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Community series in immunotherapy with checkpoint inhibitors for non-small cell lung cancer, colon cancer and esophageal cancer, volume II

Edited by
Xuelei Ma, Benjamin Frey, Udo S. Gaipl
and Hubing Shi

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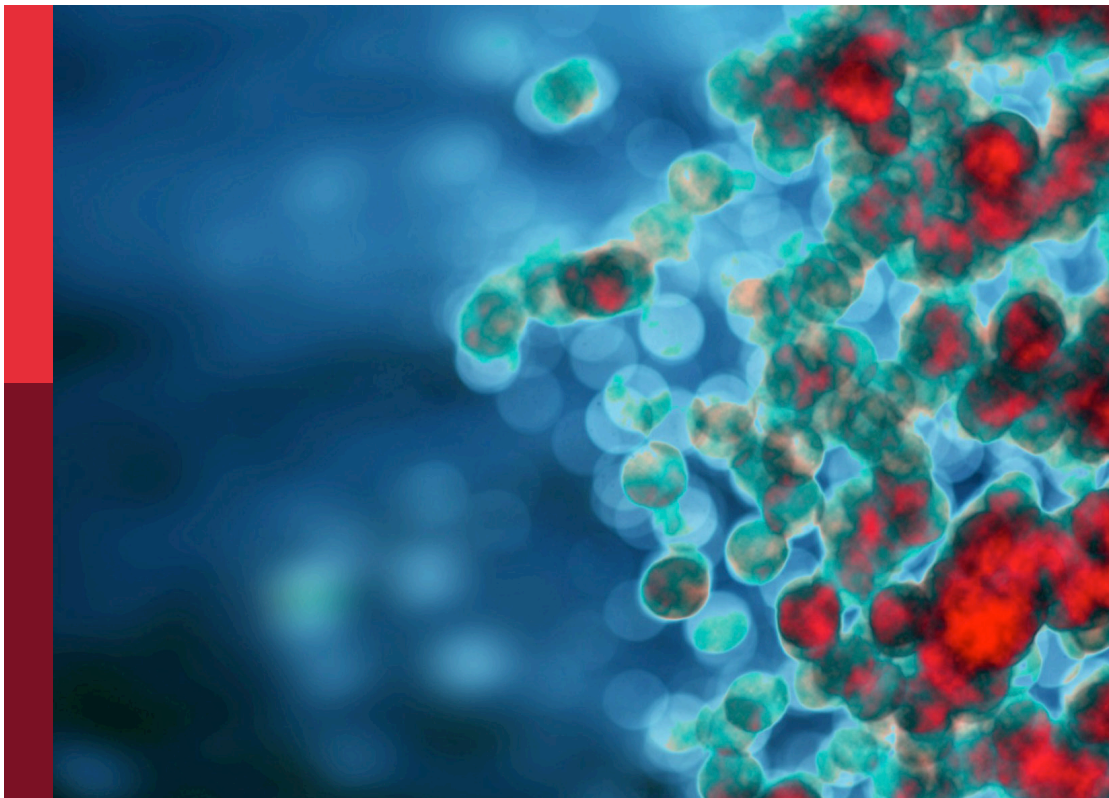

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Community series in immunotherapy with checkpoint inhibitors for non-small cell lung cancer, colon cancer and esophageal cancer, volume II

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Table of contents

06 Editorial: Community series in immunotherapy with checkpoint inhibitors for non-small cell lung cancer, colon cancer, and esophageal cancer, volume II
Yueyi Li, Yusha Wang, Jinsheng Gao, Keqin Tan, Benjamin Frey, Udo S. Gaipl, Hubing Shi and Xuelei Ma

09 ALOX12: A Novel Insight in Bevacizumab Response, Immunotherapy Effect, and Prognosis of Colorectal Cancer
Siyuan Weng, Zaoqu Liu, Hui Xu, Xiaoyong Ge, Yuqing Ren, Qin Dang, Long Liu, Jian Zhang, Peng Luo, Jianzhuang Ren and Xinwei Han

24 Efficacy and Safety of Neoadjuvant Monoimmunotherapy With PD-1 Inhibitor for dMMR/MSI-H Locally Advanced Colorectal Cancer: A Single-Center Real-World Study
Xuan Zhang, Renfang Yang, Tao Wu, Xinyi Cai, Guoyu Li, Kun Yu, Yong Li, Rong Ding, Chao Dong, Jinsha Li, Ruixi Hu, Qing Feng and Yunfeng Li

