 (0.37–0.79)
Francesca Ricciardiello, et al. (16)	2020	Italy	PGM3	Pancreatic cancer	Non-database	OS	95	1.89 (0.75–4.77)
Hyunmin Lee, et al. (17)	2022	United States	PGM3	Bladder cancer/breast cancer	OncoLnc	OS	402/1006	1.3 (1–1.7) 1.35 (1.09–1.66)
Yan Jiao, et al. (14)	2019	China	PGM5	Liver cancer	TCGA	OS/CIR	367/320	0.82 (0.61–1.11)
Yifan Sun, et al. (13)	2019	China	PGM5	Colon cancer	TCGA	OS	79	0.79 (0.27–2.29)
Zaizai Cao, et al. (18)	2020	China	PGM5	Oral cancer	TCGA	OS	516	0.93 (0.70–1.11)
Bing Chen, et al. (19)	2021	China	PGM5	Lung adenocarcinoma	TCGA	OS	478	0.65 (0.55–0.88)
Fang Ran, et al. (20)	2021	China	PGM5	Breast cancer	METABRIC	OS/DFS	145/145	0.64 (0.19–2.11)

OS, overall survival; DFS, disease-free survival; CIR, cumulative incidence of relapse; HR, hazard ratio.

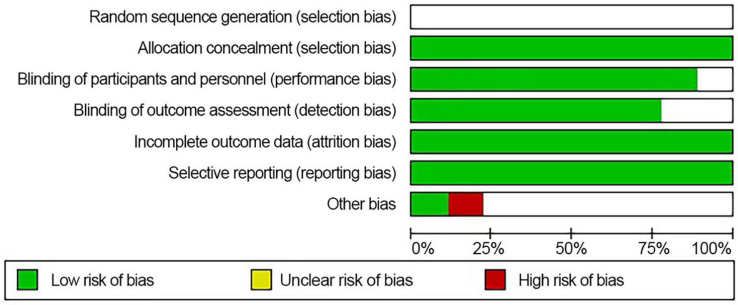


FIGURE 2
The form of literature quality evaluation.

PGM1, PGM2, PGM3, and PGM5 in PGM homologous enzymes, we hope to understand the role of PGM homologous enzymes in tumors and provide assistance for subsequent treatment and research.

PGM1 is an important key enzyme in the processes of glycogen synthesis and catabolism, which catalyzes the reversible transfer of phosphate at the α-D-glucose 1 and 6 positions. On the one hand, the G-1-P produced by glycogen catabolism is transformed into the first intermediate product G-6-P in the glycolysis process. On the other hand, G-6-P is converted into G-1-P to produce the substrate for the synthesis of uridine diphosphate glucose (UDP-glucose), which is necessary for the synthesis of many cellular components such as glycoproteins (21, 22). PGM1 inhibits cell proliferation and tumor growth by utilizing sufficient extracellular glucose for conversion to glycogen in an aerobic environment, while its deletion inhibits glycogen synthesis, leading to more glucose for glycolysis, which promotes tumor cell proliferation and tumor growth. Jin et al. (10)showed that PGM1 could inhibit the progression of hepatocellular carcinoma by regulating glucose transport. Therefore, the high expression of PGM1 can inhibit the development of tumors.

Phosphoglucomutase 2 (PGM2) also catalyzes the reversible conversion of glucose 1-phosphate to glucose 6-phosphate. PGM2 has been reported to be a biomarker for potential prognostic assessment of renal clear cell carcinoma (23). The study by Yang et al. (24) illustrated that PGM2 can be an important indicator for the prognosis of colorectal cancer. The high expression of PGM2 can inhibit the development of tumors.

PGM3 is a member of the hexose-phosphate metastable enzyme family and plays a major role in glycogenolysis and

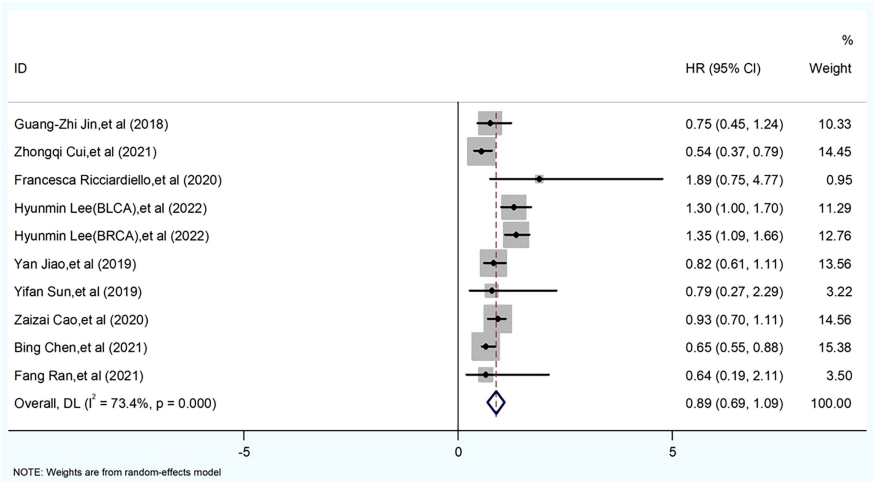


FIGURE 3
Meta-analysis of PGM homologous protein expression profile and survival prognostic risk ratio (HR) in forest plot.

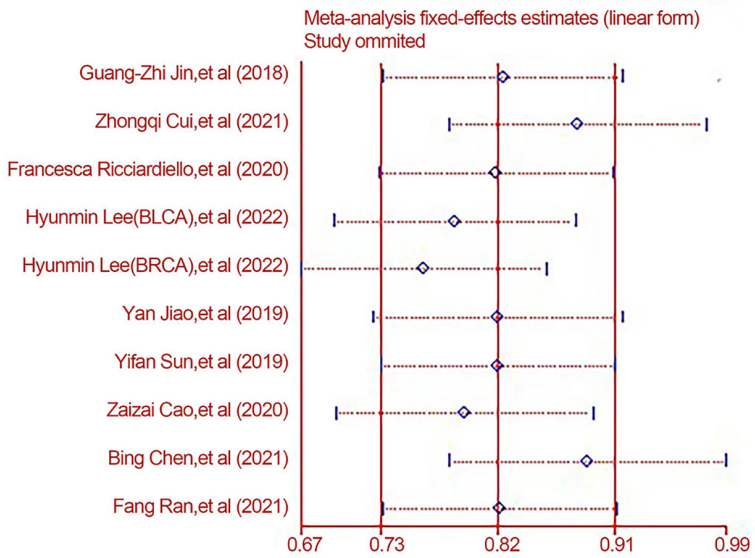


FIGURE 4
The Overall Sensitivity analysis between different groups.

gluconeogenesis. PGM3 is a *N*-acetylglucosamine triphosphatase involved in the biosynthesis of aminoalanine, which exerts anti-cancer effects (25). Lee et al. (17) showed that targeted therapy against PGM3 could be a therapeutic strategy for KRAS/LKB1 co-mutant lung cancer, validating the tumor-promoting effect of high expression of PGM3.

PGM5, also known as phosphoglycosidase-related protein (PGM-rp) or aciculin, is located on human chromosome 9 (9q21.11) (26). PGM5 shows high expression in smooth muscle, skeletal muscle, and cardiac muscle. PGM5 also has an important effect on glycolysis, and several studies have reported that high expression of PGM5 inhibits tumor development. Ran et al. (20) showed that miR-1224-3p promotes the proliferation and migration of breast cancer cells through PGM5-mediated aerobic glycolysis. Sun et al. (13) showed that PGM5 predicted the prognosis of colorectal cancer patients. Jiao et al. (14) showed that PGM5 is a biomarker for the diagnosis and prognosis of hepatocellular carcinoma.

In this paper, we included nine articles and 10 study results with a total of 3,806 patients, including 272 patients in the PGM1 group, 446 patients in the PGM2 group, 1,503 patients in the PGM3 group, and 1,585 patients in the PGM5 group. Through meta-analysis, we found that when PGM1, PGM2, and PGM5 are highly expressed, their HR is less than 1, indicating that PGM1, PGM2, and PGM5 can improve the overall survival rate of patients and have an inhibitory effect on tumors. When PGM3, which is also a PGM homologous

Study ommited	e^coef.	[95% Conf.	Interval]
Guang-Zhi Jin	2.4702017	1.9801772	3.0814905
Zhongqi Cui	2.5719776	2.0859272	3.1712847
Francesca Ricciardiello	2.4043617	1.9644897	2.9427264
Hyunmin Lee (BLCA)	2.2982235	1.8875868	2.7981927
Hyunmin Lee (BRCA)	2.2448869	1.8913016	2.6645763
Yan Jiao	2.4599109	1.9461533	3.1092937
Yifan Sun	2.4371438	1.9786648	3.0018575
Zaizai Cao	2.4181511	1.9069076	3.0664597
Bing Chen	2.5364878	2.0156133	3.1919665
Fang Ran	2.4513388	1.9902215	3.0192928
Combined	2.4275269	1.9851927	2.9684206

FIGURE 5
Specific data for sensitivity analysis between groups to the overall.

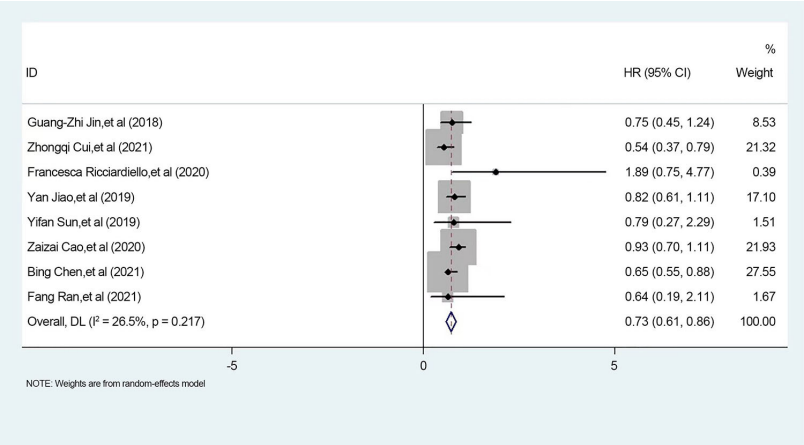


FIGURE 6
Meta-analysis of PGM homologous protein expression profile and survival prognostic risk ratio (HR) in forest plot.

enzyme, is overexpressed, its HR is >1, indicating that the overexpression of PGM3 will reduce the overall survival rate of patients and play a role in the development of tumors. Therefore, it is necessary to study PGM to assist in follow-up treatment and prognosis judgment.

The main reason for the high heterogeneity of the studies included in this paper may be related, first of all, to the expression of PGM between different tumors. The tumors selected for this experiment were different among the PGM isoenzymes, which may be one of the reasons for this high heterogeneity. Even the same tumor may have a different expression for different PGMs. In this experiment, the effect of PGM3 on tumors was opposite to that of PGM1, PGM2, and PGM5, which may be the reason for the increased heterogeneity in the literature by Lee et al.

This study has several limitations. 1) The number of included studies was too small for corresponding subgroup analysis. 2) Bias exists despite a comprehensive search.

Conclusion

In this study, based on the available clinical data, we performed a meta-analysis of the data on the expression of PGM homologous enzymes affecting the survival prognosis of tumor patients. The results showed that although PGM1, PGM2, PGM3, and PGM5 are homologous enzymes, their effects on tumors are different. The high expression of PGM1, PGM2, and PGM5 effectively prolonged the survival of patients. In contrast, high expression of PGM3 decreased the survival of patients. We

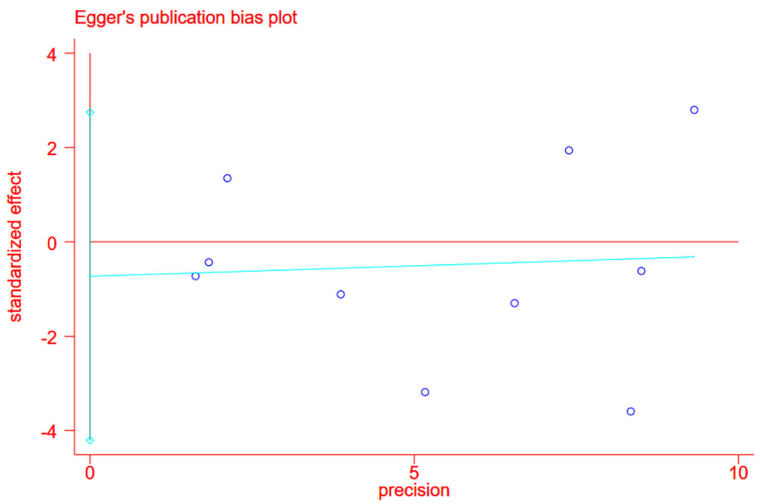


FIGURE 7
The plot of literature publication offset funnel.

hope that the meta-analysis can provide some reference for the study of clinical treatment and targeted drug therapy.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

Methodology: ZZ, XZ, JB, and YZ. Software: XZ, ZZ, and CZ. Validation: ZZ and SS. Writing—original draft preparation: XZ and ZZ. Supervision: XZ, ZZ, and YZ. Project administration: XZ, ZZ, and YZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Adenocarcinoma arising in an ectopic enterogenous cyst: A rare case report and review of literature

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Enterogenous cyst (EC) is a rare congenital lesion generally located in the central nervous system, such as in the cerebral hemispheres, posterior fossa, or spinal canal. They are usually benign lesions, and malignant transformation is rare. A 42-year-old woman felt an obvious pain in the lump and went to a local hospital for local lumpectomy. After 7 months, she again felt pain in the buttocks and difficulty in urinating and defecation. The computed tomography (CT) scan showed a mass in the pelvis. Sacrococcygeal cyst excision was performed 10 days later, and postoperative pathology showed epidermoid cyst. Shortly after, the patient recovered and was discharged from the hospital; the pain in the buttocks continued to recur. Puncture and drainage were performed five times. Later, the patient went to our hospital for treatment, and pelvic MRI showed multiple abnormal signal shadows in the presacral and sacrococcygeal regions, some of which were considered abscesses, and some were cystic lesions. She underwent tumor resection and was diagnosed with EC with locally moderately differentiated adenocarcinoma. Four months later, the patient's symptoms of swelling and pain recurred. MRI examination showed multiple high-signal T2 shadows in the anterior sacral and subcutaneous tissues of the buttocks, and enhanced scan showed partial marginal enhancement. After assessment, the patient was given a radiation dose of 60 Gy/25F. ECs in the anterior sacral and soft tissue of the buttocks are very rare, and the case of carcinomatous transformation has never been reported. Therefore, we discussed the clinicopathological features of ectopic ECs and reviewed the literature.

KEYWORDS

enterogenous cyst, malignant transformation, anterior sacral, soft tissue, adenocarcinoma

Introduction

Enterogenous cyst (EC) is considered as a rare disorder of dermal development. EC occurs during the third week of intrauterine life and evolves from the remnant or ectopic tissue of the neural tube and the gastrum (1). The tumor is mostly located in the spinal canal of the cervical and thoracic vertebrae of the central nervous system and is often accompanied by abnormal vertebral body morphology and function. In addition, ECs occurring in the mediastinum, abdominal cavity, and intracranium have also been reported. However, cyst location in the anterior sacral and soft tissue is very rare, and concurrent malignant transformation of cysts has never been reported.

We reported a case of EC with adenocarcinoma transformation in presacral and subcutaneous soft tissues and reviewed relevant literature to collect its clinical characteristics, providing reference for the diagnosis and treatment of such patients.

Case presentation

Clinical presentation

A 42-year-old woman revealed that, during childhood, a soft tissue mass near the anus was found. The mass was about 4 cm × 5 cm in size, with a slightly tough texture, and she did not experience pain; thus, she received no special attention. In 2019, she felt an obvious pain in the lump and went to the hospital for local lumpectomy. After surgery, a deep sinus was found, and the doctor repeatedly irrigated the lump with normal saline. After 7 months, she again felt pain in the buttocks and experienced difficulty in urination and defecation. She was readmitted to the hospital, and the computed tomography (CT) scan showed a mass in the pelvis. Vaginal puncture and drainage guided by B ultrasound was performed to relieve difficulty in defecation. Sacrococcygeal cyst excision was performed 10 days later, and postoperative pathology showed epidermoid cyst. The pain in the buttocks continued to recur after the patient was discharged from the hospital. After that, puncture and drainage were performed five times under the guidance of B ultrasound. The last time a drainage tube was placed in the tumor, about 25 ml of yellowish clear liquid was drawn out every day. Later, the patient went to our hospital for treatment.

Imaging and laboratory features

Pelvic magnetic resonance imaging (MRI) showed multiple abnormal signal shadows in the presacral and sacrococcygeal regions, some of which were considered abscesses. MRI scans showed a multiloculated T1 hypointense and T2 hyperintense cystic lesion in the right buttock tissue and sacrococcygeal region. Annular enhancement of the cystic wall was observed on the T2-weighted gadolinium-enhanced MRI scans (Figure 1). Colonoscopy showed no obvious abnormality. The admission laboratory examination showed that the levels of tumor serum markers, such as CEA, CA199, AFP, and CA724, were the scope of the standard. Also, there were no signs of infection. Surgical resection was performed again, and a mass of about 4 cm × 3 cm was removed (Figure 2). Unfortunately, the tumor could not be completely removed.

Pathological and immunohistochemical characteristics

The final histological diagnosis was EC with locally moderately differentiated adenocarcinoma (Figure 3). Outside the cyst wall is fibrous connective tissue and smooth muscle (Figures 3A, B). The specimen showed benign cyst lesions covered by a single layer of columnar or cuboidal epithelium. Some of the cyst walls were lined by dysplastic epithelium, with interstitial infiltration. Local cancerous lesions showed glands of different sizes and disorderly arrangement (Figures 3C, D). Immunohistochemical staining of SATB2 and CDX2 in the cyst epithelial cells showed strong nuclear staining (Figures 2E, F). In addition, MLH1, MSH2, MSH6, PMS2, CDH-17, and CK20 and other specific molecules were also positive, and CK7 was negative. All the above immunohistochemical results showed the same immune characteristics as intestinal adenocarcinoma.

Treatment options and follow-up

Four months later, the patient's symptoms of swelling and pain recurred. Considering the recurrence of the lesion, the patient was given a radiation dose of 60 Gy/25F. For the rare disease, there is insufficient evidence on the role of adjuvant chemotherapy. Given the immunohistochemical results, a fluorinated pyrimidine- and oxaliplatin-containing regimen was recommended, but the patient declined chemotherapy. We plan to identify KRAS, NRAS, and BRAF mutations and evaluate MSI status to guide subsequent individualized therapeutic and oncological prognosis for the patient, which failed due to the patient's financial reasons. As of 20 July 2022, the patient had a good outcome, and a recently repeated MRI scan did not show any evidence of cyst recurrence. This indicates that radiotherapy shows a good local control effect.

Discussion

ECs are considered congenital abnormalities. The etiology and pathogenesis of ECs are not clear. Most scholars believe that the ectoderm and endoderm are tight during the early

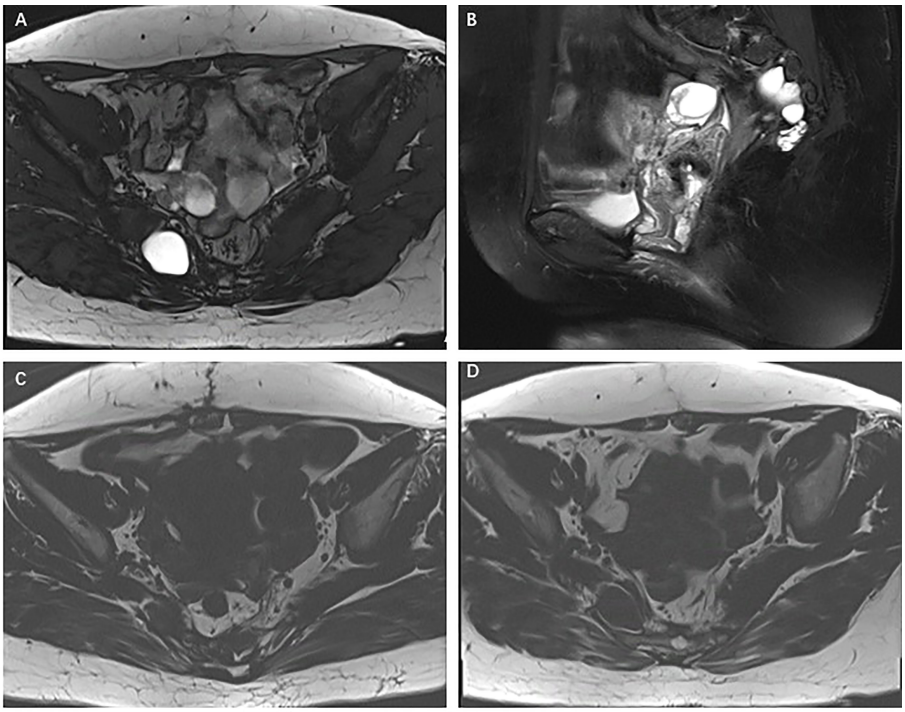


FIGURE 1
(A–C) T1- and T2-weighted MRI scans demonstrating multiple ventricular T2 hypersignal and T1 hypointense cystic lesions in the right buttock tissue and sacrococcygeal region. **(D)** T2-weighted gadolinium-enhanced MRI scans demonstrating annular enhancement of the cystic wall, but no enhancement of the cystic part of the lesion.

embryonic period. At the third week of the embryo, they separated with the development of the embryo (the ectoderm developed neural tube, the endoderm differentiated into intestinal tube), and the middle was only linked by the nerve-intestinal sac zone. If the embryo development separation disorder, residual or ectopic, it will form perineural EC, often combined with gastrointestinal, spinal, spinal cord and other malformations (2).

ECs mostly occur in the central axis, with cervical segment and upper thoracic segment being more common, lumbosacral department being rare, and other parts being rarer (3). The clinical symptoms of ECs are not typical and are often related to the site of occurrence, mainly radiculopathy and spinal cord compression at the corresponding site of the lesion. Most patients may be associated with congenital spinal malformations, such as vertebral fusion, hemivertebra, spina bifida, and butterfly vertebra. The main manifestations of intracranial ECs are intermittent headache and dizziness, which may be followed by seizures, cerebral nerve palsy symptoms, limb movement disorders, and increased intracranial pressure symptoms.

The occurrence of ECs in the anterior sacral and hip soft tissue is very rare, and ECs with malignant transformation are rarely reported. We reported a case of sacral caudal EC with carcinogenesis. After a PubMed search, we retrieved a total of nine literatures in Table 1, which had two parts. One part involved the cases with similar lesion sites to our case; only one case was located in the anterior sacral area, one case was located in soft tissue, and none of them showed signs of malignant transformation (4, 5). ECs occurring in the sacral and soft tissue are very rare. Li et al.'s surgical analysis of 33 patients with presacral tumors discovered that one patient had benign EC with abdominal mass as the initial symptom (5). Mantoo et al. reported an EC in the subcutaneous tissue of scapula with pain and a growing mass, which lasted several years. EC was diagnosed after surgery and there was no recurrence after surgery (4). Our case occurred in the subcutaneous tissue of the buttocks, with similar symptoms, such as swelling and pain. Multiple punctures were performed to discharge pus and relieve pressure. In addition, our lesions also occurred in the sacral anterior area, resulting in the patient experiencing difficulty in defecation.

The other part included seven cases of ECs undergoing malignant transformation from the central nervous system and retroperitoneum (6–12), which were not collected and discussed before. Among the seven cases of malignant transformation of ECs, the pathological type of the cases reported by Monaco et al. was intraepithelial carcinoma, and the other four cases

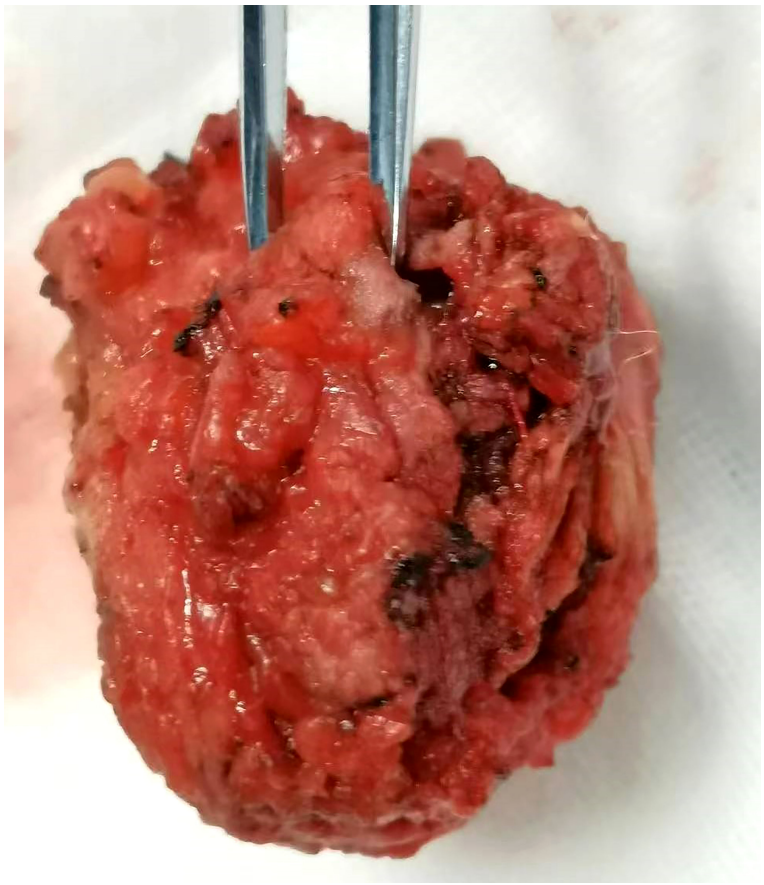


FIGURE 2
Resected lump specimens (4 cm × 3 cm).

developed adenocarcinoma and recurred. We also found that only two cases reported elevated serum tumor markers, both of which were CA199 (6, 12). It is speculated that there are no specific serum tumor markers for malignant ECs.

In our current case, several aspects are worth discussing. First, the site of occurrence is relatively special, occurring in the anterior sacrum and subcutaneous tissue of the buttocks, which we ascribed to ectopic ECs. For ectopic ECs, some scholars also named them isolated ECs, that is, cysts occurring outside the gastrointestinal tract with no direct anatomical connection and common blood supply with adjacent tissues and pathological features of gastrointestinal wall (13). The mechanism of ECs in the sacral caudal and subcutaneous tissues is unclear. Mantoo et al. speculated that the migration of cells from the foregut during intrauterine development plays an important role in the process of occurrence (4). Specifically, during the first 3 weeks of embryonic development, due to incomplete separation of mesoderm and endoderm, the unseparated endoderm tissue develops into the foregut, which then forms ECs. In addition, we hypothesized that ectopic implantation of intestinal epithelium caused by injury may be one of the possible causes of isolated ECs. In the cases of the ectopic cyst we mentioned above (4, 5), benign ECs occurring in the sacral presacral and soft tissue are characterized by a continuously growing mass, which can be cured by surgery and is not likely to recur. In our case, repeated recurrence after surgery may also be related to specific sites. Second, most of the ECs are benign and malignant transformation is rarely reported in the literature, especially in rare sites, such as the sacral caudal and subcutaneous soft tissue (14, 15). The exact molecular mechanism of malignant transformation remains unknown. A possible mechanism is that chronic inflammation is prone to malignant transformation due to repeated cyst rupture or subtotal resection of the cyst wall. Alternatively, long-term carcinoma *in situ* lesions may lead to malignant transformation (11, 16). We first report a case of ectopic EC showing malignant transformation of moderately differentiated adenocarcinoma.

Clinical and radiological examinations will likely fail in identifying malignant ECs, which can be confirmed by pathological and immunohistochemical staining. The epithelial cells forming the wall of NC cysts can be flat, cubic, columnar,

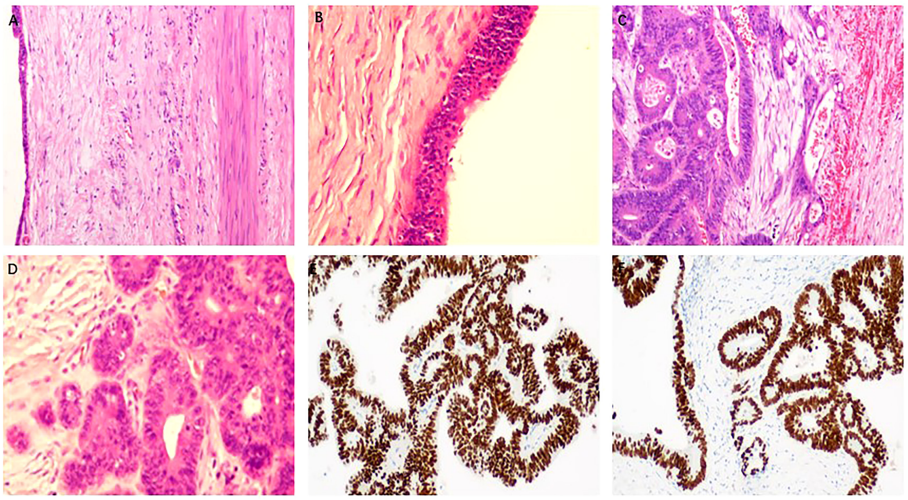


FIGURE 3
Histopathological features of malignant transformation of EC. **(A, B)** Cuboid columnar epithelium on smooth muscle tissue of cyst wall. **(C)** Focal cyst wall shows adenocarcinoma with interstitial invasion, and cancer tissue breaks through the basement membrane and invades the interstitium. **(D)** In the lesion area, glands of different sizes and disorderly arrangement can be seen, and the cancer cells are irregular in shape, different in size, and different in nuclear staining. **(E, F)** Immunostaining indicated positivity for CDX2 **(E)** and SATB2 **(F)** (original magnification: **(B, D)** ×200; **(A, C, E, F)** ×100).

TABLE 1 Characteristics of cases with ECs from published literature.

	Type								
	Similar lesion site			Malignant transformation					
Author	Mantoo et al. (4)	Li et al. (5)	Jeffrey (6)	Ho et al. (7)	Sahara et al. (8)	Monaco et al. (9)	Hill et al. (10)	Gessi et al. (11)	Tsutsumi et al. (12)
Site	Scapula soft tissue	Anterior sacral	Retroperitoneal	Right hemisphere	Foramen magnum	Posterior fossa	Retroperitoneal	Posterior fossa	Cervical spine
Published year	2008	2011	2007	1998	2001	2003	2004	2008	2020
Sex	M	M	M	F	M	M	F	M	M
Age	46	63	54	45	53	36	33	25	43
Nation	Singapore	China	UK	USA	Japan	Italy	Australia	Italy	Japan
First symptom	Pain Increasing lump	Abdominal mass	Heartburn	Abnormal sensation	Neck pain	Headaches Vomiting Drowsiness	Left loin pain	Hypoacusis	Numbness of right limb
Duration	Several years	2 months	4 years	NA	NA	Several months	NA	6 years	1 month
Elevated tumor markers	NA	NA	CA199	None	NA	NA	NA	NA	CA199
Therapy	S	S	S	S	S+R	S	S	S	S
Cancerization	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pathological type			Adenocarcinoma	Adenocarcinoma	Adenocarcinoma	Intraepithelial carcinoma	Adenocarcinoma	Adenocarcinoma	Adenocarcinoma
Recurrence	No evidence	No evidence	Yes	Yes	Yes	No	Yes	Yes	Yes
Follow-up	6 months	29 months	1 month (dead)	24 months	42 months (dead)	24 months	NA	6 months (dead)	6 months (dead)

M, male; F, female; (dead), died of the disease; S, surgery; S+R, surgery + radiotherapy; NA, Not Applicable.

pseudo-laminated columnar, laminated squamous epithelium, etc. In some cases, more than two epithelial components may be seen, with intermigration. The outer layer of the cyst wall is composed of fibrous connective tissue, and melanin deposition, cartilage, smooth muscle, mucus gland, fat, and calcification can be seen in some cases. Our immunohistochemical staining results show that intestinal epithelial-specific transcription factors CDX-2, cadherin CDH-17, special AT-rich sequence-binding protein SATB-2, and cytokeratin 20 (CK20) were all positive, while CK7 was negative, suggesting a foregut origin. CDX2 is a gut-specific nuclear transcription factor and a key regulatory protein for intestinal epithelial formation and differentiation. In gastrointestinal tumors, it is mainly expressed in the small intestine and colorectum, which can be used as a differential indicator of the primary site of tumors. CK20 and the Merkel cell-derived marker of gastrointestinal epithelial transitional epithelium, used in gastrointestinal adenocarcinoma, are often positive for bowel cancer. CK7 (cytokeratin 7), a marker of epithelial origin, is usually expressed in adenocarcinomas, expressed in glandular and transitional epithelial cells, and not expressed in cells of non-epithelial origin. Adenocarcinoma of the gastrointestinal tract was negative. SATB2 is strongly expressed in adult lower gastrointestinal tract and colorectal epithelial cells. SATB2 has a good positive rate in colorectal cancer, and its sensitivity and specificity are high. CK7–/CK20+ carcinomas make up over 90% of cases of colorectal carcinomas (17).