35 Combination of phototherapy with immune checkpoint blockade: Theory and practice in cancer
Yujie Zhao, Xu Liu, Xinyu Liu, Jing Yu, Xin Bai, Xi Wu, Xinyu Guo, Zhihui Liu and Xiaowei Liu

54 Characteristics of the immune microenvironment and their clinical significance in non-small cell lung cancer patients with ALK-rearranged mutation
Bo Zhang, Jingtong Zeng, Hao Zhang, Shuai Zhu, Hanqing Wang, Jinling He, Lingqi Yang, Ning Zhou, Lingling Zu, Xiaohong Xu, Zuoqing Song and Song Xu

66 Association between immune-related adverse events and the efficacy of PD-1 inhibitors in advanced esophageal cancer
Wenru Qin, Linlin Yang, Bingjie Fan, Bing Zou, Yanan Duan, Butuo Li and Linlin Wang

76 Advancing immune checkpoint blockade in colorectal cancer therapy with nanotechnology
Zefan Liu, Yucheng Xiang, Yaxian Zheng and Xin Kang

94 The role of immune profile in predicting outcomes in cancer patients treated with immunotherapy
Andrea Botticelli, Giulia Pomati, Alessio Cirillo, Simone Scagnoli, Simona Pisegna, Antonella Chiavassa, Ernesto Rossi, Giovanni Schinzari, Giampaolo Tortora, Francesca Romana Di Pietro, Bruna Cerbelli, Alessandra Di Filippo, Sasan Amirhassankhani, Alessandro Scala, Ilaria Grazia Zizzari, Enrico Cortesi, Silverio Tomao, Marianna Nuti, Silvia Mezi and Paolo Marchetti

108 Predictive biomarkers of colon cancer immunotherapy: Present and future
Wanting Hou, Cheng Yi and Hong Zhu

126 An *EGFR* L858R mutation identified in 1862 Chinese NSCLC patients can be a promising neoantigen vaccine therapeutic strategy
Jing Lin, Jun Liu, Shi-guang Hao, Bin Lan, Xiao-bin Zheng, Jia-ni Xiong, Ying-qian Zhang, Xuan Gao, Chuan-ben Chen, Ling Chen, Yu-fang Huang, Hong Luo, Yu-ting Yi, Xin Yi, Jian-ping Lu, Xiong-wei Zheng, Gang Chen, Xue-feng Wang and Yu Chen

139 A prognostic and therapeutic hallmark developed by the integrated profile of basement membrane and immune infiltrative landscape in lung adenocarcinoma
Kaijie Chen, Shuang Liu, Changlian Lu and Xuefeng Gu

153 Global research landscape and trends of lung cancer immunotherapy: A bibliometric analysis
Yanhao Liu, Xu Cheng, Xiaona Han, Xi Cheng, Shu Jiang, Yaru Lin, Zhen Zhang, Linlin Lu, Baozhen Qu, Yuxian Chen and Xiaotao Zhang

177 Lung cancer patients with chronic obstructive pulmonary disease benefit from anti-PD-1/PD-L1 therapy
Mao Lin, Zongyao Huang, Yingfu Chen, Hongtao Xiao and Ting Wang

189 Anti-PD-1 antibodies, a novel treatment option for advanced chemoresistant pulmonary lymphoepithelioma carcinoma
Na Zhou, Hui Tang, Shuangni Yu, Yi Lin, Yingyi Wang and Yuzhou Wang

198 PD-1/PD-L1 inhibitors plus anti-angiogenic agents with or without chemotherapy versus PD-1/PD-L1 inhibitors plus chemotherapy as second or later-line treatment for patients with advanced non-small cell lung cancer: A real-world retrospective cohort study
Shubin Chen, Haowen Wei, Wenhua Zhao, Wei Jiang, Ruiling Ning, Shaozhang Zhou, Liping Tan, Huilin Wang, Cuiyun Su, Jianbo He, Aiping Zeng, Yun Zhao and Qitao Yu

218 Acetylcholine receptor binding antibody–associated myasthenia gravis, myocarditis, and rhabdomyolysis induced by tislelizumab in a patient with colon cancer: A case report and literature review
Shengnan Wang, Danping Peng, Hao Zhu, Wanwan Min, Mengru Xue, Rui Wu, Yanqing Shao, Lin Pan and Mingqin Zhu

227 Immune-based combination therapy for esophageal cancer
Huiling Wang, Yufei Xu, Fengli Zuo, Junzhi Liu and Jiqiao Yang

242 Decreased monocyte-to-lymphocyte ratio was associated with satisfied outcomes of first-line PD-1 inhibitors plus chemotherapy in stage IIIB-IV non-small cell lung cancer
Liang Zheng, Anning Xiong, Shuyuan Wang, Jianlin Xu, Yinchen Shen, Runbo Zhong, Jun Lu, Tianqing Chu, Wei Zhang, Ying Li, Xiaoxuan Zheng, Baohui Han, Hua Zhong, Wei Nie and Xueyan Zhang

252 Meta-analysis of neoadjuvant immunotherapy for non-metastatic colorectal cancer
Long Zhou, Xiao-Quan Yang, Guang-yue Zhao, Feng-jian Wang and Xin Liu

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Editorial: Community series in immunotherapy with checkpoint inhibitors for non-small cell lung cancer, colon cancer, and esophageal cancer, volume II

Yueyi Li[1†], Yusha Wang[1†], Jinsheng Gao[1,2†], Keqin Tan[1], Benjamin Frey[3*], Udo S. Gaipl[3*], Hubing Shi[1,4*] and Xuelei Ma[1*]

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KEYWORDS

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Editorial on the Research Topic
Community series in immunotherapy with checkpoint inhibitors for non-small cell lung cancer, colon cancer, and esophageal cancer, volume II

Introduction

Immunotherapy with checkpoint inhibitors has revolutionized the treatment of various types of cancer. Non-small cell lung cancer, colorectal cancer, and esophageal cancer are among the most common cancers globally, but treatment options for advanced stages have historically been limited. However, with the advent of checkpoint inhibitors, significant progress has been made in improving patient outcomes and extending survival rates (1). This Research Topic brings together 18 studies from researchers in different countries, showcasing the latest advances in immunotherapy for these three types of cancer. The inclusion of varied perspectives and international insights enhances the depth and breadth of knowledge in the field, ultimately benefiting patients around the world.

Application of ICIs in NSCLC

In the realm of lung cancer treatment, immune checkpoint inhibitors (ICIs) have emerged as a game-changing approach. These inhibitors, such as programmed cell death-1 (PD-1) inhibitors, programmed cell death ligand 1 (PD-L1) inhibitors, and cytotoxic T-lymphocyte antigen 4 (CTLA-4) inhibitors, have demonstrated remarkable clinical

advancements, predicting outcomes and improved patient tolerability. Liu Y. et al. presented the research trends on lung cancer, emphasizing the importance of immunotherapy. In patients with advanced NSCLC, the combination of PD-1/PD-L1 inhibitors with anti-angiogenic drugs, with or without chemotherapy, was found to be superior to PD-1/PD-L1 inhibitors plus chemotherapy as a second- or later-line treatment (Chen S. et al.). Moreover, in the prediction of prognosis, a decreased monocyte-to-lymphocyte ratio (MLR) has been associated with a high objective response rate and long progression-free survival, indicating its potential predictive value for first-line PD-1 inhibitor combination chemotherapy in stage IIIB-IV NSCLC patients, as well as for all PD-L1-expressing populations. The changes in the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) may supplement the prediction of prognosis (Zheng et al.). Furthermore, Lin M. et al. reviewed the outcomes of patients with lung cancer (LC) with chronic obstructive pulmonary disease (COPD) after anti-PD-1/PD-L1 treatment and found that LC patients with COPD would benefit more from immunotherapy. In addition, PD-L1 expression has been found to be a predictor of tumor response, and PD-1/PD-L1 inhibitors have demonstrated good efficacy in treating patients with pulmonary lymphoepithelioma-like carcinoma (LELC) in retrospective cohort studies, which warrants further investigation in prospective studies as a frontline treatment option (Zhou N. et al.). Furthermore, an innovative approach has shown promise for patients with specific biomarkers and distinct tumor microenvironment (TME) characteristics. The expression of ALK/EGFR can be useful in the treatment and prognosis of non-small cell lung cancer. Patients with ALK-positive and EGFR/KRAS-positive NSCLC have been shown to exhibit an immunosuppressive TME, with high expression of PD-L1 and CTLA4 as poor prognostic factors in advanced ALK-rearranged NSCLC patients treated with ALK-TKI (Zhang B. et al.). Lin J. et al. have identified a possible cancer vaccine produced by the EGFR L858R neoantigen in non-small cell lung cancer patients with HLA A*33:03, which may serve as an effective remedy for the EGFR L858R subgroup after failed targeted therapy or ICI treatment. Moreover, the basement membranes- tumor immune microenvironment classifier has demonstrated its prognostic value in multiple cohorts. This can guide clinical decision-making and therapeutic strategies for patients with NSCLC (Lin J. et al.).