Surgery is the preferred treatment and it is widely believed the cyst should be removed regardless of the symptoms. The key to the operation is whether the capsule wall can be completely removed; otherwise, it will relapse and regenerate due to residual lesions. Once recurrence occurs, reoperation may be considered. In our case, due to the difficulty of complete resection and the malignant transformation of the lesion, postoperative local radiotherapy was combined with residual lesion. Through literature review, we found that only one patient with a tumor located in the foramen magnum received radiotherapy (50 Gy) and chemotherapy (including carboplatin and etoposide), but died 1 year after the second surgery (8). In addition, Jeffrey reported a case of a retroperitoneal EC with adenocarcinoma *in situ*, in which fluoropyrimidine and oxaliplatin were planned for the intestinal tumor, but the patient died of metastatic disease before chemotherapy (6). In the other cases, only surgical resection was performed (7, 9–12). For the treatment of malignant ECs, the choice of postoperative chemotherapy and radiotherapy is worthy of further investigation.

Since ectopic ECs are rarely reported and easily misdiagnosed as other diseases, more cases are needed to collect and summarize their characteristics. Most cases reviewed in this paper are from case reports, and the standards are not uniform, leading to limitations in our conclusions. Further observation and comparative studies are needed.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

HD and DX are the common first authors and the major contributors in writing the manuscript. SZ and ML edited the manuscript and approved the final version. XZ and MF were responsible for reviewing the literature and collecting the information of the patients. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Diagnostic value of circulating lncRNAs for gastric cancer: A systematic review and meta-analysis

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Objective: With the prevalence of next-generation sequencing (NGS) technology, a large number of long non-coding RNAs (lncRNAs) have attracted tremendous attention and have been the topic of extensive research on gastric cancer (GC). It was revealed that lncRNAs not only participate in the transduction of various signaling pathways, thus influencing GC genesis and development, but also have the potential for GC diagnosis. Therefore, we aimed to conduct a meta-analysis of previous studies on GC.

Materials and methods: An electronic search was made before August 2021 on databases including PubMed, Embase, and Web of Science. Relevant articles that compare lncRNA expression in GC patients and healthy controls were summarized. We conducted a meta-analysis with the objective of evaluating the ability of lncRNAs in diagnosing GC.

Results: A total of 40 original research studies including 6,772 participants were discussed in this meta-analysis. The overall sensitivity, specificity, and the area under the curve (AUC) were 0.78 (95% CI: 0.75–0.81), 0.79 (95% CI: 0.74–0.83), and 0.85 (95% CI: 0.81–0.87), respectively. The value of pooled diagnostic odds ratios (DORs) was 13.00 (95% CI: 10.00–17.00).

Conclusions: This meta-analysis revealed that serum or plasma lncRNAs have high sensitivity and specificity, which makes lncRNAs clinically feasible in diagnosing GC. The results from this meta-analysis demonstrated that peripheral blood lncRNAs may become novel noninvasive biomarkers in the foreseeable future. At the same time, it should be noted that a greater number of blood samples and more evidence from rigorous multicenter clinical studies are necessary to justify their applicability as cancer biomarkers.

KEYWORDS

gastric cancer, lncRNA, diagnosis, systematic review, meta - analysis

Introduction

Cancer is the leading cause of death and is a significant obstacle in the pursuit of a higher life expectancy worldwide (1). Unfortunately, the incidence and mortality of cancer are growing rapidly. Gastric cancer (GC) is an important malignant tumor in the digestive tract. According to the latest data, in 2020 alone, there are over 1 million new patients diagnosed with GC and about 769,000 cases die from it (2). It is widely accepted that chronic *Helicobacter pylori* (*H. pylori*) infection is the primary cause of GC (3, 4), and the International Agency for Research on Cancer (IARC) cited *H. pylori* as a group 1 carcinogen (5, 6).

The present treatment strategy for early GC usually depends on endoscopic surgery, while for advanced GC, the treatment methods include surgery, chemotherapy, and immunotherapy (7). Although progress has been achieved in GC treatment, challenges in terms of diagnosis remain. By the time symptoms appear in patients, most of them have already been diagnosed with an advanced stage of cancer (8), which seriously affects their prognosis and 5-year survival rate (9). Currently, gastrointestinal endoscopy operation together with biopsy is the main approach to identifying GC lesions, but detecting small lesions proved to be difficult because of the limited experience of endoscopists (5). In addition, patients find it difficult to undergo endoscopy because it is an invasive procedure and causes discomfort. Consequently, noninvasive biomarkers tend to be a better choice to solve this difficulty. From the traditional point of view, biomarkers in detecting GC can be classified from serum and gastric juice: serum biomarkers included carcinoembryonic antigen (CEA), carbohydrate antigen 199 (CA199), carbohydrate antigen 724 (CA724), and pepsinogen (PG) (10). Gastric juice biomarkers included CA724, CEA, CA199, CA242, and α1-antitrypsin (11, 12). However, the low sensitivity and specificity of these biomarkers in detecting GC limit their further application (13). Therefore, exploring novel biomarkers is of great importance in GC diagnosis.

With the increasing popularity of NGS applications, a large number of studies have been conducted to identify the role of lncRNAs in various tumors over several decades. Long non-coding RNAs, a class of non-coding RNA molecules with a length of more than 200 nt and lacking open reading frames, are closely associated with tumor invasion (14), metastasis, and drug resistance (15) of GC through multiple pathways. Moreover, studies also evaluated the diagnostic value of lncRNAs in distinguishing GC patients from healthy volunteers. These studies have demonstrated that the expression of lncRNAs could be a novel biomarker in screening GC due to their high sensitivity and specificity. Therefore, it is worthwhile to perform a systematic review and summarize the diagnostic values of these lncRNAs.

Some meta-analyses investigated the diagnostic or prognostic value of lncRNAs. However, most of them only focused on one specific lncRNA, such as lncRNA TP73-AS1 (16), lncRNA DLX6-AS1 (17), lncRNA DRAIR (18), and lncRNA HEIH (19). Furthermore, another study used a small number of lncRNAs to determine the diagnostic value of all lncRNAs in GC but ignored the heterogeneity sample differences (20). Considering the weakness of previous studies, a more integrative meta-analysis is necessary to determine GC diagnosis *via* lncRNAs.

Materials and methods

Search strategy

In order to identify potentially eligible studies that were published before August 2021, two authors (JL and QX) separately conducted an electronic database search, including PubMed, Embase, and Web of science. The following search strategy was used: (Lnc RNA OR long non-coding RNA OR lncR) AND ("stomach neoplasms"[Mesh] OR "gastric cancer" OR "stomach cancer" OR "Gastric Neoplasm" OR "gastric carcinoma" OR "stomach carcinoma" OR "gastric adenocarcinoma" OR "stomach adenocarcinoma") AND (blood OR serum OR plasma OR circulating) AND (diagnosis OR diagnostic OR diagnose).

Literature selection

For the enrolled articles, the following inclusion criteria must be fulfilled: (1) a comparison was made between GC and healthy controls; (2) the diagnosis of GC was confirmed by a pathologist; (3) the detection technique had to be quantitative real-time PCR and test samples were from serum or plasma; and (4) sufficient data were provided to calculate 2 × 2 tables including TP (true positive), FP (false positive), TN (true negative), and FN (false negative).

The exclusion criteria were as follows: (1) duplicate articles; (2) reviews, meta-analysis, bioinformatics, case reports, and laboratory studies; (3) studies irrelevant to the diagnostic value of lncRNAs or GC; and (4) the full text was not available.

Quality assessment

The Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2) (21) was applied to evaluate all enrolled articles in the meta-analysis, which mainly depend on the following domains: patient selection, index test, reference standard, and flow and timing. YZ, SB, and YD were responsible for this part of the work.

Data extraction

Two authors (YZ and YD) independently screened the full text of every study and extracted relevant information or data including (1) basic information of the enrolled articles: the first author, publication year, country of origin, ethnicity, specimen type (serum or plasma), lncRNA type, cases, and healthy control group size, mean age, and gender distribution; and (2) sensitivity, specificity, TP, FP, FN, and TN values, which were also extracted from each article.

Statistical methods

STATA 16.0 (Stata Corporation, College Station, TX, USA) and Revman 5.4 (The Nordic Cochrane Centre, Copenhagen, Denmark) were used to analyze extracted data. In this diagnostic meta-analysis, forest plots were applied to estimate sensitivity and specificity. The area under the curve (AUC) of the summary receiver operating curve (SROC) was used to calculate the diagnostic efficiency of serum or plasma lncRNAs in GC. According to a previous report, diagnostic efficiency can be divided into low, good, very good, and excellent in terms of AUC values:<0.75, 0.75–0.92, 0.93–0.96, and 0.97 or above (22). Meanwhile, Q test and Higgins I^2 statistic were used to estimate the heterogeneity among all included studies. If $I^2 >$ 50%, signifying the existence of heterogeneity, then the random-effect model was needed for data consolidation. Otherwise, the fixed-effect model was needed. Finally, the potential bias of publication was estimated by Deeks' funnel plot. $p < 0.05$ was considered statistically significant.

Registration

This article has been registered on the International Platform of Registered Systematic Review and Meta-analysis Protocols (INPLASY, https://inplasy.com/); the registration number is INPLASY2022110024.

Results

Literature search

Through the search strategy described above, there were 476 articles from PubMed, Embase, and Web of Science included. A total of 69 duplicates were removed after a review of titles and abstracts. Next, we carefully read the rest of the articles and found 364 irrelevant publications. In addition, three articles were excluded for inadequate data. Finally, 40 publications including 6,772 participants were involved in this systematic review and meta-analysis. The basic characteristics of the included articles are listed in Table 1, and the flow-process diagram for the literature is presented in Figure 1.

Quality assessment

The QUADAS-2 tool embedded in Revman 5.4 was used to assess the quality of each study. As shown in Figures 2A, B, the evaluation criteria mainly focus on patient selection, index test, reference standard, and flow and timing.

Diagnostic accuracy of circulating lncRNAs

We added all included studies to Revman 5.4, and then according to the extracted data, related figures were plotted *via* STATA 16. There were 52 lncRNAs reported among 40 studies, and their corresponding diagnostic accuracies are shown in Table 2. Overall sensitivity, specificity, and AUC were 0.78 (95% CI: 0.75–0.81), 0.79 (95% CI: 0.74–0.83), and 0.85 (95% CI: 0.81–0.87), respectively, which signifies a great performance for lncRNAs as noninvasive biomarkers to distinguish GC patients. The pooled diagnostic odds ratio (DOR) was 13.00 (95% CI: 10.00–17.00). Meanwhile, the pooled positive likelihood ratio (PLR) and negative likelihood ratio (NLR) were 3.70 (95% CI: 3.00–4.50) and 0.28 (95% CI: 0.24–0.32), respectively.

Publication bias

Deeks' funnel plot asymmetry test was used to evaluate the publication bias of the enrolled articles. The results demonstrated a low potential for publication bias ($p = 0.00$).

Discussion

In clinical practice, there are various noninvasive circulation biomarkers applied when screening GC patients from a healthy population. Of note, invasive diagnostic methods are unable to forecast prognosis and monitor the progress of GC. Meanwhile, the discomfort caused by such invasive tests makes it difficult for patients to accept them, thus limiting their further applications. In addition, traditional biomarkers lack enough specificity and sensitivity to diagnose GC, making their diagnostic efficacies questionable (23). Therefore, developing appropriate noninvasive biomarkers that can be used to diagnose and predict the prognosis of GC patients is of paramount importance. With the prevalence of next-generation sequencing (NGS) technology, a large number of

TABLE 1 Characteristics of the studies included in the meta-analysis.

Article ID	First author	Year	Country	Ethnicity	Total	Gastric cancer group			Control group			Specimen	LncRNA
						Sample size	Mean age	Gender	Sample size	Mean age	Gender		
1	Shiyi Qin	2021	China	Asian	180	98	/	57/41	82	/	/	Serum	HCP5
2	Fei Han	2021	China	Asian	159	76	57.3	52/24	83	56.1	49/34	Serum	CCAT2
3	Hao Xu	2020	China	Asian	159	109	/	81/28	50	/	/	Serum	MIAT
4	Quan Zhou	2020	China	Asian	478	200	/	/	278	/	/	Serum	C5orf66-AS1
5	Hui Zhou	2020	China	Asian	159	81	64.2	51/30	78	/	/	Serum	H19
6	Peiming Zheng	2020	China	Asian	120	60	/	38/22	60	/	/	Plasma	lnc-SLC2A12-10:1
7	Guodong Zhang	2020	China	Asian	128	68	48.2	36/32	60	48.8	32/28	Plasma	PTCSC3
8	Haiyan Piao	2020	China	Asian	361	281	/	/	80	/	/	Serum	CEBPA-AS1
9	Wenwen Liu	2020	China	Asian	162	89	/	63/26	73	/	/	Serum	FEZF1-AS1, AFAP1-AS1
10	Shibao Li	2020	China	Asian	70	43	62	32/11	27	62	20/7	Serum	GNAQ-6:1
11	Rongrong Jing	2020	China	Asian	184	104	/	/	80	/	/	Serum	RP11-731F5.2
12	Wei Feng	2020	China	Asian	194	107	/	/	87	/	/	Serum	B3GALT5 AS1
13	Guohua Zhang	2019	China	Asian	106	53	/	/	53	/	/	Plasma	ARHGAP27P1
14	Ziwei Yang	2019	China	Asian	215	109	/	82/27	106	/	51/55	Plasma	PANDAR, FOXD2-AS1, SMARCC2
15	Waleed A. Mohamed	2019	Egypt	African	60	35	45.2	28/7	25	42.7	16/9	Serum	H19
16	Ying Xu	2019	China	Asian	68	34	/	/	34	/	/	Plasma	DGCR5
17	Yun Liu	2019	China	Asian	134	94	59	57/37	40	59	26/14	Serum	HOXA11-AS
18	Hong Jiang	2019	China	Asian	417	317	/	/	100	/	/	Plasma	PCGEM1
19	Bing Ji	2019	China	Asian	242	168	/	101/67	74	/	/	Plasma	LINC00086
20	Cao Peng	2019	China	Asian	160	88	47.7	52/36	72	47.1	44/28	Serum	GASL1
21	Rui Zheng	2019	China	Asian	346	173	65	111/62	173	65	110/63	Plasma	FAM49B-AS, GUSBP11, CTDHUT
22	Chenchen Cai	2019	China	Asian	92	63	/	45/18	29	/	/	Serum	PCSK2-2:1
23	Rui Zhao	2018	China	Asian	246	126	/	66/60	120	/	/	Serum	HOTTIP
24	Haipeng Xian	2018	China	Asian	100	50	61	38/12	50	61	39/11	Serum	HULC, ZNFX1-AS1
25	Xiaojie Sun	2018	China	Asian	217	117	58.33	88/29	100	49.94	58/42	Serum	CCAT2
26	Tianhang Luo	2018	China	Asian	67	46	/	/	21	/	/	Plasma	MEF2C-AS1
27	Jingjing Liu	2018	China	Asian	100	50	/	/	50	/	/	Plasma	CTC-501O10.1, AC100830.4, RP11-210K20.5
28	Eman T. Elsayed	2018	Egypt	African	100	50	/	/	50	/	/	Plasma	HOTAIR

(Continued)

TABLE 1 Continued

Article ID	First author	Year	Country	Ethnicity	Total	Gastric cancer group			Control group			Specimen	LncRNA
						Sample size	Mean age	Gender	Sample size	Mean age	Gender		
29	Qin Lu	2017	China	Asian	152	76	63.4	50/26	76	65.4	32/44	Plasma	XIST, BCYRN1, RRP1B, TDRG1
30	Jiang Li	2017	China	Asian	180	90	66	64/26	90	60/30	64	Plasma	XIST
31	Dong Ke	2017	China	Asian	104	51	/	35/16	53	/	/	Plasma	INHBA-AS1, MIR4435-2HG, CEBPA-AS1, UCA1, AK001058
32	Yu Fan	2017	China	Asian	180	90	/	62/28	90	/	/	Serum	ANRIL
33	Lei Dong	2017	China	Asian	64	30	/	/	34	/	/	Serum	CUDR, LSINCT-5, PTENP1
34	Lin Tan	2016	China	Asian	343	263	/	/	80	/	/	Plasma	GACAT2
35	Chunjing Jin	2016	China	Asian	210	100	/	65/35	110	/	/	Serum	HULC
36	Doaa Hashad	2016	Egypt	African	62	32	43.44	19/13	30	43.53	15/15	Plasma	H19
37	Xiaoying Zhou	2015	China	Asian	140	70	/	/	70	/	/	Plasma	H19
38	Qier Li	2015	China	Asian	160	79	/	56/23	81	/	/	Plasma	LINC00152
39	Zhong Liu	2014	China	Asian	163	83	/	/	80	/	/	Plasma	FER1L4
40	Tomohiro Arita	2013	Japan	Asian	75	43	/	31/12	32	/	/	Plasma	H19

lncRNAs have attracted tremendous attention and have been the topic of extensive research. It was revealed that lncRNAs not only participate in the transduction of various signaling pathways and thus influence cancer development (24), but also have the potential for cancer diagnosis (25, 26).

In our meta-analysis, we included 40 original research studies including 6,772 participants to evaluate the diagnostic accuracies of lncRNAs for GC. The random-effect model was used in this meta-analysis due to the existence of heterogeneity. According to the AUC value, 5 lncRNAs with one panel of lncRNAs had a high diagnostic value, 30 lncRNAs had a moderate diagnostic value, and 4 lncRNAs had a low value. As shown in the forest plot (Figures 3A, B) and SROC curve (Figure 4), the overall sensitivity, specificity, and AUC were 0.78 (95% CI: 0.75–0.81), 0.79 (95% CI: 0.74–0.83), and 0.85 (95% CI: 0.81–0.87), respectively, which suggest that lncRNAs have a better diagnostic value than traditional tumor markers such as CEA and CA199 (27). Meanwhile, the PLR and NLR in our meta-analysis were 3.70 and 0.28, which implied that circulation lncRNAs had the ability to pick out GC patients from healthy people. As displayed in Figure 5, the results from Deeks' funnel plot asymmetry test demonstrated a low potential for publication bias (p = 0.00). A meta-analysis enrolled 11 studies reported that circular RNAs had a high sensitivity (0.71) and specificity (0.78) as a tumor marker in the diagnosis of GC (28). Lin et al. conducted another meta-analysis to test the diagnostic potential of circRNAs in GC, and they found that the pooled sensitivity, specificity, and ROC were 0.68, 0.70, and 0.78, respectively (29). As for the microRNAs in diagnosing GC, a meta-analysis from Wei et al. revealed that circulating miRNAs also had the potential to be biomarkers in GC, which have a sensitivity of 0.76, a specificity of 0.81, and an AUC of 0.86 (30). Although the above results suggested that circRNAs and miRNAs had promising applications, we found that lncRNAs were better than them in diagnosing GC. However, the expression level of lncRNAs is a concerning issue in GC diagnosis. Depending on their role in tumor biology, not all lncRNAs are oncogenes. Some of them play a critical role in promoting tumor genesis and regulating tumor cellular properties, while others function as inhibiting factors in the development of tumors. For instance, upregulation of C5orf66-AS1 can decrease cellular activities including proliferation, migration, and invasion (31). By contrast, high expression of CCAT2 facilitates GC cell proliferation and invasion and implies poor prognosis (32). In our meta-analysis, there were 31 lncRNAs that were highly expressed and 9 lncRNAs that were

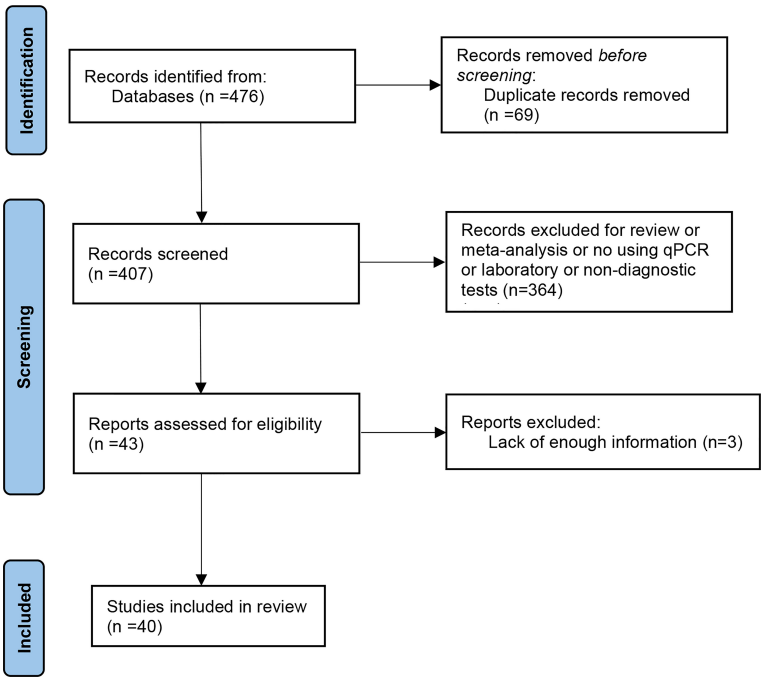


FIGURE 1
A flow diagram of the article selection process.

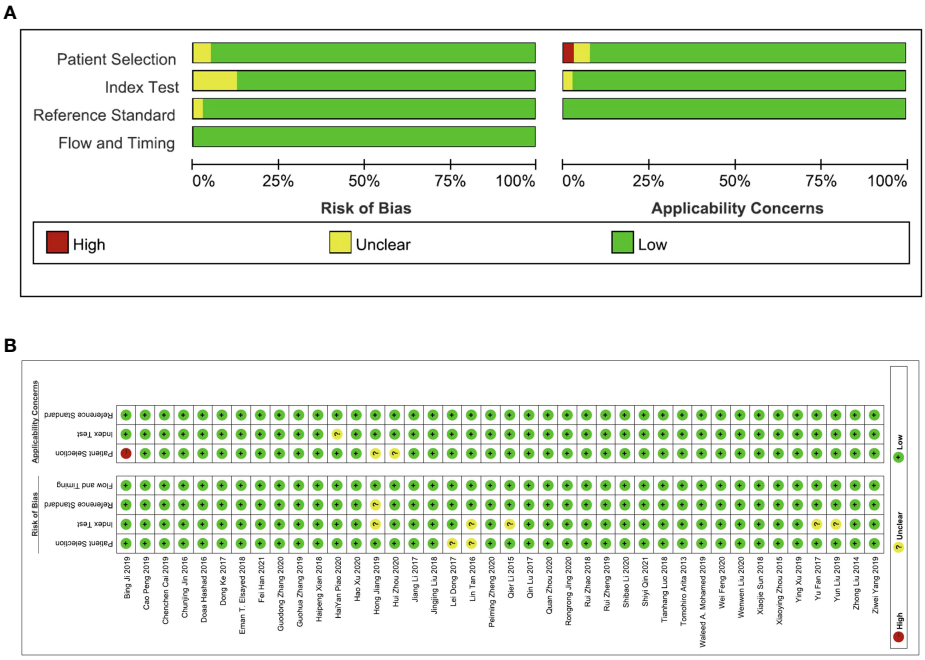


FIGURE 2
The quality assessment of the included studies *via* the QUADAS-2 tool. **(A)** Risk of bias and applicability concerns graph and **(B)** summary of quality assessment.

TABLE 2 Diagnostic accuracies of the lncRNAs mentioned in the literature.

Article ID	LncRNA	Expression	GC sample size	Control sample size	Sensitivity (%)	Specificity (%)	AUC
1	HCP5	U	98	82	0.800	0.700	0.87
2	CCAT2	U	76	83	0.8696	0.7358	0.862
3	MIAT	U	109	50	0.806	0.91	0.892
4	C5orf66-AS1	D	200	278	0.775	0.536	0.688
5	H19	U	81	78	0.7436	0.8395	0.849
6	SLC2A12-10:1	U	60	60	0.783	0.75	0.776
7	PTCSC3	D	68	60	0.897	0.846	0.92
8	CEBPA-AS1	U	281	80	0.879	0.788	0.824
9	FEZF1-AS1, AFAP1-AS1	U	89	73	0.753	0.658	0.82
10	GNAQ-6:1	D	43	27	0.837	0.556	0.736
11	RP11-731F5.2	U	104	80	0.8163	0.6364	0.78
12	B3GALT5 AS1	U	107	87	0.645	0.874	0.816
13	ARHGAP27P1	U	53	53	0.755	0.604	0.732
14	PANDAR, FOXD2-AS1, SMARCC2	U	109	106	0.797	0.846	0.839
15	H19	U	35	25	1	0.909	0.982
16	DGCR5	D	34	34	0.5939	0.8515	0.722
17	HOXA11-AS	U	94	40	0.787	0.978	0.924
18	PCGEM1	U	317	100	0.729	0.889	0.75
19	LINC00086	D	168	74	0.726	0.838	0.86
20	GASL1	D	88	72	0.841	0.81	0.8945
21	FAM49B-AS, GUSBP11, CTDHUT	U	173	173	0.775	0.739	0.818
22	PCSK2-2:1	D	63	29	0.84	0.865	0.896
23	HOTTIP	U	126	120	0.698	0.85	0.827
24	HULC, ZNFX1-AS1	U	50	50	0.58	0.8	0.85
25	CCAT2	U	117	100	0.7863	0.53	0.619
26	MEF2C-AS1	U	46	21	0.667	0.707	0.733
27	CTC-501O10.1, AC100830.4,RP11-210K20.5	D	50	50	0.99	0.49	0.764
28	HOTAIR	U	50	50	0.88	0.84	0.944
29	XIST, BCYRN1, RRP1B, TDRG1	U	76	76	0.846	0.59	0.733
30	XIST	U	90	90	0.511	0.956	0.753
31	INHBA-AS1, MIR4435-2HG, CEBPA-AS1, UCA1, AK001058	U	51	53	0.787	0.951	0.976
32	ANRIL	U	90	90	0.7444	0.889	0.83
33	CUDR, LSINCT-5, PTENP1	U	30	34	0.741	1	0.92
34	GACAT2	U	263	80	0.872	0.282	0.622
35	HULC	U	100	110	0.82	0.836	0.888
36	H19	U	32	30	0.6875	0.5667	0.724
37	H19	U	70	70	0.829	0.729	0.838
38	LINC00152	U	79	81	0.481	0.852	0.657
39	FER1L4	D	83	80	0.672	0.803	0.778
40	H19	U	43	32	0.74	0.58	0.64

downregulated in GC patients. Hence, choosing which lncRNA for early diagnosis is dependent on the actual situation and different tumors, especially when applying them as biomarkers in a clinical setting. Furthermore, more high-impact and large-scale studies are needed to illuminate the mechanism of abnormal lncRNA expression.

The research on early GC diagnosis in China began in the 1970s. With the continuous development of medical technology and the efforts of medical workers, the detection rate of early GC in China has improved, but there is still a gap compared with Japan and South Korea, because these countries have the most comprehensive GC prevention and screening programs in the

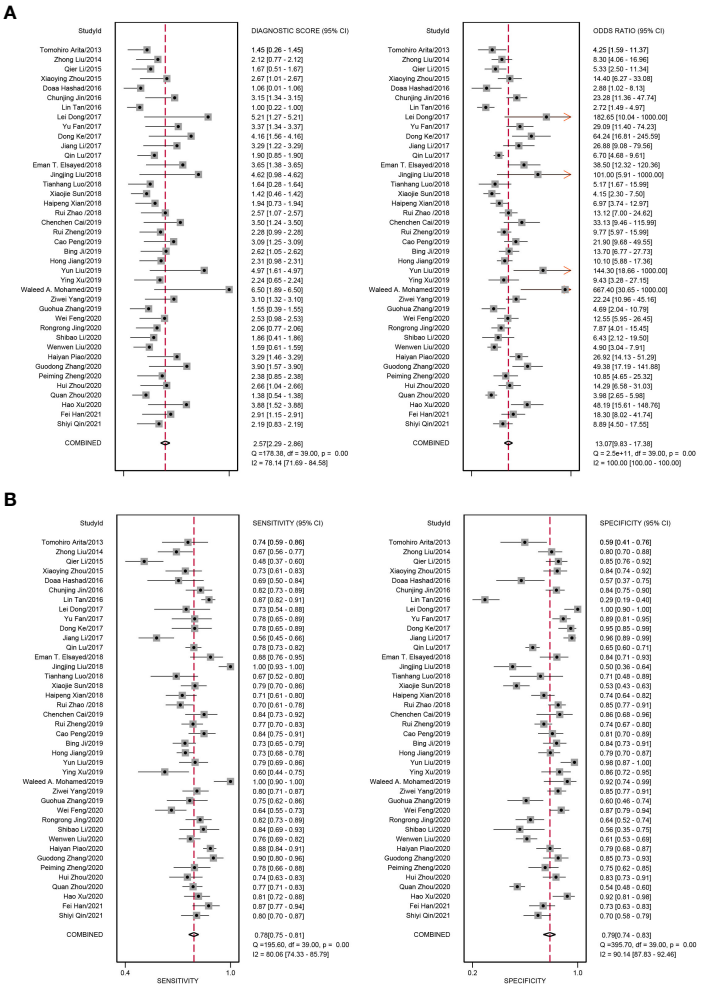


FIGURE 3
Forest plots of diagnostic accuracy of circulating lncRNAs in GC. **(A)** The pooled diagnostic score and diagnostic odds ratio (DOR) of circulating lncRNAs in the diagnosis of GC patients. **(B)** The pooled sensitivity and specificity of circulating lncRNAs in the diagnosis of GC patients.

world, and their early GC detection rates have reached 50% and 70% (33), respectively. There are advantages and disadvantages in diagnosing GC with lncRNAs. Traditionally, gastroscopy together with biopsy is the main method in detecting stomach lesions. However, the early diagnosis rate depends on many factors including the endoscopists' experience and standard operation, patient cooperation during the examination, and visual clarity using endoscopy. LncRNAs are acceptable for patients because of their invasiveness. Moreover, lncRNAs are abundant in the blood. Because of their stable properties (34) and higher sensitivity and specificity than CEA and CA199, they can replace old biomarkers and, thus, can be used as auxiliary biomarkers. This study further examined the diagnostic performance of lncRNAs in GC from the perspective of a noninvasive method, which would assist with the early diagnosis of GC. Compared with previous studies (20, 35), our study had several strengths in terms of study design and data analyses. First, we included more recent eligible articles using a comprehensive and updated search strategy, which improved the precision of the estimated effect size; second, we calculated the diagnostic efficacy in one specific cancer instead of pan-cancer, which could provide more accurate supporting information in GC diagnosis; third, we performed comprehensive analyses to explore the heterogeneity and diagnostic accuracy of circulating lncRNAs in GC. The results of this study indicate that circulating lncRNAs can be used as potential biomarkers for the diagnosis of GC. There are some limitations that should not be overlooked in the present meta-analysis. First, the number of studies included is relatively small, and more studies are needed before a solid conclusion can be drawn. Second, all included studies were case–control studies instead of randomized controlled trials, which may lead to some

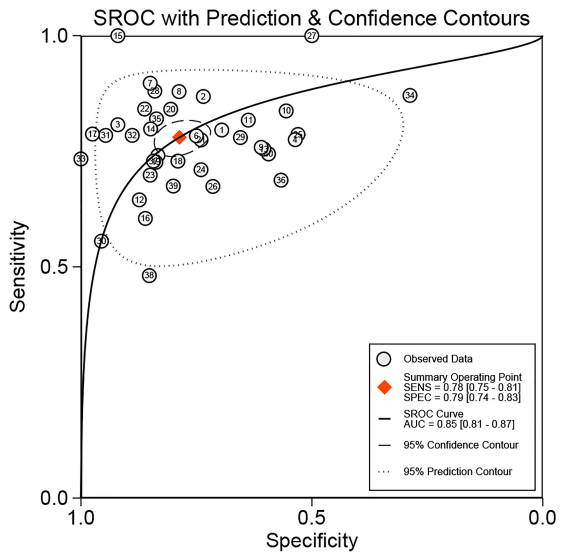


FIGURE 4
SROC of circulating lncRNAs in the diagnosis of GC patients. SROC, summary receiver operator characteristic curve; AUC, area under the curve.