Application of ICIs in colorectal cancer

In recent years, immunotherapy has made great progress in colorectal cancer. For metastatic colorectal cancer, immune checkpoint blockade therapy has been approved for the treatment of patients with mismatch-repair-deficient (dMMR) and who have high levels of microsatellite instability (MSI-H). As for early-stage colon cancer with dMMR/MSI-H, neoadjuvant immunotherapy seems to be a promising treatment. Zhou L. et al. comprehensively analyzed neoadjuvant immunotherapy and found that this type of immunotherapy could increase pCRs and MPR rates for the dMMR/MSI-H group of non-metastatic colorectal cancer (Zhou L. et al.). Furthermore, given high ORR and pCR rates, low incidence of irAE and srAE, PD-1 inhibitors have shown great efficiency as neoadjuvant mono-immunotherapy for early-stage colorectal cancer with dMMR/MSI-H (Zhang X. et al.). Predictive biomarkers that can indicate immune infiltration and immunotherapy response contribute to guiding immunotherapy for colon cancer. Hou et al. comprehensively summarized reported predictive biomarkers for colon cancer immunotherapy and discussed the prospects for technological change in colon cancer immunotherapy biomarker development (Hou et al.). Novel biomarker ALOX12 is proven to predict bevacizumab response, immunotherapy effect, and prognosis of colorectal cancer (Weng et al.). It is well known that various ICIs have different immune-related adverse events (irAEs), which may involve many organs, such as the lung, liver, skin, kidney, digestive system, or endocrine system. Wang S. et al. reported a patient with locally advanced colorectal cancer who developed tislelizumab-induced multiple organ irAEs, and treatments including intravenous immunoglobulins (IVIGs) and corticosteroids improved these symptoms (Wang S. et al.). In addition, ICIs with nanotechnology have shown to be effective to avoid undesired side effects, unsatisfactory response rates, tumor metastasis, and drug resistance (Liu Z. et al.).

Application of ICIs in esophageal cancer

Esophageal cancer (EC) is a malignant disease and remains one of the leading causes of death. Immunotherapies such as immune checkpoint inhibitors, cancer vaccines, and adoptive cell therapy are effective for patients with EC. Wang H. et al. summarized and systematically analyzed immunotherapy-based combination therapies for EC (Wang H. et al.). As mentioned above, a strong immune response may lead to more serious and multi-system irAE. However, it has been found that patients with irAEs showed markedly better efficacy in ORR, DCR, PFS, and OS in advanced EC (Qin et al.). In summary, the occurrence of adverse effects may indicate that patients may benefit from immunotherapy, but serious adverse effects should be avoided.

This Research Topic provides a series of new research findings and insights that highlight the potential of immunotherapy and checkpoint inhibitors in non-small-cell lung carcinoma, colorectal, and esophageal cancers. Zhao et al. and Botticelli et al. provided a deeper understanding of immunotherapy for cancers by providing up-to-date results, exploring new therapeutic options, and evaluating existing therapies. Several studies included in this Research Topic have shown that checkpoint inhibitors have emerged as an effective treatment option to improve survival in some patients. For example, some studies have shown that PD-1 and PD-L1 antibody combination therapy in patients with non-small-cell lung carcinoma increases survival and improves response to treatment. Overall, these articles provide an in-depth insight into the role of immunotherapy and checkpoint inhibitors in these cancer types, which can help physicians, researchers, and patients

better understand these treatments and guide future research direction and clinical practice. The significance and contribution of these articles are that they advance our understanding of the role of immunotherapy and checkpoint inhibitors in cancer therapy and provide new ideas and directions for future research and treatment.

In conclusion, this Research Topic represents a significant milestone in the field of cancer immunotherapy. It showcases groundbreaking research, novel treatment strategies, and valuable insights into the mechanisms of response and resistance to checkpoint inhibitors. By addressing clinical challenges and providing evidence-based recommendations, this article Research Topic aims to improve patient outcomes and shape the future of immunotherapeutic approaches for these specific cancer types.