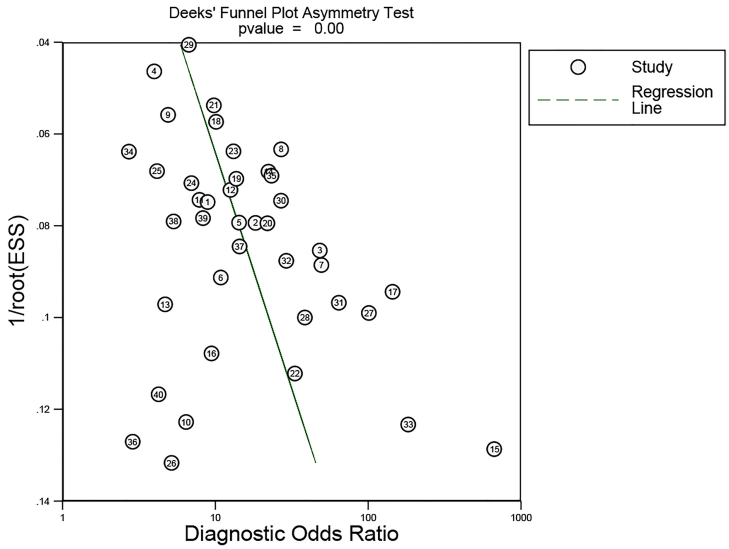


FIGURE 5
Deek's funnel plot asymmetry test was used to estimate the publication bias for discrimination of circulating lncRNAs in GC patients.

related biases. In order to acquire high-quality evidence, more randomized controlled trials are needed to avoid biases. Third, most of the included studies were from China and most of the included patients were Asian. This could further affect the generalization of the results, which could be attributed to ethnicity differences.

Collectively, our meta-analysis revealed that serum or plasma lncRNAs have high sensitivity and specificity, which makes them clinically feasible in diagnosing GC. We believe that peripheral blood lncRNAs may become novel noninvasive biomarkers in the foreseeable future. At the same time, it should be noted that a greater number of blood samples and

more evidence from rigorous multicenter clinical studies are necessary to justify their applicability as cancer biomarkers.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

Conceptualization: JL and LF. Methodology: JL, QX, YYZ, and YD. Formal analysis: JL and YQZ. Writing—original draft preparation: JL. Writing—review, and editing: JL and SB. Funding acquisition: LF. Resources: XZ. Supervision: LF and XZ. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The mechanisms and roles of melatonin in gastrointestinal cancer

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Gastrointestinal (GI) cancer is a global health problem with wide lesions and numerous cases. The increased morbidity and mortality of GI cancer is a socio-economic challenge for decades to come. Melatonin, a nature indolamine, exerts a crucial role in molecular interactions involved in multiple functional and physiological processes. Increasing evidence indicates that melatonin can modulate GI tract, decrease the occurrence of GI cancer, and enhance the sensitivity to chemoradiotherapy. However, little is known about the exact role of melatonin in anti-carcinogenesis. In this review, we discuss the action of the beneficial effects of melatonin in GI carcinogenesis. Furthermore, we compile the understanding of the role of melatonin in GI cancer, including esophageal cancer (EC), gastric cancer (GC), hepatocellular carcinoma (HCC), colorectal cancer (CRC), and pancreatic cancer (PC). In addition, the potential therapeutic application and clinical evaluation of melatonin in GI cancer are also discussed.

KEYWORDS

melatonin, gastrointestinal cancer, carcinogenesis, cellular lifecycle, immunity

1 Introduction

With the development of human civilization and social progress, the average life expectancy has increased significantly. However, cancer is a huge threat to longevity, among which gastrointestinal (GI) cancer is currently one of the major causes of death, with wide lesions and numerous cases. GI cancer includes cancer of the esophagus, stomach, colorectum, liver, and pancreas according to anatomy. It has been reported that colorectal cancer (CRC) is the most fatal and common GI cancer, followed by pancreatic cancer (PC), hepatocellular carcinoma (HCC), gastric cancer (GC) and esophageal cancer (EC) (1, 2). Due to the lack of methods for early diagnosis and effective management, as well as the properties of disease recurrence and metastasis, most GI cancers have a high fatality rate. The efficacy of GI patients has been well improved through surgery, chemotherapy, radiotherapy, etc., but

there are still problems in patient management. Therefore, efforts should be made to explore a novel preventative and therapeutic drug therapy to intervene and retard the progress of GI cancer.

L-tryptophan (L-Trp) is hydroxylated to 5-hydroxytryptophan (5-hydroxy Trp), then decarboxylated, acetylated to 5-hydroxytryptamine (5-HT) and N-acetyl-5-hydroxytryptamine (N-acetyl 5-HT), and finally methylated to N-acetyl-5-methoxytryptamine (also known as melatonin) (Figure 1). Melatonin is involved in many physiological functions (3, 4). Increasing evidence demonstrates melatonin exhibits antioxidant properties and is responsible for several diseases. It is a strong antioxidant that acts as a scavenger of free radicals (5). Besides, melatonin can activate antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPX), and superoxide dismutase (SOD), thereby reducing oxidative stress (6). It has been shown that melatonin enhances the expression of antioxidative enzyme genes, protects against depletion caused by ultraviolet radiation-induced, and prevents the formation of DNA damage (6). In addition, melatonin is also known to be an inhibitor of pro-oxidative xanthine oxidase (XO), an activator of DNA repair genes, and protector of mitochondrial membranes, which protect the body from harmful compounds (7). For example, Teixeira et al. indicated that results demonstrate the antioxidant effect of melatonin is mainly corelated with the activities of enzymes such as myeloperoxidase and XO (8). Liu et al. demonstrated that melatonin could increase DNA repair capacity *via* activating genes involved in DNA damage responsive pathways (9).

Melatonin can be produced by diverse tissues including pineal gland, GI tract, testes, retina, and lymphocytes (10). Melatonin receptors are G-protein coupled receptors, which can be divided into melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2) according to their different affinity (11). A large lines of evidence have indicated that melatonin is a vital regulator of circadian and seasonal rhythms (12, 13). Given that melatonin is a "jack-of-all-grades", it is not surprising that it affects the progression of GI cancer. For example, Parent et al. demonstrated that night shifts may affect cancer risk by inhibiting melatonin release (14). Wang et al. confirmed that melatonin is associated with the GC metastasis and poor prognosis (15). However, the mechanisms and roles by which melatonin can modulate the GI carcinogenesis are elusive. Therefore, we compile the research progress of melatonin and GI cancer (Figure 2), including EC, GC, HCC, PC, and CRC, to provide theoretical basis and ideas for further revealing melatonin and GI cancer. Moreover, the potential therapeutic application and clinical evaluation of melatonin in GI cancer are also discussed.

2 The mechanism of action of melatonin in GI carcinogenesis

Circadian rhythm disturbance is closely related to the occurrence and development of cancer. Studies have shown

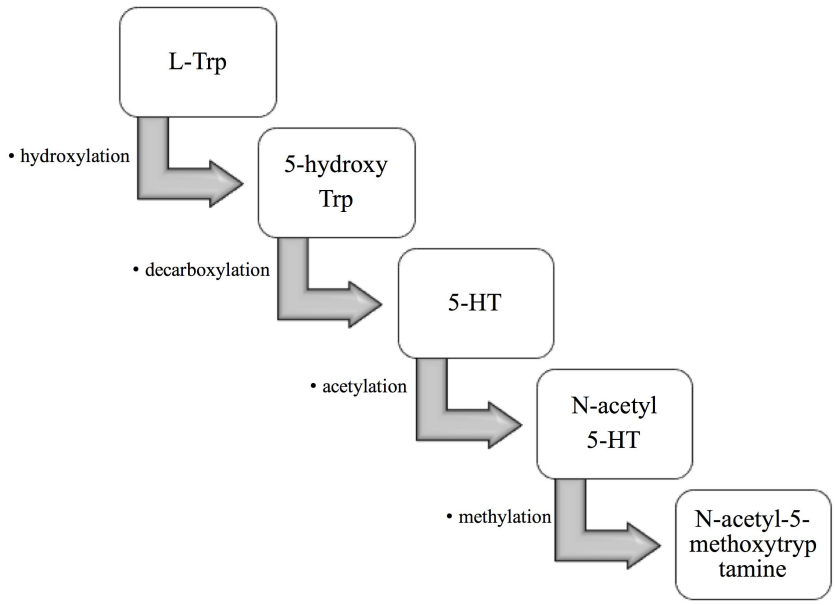


FIGURE 1
The formation of melatonin in the organism. First, L-tryptophan (L-Trp) is hydroxylated to 5-hydroxytryptophan (5-hydroxy Trp). 5-hydroxy Trp is then decarboxylated to 5-hydroxytryptamine (5-HT), which is then acetylated to N-acetyl-5-hydroxytryptamine (N-acetyl 5-HT). Finally, N-acetyl 5-HT is methylated to N-acetyl-5-methoxytryptamine, also known as melatonin.

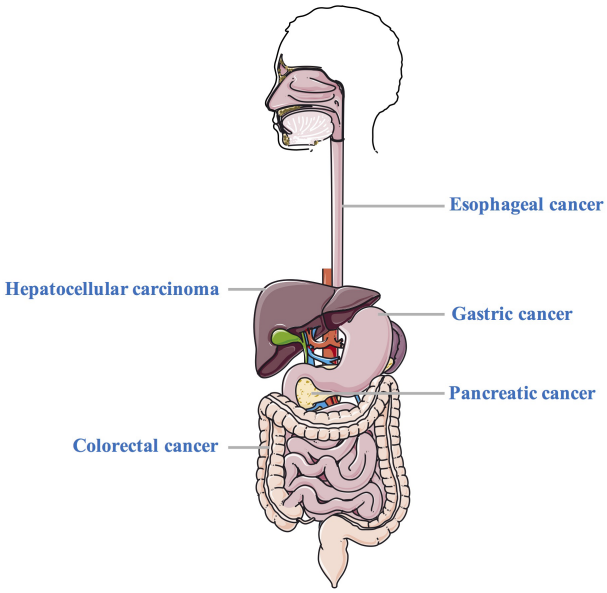


FIGURE 2
The effects of melatonin on various gastrointestinal cancer. This figure shows the examples of several common gastrointestinal cancer (including EC, GC, HCC, PC, and CRC) where melatonin exhibits protective effects EC, esophageal cancer; GC, gastric cancer; HCC, hepatocellular carcinoma; PC, pancreatic cancer; CRC, colorectal cancer.

that melatonin, normally up-regulated at night, helps to stabilize metabolic rhythm, thus involving cancer progression (16, 17). Melatonin exerts its anti-carcinogenesis role through various ways, including promoting cancer cell apoptosis, inhibiting proliferation, regulating angiogenesis and metastasis, modulating immunity, and involving several oncogenic signaling pathways (Figure 3). However, the intracellular signaling pathway of melatonin has not been clearly defined.

2.1 Melatonin and cellular lifecycle

Cell proliferation, differentiation, senescence and apoptosis are structural and functional bases of organism growth, development, aging and death, respectively (18). Cells deviate from the normal lifecycle due to internal or external factors, which may lead to the occurrence of cancer. It has been reported that melatonin act as a loyal defender against GI cancer through regulating cellular apoptosis, proliferation, metastasis, and angiogenesis. With the relevant cumulative findings, herein we investigate the action and role of melatonin in GI carcinogenesis.

Apoptosis, also known as programmed cell death (PCD), is a physiologic cell death that is distinct from necrosis. It is an active "suicide" extinction process after cells are stimulated by certain signals. Recent in-depth studies on biological cell death pathways have shown that the attenuation of apoptosis is closely related to the formation of GI cancer (19). There is increasing evidence that melatonin promotes PCD in GI cancer (20). Cytochrome c (cyt c) is release into the cytoplasm when the cells are stimulated, and triggers an enzymatic cascade that leads to apoptosis (21). The B-cell lymphoma-2 (Bcl-2) family mediates the intrinsic apoptosis pathway with both anti-apoptotic and pro-apoptotic effects (22). Fas, a transmembrane protein, binds to Fas ligand (FasL) to initiate the transduction of apoptotic signals and induce apoptosis (23). Mechanistically, melatonin can modulate the expression of multiple genes associated with apoptosis, such as cytosolic cyt c, Bcl-2, Fas, etc (20, 24).

Rampant proliferation is another important characteristics of all cancers. The carcinogenesis is the result of the imbalance between cell proliferation and apoptosis. It has been reported that melatonin exerts an obvious anti-proliferation effect *via* arresting cancer cell cycle (25). For example, Liu et al. proved that melatonin attenuated the expression of cyclin D1 and CDK4 in G1 phase, and cyclin B1 and CDK1 in G2/M phase of human osteosarcoma cells (26). Moreover, the anti-proliferative efficacy of melatonin have been demonstrated in GC and HCC cell lines (27).

Additionally, increasing evidence indicates that melatonin is also involved in angiogenesis and metastasis in GI cancer. Cancer angiogenesis is known to be an important feature of metastasis responsible for cancer death. Melatonin has the capacity to reduce the migration and neovascularization of cancer cells. For instance, Wang et al. showed that melatonin suppressed IL-1β-induced lung metastasis of GC by

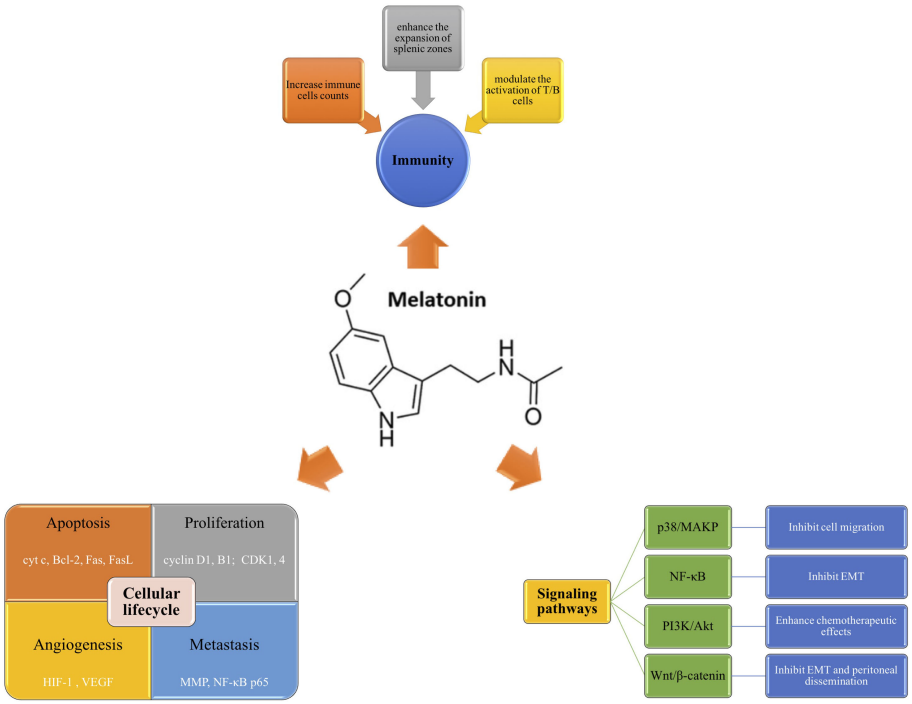


FIGURE 3
Mechanisms of melatonin in GI carcinogenesis. Melatonin plays a role in anti-carcinogenesis mainly through the following ways, including modulating cellular lifecycle, regulating immunity function, and involving several oncogenic signaling pathways. Melatonin can induce cell apoptosis *via* regulating multiple genes (cyt c, Bcl-2, Fas) and inhibit proliferation by arresting cancer cell cycle (cyclin D1, cyclin B1, CDK1, CDK 4). Moreover, it can also influence the angiogenesis and metastasis by modulating HIF-1, VEGF, MMP, etc. Secondly, melatonin is a regulator of immunity. It mediate the immune function mainly through increasing the counts of immune cells, enhancing the expansion of splenic zones, and activating the function of T/B cells. Thirdly, melatonin can inhibit carcinogenesis through specific signaling pathways, such as p38/MAPK, NF-κB, PI3K/Akt, and Wnt/β-catenin. Bcl-2, B-cell lymphoma-2; cyt c, cytochrome c; EMT, epithelial-mesenchymal transition; FasL, Fas ligand; HIF-1, hypoxia-inducible factor 1; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinases; NF-κB, nuclear factor-kappa B; PI3K, phosphoinositide 3 Kinase; VEGF, vascular endothelial growth factor.

downregulating the expression of matrix metalloproteinases (MMP), and nuclear factor-kappa B (NF-κB) p65 (28). Moreover, melatonin can also inhibits cancer angiogenesis *via* attenuating hypoxia-inducible factor (HIF)-1 and decreasing vascular endothelial growth factor (VEGF) expression (29).

2.2 Melatonin and immune function

Interactions between immune cells and cancer cells exert a crucial role in cancer development (30). Since MT are widely present in immune system, melatonin is involved in regulating immune function. Studies have shown that melatonin increases the cells of the innate immunity, such as neutrophil, macrophages, and lymphocytes counts (31). Luo et al. demonstrated that melatonin modulated the activation of T/B cells, thus playing a key role in stabilizing immune balance (32). Besides, Liu et al. indicated that melatonin inhibited GC cell growth by down-regulating the expressions of CD4 (+) and CD25 (+) regulatory T cells (Tregs) and Forkhead box p3 (Foxp3) in GC (33). Moreover, melatonin is also proved to enhance the expansion of splenic zones (34). In addition, it has been shown that melatonin participates in the regulation of cytokine production (35).

2.3 Melatonin and signaling pathways

Signaling pathways are intertwined into networks in physiological processes of systemic organs throughout the body and play important roles in human health and disease. The disruption of multiple signaling pathways is closely involved in the progression of diverse cancers. It has been indicated that melatonin could regulate the mediators in oncogenic signaling pathways, such as NF-κB, phosphoinositide 3 Kinase (PI3K)/Akt, p38/mitogen-activated protein kinase (MAPK), and Wnt/β-catenin axis (36–38). For example, Liu et al. reported that melatonin decreased Rho –associated protein kinase (ROCK) expression *via* p38/MAPK signaling pathway, thus inhibiting the migration of CRC cells (39). The transdifferentiation of epithelial cells into motile mesenchymal

cells, known as epithelial-mesenchymal transition (EMT), is a process that enhances the invasiveness and anti-apoptotic capabilities of cancer cells (40). Wang et al. proved that melatonin suppressed EMT in GC *via* attenuation of IL−1β/NF−κB/MMP2/MMP9 axis (41).

3 The role of melatonin in GI cancer

Despite tremendous scientific breakthroughs in understanding the mechanistic properties of GI cancer, therapeutic efficacy remains very limited. Increasing evidence demonstrates that melatonin has positive protective effects against both endogenous stimuli (acid and pepsin) and exogenous insults (alcohol and stress) affecting the GI tract (42). There are numerous studies assessing the roles of melatonin on health and disease. Melatonin has been seen as an adjunctive treatment for advanced cancer because of its anti-inflammatory and anti-oxidant effects. In this part, we discuss the role of melatonin in GI cancer, including EC, GC, HCC, CRC, and PC, respectively (Table 1).

3.1 The role of melatonin in esophageal cancer

Esophageal cancer (EC) refers to malignant tumors of esophageal epithelial origin, which is mainly manifested as choking sensation when swallowing food, foreign body sensation, retrosternal pain, or obvious dysphagia (67). It is one of the most common gastrointestinal cancer with regional variations in morbidity and mortality. The two major subtypes of EC are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). Studies have shown that smoking, alcohol consumption, obesity, aging, male sex, Barrett's esophagus, and gastroesophageal reflux disease (GERD) consist of the potential risk factors for EC (68, 69).

Melatonin has been shown to afford esophago-protection *via* diverse mechanisms. Firstly, it enhances tumor cell sensitivity to chemotherapy drugs. In 2016, Lu et al. demonstrated that melatonin improved ESCC cell sensitivity to 5-fluorouracil (5-FU) *via* suppressing the Erk and Akt pathway (43). Furthermore, Zhang et al. proved that melatonin significantly enhanced 5-FU-mediated suppression of esophageal cancer cell proliferation and migration by regulating enhancer of zeste homolog 2 (EZH2) (45). Histone methyltransferase EZH2, a catalytic component of polycomb repressive complex 2 (PRC2), is highly expressed in the development of esophageal cancer (70). Secondly, melatonin exerts anti-inflammatory and anti-oxidant effects in EC. NF-κB induces the expression of multiple genes through the activation of stimulating factors and produces multiple cytokines involved in inflammatory responses. Gu et al. revealed that melatonin inhibited cell invasion *via* down-regulating the NF-κB signaling pathway in EC (46). Thirdly, melatonin is involved in the regulation of cell cycle. It has been reported that melatonin

TABLE 1 Effects of melatonin against various gastrointestinal cancer.

Cancer types	Targets	Effects	Ref(s)
Esophageal cancer	Erk, Akt	improve ESCC cell sensitivity to 5-FU	(43, 44)
	EZH2	suppress EC cell proliferation and migration	(45)
	NF-κB	exert anti-inflammatory and anti-oxidant roles	(46)
	PI3K/Akt	induce cell apoptosis and cell cycle arrest	(47)
Gastric cancer	ROS, MMP2, MMP9	inhibit GC cell growth	(48)
	miR-15-5p-Smad3	suppress GC cell proliferation	(49)
	NF-kB and MAPK	induce GC cell apoptosis	(50)
	VEGF, Wnt/β-catenin	regulate angiogenesis and differentiation	(51)
	MMP2, MMP9, NF-kB p65	reduce lung metastasis	(28)
	NF-κB, C/EBPβ	block EMT and peritoneal dissemination	(52)
Hepatocellular carcinoma	ER	induce HCC cell apoptosis	(53)
	transcription factors	inhibit HCC cell proliferation and invasiveness	(54)
	ncRNAs	restrain HCC progression	(55, 56)
	RAD51-AS1	sensitize HCC cell to chemotherapy	(57)
	mTORC1	suppresses glycolysis	(58)
Colorectal cancer	miR-34a/449a cluster	regulate cell cycle	(59)
	ER	promote CRC cell apoptosis	(60)
	XIAP	increase 5-FU-mediated apoptosis	(61)
	/	sensitize CRC cell to γ-ray ionizing radiation	(62)
	lipid metabolism, gut microbiota	play a preventive and therapeutic role in CRC	(63)
Pancreatic cancer	VEGF, HSPs, caspase-3, caspase-9	stimulate PC cell apoptosis	(64, 65)
	PDGFR-β/STAT3	enhance the efficacy of chemotherapy	(66)

causes cell cycle arrest and affects the percentage of abnormalities in different phase (47).

3.2 The role of melatonin in gastric cancer

Gastric cancer (GC) is a predominant malignancy with the second leading cause of cancer death worldwide (71). The lifestyle, genetics, helicobacter pylori (H. pylori) infection, and epigenetics are potential risk factors for GC (72). H. pylori is a major cause of gastric carcinogenesis by enhancing the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), and inducing local ulceration and inflammation (73). According to the pathological types, GC can be divided into adenocarcinoma, signet ring cell carcinoma, adenosquamous carcinoma, medullary carcinoma and undifferentiated cell carcinoma, among which adenocarcinoma is the most common. Gastroscopy is the preferred method for GC, and gastric biopsy is the "gold standard" for the diagnosis of GC.

Growing evidence demonstrates that melatonin exerts gastroprotective effects in GC through multiple mechanisms, including increasing blood flow, reducing inflammation, scavenging free radicals, and inhibiting MMP (41, 74, 75). On the one hand, melatonin has been shown to play a role in affecting the growth of GC itself. Liu et al. revealed that melatonin inhibited GC cell progression *via* the NF-kB signaling pathway (48). They proved that melatonin suppressed cell growth by directly reducing ROS production, while indirectly decreasing the level of MMP2 and MMP9 in cancer-associated fibroblasts (CAFs) in gastric cancer cells. Moreover, melatonin was reported to inhibit the proliferation of GC cells *via* modulating miR-15-5p-small mothers against decapentaplegic homolog 3 (Smad3) pathway (49). Additionally, it has been demonstrated that melatonin induces GC cell apoptosis *via* regulating diverse signaling pathways (76, 77). For example, Li et al. noted that melatonin stimulated GC cell apoptosis by mediating NF-kB and MAPK signaling pathways (50). Apart from mentioned above, the effects of melatonin on angiogenesis and differentiation are also involved in the progression of GC (51). On the other hand, melatonin has also been proven to be effective in reducing metastasis exacerbations. There is increasing evidence that GC migration is significantly reduced following melatonin treatment. In a study, decreased lung metastasis in GC after melatonin treatment was associated with the downregulation of MMP2, MMP9, and NF-kB p65 (28). Wu et al. indicated that melatonin blocked EMT and peritoneal dissemination through NF-κB cleavage and calpin-mediated C/EBPβ (52). Besides, melatonin was reported to inhibit the migration of GC *via* remodeling tight junction (78).

3.3 The role of melatonin in hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a major global health challenge and the fourth leading cause of cancer-related death worldwide. HCC is prevalent to spread in the liver through the portal vein system, forming intrahepatic metastasis, and also easy to form tumor thrombus in the portal vein, causing the manifestation of portal hypertension (79). A commonly used tumor marker for HCC is alpha-fetoprotein (AFP), especially when it is significantly elevated, high vigilance should be exercised. For people with hepatitis infection, liver cirrhosis and family history of liver cancer, regular screening for HCC should be carried out for early detection, diagnosis and treatment (80).

Emerging evidence demonstrates that melatonin exerts anti-cancer activity on HCC. It is worth noting that melatonin reverses apoptosis resistance and activates both intrinsic and extrinsic pathways of apoptosis in HCC (54). Zha et al. found that melatonin stimulates endoplasmic reticulum (ER) stress-induced apoptosis in HCC (53). Moreover, melatonin is involved in the regulation of HCC by modulating a variety of transcription factors and related pathways to inhibit cell proliferation and invasiveness (54). In addition, melatonin has been proven to restrain HCC progression *via* regulating non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (55, 56). Besides, melatonin could sensitize HCC cell to chemotherapy (57). Recent studies have shown that melatonin suppresses glycolysis in HCC cells by down-regulating mitochondrial respiration and mammalian target of rapamycin complex 1 (mTORC1) activity (58).

3.4 The role of melatonin in colorectal cancer

Colorectal cancer (CRC) is considered as the second most common cancer worldwide and the incidence of CRC increases with age (81). Adenocarcinoma is the most common pathological type of CRC. In recent years, the morbidity and mortality of colorectal cancer have shown an increasing trend, which should be paid more attention to. Chemoradiotherapy is the last-line treatment for advanced CRC with significant side effects. Therefore, it is urgent to explore an anti-cancer agent in CRC.

Melatonin holds promise as an adjunctive treatment for advanced CRC. The entero-endocrine (EE) cells in GI tract mucosa are the main source of intestinal melatonin (82). Increasing evidence evaluates the effects and safety of melatonin. It has been reported that melatonin is associated with various health outcomes in heterogeneous populations (83). Mechanically, it has been reported that melatonin prevent and

delay the progression of CRC by suppressing the proliferation and inducing apoptosis of CRC cells. Ji et al. demonstrated that melatonin regulated the miR-34a/449a cluster, thus influencing the cell cycle in CRC (59). Yun et al. noted that melatonin promoted CRC cell apoptosis *via* superoxide-mediated ER stress (60). Besides, melatonin promotes chemotherapeutic drug-mediated apoptosis of CRC cells by enhancing oxidative stress (61). In addition to its role in sensitivity to chemotherapeutic agents, melatonin also sensitizes human CRC cells to γ-ray ionizing radiation both *in vitro* and *in vivo* (62). Interestingly, studies have pointed out that melatonin plays a preventive and therapeutic role in CRC by regulating lipid metabolism and gut microbiota (63). Menadione, a synthetic form of vitamin K, is known to stimulate an increase in intracellular ROS and alter the oxidative status of cancer cells (84). Collin et al. confirmed that menadione plus melatonin on Caco-2 cells could reduce cell proliferation, induce reactive nitrogen species formation, enhance superoxide anion content, and increase catalase activity, suggesting the potential as adjuvant therapy for CRC acting on different oncogenic pathways (85). Kvietkauskas et al. designed a study to explore the combined role of melatonin and glycine in CRC liver metastasis (86). They found that supplementation with melatonin and glycine reduce CRC liver metastasis growth by acting as natural antiangiogenic molecules.

3.5 The role of melatonin in pancreatic cancer

Pancreatic cancer (PC) is the source of an increasing number of cancer-related deaths with a low survival rate. The high mortality rate of PC is likely to be associated with the absence of early symptoms and therefore delayed diagnosis, as well as high resistance to chemoradiotherapy. PC is classified as resectable, borderline, locally advanced and metastatic with greatly varied treatment among them (87). The sensitivity of computed tomography (CT) and magnetic resonance imaging (MRI) in detecting PC was as high as 96% and 93.5%, respectively (88). Despite significant advances in the treatment of PC, including improved surgical techniques and refined adjuvant and neoadjuvant therapies, the incidence and mortality of PC have not decreased significantly worldwide. We therefore emphasize the historical perspective of PC treatment, highlight the prevention strategies, and identify more integrated approaches.

Growing evidence supports the therapeutic benefits of melatonin in the management of PC. Studies have shown that a variety of inflammatory pathways are closely related to the pathological process of PC (89). Melatonin stimulates cancer cell apoptosis by acting as an inflammatory inhibitor, an oxidative stress modulator, a VEGF inhibitor, a heat shock proteins (HSPs) inhibitor, etc. (64, 65). Firstly, it protects pancreatic tissue from inflammatory damage and oxidative stress *via* activating anti-oxidant enzymes and scavenging ROS and RNS. Secondly, melatonin decreases endothelial cells proliferation and reduces angiogenesis by inhibiting VEGF. Thirdly, high concentration of melatonin regulate Bax/Bcl protein balance, thus stimulating the expression of caspase-3 and caspase-9, while low level of melatonin produce anti-apoptotic HSPs, such as HSP27 and HSP90, thereby preventing the activation of caspase-3. Moreover, it has been found that melatonin is involved in enhancing the efficacy of chemotherapy and decreasing side effects. Fang et al. found that melatonin and sorafenib synergistically inhibited PC through MR and PDGFR-β/STAT3 signal pathway (66). Melatonin was also proved to enhance the chemosensitivity to gemcitabine in PC (90). The metabolites of L-Trp and melatonin are called kynuramines, of which N1-acetyl-5-methoxy-kynuramine (AMK) and N1-acetyl-N1-formyl-5-methoxykynuramine (AFMK) are the best known melatonin derivatives (91). It has been shown that melatonin precursor L-Trp and the melatonin derivatives kynuramines, may be related to the physiological and functional failure of the pancreas, leading to the impairment of pancreatic function and anti-cancer ability (92).

4 The safety evaluation of melatonin in GI cancer

Melatonin is an endogenous molecule that has its own metabolic pathway in the body. The biological half-life is short and falls to the physiological level of normal after 7~8 hours of oral administration. Therefore, it is a relatively safe substance for clinical use in humans. The underlying mechanisms of melatonin *in vitro*, such as cell apoptosis, cell proliferation, immune function, and signaling pathways, have largely been validated *in vivo* studies. For clinical application, a large number of researches have further examined the role of melatonin in GI cancer *in vivo*. On the one hand, many studies have explored the effects of melatonin on GI cancer in animal models. For example, Winczyk et al. constructed an animal model of colon cancer in mice (93). They found an increase of apoptotic cells in cancers treated with melatonin, further confirming the pro-apoptotic efficacy of melatonin on murine colon cancer cells. On the other hand, the clinical trials have also been conducted to evaluate the safety and efficacy of melatonin in GI cancer. It is well known that melatonin's sleep-inducing effects have been widely used clinically (94). Recent clinical studies have shown that melatonin can improve the survival rate of patients with GI cancers, increase the sensitivity to chemotherapeutic agents, and reduce the side effects of chemoradiotherapy (38). For example, Kouhi et al. conducted a double-blind controlled study in 60 patients with rectal cancer, in which the experimental group received 20 mg melatonin a day and the control group treated with placebo (95). Their subsequent study found that radiotherapy induced less severe reductions in blood cell counts in patients treated

with melatonin, suggesting a role for melatonin in reversing the adverse effects of radiation. The above studies all indicate that melatonin can be utilized for the treatment of GI cancer. However, the appropriate dosage and optimal duration of melatonin still require extensive studies to specifically validate the effects of melatonin on GI cancer progression in the future.

5 Conclusions and perspectives

Melatonin, a natural indolamine, is produced by a variety of tissues and is involved in the mediation of physiological functions. Given that MT are widely distributed in many organs and tissues of the body, it is not surprising that it is called a "jack-of-all-grades". GI cells can not only secrete melatonin, but also have MT on them, thus exerting important protective and regulatory roles. Studies have shown that melatonin boosts the GI immune system, regulates fecal moisture, slows intestinal peristalsis, and protects the GI tract from digestive enzymes and stomach acid. Emerging evidence proves that melatonin affects the progression of GI cancer. Despite our understanding of how melatonin exerts its anti-cancer effects is expanding, much remains to be studied. There are many challenges in translation to therapeutic applications in GI cancer, such as safety assessment and bioavailability. Future researches should continue to focus on the communication between melatonin metabolism and the development and progression of GI cancer.

Author contributions

Y-QG collected literature and wrote the manuscript. F-TH summarized the table and drew the figures. C-LX and C-LL supervised the manuscript and modified the figures. G-HH and C-WC conceived the idea and supervised the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cardia function-preserving surgery and anti-reflux anastomotic method after proximal gastrectomy for gastric cancer: Current status and future perspectives

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The incidence and mortality of gastric cancer ranked 5th and 3rd worldwide, respectively, in 2018, and the incidence of gastroesophageal junction adenocarcinoma increased over the past 40 years. Radical resection and lymph node dissection is the preferred treatment for gastric cancer. Proximal gastrectomy or total gastrectomy is usually performed for gastroesophageal junction adenocarcinoma and upper gastric cancer. Owing to the resection of the cardia structures, the incidence of reflux esophagitis increases significantly after proximal gastrectomy and total gastrectomy, resulting in poor postoperative quality of life. To reduce the incidence of reflux esophagitis and improve patients' postoperative quality of life, various methods to preserve the function of the cardia or to perform anti-reflux reconstruction have emerged. In this manuscript, we systematically introduced the advantages and problems of various anti-reflux anastomotic method after proximal gastrectomy, and cardia-preserving gastrectomy including endoscopic resection (ER), local gastrectomy by gastroscopy combined with laparoscopy, segmental gastrectomy, subtotal gastrectomy, and cardia-preserving radical gastrectomy. Cardia-preserving radical gastrectomy has the advantage of more thorough lymph node dissection and wider indications than those for subtotal gastrectomy. However, the clinical efficacy of cardia-preserving radical gastrectomy requires verification in prospective and controlled clinical trials. Cardia-preserving radical gastrectomy is a promising approach as one of the more reasonable anti-reflux surgeries.

KEYWORDS

cardia function-preserving, surgery, anti-reflux anastomotic, gastric cancer, proximal gastrectomy

1 Introduction

The incidence and mortality of gastric cancer ranked 5th and 3rd worldwide, respectively, in 2018, and the respective rates in China were 44.1% and 49.9% (1). In 2015, data from the National Cancer Center of China showed that the incidence of gastric cancer in men and women ranked 2nd and 3rd, respectively, and mortality ranked 3rd (2). Data from the Surveillance, Epidemiology, and End Results (SEER) database of the National Cancer Institute indicated that the incidence of gastroesophageal junction adenocarcinoma increased 2.5-fold over the past 35 years (3). Data from the National Cancer Center Japan showed that the incidence of gastroesophageal junction adenocarcinoma increased 7.3% from the 1960s to the early 2000s (4). In China, the incidence increased from 22.3% in 1988 to 35.7% in 2012 (5).

Radical resection and lymph node dissection is the preferred treatment for gastric cancer. Proximal gastrectomy or total gastrectomy is usually performed for gastroesophageal junction adenocarcinoma and upper gastric cancer. Anatomically, the cardia is the opening between the esophagus and the stomach, with the junction of the stomach and the esophagus as an initial segment, and the cardia is connected to the lower segment of the esophagus. There is a 2–3 cm long thickened and hypertrophic annular muscle layer in the lower esophagus containing the distributions of the spinal nerves and the vagus nerve that constitutes the lower esophageal sphincter. The sphincter mainly maintains the lower intraesophageal pressure at rest (15–30 mmHg higher than the intragastric pressure), and the sphincter can generate a pressure of approximately 100 mmHg during persistent contraction (6, 7). Owing to the resection of the cardia structures, the incidence of reflux esophagitis increases significantly after proximal gastrectomy and total gastrectomy (14.5% and 5.4%, respectively), resulting in poor postoperative quality of life (8). To reduce the incidence of reflux esophagitis and improve patients' postoperative quality of life, various methods to preserve the function of the cardia or to perform anti-reflux reconstruction have emerged. This manuscript mainly discusses the importance of preserving cardiac function, including two parts: reservation of cardia and resection of cardia.

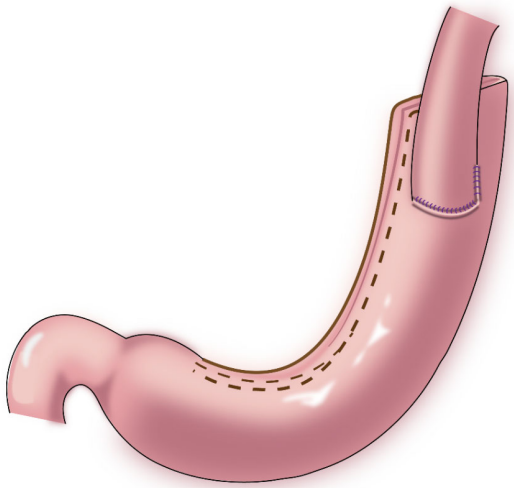


FIGURE 1
Tubular gastroesophagostomy: the esophagus was anastomosed with the anterior wall of the remnant stomach by which a fundus-like structure is created at the top of the remnant stomach.

2 Anti-reflux anastomotic method after proximal gastrectomy for gastric cancer

In first part of resection of cardia, proximal gastrectomy preserves partial stomach function but results in the loss of the anti-reflux function of the cardia, and the preserved pylorus delays gastric emptying to some extent (Table 1) (14). Thus, severe reflux esophagitis occurs easily after proximal

TABLE 1 Anti-reflux anastomotic method after proximal gastrectomy for gastric cancer.

Number	Anastomotic method	Time of first report	Disadvantage
1	Gastroesophagostomy		
1.1	Tubular gastroesophagostomy	1998, Shiraishi (9)	the incidence of reflux symptoms and anastomotic stenosis was higher
1.2	Side overlap anastomosis	2016, Yamashita (10)	retention of a long abdominal esophagus and a large remnant stomach (more than 2/3)
1.3	Double-flap anastomosis (Kamikawa anastomosis)	1998, Kamikawa (11)	double-flap technique is complicated and requires advanced suturing skills and a long operative time
2	Jejunal interposition	1993, Kameyama (12)	the operation is complicated, with a long operative time and relatively high cost, and there is the possibility of obstructed remnant stomach emptying
3	Double-tract reconstruction (DTR)	1988, Aikou (13)	the surgical procedure is relatively complicated, with many anastomotic stomas, possibly increasing the risk of stomal leakage and increased costs

gastrectomy (8). Recently, various methods of anti-reflux digestive tract reconstruction after proximal gastrectomy have emerged, which not only preserve partial gastric function, but also avoid severe reflux esophagitis.

2.1 Gastroesophagostomy

2.1.1 Tubular gastroesophagostomy

In 1998, Shiraishi et al. first reported tubular gastroesophagostomy, by which a fundus-like structure is created at the top of the remnant stomach (Figure 1) (15). The regurgitated gastric juice is temporarily stored in the "fundus" when patients are in a supine position, avoiding direct reflux to the lower end of the esophagus to some extent. Part of the gastric antrum is resected from the tubular stomach in this procedure, which reduces the secretion of gastrin and gastric acid. The tubular stomach maintains the anatomical structure of the stomach, leading to a higher quality of life than that of patients undergoing traditional anastomosis of the gastric remnant to the esophagus (16). Chen et al. found that only 14.3% of the patients undergoing tubular gastroesophagostomy presented with reflux symptoms postoperatively, and 5.7% of these patients were diagnosed with reflux esophagitis (17). Additionally, the degree of reflux esophagitis after tubular gastroesophagostomy was lower than that with traditional anastomosis of the gastric remnant to the esophagus (17). Ronellenfitsch et al. reported that 30% of patients experienced reflux symptoms after tubular gastroesophagostomy, but that symptoms were mild in all patients (18). Kukar et al. reported that 6 case of tubular gastroesophagostomy, the esophagus was anastomosed with the posterior wall of the residual stomach using a tubular stapler. All patients had negative final margins and an adequate lymph node dissection (median number of nodes examined was 15, range 12-22). The median postoperative length of stay was 7 days (range 4-7). Two patients developed anastomotic strictures requiring intervention, and 1 patient experienced significant reflux. At a median follow-up of 11 months, there was 1 recurrence. Three patients were alive without evidence of disease, and 2 patients died from other causes (Figure 2) (19). Aihara et al. reported that the incidence of reflux symptoms after tubular gastroesophagostomy was 16.7%; however, anastomotic stenosis occurred in 35% of the patients (20). Clipping of the tubular stomach is usually performed with a linear cutting stapler, which has a relatively high cost. However, the length of the tubular stomach is longer, and the method is especially suitable for patients with a higher esophageal margin.

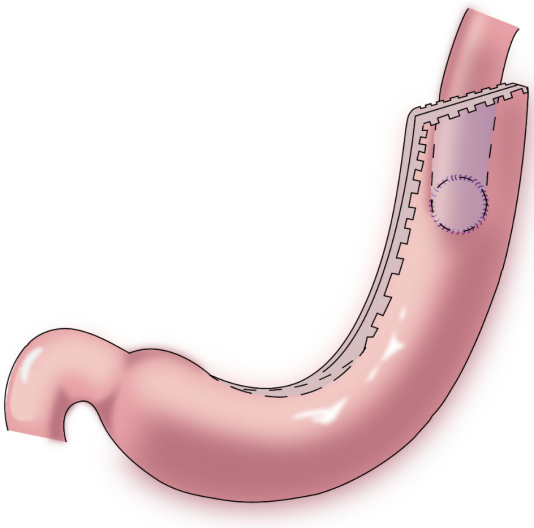


FIGURE 2
Tubular gastroesophagostomy: the esophagus was anastomosed with the posterior wall of the residual stomach using a tubular stapler.

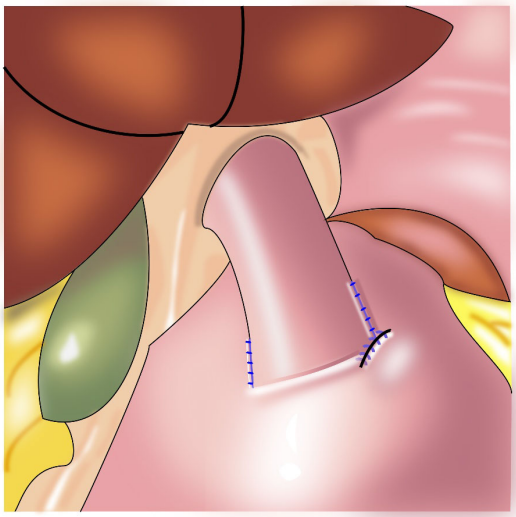


FIGURE 3
Esophagogastric side-to-side anastomosis: the opposite wall of the esophagus is fixed to the stomach to bring the esophagus close to the stomach wall.

2.1.2 Side overlap anastomosis

Yamashita et al. first reported side overlap anastomosis in 2016, which generally requires retaining the abdominal esophagus and 2/3 of the remnant stomach. The remnant stomach is fixed at the base of the left and right diaphragm to construct an artificial stomach fundus. Then, esophagogastric side-to-side anastomosis (Figure 3) is performed, and the opposite wall of the esophagus is fixed to the stomach to bring the esophagus close to the stomach wall. When the pressure in

the artificial fundus increases, the anastomotic stoma closes, which provides an anti-reflux effect (21). The incidence of reflux esophagitis after side overlap anastomosis is 10%, with a wide anastomotic stoma leading to a reduced incidence of anastomotic stenosis (21, 22). The advantages of this procedure are that it is a relatively simple operation, and it is associated with a short anastomosis time and low cost. The disadvantage is the need for retention of a long abdominal esophagus and a large remnant stomach (more than 2/3); therefore, application of this procedure is limited.

2.1.3 Double-flap anastomosis (Kamikawa anastomosis)

In 1998, Kamikawa reported double-flap esophagogastrostomy (Figure 4) to prevent reflux (23), during which a "Gong" (a Chinese character)-shaped seromuscular flap is made below the resection margin of the remnant stomach. At the lower margin of this "window," the mucosa and submucosa are cut and anastomosed to the esophageal cut margin. Finally, the two seromuscular flaps cover the lower segment of the esophagus and the upper part of the anastomotic stoma. This procedure increases the pressure in the lower esophagus and is beneficial to reduce the occurrence of reflux esophagitis. A multi-center retrospective study from Japan evaluating the efficacy and safety of the double-flap technique included 546 patients from 18 centers, of whom 464 patients underwent endoscopic evaluation of reflux esophagitis 1 year postoperatively. Grade B or higher reflux esophagitis was found in 6% of the patients under endoscopy, and the incidence of anastomotic stenosis was 5.5% (24). This surgical procedure may increase the occurrence of anastomotic stenosis; however, if the width of the seromuscular flaps is appropriately extended, the incidence of anastomotic stenosis may decrease (25). Kuroda et al. believed that the double-flap technique is promising as one of the preferred techniques for digestive tract reconstruction after proximal gastrectomy (24, 26). The double-flap technique is suitable for patients with early gastric cancer in the upper third of the stomach with a predicted residual gastric capacity of > 50%. However, the operative procedure for the double-flap technique is complicated and requires advanced suturing skills and a long operative time.

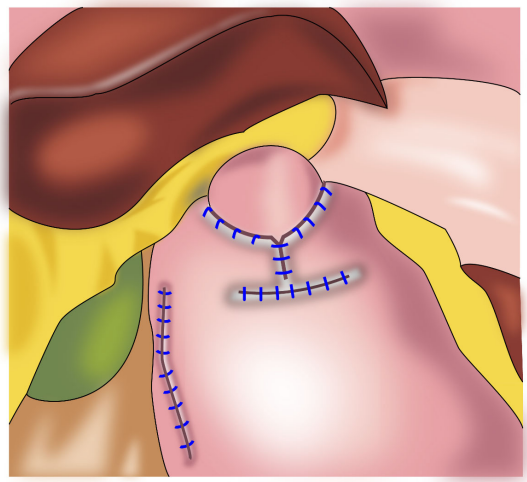


FIGURE 4
Double-flap anastomosis (Kamikawa anastomosis): This procedure increases the pressure in the lower esophagus and is beneficial to reduce the occurrence of reflux esophagitis.

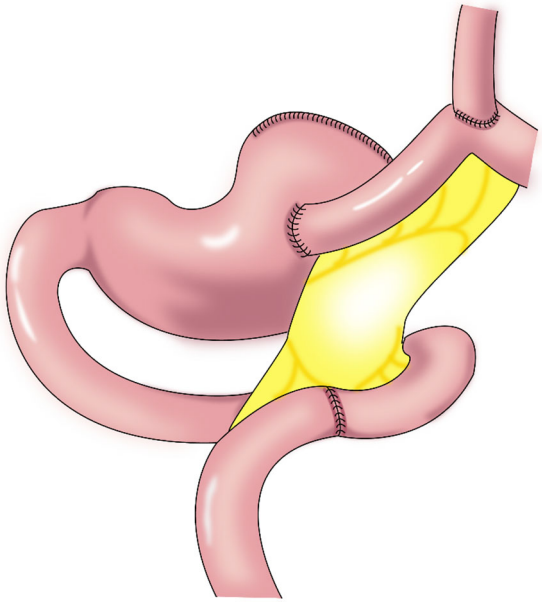


FIGURE 5
Jejunal interposition: a segment of jejunum is inserted between the esophagus and the remnant stomach to construct an anti-reflux barrier. This procedure takes advantage of intestinal peristalsis and the tolerance of the jejunum to acidic gastric juice and alkaline digestive juice.

2.2 Jejunal interposition

In jejunal interposition (Figure 5), a segment of jejunum is inserted between the esophagus and the remnant stomach to construct an anti-reflux barrier. This procedures takes advantage of intestinal peristalsis and the tolerance of the jejunum to acidic gastric juice and alkaline digestive juice. Kameyama et al. first reported that the interposition of a jejunal pouch could preserve storage capacity in the remnant stomach (9). Katai et al. reported that the incidence of reflux symptoms after jejunal interposition was 5.6%, and that of reflux esophagitis on endoscopy was 1.7%, which significantly improved the patients' postoperative quality

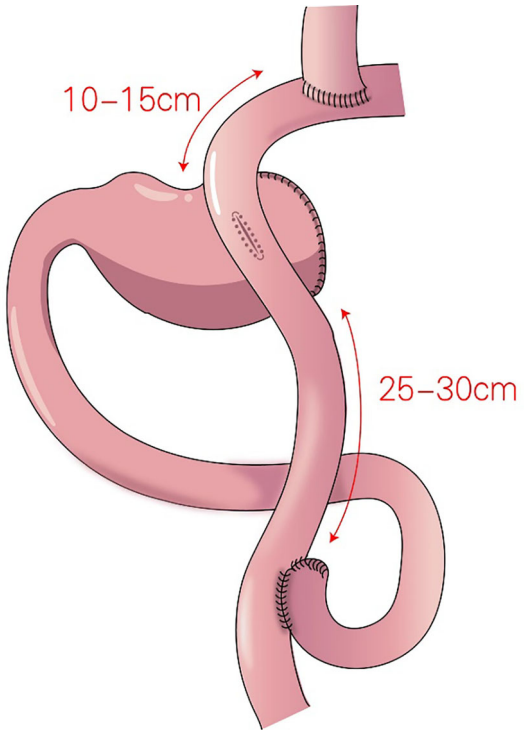


FIGURE 6
Double-tract reconstruction (DTR): Roux-en-Y anastomosis of the esophagus and jejunum is performed first, the jejunum 10–15 cm from the anastomotic stoma of the remnant stomach, and the esophagus-jejunum are anastomosed side-to-side.

of life (27). As a disadvantage, food residues are easily retained in the jejunal pouch and remnant stomach (28). To improve this situation and minimize the incidence of reflux esophagitis, the length of the jejunal pouch has been gradually shortened to the current length of approximately 10 cm (29). The small intestine replaces the upper part of the stomach. However, compared with the stomach, the jejunal pouch has thinner fascia and limited storage capacity, which is attributed to histological differences.

A type of proximal gastrectomy and piggyback jejunal interposition has been reported to block the jejunum at the distal end of the gastrojejunal anastomosis. This approach is based on double-tract anastomosis, which is a continuous interjejunal anastomosis. This procedure preserves the continuity of the interpositioned jejunal segment, reduces the possibility of obstructed food emptying, and improves the patient's nutritional status (10, 30).

The jejunal interposition has a low requirement regarding the remnant stomach size and is suitable for most reconstructions after proximal gastrectomy. However, the operation is complicated, with a long operative time and relatively high cost, and there is the possibility of obstructed remnant stomach emptying.

2.3 Double-tract reconstruction (DTR)

In 1988, Aikou et al. first reported DTR (Figure 6) as a type of proximal gastrectomy for digestive tract reconstruction (31). In this method, Roux-en-Y anastomosis of the esophagus and jejunum is performed first, after the proximal stomach is dissociated. Then, the jejunum 10–15 cm from the anastomotic stoma of the remnant stomach, and the esophagus-jejunum are anastomosed side-to-side. After esophagojejunal anastomosis, food can enter the distal jejunum through the remnant stomach and jejunum (31). Nakajima et al. found that DTR with a larger remnant stomach provided better transport and mixing of bile and food, and partial food directly entering the jejunum alleviated slow emptying or food stagnation in the remnant stomach induced by vagotomy (32). Ahn et al. showed that the incidence of reflux esophagitis in the DTR group was 4.6%, indicating a good preventive effect of DTR on reflux symptoms (11). Tomoki et al. reported that the incidences of reflux symptoms (10.5% vs. 54.5%) and anastomotic stenosis (0 vs. 27%) in the DTR group were significantly lower than those in the esophagogastric anastomosis group, respectively, 1 year postoperatively (33). Reo et al. found no difference in early complication rates between the laparoscopic proximal gastrectomy + DTR group and the total gastrectomy group (34). The incidence of reflux esophagitis was significantly higher in the proximal gastrectomy + DTR group than that in the total gastrectomy (Roux-en-Y reconstruction) group (8.0% and 0%, respectively), and the amount of weight loss and the decrease in hemoglobin concentration were significantly lower in the DTR group than in the total gastrectomy group (34).

DTR is appropriate for most reconstructions of the digestive tract after proximal gastrectomy, with low requirements regarding the remnant stomach, and DTR is especially appropriate for patients who require excessive stomach resection and who are not eligible for esophagogastrostomy. However, the surgical procedure is relatively complicated, with many anastomotic stomas, possibly increasing the risk of stomal leakage and increased costs.

3 Cardia function-preserving gastrectomy for gastric cancer

In second part of reservation of cardia, we reviewed various methods to preserve the function of the cardia including endoscopic resection (ER), local gastrectomy by gastroscopy combined with laparoscopy, segmental gastrectomy, subtotal gastrectomy, cardia-preserving radical gastrectomy (Table 2).

3.1 Endoscopic resection (ER)

Early gastric cancer means that cancer invaded into the mucosa or submucosa, regardless of lymph node metastasis (35).

TABLE 2 Function-preserving gastrectomy.

Method of operation	Indications	Disadvantage
Endoscopic resection (ER)	EMR for differentiated-type adenocarcinoma, stage T1a, no ulcerative findings, and tumor diameter ≤ 2 cm. ESD for differentiated-type adenocarcinoma, stage T1a, no ulcerative findings, and no clearly-defined tumor size. ESD for differentiated-type adenocarcinoma, stage T1a combined with an ulcer, with a tumor diameter of ≤ 3 cm.	For patients with postoperative positive resection margins or for those who underwent non-radical resection (such as vascular infiltration) need radical remedial surgery
Local gastrectomy by gastroscopy combined with laparoscopy	Suitable only for early gastric cancer	The indications for surgery are very limited
Segmental gastrectomy	Suitable only for early gastric cancer in the middle third of the stomach, preferably with the cancer located in the greater curvature of the stomach	The indications for surgery are very limited
Subtotal gastrectomy	Suitable only for tumor in the upper stomach or invading the upper stomach, preoperative stage cT1N, tumor located <5cm from the gastroesophageal junction or < 3 cm from the cut end of the remnant stomach, and negative incision margin	Lack of blood supply to the remnant stomach, worsened motility disorders in the remnant stomach, and poor anastomosis healing
Cardia-preserving radical gastrectomy	(1) early gastric cancer, the distance from the upper margin of the lesion to the cardia is ≥4 cm, 2-4 cm can be used as the relative indication. (2) In advanced middle gastric cancer, the incision margin was at least 2.0-3.0 cm distance from the cardia to ensure the anastomotic distance	The number of cases carried out is relatively small, but its clinical efficacy requires further verification in prospective and controlled clinical studies

Detection rates of early gastric cancer in Japan and Korea are 70% and 50%, respectively, compared with approximately 20% in China (36).

Endoscopic submucosal dissection (ESD) has been gradually applied to treat early gastric cancer. Isomoto et al. found that the en bloc resection rate in patients with early gastric cancer undergoing ESD was 94.9% (559/589), and the radical resection rate was 94.7% (550/581), The overall 5-year survival rate and disease-specific survival rate were 97.1% and 100%, respectively (12). Thus, ESD achieves a considerable therapeutic effect comparable to that obtained with surgery.

The 5th edition of the Japanese Gastric Cancer Treatment Guidelines recommend endoscopic mucosal resection (EMR) for differentiated-type adenocarcinoma, stage T1a, no ulcerative findings, and tumor diameter ≤ 2 cm. ESD is recommended for differentiated-type adenocarcinoma, stage T1a, no ulcerative findings, and no clearly-defined tumor size. ESD is also recommended for differentiated-type adenocarcinoma, stage T1a combined with an ulcer, with a tumor diameter of ≤ 3 cm. For patients with postoperative positive resection margins or for those who underwent non-radical resection (such as vascular infiltration), radical remedial surgery is recommended (37). The 2021 National Comprehensive Cancer Network guidelines recommend ESD for differentiated-type adenocarcinoma, tumor diameter ≤2 cm, stage T1a, and no lymphatic vascular invasion (38). A study from the UK found that the long-term prognosis of stage T1aN0 and T1bN0 gastric cancer patients undergoing ER was inferior than that of the gastrectomy group (39). However, some studies have found that the survival rate of early gastric cancer patients with lymph node metastasis is significantly lower than that of those without metastasis (40), and the recurrence risk with ESD is higher with lymph node metastasis than without (13, 41, 42).

3.2 Local gastrectomy by gastroscopy combined with laparoscopy

Abe et al. first reported endoscopic full-thickness resection in the treatment of early gastric cancer in 2008 (43). Full-thickness resection of the gastric wall can achieve vertical and horizontal tumor resection margins that meet the requirements of radical tumor treatment (43). In 2012, using laparoscopy combined with endoscopy, Nunobe et al. performed laparoscopy-assisted full-thickness ER for early gastric cancer with a wide range of lesions, and achieved good effects (44). Hur et al. found that such full-thickness resection by laparoscopy and endoscopy ensured the reliability of the tumor vertical resection margins and that laparoscopy played an important role in lymph node dissection (45). The short-term results of the sentinel node navigation oriented tailored approach from South Korea confirmed that local resection with sentinel lymph node dissection was not inferior to traditional laparoscopic gastrectomy in the treatment of early gastric cancer (46). Local resection is beneficial for preserving gastric function and for achieving better nutritional status and quality of life, but only for early gastric cancer.

3.3 Segmental gastrectomy

In 1999, Ohwada et al. first used segmental gastrectomy (Figure 7) to treat early gastric cancer located in the middle third

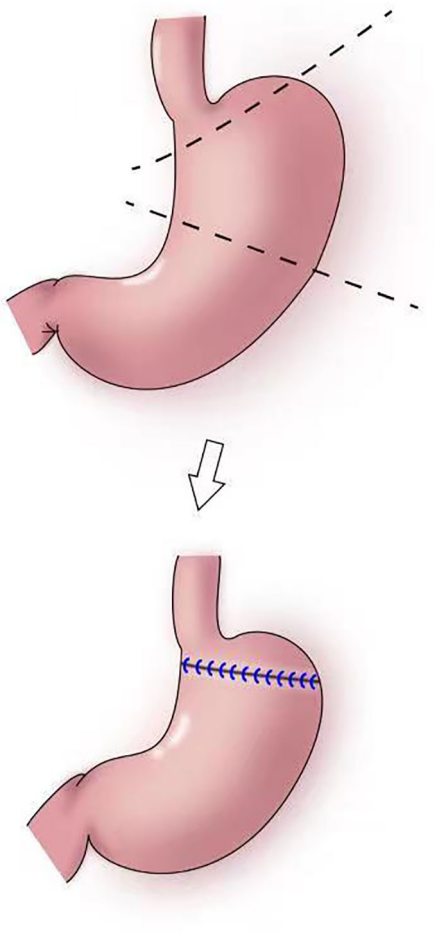


FIGURE 7
Segmental gastrectomy: The shaded area of the stomach is excised in upper figure. Anastomosis is performed between the distal remnant of the stomach and a fundic pouch in lower figure.

of the stomach (47). In 2006, Shinohara et al. reported segmental gastrectomy for early gastric cancer in the upper third of the stomach, and found that reflux symptoms and reflux esophagitis were significantly less frequent after segmental gastrectomy compared with those after proximal gastrectomy (48). In 2007, Koichi et al. reported that the incidences of early dumping syndrome and reflux gastritis were significantly lower after segmental gastrectomy compared with those after distal gastrectomy. All patients remained alive without recurrence during a mean follow-up period of 54.7 months in the segmental gastrectomy group (49). In 2010, Takeru et al. reported significantly less reflux esophagitis and reflux gastritis in the segmental gastrectomy group compared with that in the distal gastrectomy group, no recurrence or death was observed in two group following up median of 32.8 months (50). In 2012, Kim et al. proposed cardia-preserving proximal gastrectomy (51), which is a form of segmental gastrectomy. In 2017, Xiao analyzed the efficacy of laparoscopic segmental resection for early gastric cancer, and found no postoperative anastomotic fistulas, gastroparesis, or reflux (52). The number of lymph nodes obtained was 18.3 ± 7.5, and no severe gastroparesis occurred.

The indications for segmental gastrectomy are very limited, and the procedure is suitable only for early gastric cancer in the middle third of the stomach, preferably with the cancer located in the greater curvature of the stomach. Intraoperatively, lymph nodes on the lesser curvature should be resected, and hepatic and abdominal branches of the vagus nerve should be preserved. The surgical skill are also difficulties (52).

3.4 Subtotal gastrectomy

In 2011, Jiang et al. reported the first use of laparoscopy-assisted subtotal gastrectomy (with a minimal remnant stomach) (Figure 8) to treat early upper gastric cancer (53). During the procedure, 1–2 short gastric vessels near the cardia and the left inferior phrenic artery are preserved, and the distal stomach is dissociated approximately 2 cm from the tumor (53). In 2014, Toshiyuki et al. analyzed the feasibility of laparoscopy-assisted subtotal gastrectomy and the nutritional status of patients (54). The authors found that the incidence of postoperative anastomosis-related complications of laparoscopy-assisted subtotal gastrectomy was significantly lower than that with

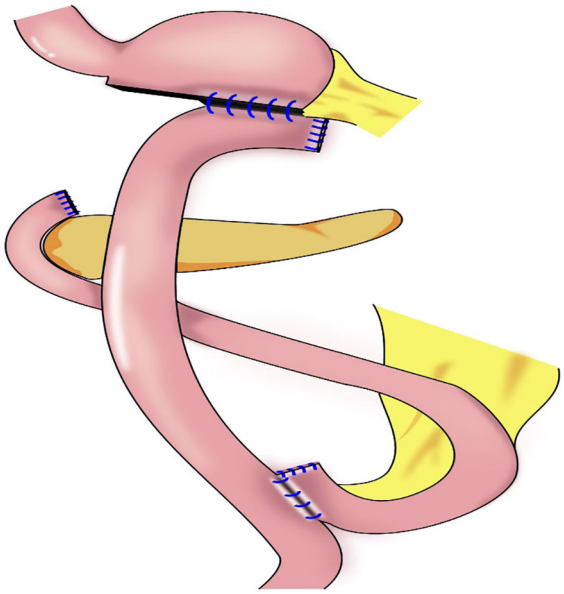


FIGURE 8
Subtotal gastrectomy: Very small remnant stomach after transection, Roux-en-Y reconstruction procedure were performed.

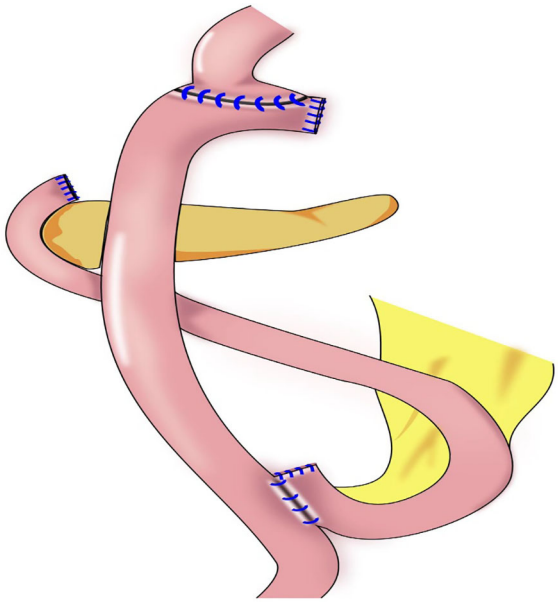


FIGURE 9
Cardia-preserving radical gastrectomy: The remnant stomach is dissociated 2–3 cm from the cardia using a linear cutting stapler, Roux-en-Y reconstruction procedure were performed.

laparoscopy-assisted total gastrectomy, and that weight gain 12 months postoperatively was significantly higher, there were none of recurrence in distant organs, remnant stomach, or lymph nodes with mean follow-up of 27.9 months (54). Souya et al. found that the serum protein concentration and the anti-esophageal reflux effect after subtotal gastrectomy were better compared with those after proximal gastrectomy, and that the hemoglobin concentration was better with subtotal gastrectomy than that with total gastrectomy (55). Itaru et al. reported that body weight and hemoglobin concentrations decreased slightly after laparoscopic subtotal gastrectomy for early upper gastric cancer compared with those after laparoscopic distal gastrectomy, and no difference in total protein and albumin concentrations was noted between the two groups (56). Hao et al. found that patients undergoing laparoscopic-assisted tailored subtotal gastrectomy for advanced gastric cancer in the middle third of the stomach had significantly lower postoperative complication rates (4.2%) compared with patients who underwent laparoscopic assisted total gastrectomy (17.8%). Furthermore, albumin, prealbumin, total protein, and hemoglobin concentrations, and red blood cell counts in the laparoscopic-assisted tailored subtotal gastrectomy group were significantly higher than the related values in the laparoscopic total gastrectomy group, The 3-year overall survival rates in the laparoscopic-assisted tailored subtotal gastrectomy and laparoscopic assisted total gastrectomy groups were 85.6% and 67.4%, respectively ($P<0.05$) (57). Jin and Liu et al. subsequently designed laparoscopic tailored subtotal gastrectomy (LTSG) to treat advanced middle gastric cancer. On the basis of the premise of guaranteeing tumor safety, tailored resection was performed according to the tumor site to retain as much stomach volume as possible. The main operation points with LTSG are to reserve 1–2 short gastric vessels without No. 2 lymph node dissection, and to ensure upper, lower, and lateral margins of > 3 cm. If the above requirements cannot be met, total gastrectomy should be performed. The study showed that the LTSG group had fewer postoperative complications, better nutritional status, no increased recurrence rate, and a long-term survival benefit compared with total gastrectomy, possibly achieved by improving nutritional status and, thereby, prolonging the patients' overall survival (57, 58). Itaru et al. analyzed the efficacy of laparoscopic subtotal gastrectomy and laparoscopic distal gastrectomy (56). After 3 years of follow-up, the authors found that body weight and hemoglobin concentration in the laparoscopic subtotal gastrectomy group were lower than the values in the laparoscopic distal gastrectomy group; however, no difference in total protein and albumin concentrations was noted between the two groups (56).