Author contributions

Literature review and data collection were performed by YL and YW. The first draft of the manuscript was written by YL, JG, and KT. The final version of the editorial was written by UG, BF, HS, and XM. All authors read and 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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ALOX12: A Novel Insight in Bevacizumab Response, Immunotherapy Effect, and Prognosis of Colorectal Cancer

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Colorectal cancer is a highly malignant cancer with poor prognosis and mortality rates. As the first biological agent approved for metastatic colorectal cancer (mCRC), bevacizumab was confirmed to exhibit good performance when combined with chemotherapy and immunotherapy. However, the efficacy of both bevacizumab and immunotherapy is highly heterogeneous across CRC patients with different stages. Thus, exploring a novel biomarker to comprehensively assess the prognosis and bevacizumab and immunotherapy response of CRC is of great significance. In our study, weighted gene co-expression network analysis (WGCNA) and the receiver operating characteristic (ROC) curves were employed to identify bevacizumab-related genes. After verification in four public cohorts and our internal cohort, ALOX12 was identified as a key gene related to bevacizumab response. Prognostic analysis and *in vitro* experiments further demonstrated that ALOX12 was closely associated with the prognosis, tumor proliferation, invasion, and metastasis. Multi-omics data analysis based on mutation and copy number variation (CNV) revealed that RYR3 drove the expression of ALOX12 and the deletion of 17p12 inhibited ALOX12 expression, respectively. Moreover, we interrogated the relationship between ALOX12 and immune cells and checkpoints. The results exhibited that high ALOX12 expression predicted a higher immune infiltration and better immunotherapy response, which was further validated in Tumor Immune Dysfunction and Exclusion (TIDE) and Subclass Mapping (SubMap) methods. Above all, our study provides a stable biomarker for clinical protocol optimization, prognostic assessment, precise treatment, and individualized treatment of CRC.

Keywords: ALOX12, colorectal cancer, bevacizumab, prognosis, immunotherapy, multi-omics, biomarker

INTRODUCTION

Colorectal cancer (CRC) is reported to be the third leading cause of tumor mortality around the world with more than 850,000 deaths and 1.85 million cases annually. Twenty percent of patients with newly diagnosed CRC already have metastases. More seriously, nearly a quarter of CRC patients develop metastases after the onset of local disease (1). With the development of multidisciplinary and comprehensive treatment options, the survival of CRC patients has been considerably prolonged, while the long-term survival rate of CRC, especially metastatic patients, is still unsatisfactory (2). Bevacizumab is an anti-VEGF monoclonal antibody targeting angiogenesis (3). As a standard-of-care therapy in metastatic colorectal cancer (mCRC) patients and the first biological agent approved for mCRC, bevacizumab combined with chemotherapy (leucovorin, irinotecan, and fluorouracil) was confirmed to illustrate surprising performance (1, 3). In addition, studies with bevacizumab in mCRC have also shown the dramatic benefits of combining bevacizumab with new chemotherapy regimens (capecitabine/oxaliplatin or fluorouracil/leucovorin) as the first-line treatment and with leucovorin/fluorouracil and oxaliplatin as the second-line treatment (4–7). Unfortunately, despite the dramatic benefits in mCRC treatment, the molecular mechanism of bevacizumab is still unclear. Recently, Quintanilha et al. have reported that rs3795897 (G>A) in *AGAP1* might be a potential predictor related to bevacizumab and patient survival (8). It is worth noting that no bevacizumab response biomarker for CRC was reported before (8, 9). Nevertheless, just as the authors said, this study displayed many limitations and a lack of further validation, which make the result questionable. Considering this, exploring a novel biomarker to predict the response of bevacizumab for CRC is still warranted.

In recent years, immunotherapy has exhibited a great sensation due to the dramatic benefits of solid cancer treatments (2, 10). Immune checkpoint inhibitor (ICI) can promote the immune system to recognize and suppress basic targets of tumor cells such as PD-1, CTLA-4, and PD-L1 (11). In CRC, ICI therapy was approved for the treatment of patients with advanced microsatellite instability (MSI) or DNA mismatch repair (dMMR) deficiency in 2017 (12). Apart from this, there are other classification tools to stratify patients, such as molecular subtypes, *PD-1*, *PD-L1*, *CTLA-4*, and tumor mutation burden (TMB) (2). However, these classification systems do not perfectly predict response to ICI therapy and only a small proportion of CRC patients can benefit from them (13). Given the enormous cost and serious adverse effects of immunotherapy, exploring new biomarkers for effective immunotherapy management in CRC is warranted.