The indications for laparoscopic subtotal gastrectomy comprise a tumor in the upper stomach or invading the upper stomach, preoperative stage cT1N, tumor located < 5 cm from the gastroesophageal junction or < 3 cm from the cut end of the remnant stomach, and negative incision margin (55). At least 1–2 short gastric vessels and posterior gastric vessels from the cardia should be preserved during subtotal gastrectomy. Blood flow to the remnant stomach is mainly supplied by the left inferior phrenic artery and 1–2 short gastric and posterior gastric vessels (54, 57). Subtotal gastrectomy performed after complete dissection of the No. 1 and No. 2 lymph nodes may result in a lack of blood supply to the remnant stomach, worsened motility disorders in the remnant stomach, and poor anastomosis healing.

3.5 Cardia-preserving radical gastrectomy

Our team began to perform cardia-preserving radical gastrectomy (Figure 9) in November 2020. No.s 1, 2, 3, 4, 5, 6, 7, 8, and 9 lymph nodes are dissected by laparoscopy; the No. 11 lymph node is dissected along the splenic artery; and one short gastric artery is reserved during dissection of the No. 10 lymph node to complete D2 lymph node dissection. After lymph node dissection is complete, 2–3 cm of the lower esophagus is dissociated, and the remnant stomach is dissociated 2–3 cm from the cardia using a linear cutting stapler. The specimen is extracted through a small incision approximately 4 cm from the umbilicus to determine sufficient incision margins and to confirm negative margins by frozen section. The gastric stump

and jejunum are sutured manually and anastomosed (Roux-en-Y). To date, cardia-preserving radical gastrectomy has been performed successfully in 10 cases, without conversion to laparotomy and without severe surgery-related complications, such as postoperative bleeding, anastomotic fistula, or anastomotic stenosis. The proximal and distal margins of the resected specimens were negative in all 10 cases. The patients were followed-up for 2–15 months, with no deaths or tumor recurrence and metastasis during the follow-up period. There were also no postoperative reflux symptoms. Subtotal gastrectomy performed after complete dissection of the No. 1 and No. 2 lymph nodes may result in a lack of blood supply to the remnant stomach, which leads to further motility disorders in the remnant stomach. During cardia-preserving radical gastrectomy, approximately 2–3 cm of the gastric wall away from the dentate line is preserved, with little residual gastric tissue; therefore, the blood supply is relatively better. Complete cardia-preserving radical gastrectomy can reduce the incidence of reflux esophagitis, but its clinical efficacy requires further verification in prospective and controlled clinical studies (59).

The indications for cardia-preserving radical gastrectomy comprise (1) Siewert Type III(2-5cm below the dentate line) for early gastric cancer with the upper edge of the lesion is more than 4 cm from the cardia. (2) Advanced middle gastric cancer, the incision margin is more than 4 cm from the tumor, and the incision margin is at least 2.0-3.0cm below the cardia to ensure the anastomosis distance. (3) Rapid pathological examination should be performed to confirm that the surgical margins was negative.

4 Summary and prospects

The gastric cardia has an anti-reflux function, the loss of which significantly increases the incidence of reflux esophagitis and reduces patients' quality of life. Currently, although various anti-reflux reconstruction methods after proximal gastrectomy reduce the incidence of reflux esophagitis to a certain extent, the incidence is still quite high. The reconstruction process is complicated and postoperative complications increase correspondingly. There are many anti-reflux gastrectomy procedures, but the indications for each procedure are limited.

Cardia-preserving radical gastrectomy has the advantage of more thorough lymph node dissection and wider indications than those for subtotal gastrectomy. However, the clinical efficacy of cardia-preserving radical gastrectomy requires verification in prospective and controlled clinical trials. Cardia-preserving radical gastrectomy is a promising approach as one of the more reasonable anti-reflux surgeries.

Author contributions

The review was designed and revised by Y-YW, LL, and Y-PM. LL, YR, Z-HL, and Q-TJ conducted the literature collection. X-FC draw the figure and Z-HL, Y-YW, and LL wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prognostic value of pan-immune-inflammation value in colorectal cancer patients: A systematic review and meta-analysis

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Background: The pan-immune-inflammation value (PIV) has been reported as a novel prognostic biomarker in multiple malignancies. The aim of this study is to investigate the prognostic value of the PIV in patients with colorectal cancer.

Methods: We comprehensively searched electronic databases including PubMed, Embase and Web of Science up to August 2022. The endpoints were survival outcomes. Hazard ratios (HRs) with 95% confidence intervals (CIs) for survival data were collected for analysis.

Results: Six studies including 1879 participants were included. A significant heterogeneity in the PIV cut-off value among studies was observed. The combined results indicated that patients in the high baseline PIV group had a worse overall survival (HR=2.09; 95%CI: 1.67-2.61; P<0.0001; I^2 = 7%) and progression-free survival (HR=1.82; 95%CI: 1.49-2.22; P<0.0001; I^2 = 15%). In addition, early PIV increase after treatment initiation was significantly associated with decreased overall survival (HR=1.79; 95%CI: 1.13-2.93; P=0.01; I^2 = 26%), and a trend toward poor progression-free survival (HR=2.00; 95%CI: 0.90-4.41; P=0.09; I^2 = 70%).

Conclusion: Based on existing evidence, the PIV could act as a valuable prognostic index in patients with colorectal cancer. However, the heterogeneity in the PIV cut-off value among studies should be considered when interpreting these findings.

KEYWORDS

colorectal cancer, pan-immune-inflammation value, overall survival (OS), progression-free survival (PFS), meta-analysis

1 Background

Colorectal cancer is one of the most common malignancies in the world, accounting for about 10% of newly diagnosed cancers and cancer-related deaths (1). Despite significant advances in surgery-based multimodal therapy for colorectal malignancy, the prognosis of most patients, especially those with advanced stages, is still unsatisfactory (2–4). Consequently, it is essential to develop a useful prognostic index to predict postoperative recurrence and survival in colorectal cancers, aiming to formulate treatment plans for patients in the clinic.

Cancer-related inflammation is prevalent in most patients with malignancy, which can promote tumor progression and suppress treatment response (5, 6). Increasing evidence has reported that cancer-related inflammation plays an important role in postoperative recovery and prognosis of cancer patients (7, 8). Therefore, inflammation-based biomarkers are expected to be valuable predictors of surgical and long-term outcomes. For example, as the most common indicators of systemic inflammation, neutrophil (9), platelet (10) and monocyte (11) have been reported as strong indicators for increased postoperative complications, prolongation of hospital stays and poor survival outcomes in several types of malignancies. On the contrary, tumor-infiltrating lymphocyte subsets, such as CD8+ T cells and memory T cells, are associated with better prognosis in various tumors (12, 13).

In recent years, a novel biomarker, the pan-immune-inflammation value (PIV), which integrates peripheral neutrophil, platelet, monocyte and lymphocyte (neutrophil x platelet x monocyte/lymphocyte), has been reported as a promising predictor of long-term outcomes in cancers, because it can precisely reflect the inflammatory and immune status of patients with malignancy (14–17). A recent meta-analysis demonstrated that high PIV before treatment indicates poor prognosis in cancer patients (18). Nevertheless, the role of the PIV in survival outcomes of colorectal cancer remains inconclusive and no meta-analysis is available so far. In addition, emerging studies on the PIV and survival outcomes in colorectal cancer have been reported in recent years. Thus, we performed a systematic review and meta-analysis based on existing evidence to investigate the value of the PIV in long-term survival outcomes in patients with colorectal cancer.

2 Methods

2.1 Search strategy

The current study was performed in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines to identify studies that assess the association of PIV with survival outcomes in colorectal cancer patients. Relevant studies from PubMed, Embase and Web of Science were comprehensively examined up to August 20, 2022. Published language was not restricted during the search process. The MeSH term "pan-immune-inflammation value" was used to comprehensively identify potential studies. In addition, the references of the included studies were scanned for additional reports. The search was independently performed by two investigators (XC-Y and H-L).

2.2 Inclusion and exclusion criteria

The inclusion criteria were as follows:

(1) Studies examined the relationship between the PIV and long-term survival of patients with colorectal cancer;
(2) Hazard ratios (HRs) with 95% confidence intervals (CIs) were available;
(3) The cutoff value of the PIV was clearly reported.

The exclusion criteria were as follows:

(1) Studies were reported as case reports, reviews and letters;
(2) Duplicated data.

2.3 Data extraction and quality assessment

Two reviewers (XC-Y and H-L) conducted the data extraction independently and cross-checked all the results. The extracted data included first author, publication year, study interval, country, study design and sample size, selection method, cut-off value, clinicopathological features like age, sex and tumor stage, and survival data.

The quality of included studies were also evaluated following this method described by Lin et al. (19), which contains predefined nine items. A study could get a final score from 0 to 9 after assessment.

2.4 Outcomes

In the present study, the primary outcomes were to investigate the relationship between the PIV and long-term survival in patients with colorectal cancer. Long-term outcomes included OS and PFS. Of note, since disease-free survival (DFS), recurrence-free survival (RFS) and PFS share the similar endpoints, they were analyzed together as one outcome, PFS, as previously suggested (20).

2.5 Statistical analysis

The HRs with their 95% CIs were used as the effect size for OS and PFS. Statistical heterogeneity among enrolled studies was assessed using I^2 statistic. All pooled analyses were conducted assuming the random-effects model, which accounts for variance across included studies. Subgroup analysis and sensitivity analysis were utilized to evaluate the credibility of pooled results. Begg's funnel plot was applied to assess the possibility of publication bias. A two-tailed P value <0.05 was considered statistically significant. All of these statistical analyses were performed by Review Manager Software, version 5.3 (Cochrane, London, UK) and Stata, version 12.0 (Statacorp, College Station, TX).

3 Results

3.1 Study characteristics

As shown in Figure 1, the search strategy yielded a total of 89 records. After careful title, abstract assessment and full text assessment, 6 studies (21–26) were finally included in the present study. The basic information of the included studies was shown in Tables 1, 2. A total of 1879 patients from Italy (21, 23), Turkey (22), Spain (24) and Japan (25, 26) were included in this study. These studies were published from 2020 to 2022 with a sample size ranging from 86 to 758. Among these studies, two studies (21, 23) were designed as multicenter studies, and another four studies (22, 24–26) were single-center studies. In addition, five (21–24, 26) and six studies (21–26) reported the relationship between baseline PIV and OS and PFS, respectively; and two studies (21, 24) reported the relationship between early PIV increase after treatment initiation and OS and PFS, respectively. Moreover, the cut-off value of the PIV varies a lot among these studies, ranging from 209 to 492. The quality of the included studies was good with a median score of 8 (range: 6-9, Figure 2 and Table S1).

3.2 Relationship between baseline PIV and OS

Five studies (21–24, 26) involving 1793 patients described the association between the baseline PIV and OS. The pooled HR was 2.09 (95%CI: 1.67-2.61; P<0.0001; I^2 = 7%), which indicated that a high PIV was significantly associated with decreased OS in patients with colorectal cancer (Figure 3 and Table 3). Furthermore, subgroup analyses based on country, study design, sample size, and tumor stage were performed. As shown in Table 3 and Figure S1, the pooled results of all subgroup analyses revealed that patients in the high PIV group had a substantially reduced OS when compared with these in the low PIV group. Additionally, sensitivity analysis by deleting one study at a time showed that the pooled outcome did not substantially change (Figure S3A).

3.3 Relationship between baseline PIV and PFS

A total of six studies (21–26) involving 1879 patients reported on PFS. The pooled HR was 1.82 (95%CI: 1.49-2.22; P<0.0001; I^2 = 15%), which suggested that patients in the high PIV group had a worse PFS when compared with patients in the low PIV group (Figure 4 and Table 3). Similarly, subgroup analyses based on country, study design, sample size, and tumor stage demonstrated that the pooled results remained consistent in each subgroup (Table 3 and Figure S2). Sensitivity analysis showed that the combined effect was not significantly changed (Figure S3B).

3.4 Relationship between early PIV increase and OS/PFS

Only two studies (21, 24) involving 277 cases reported the relationship between early PIV increase after the treatment initiation and survival outcomes. As shown in Figure 5, the combined results suggested that early PIV increase was substantially correlated with decreased OS (HR=1.79; 95%CI: 1.13-2.93; P=0.01; I^2 = 26%), and a trend toward poor PFS (HR=2.00; 95%CI: 0.90-4.41; P=0.09; I^2 = 70%).

3.5 Publication bias

The Begg's funnel plot was performed to assess the possibility of publication bias. As shown in Figure S4, the funnel plots of OS and PFS were symmetric, and the P values of Begg's test were 0.130 and 0.060, respectively, indicating that these pooled outcomes were absence of publication bias.

4 Discussion

In 2020, Fuca et al. (23) first developed the PIV based on commonly used peripheral blood count parameters as a systemic inflammation-related prognostic biomarker for metastatic colorectal cancer. Since then, the PIV has been widely used as a cheap, readily available and reliable index to evaluate the prognosis of various cancers (27–29). In the present study, we included six studies with 1879 patients with colorectal cancer and found that high PIV was significantly associated with decreased OS and PFS. Meanwhile, we have further identified

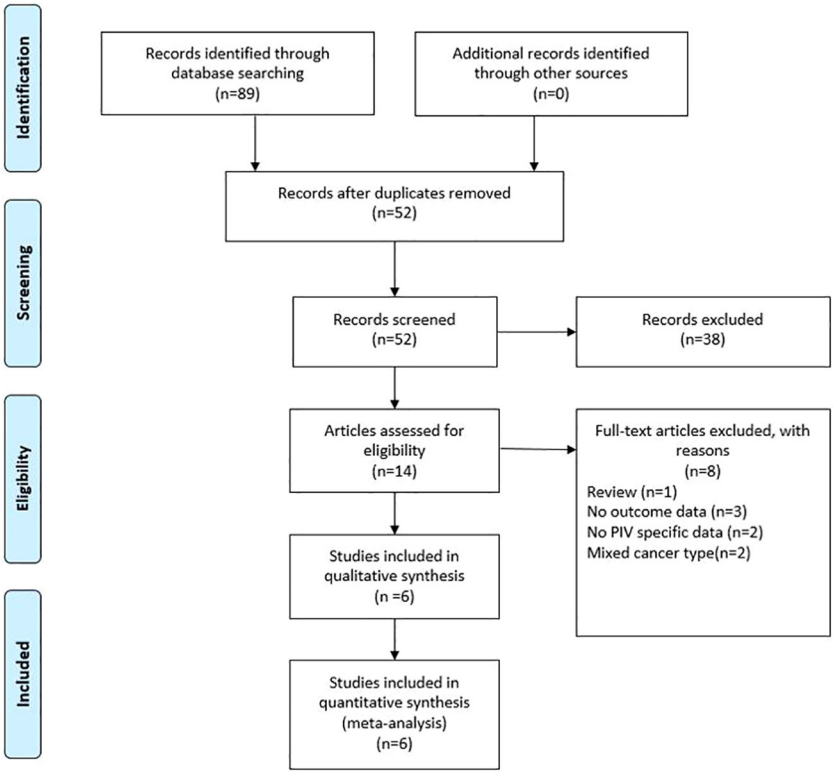


FIGURE 1
The PRISMA Flowchart of study selection.

that the early PIV increase after the treatment initiation was also associated with significantly poor OS and a trend toward worse PFS in colorectal cancer patients. Therefore, the PIV may have a good discriminatory value and remains an effective inflammatory index for predicting long-term survival outcomes in colorectal cancer.

Systemic inflammatory reflection has been well confirmed to be closely associated with the occurrence and progression of

TABLE 1 Basic information of included studies.

Reference	Country	Study design	Study interval	Sample size	Age, years	Sex(Male/ Female)	Tumor stage	Survival analysis
Corti, 2021[21]	Italy	M; R	2014-2020	163	NR	90/73	Metastatic	OS; PFS
Efile, 2021 [22]	Turkey	S; R	2008-2016	304	NR	NR	Non-metastatic	OS; DFS
Fuca, 2020 [23]	Italy	M; R	2008-2018	438	62(IQR:53-68)	275/163	Metastatic	OS; PFS
Perez-Martelo, 2022 [24]	Spain	S; R	2015-2018	130	68.8 (range:26-88)	96/34	Metastatic	OS; PFS
Sato (1), 2022 [25]	Japan	S; R	2013-2020	86	70(range:37-93)	50/36	Non-Metastatic	RFS
Sato (2), 2022 [26]	Japan	S; R	2000-2019	758	NR	466/292	Non-metastatic	OS; RFS

R, retrospective; S, single center; M, multiple center; NOS, Newcastle Ottawa Scale; NR, not report; IQR, inter-quartile range.
OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; DFS, disease-free survival.

TABLE 2 Survival information of included studies.

Reference	Sample size (High / Low)	Selection method	Cut-off value	Survival analysis	Median follow-up (months)	Analysis method	OSHR (95%CI)	RFS/PFS/ DFSHR (95% CI)
Corti, 2021[21]	163(63:100)	MSR	492	OS; PFS	31	Multivariate	3.00 (1.49-6.04)	1.91 (1.06-3.44)
Efile, 2021 [22]	304(152:152)	Median	491	OS; DFS	NR	Multivariate	2.43(1.55-3.79)	2.28 (1.51-3.45)
Fuca, 2020 [23]	438(230:208)	MSR	380	OS; PFS	38.4(IQR:27.4-50.9)	Multivariate	1.55 (1.02–2.37)	1.53 (1.09–2.15)
Perez-Martelo, 2022 [24]	130(70:60)	Literature	380	OS; PFS	NR	Multivariate	1.82 (1.15–2.90)	1.56 (1.05–2.31)
Sato (1), 2022 [25]	86(63:23)	ROC	209	RFS	35(range:1-104)	Multivariate	NR	3.99 (1.69–9.45)
Sato (2), 2022 [26]	758(190:568)	ROC	376	OS; RFS	63.5	Multivariate	2.49(1.55–3.98)	1.70 (1.10–2.62)

OS, overall survival; PFS, progression-free survival; RFS, recurrence-free survival; DFS, disease-free survival; ROC, receiver operating characteristic curve. MSR, maximally selected rank; IQR, inter-quartile range NR, not report.

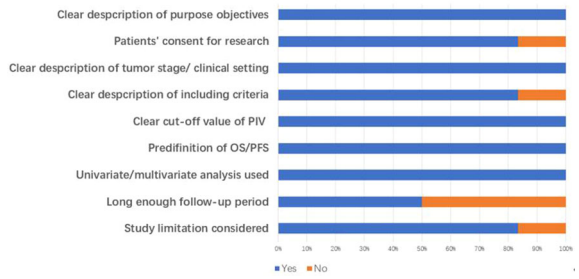


FIGURE 2
Quality assessment of included studies.

malignancies (5). Increased neutrophils and monocytes in the tumor microenvironment have been reported to induce myeloid-derived suppressor cells, thereby suppressing the host immunity and prompting the tumor growth (30, 31). In addition, monocytes can differentiate into tumor-associated macrophages, which is associated with creating a favorable microenvironment for cancer development (32). Platelets are reported to secrete TGF-β, FGF and VEGF, which contribute to the epithelial–mesenchymal transition and angiogenic process (33, 34). Moreover, the interaction between platelets and tumor cells recruits and activates neutrophils and monocytes, which is required for the formation of distal metastasis sites (34). While lymphocytes, especially cytotoxic T lymphocytes, as the most important cell-mediated anti-tumor immune cells, inhibit tumor cell proliferation and metastasis by inducing the lysis and apoptosis of tumor cells (35, 36). Low lymphocyte counts have been demonstrated to lead to poor prognosis in colorectal cancer patients (37). Reasonably, the PIV, combined with neutrophils, monocytes, lymphocytes, and platelets, may enable better understanding of the functional state of patients and predict the prognosis of patients with colorectal cancer.

In our combined analysis involving 1793 samples, we identified that the baseline PIV is an independent

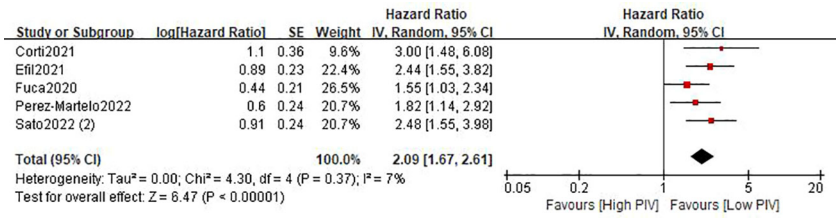


FIGURE 3
Forest plot assessing the relationship between PIV and OS.

TABLE 3 Subgroup analyses for OS and PFS of PIV-high patients vs. PIV-low patients.

		Studies, n	Patients, n	HR (95%CI)	P value	I^2 (%)
Overall survival						
	Total	5	1793	2.09(1.67-2.61)	<0.0001	7
Country	Eastern	1	758	2.48(1.55-3.98)	0.0001	–
	Western	4	1035	2.00(1.53-2.61)	<0.0001	17
Sample size	>200	3	1500	2.08(1.52-2.83)	<0.0001	32
	≤200	2	293	2.18(1.36-3.48)	0.001	25
Study design	Multi-center	2	601	2.02(1.07-3.82)	0.03	60
	Single-center	3	1192	2.23(1.71-2.92)	<0.0001	0
Tumor stage	Non-metastatic	2	1062	2.46(1.78-3.40)	<0.0001	0
	Metastatic	3	731	1.86(1.35-2.58)	0.0002	20
Progression-free survival						
	Total	6	1879	1.82(1.49-2.22)	<0.0001	15
Country	Eastern	2	844	2.39(1.06-5.40)	0.04	67
	Western	4	379	1.74(1.42-2.14)	<0.0001	0
Sample size	>200	3	1500	1.77(1.41-2.23)	<0.0001	6
	≤200	3	449	2.02(1.28-3.20)	0.003	46
Study design	Multi-center	2	601	1.62(1.21-2.17)	0.001	0
	Single-center	4	1278	1.97(1.46-2.64)	<0.0001	36
Tumor stage	Non-metastatic	3	1148	2.20(1.51-3.20)	<0.0001	37
	Metastatic	3	731	1.60(1.27-2.02)	<0.0001	0

prognostic factor of OS in patients with colorectal cancer. Furthermore, subgroup analyses based on country, study design, sample size and tumor stage showed our results were consistent and robust. Meanwhile, the sensitivity analysis showed that there was no significant change in the correlation between high PIV and decreased OS. Additionally, we have further investigated the relationship between the PIV and PFS. The pooled result including 1879 patients showed that patients in the high PIV group has a substantially decreased PFS. Similarly, the subgroup analyses and sensitivity analysis supported the reliability of this incorporated result. Furthermore, we have also preliminarily explored the relationship between the early PIV increase after the treatment initiation and survival outcomes. The integrated results showed that the early PIV increase was correlated with decreased OS and tended to have a poor PFS. However, given that there were only two studies with small samples included, these results should be interpreted with caution and more studies with big sample size were required to further classify this issue. Based on these results, the PIV may be regarded as

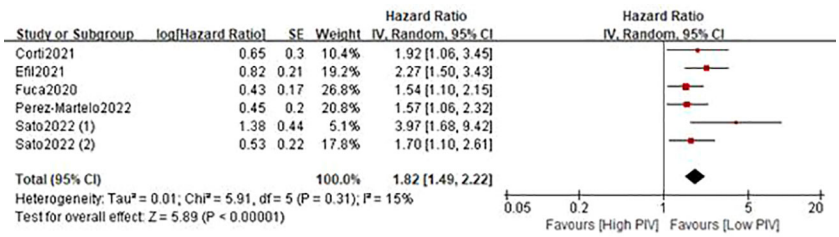


FIGURE 4
Forest plot accessing the relationship between PIV and PFS.

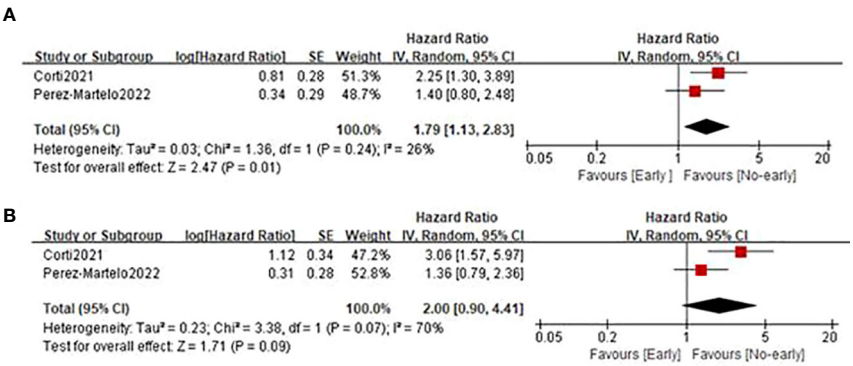


FIGURE 5
Forest plot assessing the relationship between PIV dynamics and survival outcomes including OS **(A)** and PFS **(B)**.

an effective prognostic indicator of long-term results of colorectal cancer.

There are some limitations to be noted in the present study. First, all involved studies were retrospective in nature, which may increase the risk of bias, and more prospective studies and randomized controlled trials are required to further investigate this issue. Second, due to the limited number of included studies, the value of the PIV dynamics in survival outcomes needs to be further clarified. Third, the cut-off value of PIV varies greatly among studies, which might affect the clinical utility of these findings. Finally, we were also unable to compare the prognostic predictability of PIV with other biomarkers in colorectal cancer patients, with few data eligible.

5 Conclusions

The findings of the meta-analysis suggested that the PIV is of great significance in predicting long-term survival results in patients with colorectal cancer. However, further research is still required to validate the value of PIV in colorectal malignancy.

Author contributions

X-CY wrote the manuscript. X-CY and HL performed the data search and data analysis. X-CY and HL and D-CL prepared figures and tables. All authors reviewed the manuscript. X-WL and R-HC approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Tumor budding as a predictor for prognosis and therapeutic response in gastric cancer: A mini review

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In recent years, the role of tumor budding in gastric cancer has received increased attention across a number of disciplines. Several studies have found associations between tumor budding and the prediction of lymph node metastasis in early gastric cancer, prognosis of advanced gastric cancer, predictors of therapeutic response to immune checkpoint inhibitors, such as microsatellite instability (MSI), and therapeutic targets of molecular targeted therapy, such as human epidermal growth factor receptor 2 (HER-2). Therefore, tumor budding is a major element in the formulation of risk stratification and precision medicine strategies for patients with gastric cancer.

KEYWORDS

EMT, gastric cancer, prognosis, tumor budding, therapeutic response

1 Introduction

According to the 2020 WHO statistics (1), gastric cancer ranks fifth in incidence and fourth in mortality globally among all cancers. The choice of treatment modality and prognostic criteria for gastric cancer often depends on the TNM staging system. In recent years, tumor budding, a general clinicopathological feature of tumor aggressiveness, invasion, and poor prognosis, has attracted increasing attention. This pathologic phenomenon has also been observed in other tumors, such as extrahepatic cholangiocarcinoma (ECA) (2), pancreatic ductal carcinoma (PDC) (3), oral cancer (4), and cervical squamous cancer (ECSC) (5); thus, tumor budding is not unique to gastric cancer but is widespread among all tumors.

This review will address the concept of tumor budding, the molecular mechanism underlying this pathologic phenomenon in gastric cancer, and its role in predicting the prognosis and therapeutics of gastric cancer, to provide a new modality and reference for the individualized diagnosis and treatment of patients with gastric cancer.

2 Overview

According to the recommendations for reporting tumor budding in colorectal cancer based on the International Tumor Budding Consensus Conference (ITBCC) 2016 (6), tumor budding is defined as a single cell or clusters of up to four cells at the cancer invasion margin and can be stratified into peritumoral budding [(PTB), tumor budding at the tumor front] and intra-tumoral budding [(ITB), tumor budding in the tumor center and surrounded by tumor stroma (7)] (i.e., "Figure 1"). PTB can only be assessed using surgical resection specimens, whereas ITB can be assessed using biopsies and resection specimens.

Although the ITBCC provides a definition of the cell number and location of tumor budding, it does not specify the pathologic changes that accompany tumor budding. Thus, there has been minimal consensus about the histopathological changes related to tumor budding. Gabbert et al. (8) reported the pathologic changes of tumor budding visualized by light microscopy as early as 1985; at the invasion front, the regular architecture of differentiated carcinomas is lost. Here, the tumor glands are separated from each other and are composed of flat-to-cuboidal tumor cells. At the foremost border of the invasion front, there are no tumor glands, but there are isolated tumor cells. Some of these tumor cells undergo mitosis and aggregate into very small tumor cell complexes. The cell shape the isolated tumor cells at the foremost invasion front is extremely variable and ranges from round or oval to sandglass-like or arrow-like. Unlike the study of Gabbert et al., in 2014, Bronsert et al. (9) found that many tumor buddings were interconnected and ultimately connected to the main tumor branches in pancreatic ductal adenocarcinoma, colorectal adenocarcinoma, liver metastasis of colorectal adenocarcinoma, lung adenocarcinoma and invasive ductal breast cancer, when reconstructed in 3D. In 2020, Yoshizawa et al. (2) confirmed that high-grade tumor budding had more branch points than low-grade tumor budding (median, 26 vs. 20, $p = 0.021$) and longer mean protrusion length (median, 53.3 vs 32.1 μm, $p < 0.001$). Some scholars (10) have also proposed that tumor nodules within 1 mm of the tumor edge should not be regarded as tumor budding, because tumor nodules may be linked to tumor tissue in deeper sections and should be regarded as "discontinuous diffusion" of tumor tissues.

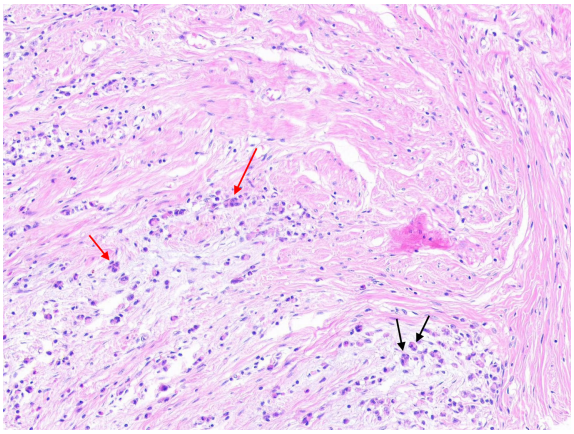


FIGURE 1
The black arrow represents tumor budding in gastric cancer (single tumor cells or clusters of up to four tumor cells), and the red arrow represents poorly differentiated cluster (PDC) in gastric cancer (five or more cells).

Currently, the ITBCC group (6) recommends the use of the following three-tier system, as used by the Japanese Society for Cancer of the Colon and Rectum: low-grade budding (Bd 1), 0-4 buds; intermediate grade budding (Bd 2), 5-9 buds; and high grade budding (Bd 3), 10 or more buds. However, in practical applications, different tumor have different grading criteria for tumor budding. For example, tumor budding is divided into two grades in head and neck, oral, and cervical cancers (11–13) [low grade of budding (0-5 buds) and high grade of budding (more than 5 buds)]. In upper urothelial carcinoma and pancreatic ductal adenocarcinoma (14, 15), less than 10 buds is considered low grade of budding, while more than 10 buds is considered high grade. In colon cancer, Zlobec et al. (16) found that there was a significant difference when BD0(0 buds) was compared statistically to BD1 (1–4 buds) for pT, TNM, tumor grade, and lymphatic, venous, and perineural invasion ($p < 0.01$, all). Because of these findings, they recommend that BD0 should be considered for inclusion in future ITBCC guidelines. In muscle invasive urothelial carcinomas, Lorenzo Soriano et al. (17) and Seker et al. (18) determined the critical value of tumor budding through receiver operating characteristics (ROC) curve, which also provided a new idea for the formulation of grading standard for tumor budding in gastric cancer in the future. Furthermore, there is little agreement on whether the type of tumor budding should be evaluated as PTB or ITB. Some scholars believe that for certain tumors, such as PDC, the scope of the tumor invasion edge cannot be clearly defined because of the small extent of resection. Thus, ITB should be used to evaluate the amount of tumor budding.

In gastric cancer, the grading system recommended by ITBCC has been used in many studies. However, due to the inability to distinguish the pathologic differences between diffuse-type gastric cancer and tumor budding, many studies cannot apply the tumor budding classification system to diffuse-type gastric cancer. Therefore, many studies are limited to intestinal-type gastric cancer, which can be distinguished from tumor budding (19). Although the number of patients with intestinal-type gastric cancer account for more than 50% of the total number of those with gastric cancers, the prognosis of patients with diffuse-type gastric cancer is worse than those with intestinal-type gastric cancer (20), which introduces bias in studies of the relationship between tumor budding and patient prognosis. Therefore, it is necessary to establish standard grading system for tumor budding in the future research of gastric cancer

3 Molecular mechanism underlying the pathogenesis of tumor budding in gastric cancer

3.1 Epithelial-mesenchymal transformation is the initiating process of tumor budding

Sun et al. (21) reported that ZBTB7A is highly expressed at the edge of enteric-type gastric cancers. ZBTB7A acts as a transcription factor that inhibits the expression of the Arf tumor suppressor gene, which results in decreased P53 activity. At the same time, high Arf

expression highly correlates with tumor budding, but negatively correlates with E-cadherin expression. The loss of E-cadherin expression will be manifested by tumor cells dissociating from each other, as they lose cell-cell adhesive junctions and acquire mesenchymal characteristics, which also contributes to the phenomenon of tumor budding (22). Furthermore, E-cadherin expression is also inhibited due to the high expression of the upstream TGF-β signaling pathway. Increased levels of TGF-β may also contribute to acquiring metastatic ability, as it enables gastric cancer cells to destroy and penetrate basement membrane barriers (23), enabling tumor cells to "escape" into the stroma and eventually form tumor buds. The change in E-cadherin expression is not simply quantitative, however, because although tumor cells lose E-cadherin membrane expression, there is a simultaneous increase in cytoplasmic expression of the protein. This allows to study EMT directly during tumor budding in tumor cell clusters of different cell numbers, demonstrating that the fraction of cells with cytoplasmic E-cadherin staining is significantly increased in smaller cell clusters, whereas the fraction of cells with mixed (cytoplasmic/membrane) and membrane expression patterns decreased with decreasing tumor cell cluster size (24).

3.2 Anoikis resistance promotes tumor bud survival

Cells express a variety of cell-surface adhesion molecules that mechanically act as contact points between cells and the extracellular matrix or adjacent cells and initiate intracellular signaling pathways that regulate important cellular events, including survival and proliferation. Normal cells undergo apoptosis in the absence of extracellular matrix attachment. This type of cell death is known as anoikis (25). Tanaka et al. (26) showed that the level of Trkb expression at the gastric cancer invasion front and in tumor budding cells was significantly higher than that in tumor cells in the gastric cancer center, with a significant positive correlation between the level of Trkb expression at the tumor invasion front and tumor budding ($p = 0.0023$). However, there was no significant correlation between tumor budding and Trkb expression in the gastric cancer center ($p = 0.0997$). Another study (27) showed that the BDAF/Trkb pathway inhibits the expression of E-cadherin in cells and promotes epithelial-mesenchymal transformation, the proliferative activity of tumor cells, and anoikis resistance. Thus, these results suggest that after tumor cells lose their attachment sites and attain mesenchymal cell characteristics through epithelial-mesenchymal transformation, the tumor cells can continue to survive in the mesenchyme and metastasize to distant places through the high expression of Trkb, eventually forming tumor buds.

3.3 Changes in the immune microenvironment inhibit tumor buds clearance

Zhang et al. (28) analyzed immune cell infiltration in the tumor budding microenvironment of gastric cancer. They observed a negative correlation between the density of tumor budding and tumor-infiltrating lymphocytes (TILs) in the budding area, tumor stroma, and parenchyma. The number of TILs around the tumor budding was reduced compared with TILs in the non-budding region ($p < 0.001$). Additionally, the number of TILs in turn changed from non-budding area CD8+>FOXP3+>OX40+> GrB + T cells to FOXP3+>CD8+>OX40 + T > GrB + T cells in budding area. CD8 surface antigen-expressing cytotoxic T lymphocytes are the most effective cells in the antitumor immune response. The abundance of CD8 + TILs positively correlates with better prognosis (HR = 0.77, 95% CI = 0.63-0.95) (29). Regulatory T cells (Tregs) are characterized by the expression of the transcription factor Foxp3, which is essential for the prevention of autoimmunity, maintenance of immune homeostasis, and regulation of immune responses to self and foreign antigens (30). Both tumor cells and Tregs can have high expression levels of TGF-β, which upregulates Foxp3 and Treg functional polarization in CD4+T cells and transforms macrophages from the M1-to-M2 type (31). Therefore, in the tumor budding area, tumor cells can increase TGF-β levels to increase Treg levels and the number of M2-type macrophages. This further reduces the immune response and the immune microenvironment conducive to M2-type macrophage growth, to benefit tumor cells for peripheral transfer and not be cleaned by immune cells.

These experiments revealed that during the early tumor budding process in gastric cancer, all steps are not isolated but are rather closely related and complementary through a complete and continuous process (Figure 2).

3.4 Prognosis and treatment of tumor budding in gastric cancer

3.4.1 Prognosis of early gastric cancer

Based on the 2016 ITBCC study (6), tumor budding was identified as an independent predictor of lymph node metastasis in patients with pT1 colorectal cancer. Simultaneously, it was widely recognized by academics that the occurrence and development of tumor budding is significantly correlated with the highly invasive properties of tumor cells, and the degree of occurrence and development is highly correlated with lymph node metastasis in early gastric cancer. As early as 2000, Matsumoto et al. (32) indicated a significant relationship between irregular narrowing or tumor buddings in the third layer on EUS and submucosal tumor invasion($p < 0.01$). The investigators recommended for lymph node removal to be considered, even when the endoscopy and biopsy show that the lesions present indications for therapeutic endoscopic treatment. In 2015, Gulluoglu et al. (33) conducted a study that involved 126 patients with early gastric cancer after radical total and subtotal gastrectomy. The clinicians reported that tumor budding was the only independent risk factor for lymph node metastasis in pT1a and pT1b gastric cancer. In 2019, Du et al. (34) also showed that tumor budding was a significant risk factor for lymph node metastasis in patients with early gastric cancer. Furthermore, in some early gastric cancers, lymph node metastasis was absent when there was no tumor budding (47 patients with submucosal early gastric cancer from the cardia, 15 with submucosal early stage gastric cancer <1.0 cm in size,

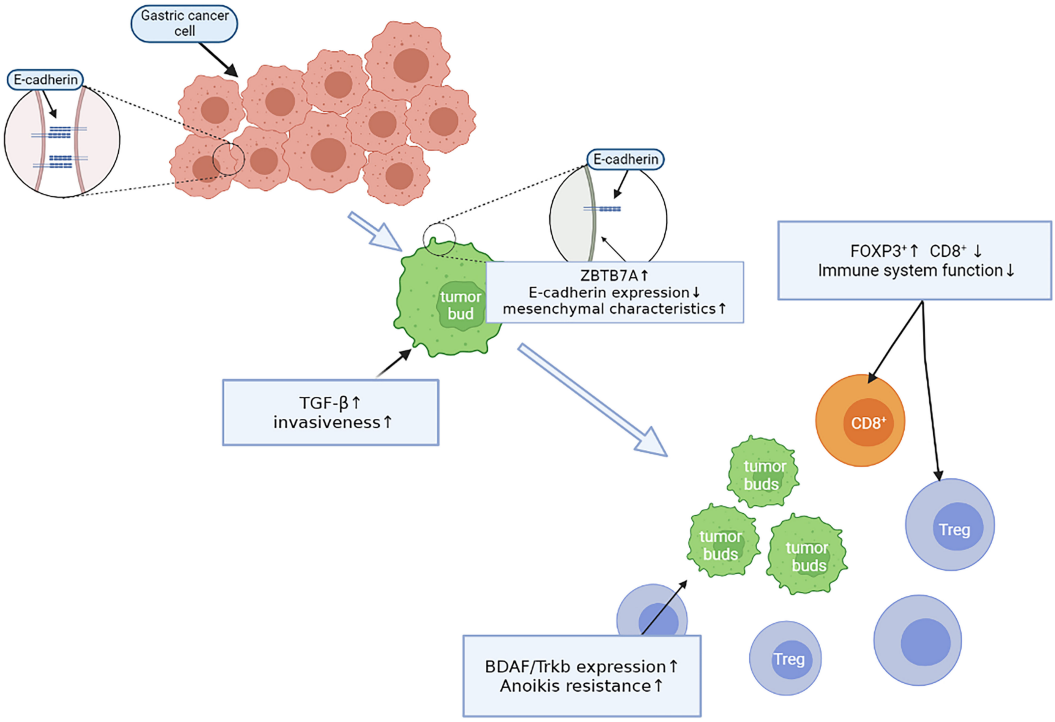


FIGURE 2
The continuous process of tumor budding.

and 17 cases of well-differentiated tubular/papillary early stage gastric cancer <1.0 cm in size). In 2021, Yim et al. (35) found that mTB (modified tumor budding, which excludes the signet ring cell component) was superior to traditional tumor budding (dAUC, 0.085 and 0.087 vs. 0.054 and 0.057) in predicting lymph node metastasis,which can significantly increase lymph node metastasis prediction accuracy in early gastric cancer.

Overall, these results indicate that tumor budding can be used as a predictor of lymph node metastasis in early gastric cancer and as a potential predictive factor to provide precise treatment strategies for patients with early gastric cancer (Table 1).

3.4.2 Prognosis of advanced gastric cancer

Nearly all of the studies on tumor budding in advanced gastric cancer have indicated that tumor budding is an important predictor of gastric cancer prognosis; however, each study had a different focus. Kucuk et al. (35) and Pun et al. (36) discovered that tumor budding was significantly related to pathologic stage and lymph node involvement ($p < 0.01$ & $p < 0.001$ and $p < 0.05$ & $p = 0.004$). In a study that involved 104 patients with surgically-resected gastric adenocarcinoma, Olsen et al. (36) reported that patients with high tumor budding were more likely to have nerve infiltration than patients with low budding (52% vs. 11%, $p = 0.002$), lower T stage

TABLE 1 Results of tumor budding in early gastric cancer.

Researcher	Year	Sample source	Pathologic stage	Number of samples	P value of lymph node metastasis	Conclusion
Du	2019	Surgical specimen	pT1b EGC	621	<0.01	Tumor budding is a significant high-risk factor for lymph node metastasis in submucosal early gastric carcinoma
Yusuke	2000	Endoscopically resected specimen	pT1a & pT1b EGC	75	\	The findings of irregular narrowing or tumor buddings in the third layer on EUS and submucosal invasion of the tumor has a significant relationship (P<0.01)
Yim	2021	Surgical specimen	pT1a & pT1b EGC	289	0.03	Tumor budding was the most predictive independent factor for lymph node metastasis, for groups containing all kinds of EGCs. Meanwhile, excluding signet ring cell from tumor budding significantly increased its lymph node metastasis prediction ability, compared to conventional tumor budding.
Gulluoglu	2015	Surgical specimen	pT1a & pT1b EGC	126	0.04 in pT1a <0.0001 in pT1b	Presence of tumor budding was the only variable that remained statistically significant as an independent marker for node pT1a & pT1b positivity of EGC

(70% vs. 10%, $p < 0.001$), and higher recurrence rate (27% vs. 0, $p = 0.007$). In the study on the relationship between diffuse gastric cancer with a high budding grade and intestinal gastric cancer, while no significant differences in the number and recurrence rate of lymph node metastasis was observed, intestinal gastric cancer had higher lymphovascular infiltration than diffuse gastric cancer (76% vs. 39%, $p = 0.002$). Thus, gastric cancer with a higher tumor budding grade has a stronger invasion ability, worse prognosis, and higher recurrence probability. Dao et al. (37) found that patients with a lower budding grade (grade 1 and 2) had a longer overall survival time than those with a higher budding grade (grade 3 and above) [(OS), 78.07 ± 2.15 vs. 33.87 ± 3.48 months]. With respect to 5-year disease-free survival (DFS), patients with budding grades 1 and 2 showed DFS rates of 95.0% and 84.7%, respectively, while all patients with a higher budding grade died before 5-years, with a statistically significant difference between the low and high budding grade groups ($p < 0.001$). Based on the connection between tumor budding and clinicopathology, Dao et al. proposed that tumor budding could be used as a tool for risk stratification prediction, which would be of great significance for guiding tumor follow-up treatment.

With regard to pathological characteristics, Qi et al. (38) studied PTB and ITB of gastric adenocarcinoma specimens and discovered that ITB was present in more patient tumor tissues than PTB (92.8% vs. 33.3%). Compared with ITB alone, patients with PTB and ITB had lower overall survival (42.43 vs. 54.62 months, $p = 0.033$) and a worse prognosis ($p < 0.001$). Therefore, ITB has application value in pathological biopsies, prediction of lymph node metastasis, and prediction of preoperative staging. However, the prognostic difference between ITB and PTB has not been compared in detail; therefore, it remains necessary to further explore which type of tumor budding form should be used for pre- and postoperative prognosis and staging evaluations. Furthermore, Szalai et al. (39) compared the ability of tumor budding and poorly differentiated clusters to predict prognosis and lymph node metastasis. The analyses showed higher tumor budding has poorer overall survival and more lymph node metastasis in the total cohort ($p = 0.014$ & $p = 0.038$) and in intestinal-type adenocarcinomas ($p = 0.005$ & $p = 0.019$). In contrast to tumor budding, no significant association was found between poorly differentiated clusters and the occurrence of lymph node metastasis, tumor stage, or survival. The results of this study further reveal the superiority of tumor budding in predicting prognosis and lymph node metastasis in patients with gastric cancer.

In patients receiving adjuvant chemotherapy, Jesinghaus et al. (40) reported that tumor budding was associated with many clinicopathological characteristics after neoadjuvant chemotherapy (ypT ($p < 0.001$), ypN ($p = 0.045$), and ypM stage ($p = 0.050$)). In parallel, tumor budding can stratify the prognosis of patients after adjuvant chemotherapy. Notably, a Kaplan-Meier survival curve analysis revealed significant differences in survival between the three grades of tumor budding in patients after adjuvant chemotherapy ($p < 0.001$). Patients whose tumors were assigned to the Bd1 subgroup had a mean OS of 51.7 months (95% CI:46.5–56.8 months) compared to 37.4 months for Bd2 (95% CI:30.3–44.5 months; HR:3.48, 95% CI:1.57–7.73) and 28.1 months for Bd3 carcinomas (95% CI:23.2–33.1 months; HR:6.26, 95% CI:3.06–12.81).

Therefore, tumor budding in patients who are operable (with or without neoadjuvant chemotherapy) can effectively predict prognosis and lymph node metastasis and stratify patient prognosis. However, few studies have investigated the prognostic relationship between tumor budding and advanced unresectable gastric cancer, and further research is needed in this regard (Table 2).

3.5 Therapeutic targets of tumor budding in gastric cancer

Ulase et al. (41) analyzed tumor budding in 456 surgically resected specimens and found that tumor budding grade significantly correlated with MSI and HER-2. At the same time, there was a significant association between tumor budding and MET status, but in contrast to HER-2 and MSI, gastric cancer with a high budding grade tended to be MET-positive more frequently than tumors with a low budding grade. Heckl et al. (42) studied the relationship between insulin receptors and gastric cancer and found that the expression of insulin receptors in gastric cancer cells negatively correlated with tumor budding ($p < 0.001$) and significantly correlated with HER-2 status ($p = 0.002$). Insulin receptor expression was found to be higher in HER-2+ tumor cells, which suggests that tumor budding not only predicts insulin receptor status, but also that the combination of HER-2 inhibitors and insulin receptor blockers (or metformin) may provide a potential treatment for patients with tumor budding at the corresponding grade in the future.

At present, there are few studies on the relationship between tumor budding, MSI, and PD-1 in gastric cancer, however, it has been widely described in colorectal cancer and other tumor types. A study in colon cancer by Jass et al. (43) found that the frequency of both somatic APC mutation and tumor budding increased pari passu in cancers stratified as sporadic MSI high (MSI-H), hereditary non-polyposis colorectal cancer (HNPCC), MSI low (MSI-L), and microsatellite stable (MSS). Notably, this finding explains why a lack of tumor budding correlates with improved prognosis in MSI-H colorectal cancer. However, while few studies have investigated the relationship between tumor budding and MSI and PD-1 in gastric cancer, the relationship has been widely described in colorectal cancer and other tumor types. In colorectal cancer, tumor budding was found to strongly correlate with PD-L1 positive MSI-H. The study of Korehisa et al. (44) reported that PD-L1 expression in tumor cells (PD-L1 (T))-positive MSI-H CRCs did not correlate with budding graded 2 or 3 ($p = 0.34$); however, PD-L1 expression in tumor-infiltrating myeloid cells in stroma (PD-L1 (I))-positive MSI-H colorectal cancers significantly correlated with budding grades 2 or grade 3 ($p = 0.043$). However, the investigation of Kim et al. (45) did not observe such a correlation. The researchers reported that PD-L1+(T) tumors in MSI-H colorectal cancers significantly correlated with tumor budding-positivity ($p < 0.001$). The differential findings may be due to the different grades of tumor budding that were investigated in the two studies. Therefore, it is necessary to further analyze the relationship between tumor budding, MSI, and PD-L1 expression according to the standard classification criteria for tumor budding, in both colorectal and gastric cancers.

Thus, the therapeutic targets associated with tumor budding explains how tumor budding appears as a proliferative phenotype and invasive phenomenon and provides a more perfect risk

TABLE 2 Results of tumor budding in advanced gastric cancer.

Researchers	Year	Sample source	Number of samples	Pathological classification	Budding type	Budding grade	P value of lymph node metastasis	OS (low grade VS high grade, month)	Conclusion
Qi	2020	Surgical specimen	170	Intestinal carcinomas	ITB	0-12 low grade ≧13high grade	<0.001	\	The presence of ITB predicts lymph node metastasis, which suggests that ITB is clinically relevant for predicting lymph node status
				Intestinal carcinomas	PTB	0-8 low grade ≧9 high grade	0.003	\	
Olsen	2017	Surgical specimen	52	Intestinal carcinomas	ITB & PTB	High grade tumor bud score ≧1 Low grade tumor bud score <1	<0.001	\	Tumor budding was significantly associated with tumor differentiation, lymphovascular space invasion, T-stage, and N-stage in a univariate analysis of the cohort
Dao	2020	Surgical specimen	109	Gastric adenocarcinoma	ITB & PTB	0-9 low grade >10 high grade	<0.001	78.91 ± 2.00 VS 36.69 ± 3.45	Tumor budding may provide a unique predictive hallmark to stratify risk categories of patients with gastric cancer
Ulase	2019	Surgical specimen	456	Gastric adenocarcinoma	ITB & PTB	0 tumor budding absent 1-9 low grade ≧10 high grade	<0.001	48.0 vs 12.7 vs 12.8	Tumor budding was associated with various adverse clinicopathological features and patient outcomes
Kemi	2019	Surgical specimen	583	Intestinal carcinomas	ITB & PTB	0-9 low grade ≧10 high grade	\	\	High tumor budding is an independent prognostic factor in gastric adenocarcinoma, more specifically, in intestinal-type gastric adenocarcinoma. Assessment of tumor budding in diffuse-type gastric adenocarcinoma is not recommended
Jesinghaus	2022	Patients received neoadjuvant chemotherapy prior to their resection	167	Intestinal carcinomas	ITB & PTB	0-4 low grade 5-9 intermediate grade ≧10 high grade	\	51.7 vs 37.4 vs 28.1	The assessment of tumor budding according to the ITBCC criteria provides valuable prognostic information in the post-neoadjuvant setting of intestinal-type gastric cancer and may be a considerable substitute for the conventional grading system in gastric cancers after neoadjuvant therapy
Pun	2022	Surgical specimen	76	Gastric adenocarcinoma	ITB & PTB	0-4 low grade 5-9 intermediate grade ≧10 high grade	0.005	\	Tumor budding has prognostic value in intestinal-type gastric adenocarcinoma
Jesinghaus	2021	Surgical specimen	43	Gastric adenocarcinoma	ITB & PTB	1-4 low grade 5-9 intermediate grade ≧10 high grade	\	\	Tumor budding was statistically significantly related to pathologic stage, lymph node involvement, and grade. The finding also suggest that tumor budding can be applied to gastric cancer and it might contribute to the standardisation of diagnosis and prognostic factors
Szalai	2022	Surgical specimen	290	Gastric adenocarcinoma	ITB & PTB	0 Bd 0 1-4 Bd 1 5-9 Bd 2 ≧10 Bd 3	<0.0001	\	Tumor budding is an independent prognostic factor for survival in gastric cancer, especially in intestinal-type adenocarcinomas. However, there was no significant association between PDC and the occurrence of lymph node metastasis, tumor stage, and survival

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assessment and grading treatment strategy for patients with tumor budding. Thus, the treatment of patients with tumor budding follow-up may provide a possible direction. Meanwhile, although tumor budding has been correlated with immunotherapy targets such as PD-1/PD-L1 and MSI in other tumors, due to the particularity of gastric cancer histopathology, it remains necessary to prove the potential relationship between gastric cancer tumor sprouting and immunotherapy targets through further experiments. Such investigations may also help to resolve controversial issues in related fields.

4 Discussion

At present, tumor budding is an important prognosticator in gastric cancer, but further investigation is warranted to address outstanding questions. The classification system for tumor budding in gastric cancer is imperfect, and many studies have used the colon cancer staging system for prognosis assessment. Owing to the histologic classification of gastric cancer, this system cannot be applied to all patients with gastric cancer, which also presents certain limitations. The classification system for tumor budding is also of great significance for the precise treatment of patients with gastric cancer. At the same time, the evaluation of tumor specificity should choose the type of tumor budding (i.e., only evaluate ITB or PTB, simultaneously evaluate PTB and ITB, or exclude certain types of cells, such as signet ring cells), and the specific parameters still need further evaluation and formulation. Yim et al. provided a possible solution for early gastric cancer, however, this solution needs to be investigated for relevance in advanced gastric cancer. Third, there is a relative paucity of high-quality research into the molecular biological mechanisms underlying tumor budding in gastric cancer. Several unresolved questions also remain regarding the continuous pathologic development process of tumor budding. There is also a paucity of literature specifically relating to predisposing factors for tumor budding, which will contribute to our understanding of tumor budding.

With the growing recognition of tumor budding in gastric cancer and the development of related technologies, many questions related to this will be answered in the near future, which may promote and improve the continuous development of gastric cancer diagnosis and treatment.

Author contributions

CX and YD drafted the manuscript; HX and ZZ polished the manuscript multiple times to significantly enhance the quality; YL, YD, and ZZ gave useful suggestions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ferroptosis open a new door for colorectal cancer treatment

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Colorectal cancer (CRC) is the third highest incidence and the second highest mortality malignant tumor in the world. The etiology and pathogenesis of CRC are complex. Due to the long course of the disease and no obvious early symptoms, most patients are diagnosed as middle and late stages. CRC is prone to metastasis, most commonly liver metastasis, which is one of the leading causes of death in CRC patients. Ferroptosis is a newly discovered cell death form with iron dependence, which is driven by excessive lipid peroxides on the cell membrane. It is different from other form of programmed cell death in morphology and mechanism, such as apoptosis, pyroptosis and necroptosis. Numerous studies have shown that ferroptosis may play an important role in the development of CRC. For advanced or metastatic CRC, ferroptosis promises to open a new door in the setting of poor response to chemotherapy and targeted therapy. This mini review focuses on the pathogenesis of CRC, the mechanism of ferroptosis and the research status of ferroptosis in CRC treatment. The potential association between ferroptosis and CRC and some challenges are discussed.

KEYWORDS

ferroptosis, colorectal cancer, molecular mechanism, inducer, nanomedicine 1

1 Introduction

CRC is a common gastrointestinal malignancy, CA: A Cancer Journal for Clinicians usually combines colon, rectum, and anus cancers as CRC for statisticsor. The latest data showed that in 2020, there were more than 1.9 million new cases of CRC and about 935,000 deaths in the world. The incidence and mortality of CRC ranked the third (10.0%) and

second (9.4%) worldwide, respectively (1). The National Cancer Center also released the cancer report in 2022. The data showed that there were about 560,000 new cases and 290,000 deaths of CRC in China. Incidence and mortality ranked second (12.2%) and fifth (9.5%) in China, respectively (2). CRC has posed a major threat to human health. The 5-year survival rate for advanced CRC is only 14% (3). Due to the unsatisfactory effects of traditional chemotherapeutic drugs or targeted drugs, it is urgent to explore new treatment strategies. Ferroptosis is a research hotspot in recent years. It has a unique mechanism that distinguishes it from other cell death manners. More importantly, studies have found that ferroptosis is closely related to multiple cancer including CRC, which can open a new door for CRC treatment.

2 Colorectal cancer

2.1 Symptoms and etiology

The symptoms of CRC are not obvious in the early stage. As the tumor grows, it shows symptoms such as changes in bowel habits, bleeding per rectum, diarrhea, local abdominal pain, anemia and weight loss (Figure 1) (4). CRC can occur anywhere in the colon or rectum, but the sigmoid colon and rectum are the most common case (5). At present, the etiology of CRC is not completely clear. It is generally believed that the following factors are closely related to the disease (Figure 1).

2.1.1 Dietary habit

It mainly includes the intake of high fat, high protein, low dietary fiber and nitrite compounds; vitamin deficiency; intestinal flora imbalance and other factors. Obesity induced by high fat diet has been a high risk factor for CRC.

High fat diet not only can stimulate the increase of bile secretion, but also promotes the growth of some anaerobic bacteria in the intestine. Bile alcohol and bile salt are decomposed by anaerobic bacteria to form unsaturated cholesterol, such as deoxycholic acid and lithocholic acid, which is carcinogens or co-carcinogens (6). Studies have shown that high fat diet can reduce the expression of the major histocompatibility complex class II (MHC class II) genes in intestinal epithelial cells (including intestinal stem cells), thereby disrupting the intestinal flora and promoting intestinal tumorigenesis (7).

High protein diet can increase harmful metabolites in the gut, such as N-nitroso compounds (NOCs) and H_2S. NOCs can lead to DNA alkylation, which can induce genetic mutations that ultimately lead to cancer (8, 9). After ingestion, protein is decomposed into various absorbable amino acids including

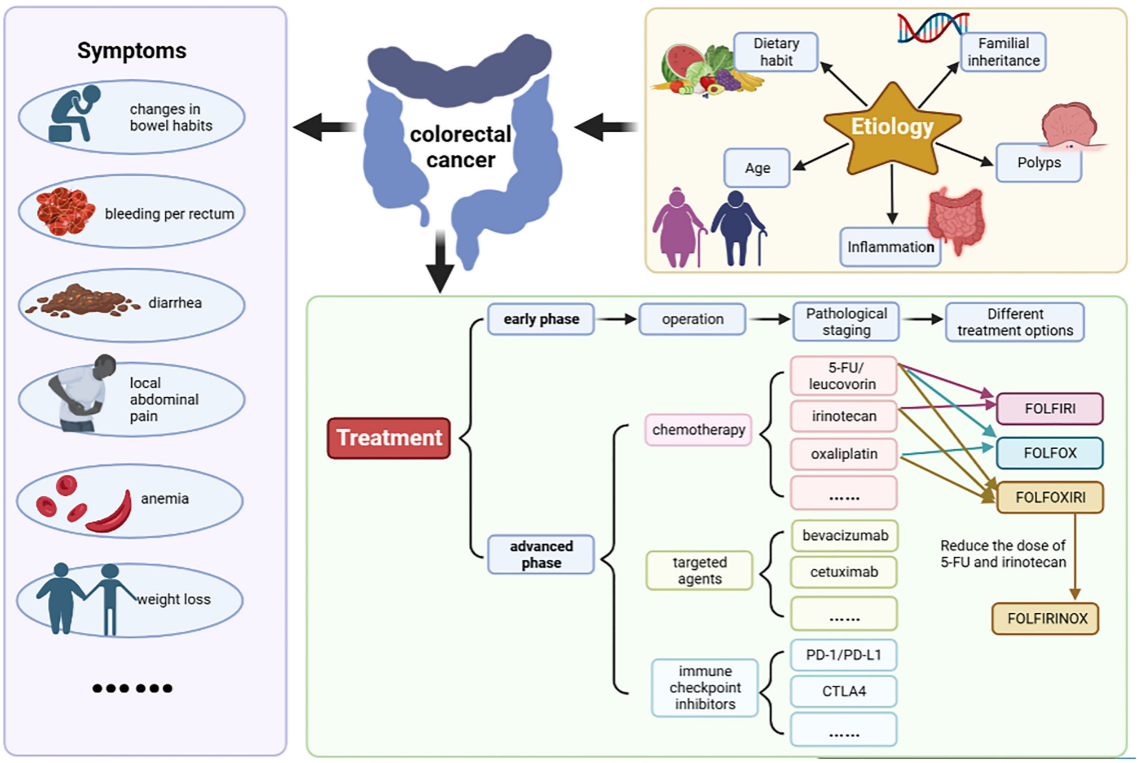


FIGURE 1
The symptoms, etiology and treatment of CRC. The symptoms include changes in bowel habits, bleeding per rectum, diarrhea, local abdominal pain, anemia and weight loss etc. At present, the etiology of CRC is not completely clear. It is generally believed that dietary habit, familial inheritance, polyps, inflammation, age are closely related to the disease. Surgery is the preferred treatment for patients with early-stage CRC. Commonly used chemotherapy drugs mainly include 5-fluorouracil (5-FU)/leucovorin (LV), irinotecan, and oxaliplatin etc. Targeted therapeutic drugs mainly include cetuximab and bevacizumab. Basic chemotherapy regimens included FOLFIRI (irinotecan+5-FU+LV), FOLFOX (oxaliplatin+5-FU+LV), FOLFOXIRI (oxaliplatin+irinotecan+5-FU+LV) and its modified regimen FOLFIRINOX (reduced doses of irinotecan and 5-FU).

methionine and cysteine that can be further decomposed by sulfate-reducing bacteria to produce H_2S, which can promote the occurrence of CRC by inhibiting butyrate oxidation, destroying the intestinal barrier, and interacting with ROS to induce DNA damage (10, 11).

Dietary fiber can increase intestinal peristalsis, promote stool excretion, and reduce the food transit time in the colon, thus reducing the contact time of potential carcinogens with intestinal mucosa (12, 13). Moreover, the residues of dietary fiber can be fermented by intestinal microbiota to produce short chain fatty acids (SCFA), especially butyrate, which has important physiological effects. Butyrate is not only the main energy source of intestinal microbiota and intestinal epithelial cells, but also plays an important role in colon health by inhibiting cell proliferation, inducing cell differentiation, promoting cell apoptosis, and reducing tumor cell invasiveness (14, 15). Therefore, lack of dietary fiber will increase the risk of intestinal diseases.

2.1.2 Familial inheritance

Approximately 20-30% of CRC cases are associated with genetic factors, 2-5% of CRC is caused by inherited syndrome, mainly including Lynch syndrome, familial adenomatous polyposis, juvenile polyposis syndrome, *MUTYH*-associated polyposis, and Peutz-Jeghers syndrome etc (16, 17).

2.1.3 Polyps

The incidence of CRC is closely related to polyps. Colorectal polyps including adenomatous polyps (adenomas), hamartomatous polyps and serrated polyps are early lesions of cancer (18). It takes at least 10 years for the progression of "polyp-adenomas-CRC" (19). The formation factors of polyps mainly include genetic factors, obesity, smoking, drinking, age and so on (20). Diet affects the development of polyps and inflammation. Studies have reported that high fat and protein (especially red meat) intake can significantly increase intestinal polyp formation and inflammation (21–23). However, the intake of dietary fiber can promote stool excretion, reduce the contact of carcinogens with the intestinal mucosa, and produce short-chain fatty acids, thereby reducing the formation of intestinal polyps and inflammatory response (24).

2.1.4 Inflammation

Chronic inflammatory stimulation can lead to the development of CRC. Inflammatory bowel disease (IBD) mainly includes ulcerative colitis and Crohn's disease, which is one of the high risk factors for the development of CRC (25). Compared with the general population, patients with IBD have an approximately 2-6 folds increased risk of developing CRC (26).

2.1.5 Age

Population aging has a significant impact on the incidence of CRC. Surveys show that the incidence of CRC worldwide increases with age. In the United States, the incidence of CRC is three times higher in people over 65 years old than in 50-64 years old, and approximately 30 times higher than in 25-49 years old (27).

2.2 Pathogenesis

The pathogenesis of CRC is a multi-gene, multi-step and multi-pathway process, which is caused by the gradual accumulation of complex genetic and epigenetic events. CRC can be divided into three classifications by etiology: sporadic CRC (~80% cases), hereditary CRC (~20% cases), and inflammatory CRC (<2% cases) (28). The most classic pathological process is the progression from polyps to adenomas and finally to adenocarcinoma. Intestinal polyps are benign precursors of most CRC. Adenomas and serrated polyps are the two main subtype. Approximately 85-90% of sporadic CRCs develop from adenomas. The serrated polyps pathway accounts for only 10-15% of sporadic CRC (29).