Due to the poor prognosis and high mortality rate of CRC patients, considerable effort has been invested to develop markers for assessing the prognosis of CRC over the past decade. It has been reported that mutations in *KRAS*, *PIK3CA*, and *BRAF*, amplification of *HER2*, and the loss of *SMAD4* were significantly associated with the relapse of CRC (14). The consensus molecular subtype (CMS) classification was confirmed to be related to the clinical outcome of CRC, and CMS4 tumors had a frustrating recurrence and overall survival (OS) (15). In addition, MSI-H patients were reported to illustrate a significantly reduced risk of recurrence and death (16). Recently, Liu et al. have reported that the double hit of TTN and OBSCN demonstrated a better prognosis in CRC (17). However, these biomarkers possess limited clinical utility and only a moderate accuracy of prediction (18, 19).

In the present study, considering the fundamental role of bevacizumab in mCRC treatment, the weighted gene co-expression network analysis (WGCNA) algorithm and receiver operating characteristic (ROC) curves were employed to identify bevacizumab-related genes. Subsequent validations in our internal cohort and four independent cohorts demonstrated the robust and accurate ability of ALOX12 in bevacizumab response prediction. Univariate and multivariate Cox analyses and survival analysis revealed that the high expression of ALOX12 predicted worse overall survival (OS), progression-free survival (PFS), and relapse-free survival (RFS). Additionally, gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA) algorithms were employed to explore the potential functions, and two potential driving targets of ALOX12 (*RYR3* and *17p12*) were further determined based on multi-omics data analysis. Besides, we also investigated the tumor mutation burden (TMB), immune landscape, immune subtype, immune checkpoint profile, and potential drug targets of ALOX12. In conclusion, our study provides a stable and powerful biomarker for CRC patients to predict the bevacizumab response, prognosis (OS, RFS, and PFS), immunotherapy effects, and potential therapeutic agents, which performed a dramatic significance in clinical therapeutic regimen optimization, prognostic risk assessment, precision treatment, and the individualized treatment regimen formulation of CRC.

MATERIALS AND METHODS

Data Collection and Processing

The flowchart of our study is illustrated in **Figure 1**. Three independent CRC cohorts were retrieved from the GEO website (http://www.ncbi.nlm.nih.gov/geo/), namely, GSE72970, GSE19860, and GSE19862. Two different CRC datasets were obtained from the UCSC Xena browser (https://xenabrowser.net/datapages/), namely, TCGA-COAD (n = 512) and TCGA-READ (n = 177). The somatic mutation (VarScan2 variant aggregation and masking) and HumanMethylation450 array were downloaded from TCGA GDC website (https://portal.gdc.cancer.gov/), and TMB was obtained by calculating the count of non-silent somatic mutation in every patient. Copy number variation (CNV) data processed by the Genomic Identification of Significant Targets in Cancer 2.0 (GISTIC2.0) algorithm were retrieved from FireBrowse (http://firebrowse.org/) (20). Of note, the robust multiarray averaging (RMA) algorithm implemented in the *affy* R package was utilized to process the raw data obtained from GEO, and the FPKM-normalized data from UCSC were further converted into log2 (TPM + 1).

Samples in TCGA cohort were screened according to the following conditions: (1) all samples were obtained from primary cancer tissues; (2) no preoperative radiotherapy or chemotherapy was received; (3) survival information was available; and (4) RNA expression data were available. Besides, in the GEO cohort, only samples with bevacizumab treatment response information were