It is currently believed that the pathogenesis of CRC is mainly related to the abnormality of five cell signal pathways: epidermal growth factor receptor (EGFR) signal pathway (including MAPK and PI3K signal pathway), Wnt/β-catenin signal pathway, Notch signal pathway, p53 signal pathway and Transforming growth factor-beta (TGF-β) signal pathway (30). Further analysis shows that the molecular mechanism of CRC can be divided into three type (Figure 2): chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylation phenotype (CIMP) (31). These three pathways are not mutually exclusive.

2.2.1 CIN mechanism

The CIN is the most classical pathway, accounting for 80-85% of all CRC cases. Aneuploidy or structural chromosomal abnormalities, frequent loss of heterozygosity at tumor suppressor loci, and chromosomal rearrangement are the main features (32). These changes will affect important genes related to the maintenance of cell function, such as adenomatous polyposis coli (APC), KRAS and TP53. About 80% of CRC patients have APC mutations, which can active Wnt signal pathway. There are three characteristic genes related to human tumors in RAS gene family including HRAS, NRAS and KRAS. Among them, KRAS is the most mutated in 40% of sporadic CRC (33). KRAS mutations can active MAPK and PI3K signaling pathway, which increases cell proliferation (34). It is well known that p53 is a tumor suppressor protein, which is encoded by the TP53 gene. TP53 mutations occurs in 40-50% of sporadic CRC, which is a key step driving the development of CRC (35).

2.2.2 MSI mechanism

Microsatellite is short nucleotide tandem repeat in the DNA sequence, which is easy to get a replication error due to repeated structure. Mismatch repair (MMR) system mainly including MLH1, MSH2, MSH6, PMS1 and PMS2 proteins can repair these errors. MSI is driven by the loss of function of MMR system, and promoter

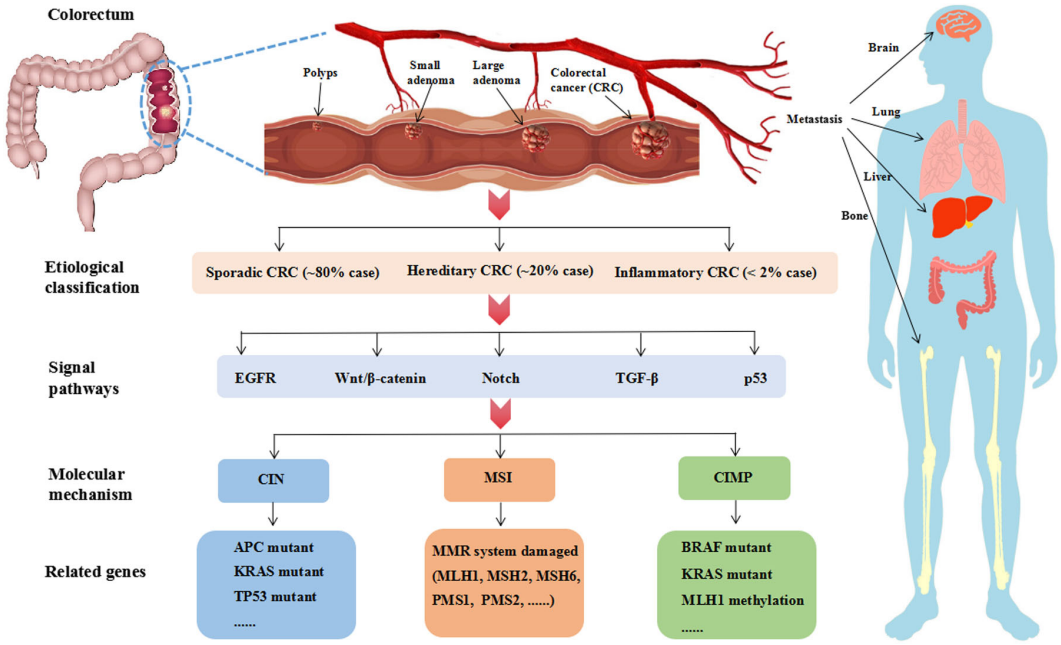


FIGURE 2
The pathological process, classification, signal pathway and molecular mechanism of CRC. The classic pathological process of CRC is "polyps-adenoma-adenocarcinoma", which needs over 10 years. Advanced CRC is prone to metastasis. Liver metastasis is the most common CRC more than 50%. According to the etiology, CRC can be divided into three classifications. Sporadic CRC is the most common clinical case accounting for approximately 80%. Hereditary CRC is associated with genetic factors accounting for approximately 20%, in which 2-5% cases are caused by inherited syndrome such as Lynch syndrome and familial adenomatous polyposis. Inflammatory CRC is rare in clinical, which is less than 2%. As reports, EGFR, Wnt/β-catenin, Notch, TGF-β and p53 are the main signal pathways at present. And CIN, MSI, CIMP are the major molecular mechanism. CIN is related to high gene mutant of APC, KRAS and TP53. MSI is driven by the impairment of DNA mismatch repair (MMR) system. CIMP is a form of epigenetic modification and relates to the hypermethylation of CpG islands. CIMP-high subtype is BRAF mutations and MLH1 methylation and CIMP-low subtype is KRAS mutations.

hypermethylation is considered to be the main cause of gene silencing. MSI accounts for about 15% of sporadic CRC. Moreover, germ-line mutations in these genes causes Lynch syndrome, which is the most common inherited type of CRC (36).

2.2.3 CIMP mechanism

Site specific DNA hypermethylation of CG dinucleotides (CpG islands) associated with promoters of tumor suppressor genes and DNA repair genes leads to transcriptional silencing that promotes cancer initiation and progression. CIMP is first identified in CRC, which is related to the hypermethylation of CpG islands in the promoter region of tumor suppressor genes such as MINT1, MINT2, MINT3 and MLH1 (37, 38). It is a form of epigenetic modification and can be divided into two type. CIMP-high subtype is BRAF mutations and MLH1 methylation. CIMP-low subtype is KRAS mutations. Studies have shown that BRAF and KRAS direct the assembly of distinct corepressor complexes on a common promoter through different pathways leading to promoter hypermethylation and transcriptional silencing of CIMP (39). BRAF increases phosphorylation of MAFG (a small MAF protein) *via* the MEK/ERK signaling pathway, thereby protecting MAFG from polyubiquitination and subsequent proteasome-mediated degradation. Subsequently, MAFG recruits corepressor complexes including BACH1, CHD8 and DNMT3B, resulting in promoter hypermethylation and transcriptional silencing of CIMP (40). KRAS increases the level of zinc finger protein 304 (ZNF304) by transcriptionally upregulating the serine/threonine kinase protein kinase D1 (PRKD1) and ubiquitin-specific peptidase 28 (USP28). Subsequently, ZNF304 recruits corepressor complexes including KAP1, SETDB1 and DNMT1, resulting in promoter hypermethylation and transcriptional silencing of CIMP (41). CIMP is highly associated with the CRC developing from serrated polyps pathway, but the underlying mechanism remains to be further explored.

2.3 Metastasis and treatment of CRC

CRC treatment mainly involves surgery, chemotherapy and radiotherapy. According to clinical guidelines, surgery is the preferred treatment for patients with early-stage CRC, and chemotherapy or targeted therapy should be used for patients with advanced or metastatic CRC (42). Commonly used chemotherapy drugs mainly include 5-fluorouracil (5-FU)/leucovorin (LV), irinotecan, oxaliplatin, capecitabine, trifluridine, tippiridine and raltitrexed. Targeted therapeutic drugs mainly include cetuximab, bevacizumab, regorafenib, panitumumab and so on. Clinically, it is mainly a comprehensive treatment based on chemotherapy. Basic

chemotherapy regimens includes FOLFIRI (irinotecan+5-FU+LV), FOLFOX (oxaliplatin+5-FU+LV), FOLFOXIRI (oxaliplatin +irinotecan+5-FU+LV) and its modified regimen FOLFIRINOX (reduced doses of irinotecan and 5-FU) (Figure 1) (43). The latest NCCN guidelines (version 1. 2022) recommended FOLFIRINOX instead of FOLFOXIRI because the FOLFOXIRI regimen at high doses of 5-FU showed greater toxicity in American patients.

Surgery is the most effective way to cure CRC (44). However, the surgical cure rate and 5-year overall survival rate of CRC have been hovering around 50% (45), and the main reasons for treatment failure are the high recurrence and metastasis rates.

CRC is prone to metastasis. It usually occurs in the liver, lung and abdominal cavity. In addition, bone and brain metastasis may occur. Among them, liver metastasis is the most common CRC, and the rate of liver metastasis can over 50%. It is worth noting that 15-25% of CRC patients appeared liver metastasis at initial diagnosis (46). Without any treatment, most people with liver metastasis survive only a few months. Liver metastasis is considered as the leading direct cause of CRC-related death. It is fewer than 20% of 5-year survival rate for metastatic CRC patients (47). Due to the clinical benefit rate remaining at a low level, it is urgent to explore new treatment strategies for CRC. Ferroptosis is a novel manner of programmed cell death, which plays an important role in multiple diseases, such as neurological diseases, liver diseases, gastrointestinal diseases and cancer (48, 49).

3 Ferroptosis

3.1 Characteristics of ferroptosis

Programmed cell death is an active and orderly way of cell death determined by genes, which is closely related to the maintenance of life homeostasis and the occurrence of diseases, mainly including apoptosis, pyroptosis, necrosis and autophagy (50, 51). Cellular ferroptosis is a term coined in 2012 by Dixon et al. It is referred to induce cell membrane breakage through excess lipid peroxides on cell membranes, a process in which iron is involved in regulation (52). Ferroptosis is different from other type of cell death in morphology and mechanism, and its main characteristics are as follows.

3.1.1 Morphological characteristics

The main features are decreased mitochondrial volume, decreased or disappearance crista, increased mitochondrial membrane density, disruption of mitochondrial membrane and without chromatin condensation. However, the nucleus structure is intact and morphological changes are not obvious (53).

3.1.2 Biological characteristics

It is mainly manifested in the increase of reactive oxygen species (ROS), the aggregation of iron ions, the increase of oxidation level of reduced coenzyme II (NADPH), the inhibition of glutathione peroxidase 4 (GPX4) and cystine/glutamic acid antiporter transporter (system Xc^-) (54).

3.1.3 Immunological characteristics

Ferroptosis not only affects innate immunity by affecting the number and function of immune cells (e.g. macrophages and neutrophils) but also affects adaptive immunity through triggering inflammatory or specific responses after ferroptotic cells recognized by immune cells (e.g. T and B lymphocytes). The death of immune cells caused by ferroptosis may impair the immune response. In contrast, non-immune cell death caused by ferroptosis can activate damage-associated molecular patterns (DAMP), thereby activating immune responses (55).

3.1.4 Genetic characteristics

The genes/proteins that regulate ferroptosis can be mainly divided into three categories, among which SLC7A11, SLC3A2, ATF3, p53, etc. mainly regulate cystine uptake; GPX4, ACSL4, LPCAT3, FSP1, NRF2, etc. mainly regulate lipid metabolism; TFR1, HSPB1, IREB2, NFS1 and others mainly regulate iron metabolism (56).

3.2 Mechanism of ferroptosis

The occurrence and execution of ferroptosis involve multiple metabolic pathways and processes, including lipid metabolism, amino acid metabolism, iron metabolism, and other metabolic pathways (Figure 3). Each aspect does not exist independently, but influences and penetrates each other.

3.2.1 Lipid metabolism

Lipid peroxidation is key to the occurrence of ferroptosis. Compared with monounsaturated fatty acids, polyunsaturated fatty acids (PUFA) have diallyl moiety. Therefore, phospholipids containing polyunsaturated fatty acids (PUFA-PL) are very susceptible to peroxidation (57). The peroxidation of PUFA-PL is mainly catalyzed by non-enzymatic autooxidation, which is actuated by Fenton reaction and catalyzed by iron. Lipid peroxides can be formed by capturing an unstable hydrogen atom in diallyl group on PUFA-PL (58).

Acyl-coa synthetase long-chain family member 4 (ACSL4) can catalyze the conversion of long-chain fatty acids to fatty acyl-coenzymes in an ATP-dependent manner, which plays a crucial role in the process of iron-dependent oxidative stress (59). ACSL4 tends to bind long-chain polyunsaturated fatty acids such as arachidonic acid (AA) and adrenic acid (ADA), and then specifically esterifies PUFA to PUFA-CoA such as AA-CoA and ADA-CoA under the action of acetyl coenzyme A (CoA). Then, under the catalysis of lysophosphatidylcholine acyltransferase 3 (LPCAT3), PUFA-CoA combines with phospholipids on the cell membrane to form PUFA-PL, which is peroxidized to PUFA-PL-OOH under the regulation of dioxygenase (ALOX) (60–62). On the one hand, the accumulation of phospholipid peroxides makes the cytomembrane thin and increases its curvature, and the accelerated influx of oxidants intensifies the oxidation, which eventually leads to membrane perforation, rupture and the release of cellular contents (63). On the other hand, lipid peroxides are further

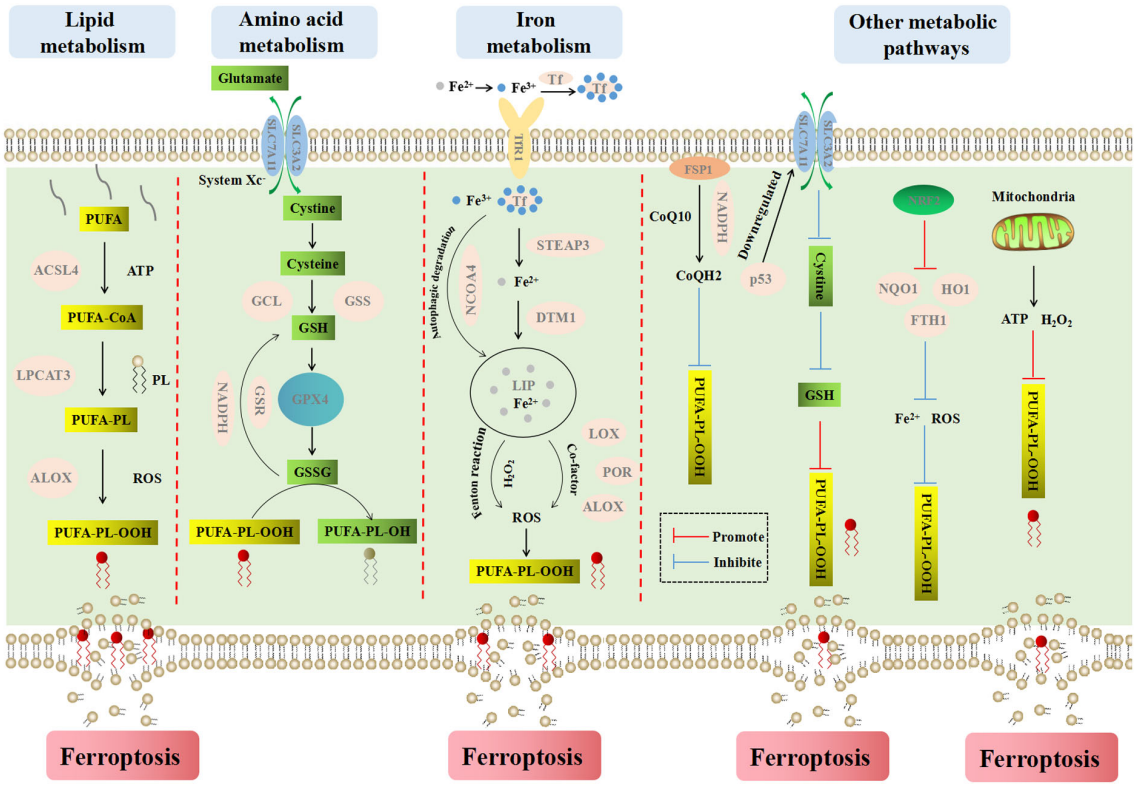


FIGURE 3
The main metabolic pathways and processes of ferroptosis. Ferroptosis is a iron-dependent cell death type, which causes cell membrane rupture by lipid peroxidesn. Lipid metabolism explains the formation process of lipid peroxides from PUFA to PUFA-PL-OOH. Amino acid metabolism explains the process of cell inhibiting lipid peroxidation by GSH. Iron metabolism explains the important role of iron ions for inducing ferroptosis. Fe^{2+} reacts with endogenous H_2O_2 in Fenton reaction to produce abundant ROS, which can promote lipid peroxidation and ferroptosis. Other metabolism ways mainly explain the influence of FSP1, NADPH, CoQ10, p53, NRF2 and mitochondria on ferroptosis.

broken down into active substances that can deplete nucleic acids and proteins, or accelerate the destruction of membrane structural integrity, leading to cell ferroptosis (64, 65).

3.2.2 Amino acid metabolism

System Xc^- is a sodium independent transmembrane cystine/glutamate antiporter located on the plasma membrane. It belongs to heteromeric amino acid transporters (HATs), which are composed of the light chain subunit SLC7A11 (xCT) and the heavy chain subunit SLC3A2 (4F2hc) linked by covalent disulfide bonds. SLC7A11 is a member of the SLC7 family, which mainly includes two subfamilies of cationic amino acid transporters (CATs, SLC7A1-4 and SLC7A14) and L-type amino acid transporters (LATs, SLC7A5-13 and SLC7A15) (66). LATs can bind specifically to two members of SLC3 (SLC3A1 and SLC3A2) to form HATs (67).

The activity of system Xc^- is mainly determined by SLC7A11, which is highly specific to cystine and glutamate. It can pump glutamate out of the cell in a 1:1 ratio while transferring extracellular cystine into the cell (68). Excessive release of glutamate will increase the concentration of extracellular glutamate, which in turn regulates the function of the system Xc^-, reducing the intake of cystine and the excretion of glutamate. At the same time, released glutamate is an excitatory neurotransmitter with dual effects of neurotoxicity and excitabilit (69).

The cystine that enters the cell is decomposed into cysteine in a highly reduced environment. Then cysteine is converted to GSH under the catalysis of glutamate cysteine ligase (GCL) and glutathione synthetase (GSS) (70). GPX4 is a kind of intracellular antioxidant enzymes, which can catalyze the conversion of GSH into its oxidized form GSSG, and convert harmful lipid peroxide (L-OOH) into non-toxic lipid alcohol (L-OH), so as to inhibit lipid peroxidation and prevent the occurrence of ferroptosis. With the help of glutathione reductase (GSR), excess GSSG is reduced to GSH by NADPH and enters the next cycle. On the contrary, when system Xc^- or GPX4 is inhibited or inactivated, the redox balance in the cell is dysregulated, which will promote the ferroptosis (71).

3.2.3 Iron metabolism

Fe^{2+} and Fe^{3+} are two oxidation states of iron. Iron bines with transferrin in blood circulation and exists in the form of Fe^{3+}. After entering cells *via* the transferrin receptor 1 (TfR1) on the cell membrane, Fe^{3+} is deoxidized and converted to Fe^{2+} under the action of iron oxide reductase six transmembrane epithelial antigen of the prostate 3 (STEAP3) (72). After that, Fe^{2+} is transported to the labile iron pool (LIP) in the cytoplasm by divalent metal transporter 1 (DMT1) (73). Intracellular iron is mainly stored in ferritin, and the autophagic degradation of ferritin, which is mediated by nuclear receptor coactivator 4 (NCOA4) (74), can release iron into LIP. Thus,

blocking NCOA4 can reduce the level of LIP and inhibit ferroptosis (75). In contrast, enhanced ferritin phagocytosis can increase the LIP and promote ferroptosis. Dynamic iron pools can maintain iron balance under normal physiological conditions while Fe^{2+} accumulates in cells under pathological conditions. Excessive Fe^{2+} can not only react with endogenous H_2O_2 in Fenton reaction to produce a large amount of ROS, but also enhance the activities of multiple metabolic enzymes (such as LOXs, PDH1, ALOX and POR) with iron as a co-factor to promote the generation of ROS (76). ROS can promote the peroxidation of PUFA-PL on the cell membrane to generate lipid peroxides and cause ferroptosis. Therefore, factors related to iron metabolism are potential target sites for inducing ferroptosis.

3.2.4 Other metabolic pathways

Several metabolic processes in mitochondria are also significant in triggering ferroptosis. Mitochondria are the main sites for the production of intracellular ROS, which is critical for lipid peroxidation and ferroptosis. Complexes I, II and III in mitochondria are mainly located in the respiratory chain and generate superoxide, which is then converted to H_2O_2 by superoxide dismutase (77). H_2O_2 reacts with labile iron to generate hydroxyl radicals (·OH) and drives PUFA-PL peroxidation through Fenton reaction. In addition, electron transport and proton pumps in mitochondria are vital to produce ATP, which also contributes to ferroptosis (78). When ATP is depleted, PUFA-PL and ferroptosis are inhibited by activation of AMP-activated protein kinase (AMPK) and inactivation of acetyl-CoA-carboxylaze (ACC). In contrast, in the condition of sufficient energy, PUFA-PL synthesis and ferroptosis are promoted (79).

Other factors affecting ferroptosis include coenzyme Q10 (CoQ10), NADPH, p53, nuclear factor E2 related factor 2 (NRF2), etc. Ferroptosis suppressor protein 1 (FSP1) is localized to the plasma membrane and acts as an NADPH dependent oxidoreductase that reduces ubiquinone CoQ10 to ubiquinol (CoQH2) to prevent lipid oxidation and inhibits ferroptosis (80, 81). p53 can inhibit cystine uptake and ferroptosis by down-regulating the expression of SLC7A11 (82). NRF2 plays a significant role in maintaining intracellular redox equilibrium. It up-regulates the level of many genes (NQO1, HO1 and FTH1, etc.) involving in the metabolism of iron and ROS through the p62-Keap1-NRF2 pathway, then inhibits ferroptosis (83).

4 Research status of ferroptosis in CRC

4.1 Potential association between ferroptosis and CRC

4.1.1 The role of ferroptosis in liver disease

Liver metastasis is the most common cause of CRC, and the liver is a favorable site for ferroptosis. Liver disease is currently the most studied type of ferroptosis. On the one hand, programmed cell death triggered by lipid accumulation in hepatocytes is considered as a possible cause of liver tissue damage and inflammation (84). On the other hand, the liver is the main organ of iron deposition, and the dysregulation of iron metabolism leads to the production of a large amount of free iron, which can significantly increase the sensitivity of hepatic cells to ferroptosis (85). We know that nonalcoholic steatohepatitis (NASH) is a severe chronic liver disease characterized by lipid droplet accumulation, hepatocyte death, and inflammatory cell infiltration. It is an important risk factor for liver cirrhosis and carcinogenesis. Studies have shown that ferroptosis is the preferential form of cell death in NASH. Liver tissue inflammation is significantly inhibited by specific inhibition of ferroptosis (86), thereby reducing the possibility of CRC.

4.1.2 The gastrointestinal tract provides favorable conditions for ferroptosis

In fact, dietary fat is decomposed into fatty acids and monoacylglycerols by pancreatic lipase in the intestinal cavity. Fatty acids penetrate the mucus layer on the surface of microvilli and are absorbed by villous intestinal epithelial cells (CD36, FABP, etc.) then transport to the endoplasmic reticulum (87). With the action of multiple enzymes such as fatty acid synthase, long-chain fatty acids and triglycerides are resynthesized, which participates in the formation of cell membranes or reserve energy for the body (88). Based on studies of ferroptosis in liver disease, we can make reasonable assumptions. Since the gastrointestinal tract is the primary site for the metabolism and absorption of nutrients, including fat and iron, it provides favorable conditions for ferroptosis of gastrointestinal cells (89).

4.1.3 CRC tumor suppressor p53 regulates ferroptosis

In addition, in sporadic CRC, p53 is mutated in the late stage of carcinogenesis, which promotes the further progression of adenoma to adenocarcinoma (90). In colitis associated CRC, p53 is more important and the mutating early can initiate tumorigenesis (91). As the most important tumor suppressor gene, p53 can not only cause apoptosis, cell cycle arrest and senescence, but also play a tumor suppressor function by inducing ferroptosis as described above.

4.1.4 Ferroptosis and CRC prognosis

There may be a correlation between ferroptosis and prognosis of CRC. Biomarkers of ferroptosis, including GPX4, NOX1 and ACSL4, are important prognostic markers in CRC. Moreover, IFN-γ may be involved in tumor death, suggesting that patients with CRC have a good prognosis (92). Obesity is closely related to poor prognosis in patients with advanced CRC, because fat-derived exosomes reduce the susceptibility to ferroptosis in CRC and promote chemotherapy resistance to oxaliplatin (93).

4.2 Medications associated with CRC ferroptosis

4.2.1 Ferroptosis inducers

Are ROS-promoting compounds effective in inducing ferroptosis? We knew that endogenous ROS were mainly formed

in the mitochondrial respiratory chain, and low levels of ROS played an important role in regulating the biological functions of cells. However, excessive ROS could oxidize proteins, lipids and DNA, break redox homeostasis, and lead to cell death. ROS-induced lipid peroxidation could induce programmed cell death in the form of apoptosis, autophagy and ferroptosis.

Firstly, the formation of lipid peroxides can be achieved through different pathways, mainly including non-enzymatic and enzymatic pathways. The non-enzymatic pathway was an iron-dependent process and an important link in the formation of ferroptosis. As the name implied, the formation of lipid peroxides by lipoxygenase catalysis was the enzymatic pathway. Secondly, lipid peroxides could initiate different forms of programmed cell death through different signaling pathways. They could not only initiate apoptosis through pathways such as NF-κB, MAPK and PKC, but also initiate autophagy through AMPK/mTORC and JNK-Bcl-2/Beclin 1 (94). For example, knockdown of protocadherin 7 (PCDH7) affects autophagy and induces ferroptosis, which enhances the sensitivity of colon cancer cells to chemotherapy by inhibiting the MEK1/2/ERK/c-FOS axis (95). In addition, they could achieve ferroptosis by interacting directly with the cell membrane and disrupting membrane integrity. Therefore, ferroptosis was different from other forms of cell death in cell morphology and mechanism.

The changes of ferrous ion concentration, lipid peroxides and ferroptosis-related proteins were the key indicators to identify the occurrence of ferroptosis (64). For example, Liu et al. demonstrated that oxaliplatin could promote ferroptosis by inhibiting the NRF2 signaling pathway. The results showed that after HT29 cells were treated with oxaliplatin, the Fe^{2+} concentration in the cells was significantly increased and the ferroptosis-related protein GPX was significantly decreased, thus contributing to the production of a large amount of ROS and lipid peroxides (95). Dichloroacetic acid (DCA) can cause iron death in colorectal cancer stem cell (CSC), manifested as increased iron concentrations, lipid peroxides, and glutathione levels (96).

Although many chemotherapeutic drugs could induce ROS production, there was no significant change in the ferroptosis-related characteristics during cell death. Therefore, it did not belong to ferroptosis. They may cause the destruction of proteins or DNA by ROS or the production of lipid peroxides by enzymatic pathways, thereby causing cell death through apoptosis and autophagy. For example, Liu et al. found that the small molecule compound VB1 (extracted from *Vitex negundo*) showed anti-tumor activity, which led to DNA damage and apoptosis by promoting the accumulation of ROS in cells (97).

Ferroptosis inducers (FIN) are compounds that can induce ferroptosis in cells. In tumor therapy, they can be mainly divided into two kinds according to their mechanism of action. 1) *FIN associated with System* Xc^-. These inducers act primarily by inhibiting SLC7A11-mediated cystine uptake in system Xc^-. It mainly includes erastin and its derivatives (such as imidazole ketone erastin, perazine erastin), sulfasalazine, sorafenib, etc. 2) *FIN associated with GPX4*. These inducers can be further subdivided into three kinds: a) directly blocking GPX4 enzyme activity, including RSL3, ML162, ML210, DPI7, etc.; b) depleting GPX4 protein, binding squalene synthase (SQS) and depleting antioxidant CoQ10, such as FIN56; c) directly oxidizing Fe^{2+} and indirect inactivating GPX4, such as FINO2 (90, 98).

According to reports, there are mainly 14 compounds (such as erastin, RSL3, talaroconvolutin-A, etc.) that can be used in CRC treatment (Table 1). RSL3 is a classical inhibitor by directly inhibiting the catalytic activity of GPX4. It utilizes the electrophilic chloroacetamide fraction to covalently bind to the selenocysteine residue of GPX4, which promotes the accumulation of intracellular ROS and lipid peroxidation, thereby triggering ferroptosis (99). Cetuximab inhibits the Nrf2/HO-1 axis by activating p38 MAPK and enhances RSL3-induced ferroptosis (111). Erastin was the first FIN to be discovered. By binding with SLC7A11, erastin can inhibit its activity and affect cystine transport, thereby reducing GSH synthesis, which results in the failure of removing lipid peroxides in time, then causing cell membrane damage and triggering ferroptosis (112). Talaroconvolutin-A (TalaA) is a novel ferroptosis inducer, which is more effective than erastin according to report. TalaA can promote ferroptosis through not only promoting ROS production but also down-regulating SLC7A11 and up-regulating arachidonate lipoxygenase 3 (ALOXE3) (103). Lysionotin (Lys) is a flavonoid compound that promotes the accumulation of ROS in CRC cells and increases the degradation rate of Nrf2 protein, resulting in ferroptosis (113). Petunidin 3-O-[rhamnopyranosyl-(trans-p-coumaroyl)]-5-O-(β-D-glucopyranoside) (Pt3R5G) further inhibits the proliferation of human colonic adenocarcinoma cells (RKO) primarily by down-regulating SLC7A11 to inhibit ferroptosis (114). Auriculasin promotes CRC apoptosis, ferroptosis, and oxidative apoptosis by inducing ROS production, thereby inhibiting cell infiltration (114). Double-targeted PI3K and HDAC inhibitor BEBT-908 developed by Fan et al. can induce cancer cell ferroptosis and effectively inhibit tumor cell growth by up-regulating MHC class I molecule of tumor cell and activating endogenous IFN- γ signal through STAT1 signaling pathway (115). Tagitinin C induces ferroptosis through endoplasmic reticulum stress-mediated activation of the PERK-Nrf2-HO-1 signaling pathway (110).

Since the concept of ferroptosis was proposed in 2012, the initial design ideas of some drugs like those listed in Table 1 were not based on the mechanism of ferroptosis. For example, oxaliplatin exerted anti-tumor effect mainly by inhibiting DNA replication and transcription. It produced biologically active hydrated derivatives in body fluids that form intra- and inter-strand crosslinks with DNA (116). However, its ferroptosis-inducing effect has been found in recent studies. At present, there is no clear evidence that oxaliplatin has a more obvious effect on inhibiting DNA synthesis or inducing ferroptosis. However, according to the existing research results, ferroptosis is an effective adjuvant treatment method at least, which can enhance the overall treatment effect (117).

In addition to the drugs mentioned above, some other proteins and enzymes have extensive effects, including miR-19a, OTUD1, miR-15a-3p, HSPA5 and N-acetyltransferase 10 (NAT10), etc.,

TABLE 1 Ferroptosis inducers for CRC treatment.

Drug/compound	Mechanisms	References
RSL3	Inhibited GPX4	(99)
Resibufogenin	Inhibited GPX4	(100)
Erastin	Inhibited SLC7A11	(101)
Benzopyran derivative 2-imino-6-methoxy-2H-chromene-3-carbothioamide (IMCA)	Down-regulated SLC7A11	(101)
Paclitaxel	Down-regulated SLC7A1 and up-regulated p53	(102)
Oxaliplatin	Inhibited NRF2	(95)
Talaroconvolutin A	Promoted ROS production, down-regulated SLC7A11, up-regulated ALOXE3	(103)
β-elemene	Combined with cetuximab to promoted ROS production, up-regulated HO-1 and transferrin, down-regulated of GPX4, SLC7A11 in KRAS mutant CRC cells	(104)
Vitamin C	Promoted ROS production and combined with cetuximab for anti-EGFR therapies in CRC	(105)
Bromelain	Promoted ROS production by up-regulating ACSL4 in KRAS-mutant CRC cells	(106)
Sulfasalazine	Enhanced sensitivity of cisplatin to CRC by inhibiting system Xc^-	(107)
Sorafenib	Induced lipid peroxidation and adjusted iron metabolism	(108)
Artemisinin and its derivatives (Artesunate, dihydroartemisinin)	Adjusted iron metabolism by targeting ferritinophagy	(109)
Tagitinin C	Up-regulated HO-1 and promoted lipid peroxidation	(110)

which can also be involved in the regulation of iron death and thus affect the occurrence and development of colorectal cancer. For example, oncogenic miR-19a negatively regulates ferroptosis inducer iron responsive element binding protein 2 (IREB2), inhibiting the growth of CRC cells and reducing the risk of ferroptosis (118). OTUD1 is a deubiquitinating enzyme of IREB2 that stabilizes IREB2-mediated iron transport, leading to increased ROS production and ferroptosis (119). Overexpression of miR-15a-3p can inhibit GPX4, resulting in increased levels of ROS, intracellular Fe^{2+} and malondialdehyde (120). HSPA5 protects cells against ferroptosis, which promotes the occurrence and development of CRC by maintaining the stability of GPX4 and inhibiting ferroptosis (121). N-acetyltransferase 10 (NAT10) can affect the mRNA stability and expression of ferroptosis suppressor protein 1 (FSP1). The overexpressed of NAT10 can enhance the cells proliferation, migration, invasion, tumorigenesis and metastasis, and reduce the patient survival time (122).