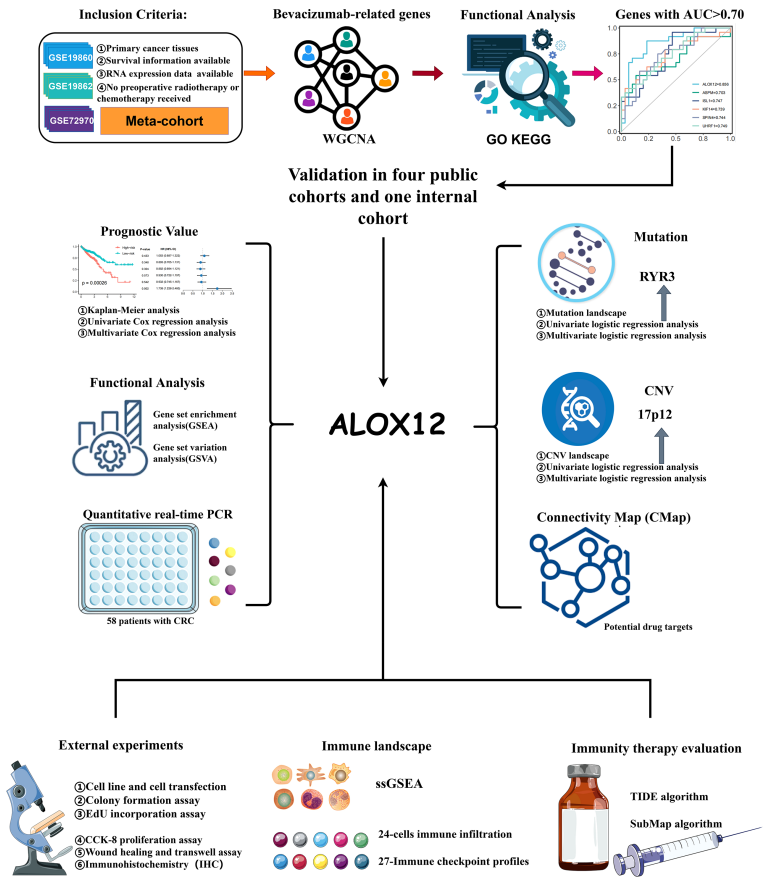

FIGURE 1 | The flowchart of this study.

retained. For the detailed baseline data of all patients, please refer to **Table S1**.

Construction of WGCNA

WGCNA is an R package available for weighted correlation network analysis. Before the construction of WGCNA, batch effects were removed from the meta-cohort (including GSE72970, GSE19860, and GSE19862) by the ComBat algorithm implemented in the *sva* package. In order to achieve the condition of a scale-free network, the optimal soft threshold β was identified and the adjacency matrix was transformed to a topological overlap matrix (TOM). Further, the corresponding dissimilarity (1-TOM) was calculated and modules were determined using the dynamic tree cutting method.

Determination of Bevacizumab-Related Genes

After obtaining the modules, the relationship of the modules and bevacizumab treatment response was calculated. Afterward, three modules with the strongest correlation were selected and genes in these modules was defined as bevacizumab-related genes.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analysis

The molecular functions, biological processes, cellular components, and potential mechanism of bevacizumab-related genes were further explored by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses, which were conducted by the *clusterProfiler* package. Pathways with $P < 0.05$ were considered significant.

ROC Curves, Cox Regression, and Survival Analysis

ROC curves and the area under the ROC curve (AUC) were employed to estimate the accuracy of bevacizumab-related genes for predicting bevacizumab treatment response, and genes with AUC >0.7 were retained, subsequently. After validating in GSE72970, GSE19860, GSE19862, meta-cohort, and our internal cohort, ALOX12 was identified as an accurate and stable predictor of bevacizumab response in CRC. Additionally, Kaplan–Meier survival analysis and univariate and multivariate Cox regression analyses were further employed to investigate the prognostic value of ALOX12.

Gene Set Enrichment Analysis

Correlations between ALOX12 expression and all mRNA genes were evaluated by the Pearson correlation. All genes were arranged in descending order of correlation coefficient. Afterward, GSEA was conducted by the *clusterProfiler* R package to recognize remarkably enriched terms associated with the GO and KEGG pathways (21).

Gene Set Variation Analysis

To investigate whether ALOX12 expression was associated with tumors, we performed GSVA *via* the *GSVA* R package (22). The hallmark gene set was obtained from the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb/index.jsp). Patients were divided into two groups according to the median expression of ALOX12. To reduce the overlap and redundancy of pathways, the gene set associated with a pathway was screened to contain only unique genes, and all genes related to multiple pathways were removed (23). The *limma* package was employed to recognize the remarkably altered pathways between the high and low groups, and the pathway with $|t| > 1$ was regarded significant.

The Mutation Landscape of CRC

The TMB of each patient was assessed using the *maftools* R package (24). To explore whether there were differences in genomic mutations between high and low ALXO12 expression groups, the mutation waterfall plot of the top 30 genes with the highest mutation number in CRC was visualized using the *maftools* and *ComplexHeatmap* packages. Subsequently, Wilcoxon test and univariate and multivariate logistic regression analyses were performed to assess and verify the correlation between 30-gene mutation status and ALOX12 expression, respectively. It is worth noting that apart from age, gender, and stage, TMB was also included in the multivariate logistic regression to ensure that the relationship between mutation and ALOX12 was independent.