4.2.2 Nanomedicine based on ferroptosis

Nanotechnology has been extensively studied to develop advanced nanoparticle drug delivery systems for anticancer drug delivery, which has significant advantages in improving drug availability and targeted delivery properties. The disadvantages of low solubility and poor membrane permeability can be well overcome by preparing anticancer drugs into nanoparticles (123). More importantly, nanomedicine has unique advantages in cancer treatment due to the enhanced permeability and retention (EPR) effect and easy surface modification of nanoparticles (124). Recently, there has been increasing interest in ferroptosis driven nanomedicine due to the potent antitumor activity offered by the combination of ferroptosis and nanotechnology (125). Pan et al. reported that zinc oxide nanoparticles could inhibit GSH synthesis by scavenging H_2S from CRC to induce ferroptosis (126). Li et al. designed a nanoplatform (GCMNPs) based on glycyrrhetinic acid (GA)/poly (lactic-co-glycolic acid), which could induce ferroptosis by increasing intracellular H_2O_2, Fe^{2+} and lipid peroxidation levels and suppressing GPX4 expression. Moreover, they confirmed that combination of GCMNPs and ferrotherapy could enhanced Fenton reaction and immune response in CRC mouse models (127). Chen et al. constructed nanoelicitors, which included two immune-elicitable polyphenols (Chlorogenic acid and Mitoxantrone) and Fe^{3+} ions. The results showed that nanoelicitors could activate tumoricidal immunityto enhance ferroptosis in CRC treatment (128). Han et al. reported core-shell structure nanoparticles of ZnP@DHA/Pyro-Fe, which was used to co-deliver dihydroartemisinin (DHA) and pyropheophorbide-iron (Pyro-Fe). This strategy could remarkably enhance ferroptosis and therapeutic effect of DHA in CRC mouse models (128). Carbon dots (CDs) are zero-dimensional nanomaterials. Tian et al. prepared pentacyclic triterpenes (PTs), a natural anticancer product, into PTs-CDs, which can induce and selectively kill cancer cells by targeting apoptosis, autophagy and ferroptosis of tumor mitochondria (129).

As mentioned above (Table 2), there are few reports on the nanoparticles by promoting ferroptosis for CRC treatment. Moreover, due to poor effect of monotherapy in tumor treatment,

TABLE 2 Nanoparticles based on ferroptosis for CRC treatment.

Nanoparticles	Components	Mechanisms	References
Zinc oxide nanospheres	ZnO; Virus-like mesoporous silica nanoparticles;	Inhibited GSH synthesis by scavenging H_2S	(126)
Glycyrrhetinic acid nanoparticles	Glycyrrhetinic acid; Poly (lactic-co-glycolic acid); Leukocyte membrane; Ferumoxytol	Inhibited GPX4 Increasd intracellular H_2O_2, Fe^{2+} and lipid peroxidation levels Enhanced Fenton reaction by introducing exogenous Fe iron	(127)
Self-assembly nanoelicitors	Chlorogenic acid (CA); Mitoxantrone (MIT); Fe^{3+} ions; PEG-AZB-O; HSPC; Cholesterol	CA and MIT induced tumoricidal immunity and promoted the cytotoxic T lymphocyte to release IFN-γ, which could inhibit system Xc^- to GPX4 pathway. Enhanced Fenton reaction by introducing exogenous Fe iron.	(128)
ZnP@DHA/Pyro-Fe nanoparticles	Zin phosphates; DOPA; DOPC; DSPE-PEG2k; Cholesterol; Cholesterol derivative of dihydroartemisinin (Chol-DHA); pyropheophorbide-iron (Pyro-Fe);	Enhanced Fenton reaction by introducing exogenous Fe iron	(128)

the combination of ferroptosis with tumor targeted imaging, phototherapy, chemotherapy, autophagy or immune regulation is going to be a new trend. Therefore, the nanomedicine based on ferroptosis for CRC treatment has great space for development.

5 Summary and prospect

This mini review summarizes the epidemiology, symptoms, etiology and pathogenesis of CRC, focusing on three molecular mechanisms (CIN, MSI and CIMP) of CRC. The basic characteristics, mechanisms of ferroptosis and its potential association with CRC are also summarized. The mechanisms including lipid metabolism, amino acid metabolism, iron metabolism and other metabolic methods that affect the occurrence and execution of ferroptosis are highlighted. In addition, the research status of FIN and nanomedicine in CRC treatment are emphatically introduced.

In recent years, although significant progress has been made in the research of ferroptosis in cancer treatment, it is still in the initial stage, mainly focusing on basic research. However, the mechanism between ferroptosis and CRC remains unclear. Whether there are specific regulatory factors or signaling pathways. Ferroptosis and other forms of programmed cell death, which have a greater impact on CRC, require further research and confirmation.

The ultimate goal of research is to achieve clinical transformation and benefit patients. But there is still a long way to go before clinical application, and there are still many problems to be solved. Which patients are more likely to benefit from ferroptosis-related treatment. Which gene or protein can be used as biomarkers for patients' response to ferroptosis treatment. What are the adverse effects of ferroptosis treatment and how to control adverse reactions. There is still no clear answer to the above question, which needs further exploration.

Although ferroptosis faces many difficulties and challenges on the road to clinical application, as an emerging cell death type, ferroptosis-based therapeutic strategies still have great potential. Although there are few studies on nanoparticles involving ferroptosis for CRC treatment, the combination of ferroptosis with nanotechnology and various theranostic modalities is inevitable development trend. Overall, ferroptosis promises to open a new door for cancer treatment including CRC.

Author contributions

HL, XH and YT wrote the manuscript. NB, YP and KH prepared tables and figures. YW designed and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Clinical significance of the advanced lung cancer inflammation index in gastrointestinal cancer patients: a systematic review and meta-analysis

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Background: The advanced lung cancer inflammation index (ALI) has been identified as a scientific and clinical priority in multiple malignancies. The aim of this study is to investigate the value of the ALI before treatment in evaluating postoperative complications (POCs) and survival outcomes in patients with gastrointestinal (GI) cancer.

Methods: Electronic databases including PubMed, Embase and Web of Science were comprehensively reviewed up to June 2022. The endpoints were POCs and survival outcomes. Subgroup analyses and sensitivity analyses were also performed.

Results: Eleven studies including 4417 participants were included. A significant heterogeneity in the ALI cut-off value among studies was observed. Patients in the low ALI group showed increased incidence of POCs (OR=2.02; 95%CI:1.60-2.57; P<0.001; I^2 = 0%). In addition, a low ALI was also significantly associated with worse overall survival (HR=1.96; 95%CI: 1.58-2.43; P<0.001; I^2 = 64%), which remained consistent in all subgroups based on country, sample size, tumor site, tumor stage, selection method and Newcastle Ottawa Scale score. Moreover, patients in the low ALI group had an obviously decreased disease-free survival compared to these in the high ALI group (HR=1.47; 95%CI: 1.28-1.68; P<0.001; I^2 = 0%).

Conclusion: Based on existing evidence, the ALI could act as a valuable predictor of POCs and long-term outcomes in patients with GI cancer. However, the heterogeneity in the ALI cut-off value among studies should be considered when interpreting these findings.

KEYWORDS

gastrointestinal cancer, advanced lung cancer inflammation index, postoperative complications, prognosis, meta-analysis

1 Introduction

Gastrointestinal (GI) cancers are one of the most common malignancies worldwide, accounting for about 25% of newly diagnosed cancer cases and more than 35% of cancer-related deaths (1). Although significant advances in surgery-based multimodal therapy for gastrointestinal cancers, the clinical efficacy of most of these patients is still poor (2, 3). Consequently, it is essential to develop a useful index to predict the short-term and long-term therapeutic outcomes in GI cancers.

Increasing evidence indicates that cancer-related inflammation and malnutrition status are prevalent in most patients with malignancy, which play an important role in postoperative recovery and prognosis (4, 5). Therefore, inflammation/nutrition-based biomarkers are expected to be valuable predictors of surgical and long-term outcomes. For example, preoperative neutrophil-to-lymphocyte ratio (NLR), based on two blood inflammatory factors, has been reported as a strong indicator for increased postoperative complications (POCs), prolongation of hospital stays and poor survival outcomes in breast cancer (6), lung cancer (7) and GI malignancies (8, 9). Meanwhile, reduced body mass index (BMI) and serum albumin (ALB), which reflect the nutritional status, have also been proven to be associated with adverse therapeutic outcomes in multiple cancers (10–12).

In recent years, a novel biomarker, the advanced lung cancer inflammation index (ALI), which integrates BMI, ALB and NLR (BMI *ALB/NLR), has been reported as a more promising predictor of surgical and long-term outcomes in cancers, because it incorporates multiple nutritional and inflammatory indicators (13, 14). A previous meta-analysis reported that low ALI before surgery indicates poor prognosis in Lung cancer patients (15). Another meta-analysis focusing on the relationship between the ALI and survival outcomes also found a similar conclusion in cancer patients (16). Nevertheless, the role of the ALI in POCs and survival outcomes of GI cancers remains inconclusive and no meta-analysis is available so far. In addition, a number of other studies on the ALI and therapeutic outcomes in GI cancers have been addressed in recent years.

Herein, we performed a systematic review and meta-analysis based on existing evidence to investigate the value of the ALI in POCs and long-term results in GI cancers.

2 Methods

The present study was conducted according to the requirements from Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines to identify studies that assess the association of pretreatment ALI with POCs and survival outcomes in GI cancer patients.

2.1 Search strategy

Relevant studies from PubMed, Embase and Web of Science were comprehensively examined up to June 30, 2022. Published language was not restricted during the search process. The following combination of key words in title/abstract were used to identify potential studies: ["advanced lung cancer inflammation index" OR "ALI"] AND ["cancer" OR "carcinoma" OR "neoplasm" OR "tumor"] AND ["survival" OR "prognostic" OR "prognosis" OR "mortality" OR "postoperative complications" OR "morbidity"]. In addition, the references of the included studies were scanned for potentially related reports. The search was independently performed by two investigators (HY-P and XF-C).

2.2 Inclusion and exclusion criteria

Studies that met the following criteria were included: (1) studies examined the relationship between the pretreatment ALI and POCs or long-term survival of patients with GI cancer; (2) Hazard ratio (HR) with 95% confidence interval (CI) was available or could be calculated based on survival curve; (3) The cutoff value of the ALI was clearly reported.

The exclusion criteria were studies (1) reported as case reports, reviews, letters and conferences; (2) with overlapping data.

2.3 Data extraction and quality assessment

Two reviewers (HY-P and XF-C) conducted the data extraction independently and cross-checked all the results. The extracted data included first author, publication year, study interval, country, study design and sample size, selection method, cut-off value, clinicopathological features like age, sex, tumor site and tumor stage, POCs and survival data.

The Newcastle Ottawa Scale (NOS) (17) was used to assess the quality of included studies and a study with NOS score >6 is regarded as of high quality.

2.4 Outcomes

In the present study, the primary outcomes were to investigate the relationship between the ALI and POCs or long-term results in patients with GI malignancy. POCs were defined as any morbidity classified as Clavien–Dindo (18) grade I or higher. Long-term outcomes included OS (from the date of surgery to the date of any cause of death) and DFS (from the date of surgery to the date of recurrence or the date of death from any cause).

Of note, since progression-free survival (PFS: from the date of surgery to the date of recurrence or any cause of death), recurrence-free survival (RFS: from the date of surgery to the date of recurrence), cancer-specific survival (CSS: from the date of surgery to the date of cancer-related death) and DFS share the similar endpoints, they were analyzed together as one outcome, DFS (19, 20).

2.5 Statistical analysis

The hazard ratios (HRs) and odds ratios (ORs) with their 95% confidence intervals (CIs) were used as the effect size for time-

dependent outcomes and dichotomous variables, respectively. For studies that HR with 95%CI was not reported, the indirect data were calculated following the methods reported by Tierney et al. (21) Statistical heterogeneity among studies was assessed using I^2 statistic. All pooled analyses were performed assuming the random-effects model, which accounts for variance across included studies. Subgroup analysis was used to explore the source of heterogeneity. Sensitivity analysis was conducted to evaluate the credibility of results. Publication bias was evaluated using Begg's funnel plot. A two-tailed P value <0.05 was considered statistically significant. All of these statistical analyses were performed by Review Manager Software, version 5.3 (Cochrane, London, UK) and Stata, version 12.0 (Statacorp, College Station, TX).

3 Results

3.1 Study characteristics

A flow chart of the selection process was shown in Figure 1. The search strategy yielded 1407 potential studies. After title, abstract assessment and full text assessment, 11 retrospective cohort studies (22–32) were finally included in the present study.

The baseline information of the included studies was shown in Tables 1, 2. A total of 4417 patients from China, Korea and Japan were included in this study. These studies were published from 2014 to 2022 with a sample size ranging from 132 to 813. Among these studies, two studies reported esophageal cancer, three studies reported gastric cancer, and six studies reported colorectal cancer. In addition, three studies reported overall POCs, ten studies reported OS, and seven studies reported DFS. Moreover, the cut-off value of the ALI varies a lot among these studies. The details of quality assessment of the included studies were shown in the supplementary file (Table S1), and the scores of these studies ranged from 6 to 7 after careful assessment with NOS.

3.2 Overall survival

Ten studies involving 4124 patients (2543 in the high ALI group and 1581 in the low ALI group) reported the OS outcome. The pooled HR was 1.96 (95%CI: 1.58-2.43; P<0.001), which indicated that a low ALI was associated with decreased OS in patients with GI cancer (Figure 2A, Table 3). Given the presence of moderate heterogeneity (I^2 = 64%; P=0.003), subgroup analyses based on country, sample size, tumor site, tumor stage, selection method and NOS score were performed. As shown in Table 3 and Figure S1, the pooled results of all subgroup analyses revealed that patients in the high ALI group had a significantly better OS when compared with these in the low ALI group. Additionally, sensitivity analysis by deleting one study at a time showed that the pooled outcome did not substantially change (Figure 2B).

3.3 Disease-free survival

A total of seven studies involving 3433 patients (2195 in the high ALI group and 1238 in the low ALI group) reported on DFS. The pooled HR was 1.47 (95%CI: 1.28-1.68; P<0.001; I^2 = 0%), which suggested that patients in the low ALI group had a worse DFS compared with patients in the high ALI group (Figure 3A). Sensitivity analysis showed that the combined effect was not significantly changed (Figure 3B).

3.4 Postoperative complications

Three studies involving 1607 patients (987 in the high ALI group and 620 in the low ALI group) reported this outcome. The

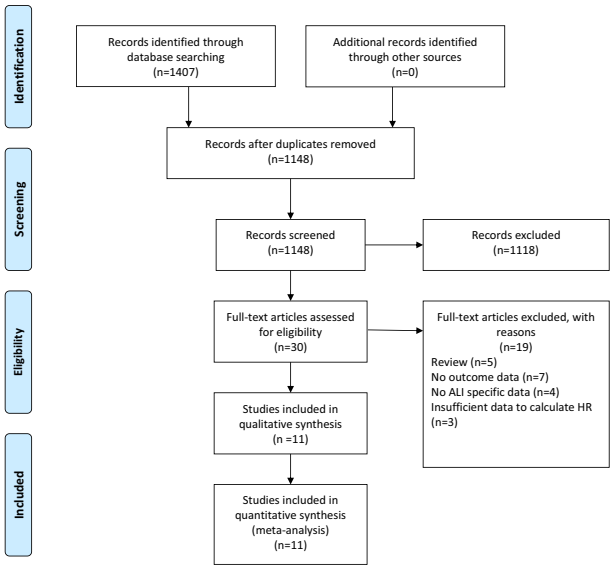


FIGURE 1
The PRISMA flowchart of study selection.

TABLE 1 Basic information of included studies.

Reference	Country	Study design	Study interval	Tumor site	Sample size	Age, years (Median or mean)	Sex (Male/ Female)	Tumor stage	Survival analysis	NOS score
Chen,2022 (20)	China	Retro; S	2012-2016	Colorectal cancer	309	61	178/131	Mixed	OS	7
Feng,2014 (21)	China	Retro; S	2006-2008	Esophageal Cancer	293	59.5	259/34	Non-metastatic	CSS	7
He,2022 (22)	China	Retro; S	2009-2014	Gastric cancer	358	61	284/74	Non-metastatic	OS	7
Horino,2021 (23)	Japan	Retro; S	2005-2019	Colorectal cancer	813	NR	464/349	Non-metastatic	OS; RFS	6
Kusunoki,2020 (24)	Japan	Retro; S	2005-2011	Colorectal cancer	298	67	171/127	Mixed	OS; DFS	7
Pian,2020 (25)	Korea	Retro; S	2009-2018	Colorectal cancer	132	62	88/44	Metastatic	OS; DFS	6
Shibutani,2019 (26)	Japan	Retro; S	2008-2016	Colorectal cancer	159	65	87/72	Metastatic	OS	6
Tan,2021 (27)	China	Retro; S	2013-2018	Esophageal Cancer	158	69.5	126/32	Non-metastatic	OS	6
Xie,2020 (28)	China	Retro; S	2012-2014	Colorectal cancer	662	NR	408/254	Mixed	OS; PFS	7
Yin,2020 (29)	Japan	Retro; S	1992-2011	Gastric cancer	620	NR	424/196	Non-metastatic	OS; DFS	7
Zhang,2022 (30)	China	Retro; S	2010-2017	Gastric cancer	615	NR	469/146	Non-metastatic	OS; DFS	7

Retro, retrospective; S, single center; NOS, Newcastle Ottawa Scale; NR, not report.
CSS, cancer specific survival; RFS, recurrence-free survival; PFS, progression-free survival; OS, overall survival; DFS, disease-free survival.

pooled OR was 2.02 (95%CI: 1.60-2.57; P<0.001; I^2 = 0%), which suggested that patients with a low ALI had a higher risk of POCs than those with a high ALI (Figure 4A). Similarly, sensitivity analysis did not show significant change for the pooled outcome (Figure 4B).

3.5 Publication bias

The Begg's funnel plot was performed to assess the potential publication bias. As shown in Figure 5, the funnel plots of OS, DFS and POCs were virtually symmetric, and the P

TABLE 2 Survival information of included studies.

Reference	Sample size (High / Low)	Selection method	Cut-off value	Survival analysis	Median follow-up (months)	OS HR (95%CI)	CSS/RFS/ PFS/DFS HR (95%CI)
Chen,2022 (20)	309(130/179)	ROC	25.71	OS	60	2.62(1.87-3.66)	NR
Feng,2014 (21)	293(173/120)	Literature	18	CSS	36.8	NR	1.44(1.05-1.96)
He,2022 (22)	358(242/116)	ROC	40.5	OS	101	1.34(0.75-2.44)	NR
Horino,2021 (23)	813(532/281)	classification and regression tree	Male:43.1; Female:13.2	OS; RFS	NR	2.30(1.52-3.50)	1.73(1.22-2.44)
Kusunoki,2020 (24)	298(224/74)	lowest quartile value	20.53	OS; DFS	36.8	3.21(1.97-5.19)	2.13(1.23-3.63)
Pian,2020 (25)	132(32/100)	X-tile	70.4	OS; DFS	NR	2.98(1.32-6.71)	1.46(0.81-2.60)

(Continued)

TABLE 2 Continued

Reference	Sample size (High / Low)	Selection method	Cut-off value	Survival analysis	Median follow-up (months)	OS HR (95%CI)	CSS/RFS/ PFS/DFS HR (95%CI)
Shibutani,2019 (26)	159(92/67)	ROC	28.9	OS	21.6	2.77(1.77-4.34)	NR
Tan,2021 (27)	158(57/101)	ROC	31.24	OS	NR	1.86(1.04-3.33)	NR
Xie,2020 (28)	662(423/239)	X-tile	Male:31.6; Female:24.4	OS; PFS	63	1.45(1.11-1.90)	1.37(1.06-1.78)
Yin,2020 (29)	620(449/171)	ROC	30	OS; DFS	NR	1.59(1.15-2.19)	1.26(0.51-3.11)
Zhang,2022 (30)	615(362/253)	ROC	39.77	OS; DFS	NR	1.33(1.01-1.76)	1.36(1.04-1.77)

CSS, cancer specific survival; RFS, recurrence-free survival; PFS, progression-free survival; OS, overall survival; DFS, disease-free survival; NR, not report; ROC, Receiver operating characteristic curve.

values of Begg's test were 0.239, 0.230, and 1.000, respectively, indicating that these pooled outcomes had a low risk of publication bias.

4 Discussion

In 2013, Jafri et al. (13) first established the ALI based on commonly used clinical parameters as a systemic inflammation-related prognostic score tool for metastatic non-small cell lung cancer. Since then, the ALI has been widely used as a readily available and reliable biomarker to evaluate the prognosis of coronary artery disease (33), lung cancer (34) and pancreatic cancer (35). As shown in Table 1, most of included studies were published after 2019, indicating that the ALI is still a very new index in the field of GI cancer.

In this study, we included eleven studies with 4417 patients with GI cancer and found that a low ALI was significantly associated with decreased OS (HR=1.96; 95%CI: 1.58-2.43; P<0.001) and DFS (HR=1.47; 95%CI: 1.28-1.68; P<0.001). Meanwhile, we have further identified that the ALI could also act as a predictor for POCs (OR=2.02; 95%CI:1.60-2.57; P<0.001) in patients with GI cancer. Therefore, the ALI may have a good discriminatory value and remains an effective inflammatory/nutrition factor for predicting therapeutic outcomes in GI cancer.

Systemic inflammatory reflection (SIR) is recognized as the 7th hallmark of cancer which is closely associated with the occurrence and progression of malignancies (36). Consistent with this evidence, the SIR as a potential prognostic marker is also demonstrated for various

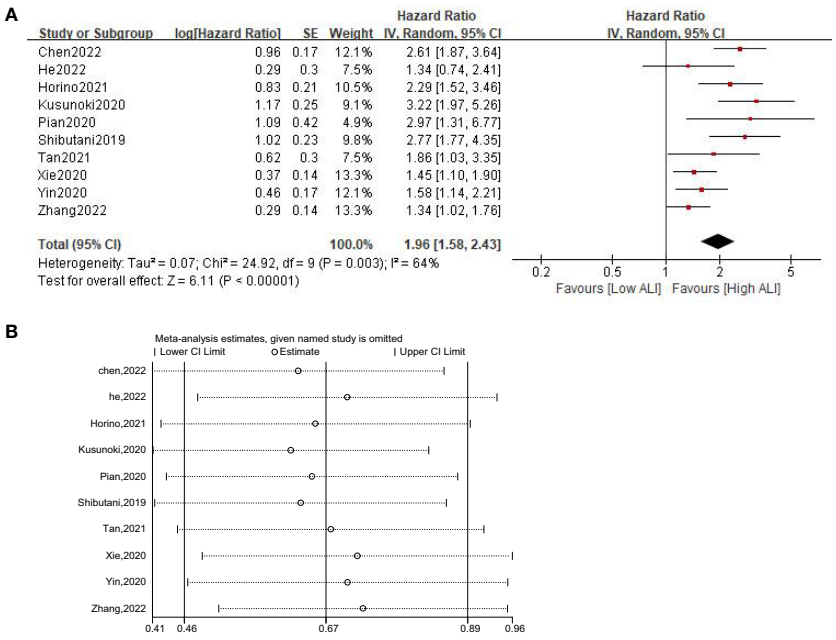


FIGURE 2
Forest plot **(A)** and sensitivity analysis **(B)** assessing the relationship between ALI and OS.

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TABLE 3 Subgroup analysis for OS of ALI-high patients vs. ALI-low patients.

		Studies, n	Patients, n	HR (95%CI)	P value	I^2 (%)
	Total	10	4124	1.96 (1.58-2.43)	<0.0001	64
Country	China	5	2102	1.66 (1.26-2.19)	0.0004	64
	Japan	4	1890	2.31 (1.68-3.19)	<0.0001	58
	Korea	1	132	2.97 (1.31-6.77)	<0.0001	–
Sample size	>200	7	3675	1.84 (1.43-2.36)	<0.0001	70
	≤200	3	449	2.48 (1.78-3.44)	<0.0001	0
Selection method	ROC	6	2219	1.84 (1.39-2.42)	<0.0001	65
	Others	4	1905	2.24 (1.47-3.39)	0.0002	70
Tumor site	Esophageal	1	158	1.86 (1.03-3.35)	0.04	–
	Gastric	3	1593	1.42 (1.16-1.73)	0.0006	0
	Colorectal	6	2373	2.36 (1.78-3.14)	<0.0001	64
Tumor stage	Mixed	3	1269	2.24 (1.37-3.66)	0.001	83
	Non-metastatic	5	2564	1.60 (1.31-1.97)	<0.0001	23
	Metastatic	2	291	2.40 (1.86-3.11)	<0.0001	0
NOS score	6	4	1264	2.40 (1.86-3.11)	0.002	0
	7	6	2860	1.78 (1.35-2.35)	<0.0001	72

NOS, Newcastle Ottawa Scale; HR, Hazard ratio; CI, Confidence interval.

malignancies, and the NLR is one of the well-established SIR markers (37, 38). Increased neutrophils in the tumor microenvironment have been reported to prompt the tumor growth and metastasis by releasing chemokines and cytokines (39). Besides, neutrophils can also inhibit the activation of T lymphocytes, thereby inhibiting the anti-tumor immunity of the host (39, 40). While lymphocytes, especially CD4+ T lymphocytes and CD8+ T lymphocytes, as the most important immune cells, play an anti-tumor role by inducing the lysis and apoptosis of tumor cells (41). Lymphopenia has been demonstrated to be associated with poor prognosis in cancer patients (42, 43). On the other hand, nutritional status is also reported as an important predictive factor of therapeutic outcomes in several types of malignancies (44, 45). Studies have proven that malnutrition leads to an inadequate anti-tumor immune response and reduces wound healing ability, thereby

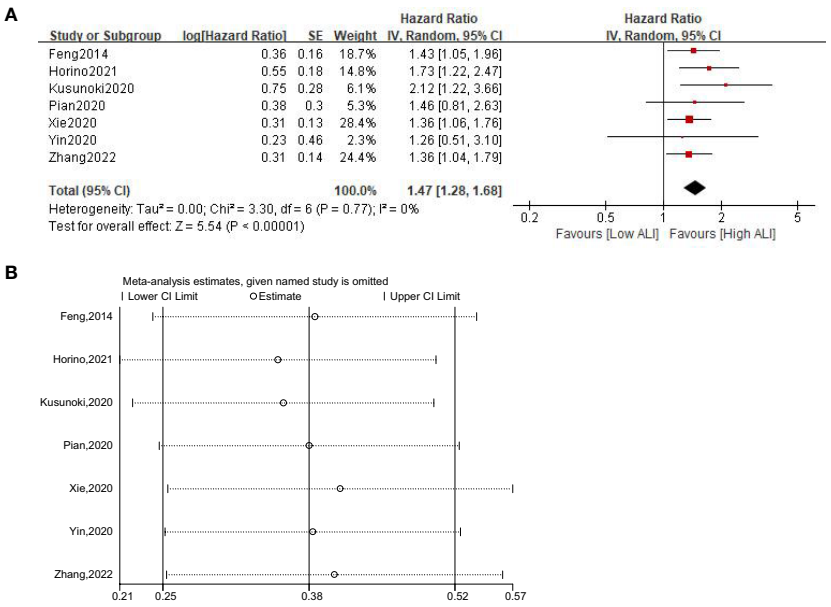


FIGURE 3
Forest plot **(A)** and sensitivity analysis **(B)** assessing the relationship between ALI and DFS.

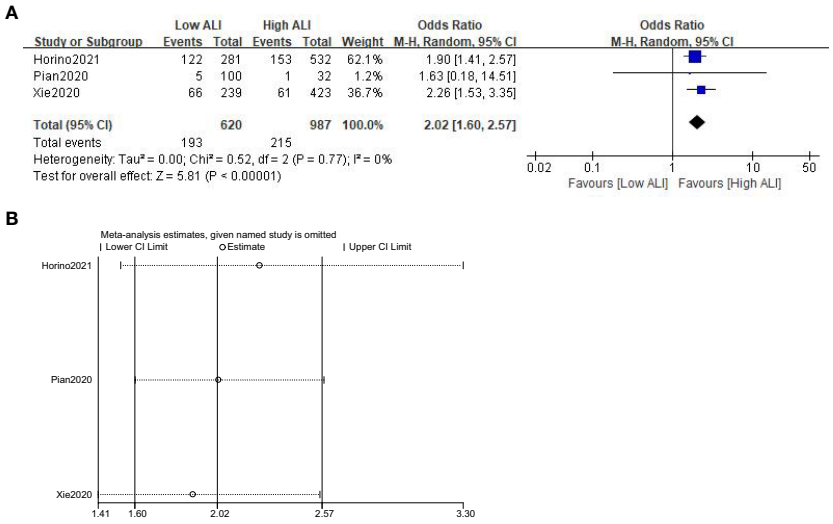


FIGURE 4
Forest plot **(A)** and sensitivity analysis **(B)** assessing the relationship between ALI and POCs.

reducing treatment efficacy and leading to severe POCs (46, 47). As an objective and common measurement reflecting patients' nutritional status, baseline BMI and ALB have been indicated to be positively associated with the short-term and long-term outcomes of cancer patients and have been used to triage patients in clinic care (48). Reasonably, the ALI, combined with these factors, is a useful comprehensive indicator of nutritional and inflammatory status, may enable better understanding of the functional state of patients and predict the therapeutic results of patients with GI cancer.

In our pooled analysis involving 4124 participants, we found that the ALI is an independent factor influencing the OS of patients with GI cancer. Even though significant heterogeneity existed, our subgroup analyses based on country, sample size, tumor site, selection method, tumor stage and NOS score showed our results were reliable and robust. Meanwhile, the sensitivity analysis showed that there was no change of the evident correlation between low ALI and poor OS. In addition, we have further investigated the relationship between the ALI and DFS. The combined result including 3433 patients showed that a low ALI was significantly associated with decreased DFS in GI cancer patients without obvious heterogeneity. Likewise, the sensitivity analysis supported the consistence and reliability of the result. We have also explored the correlation between ALI and POCs. The integrated result demonstrated that low ALI could act as a predictor of the incidence of POCs without heterogeneity. Furthermore, sensitivity analysis suggested that the result of meta-analysis for POCs was reliable. Based on these results, the ALI may be regarded as an effective clinical indicator predicting the short-term and long-term results of GI cancer patients.

There are some limitations in our study. First, all of the involved studies were retrospective studies performed at a single center, which may increase the risk of bias, and more prospective studies are required to further investigate this issue. Second, all included studies are Asian cohorts and studies from western countries are lacking, which may lead to potential publication bias and limit the generalization of the results. Third, due to the limited number of included studies, the value of the ALI in POCs, especially in specific POCs, needs to be further clarified. Finally, the cut-off value of the ALI among studies varied a lot, which might affect the validity and clinical utility of these findings; and further studies that use ALI as a continuous variable are warranted to verify this issue.

5 Conclusions

The findings of the meta-analysis suggested that the ALI prior to initial treatment is of great significance in predicting POCs and long-term survival results in patients with GI cancer. However,

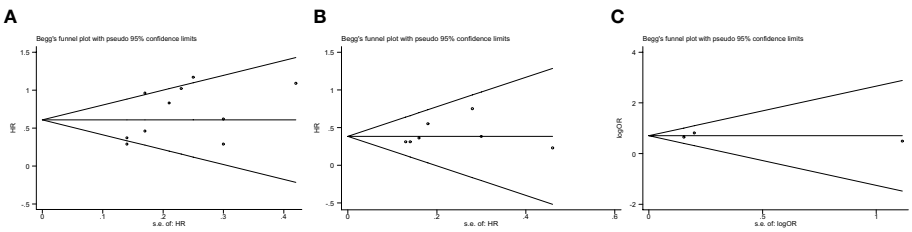


FIGURE 5
Begg's funnel plot assessing publication bias between ALI and therapeutic outcomes, including **(A)** OS, **(B)** DFS, **(C)** POCs. The Begg's P values were 0.239, 0.230, and 1.000, respectively.

high-quality prospective studies with large sample size are still required to further validate the value of ALI in GI cancer.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

H-YP wrote the manuscript. H-YP, X-FC and M-HY performed the data search and data analysis. H-YP, M-HY and L-HC prepared figures. All authors reviewed the manuscript. HS approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1021672/full#supplementary-material

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