Copy Number Variation in CRC Patients

To investigate the proportion of genomic alterations in CRC, the fraction of genomic alterations (FGA), genomes gained (FGG), and genomes lost (FGL) were calculated, respectively. The *ComplexHeatmap* package was employed to visualize the CNV waterfall chart of the top 15 amplification (AMP) and homozygous deletion (Homdel) chromosome fragments in CRC. In addition, Wilcoxon test and univariate and multivariate logistic regression analyses were performed to calculate and confirm the correlation between the CNV of 30 fragments and ALOX12 expression. Of note, in addition to age, gender, and stage, FGG was contained in the multivariate logistic regression analysis to ensure that the correlation between ALOX12 expression and the AMP fragments was independent. Similarly, FGL was contained in the multivariate logistic regression analysis to ensure that ALOX12 was an independent factor of Homdel fragments.

Comprehensive Analysis Based on Immune Infiltration and Immune Checkpoints

The single-sample gene set enrichment analysis (ssGSEA) algorithm was conducted to estimate the infiltration abundance of 24 immune cells in the tumor immune microenvironment *via* the *GSVA* package (25). The gene set of 24 immune cell types was obtained from the previous study (26). We also retrieved 27 immune checkpoints from the published studies, including the member of the B7-CD28 family (27), TNF superfamily (28), and other molecules (29, 30). Studies of the relationship between the ALOX12 expression and immune infiltration and checkpoints were employed subsequently.

Immunotherapeutic Response Prediction

The tumor immune dysfunction and exclusion (TIDE) and subclass mapping (SubMap) algorithm were employed to predict the responses to ICB therapy (31, 32). Actually, TIDE evaluates immune evasion by integrating the expression characteristics of T-cell exclusion and T-cell dysfunction. In parallel, the GSEA algorithm was implemented in SubMap to derive the degree of commonality between high and low groups, and the adjusted *P*-value was employed to assess the similarity. A lower adjusted *P* value represents higher similarity.

Connectivity Map Analysis Identified Potential Compounds/Inhibitors for CRC

Connectivity Map (CMap) is a public online tool that allows users to predict compounds that can activate or inhibit based on a gene expression signature (33). Based on the key gene expression, we performed CMap to screen potential therapeutic agents to further identify which target drug might be helpful against CRC. Agents with $P < 0.001$ were considered significant.

Human Tissue Specimens and qRT–PCR Analysis

A total of 58 paired CRC tissues and matched adjacent non-tumor tissues were collected from the First Affiliated Hospital of Zhengzhou University. All patients signed informed consent. After radical surgery, patients received available standard systemic bevacizumab therapies. Drug responses were evaluated based on the Response Evaluation Criteria in Solid Tumors (RECIST, version 1.1). The detailed baseline characteristics of the patients are illustrated in **Table S1**. In the qRT-PCR analysis, six bevacizumab-related genes with AUC >0.7 were detected. Gene expression values were normalized to GAPDH and further log2 transformed. The primer sequences of the included six genes and GAPDH are exhibited in **Table S2**. See **Supplementary Material** for a detailed description.

Immunohistochemistry

The anti-ALOX12 (Ab211506, 1:100) antibody was employed to conduct immunohistochemistry (IHC). Percent staining was scored as follows: 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%), and staining intensity was scored on a scale of 0 (signal-less color) to 3 (light yellow, brown, and dark brown). Stained tissue was scored by three individuals blinded to clinical parameters, and IHC scores were determined by percentage and intensity scores.

Cell Lines and Cell Transfection

Two cell lines, namely, human CRC HCT-116 and SW480, were used in our research, which were cultured in RP1640 (Solarbio,

Beijing, China, 31800-500) containing 10% FBS (04-001-1ACS, Bioind, Beit Haemek, Israel) at 37°C with 5% CO_2. Silencer Select small interfering RNAs (siRNAs) specific for ENSG00000108839 (ALOX12) and inhibitor control were generated from RiboBio (Guangzhou, China). To silence mRNA in cancer cells, specific siRNA and control siRNA were transfected into HCT-116 and SW480 cells. Lipofectamine 3000 (Invitrogen, Carlsbad, USA) was utilized as a transfection carrier, and qRT-PCR analysis was employed to confirm the transfection efficiency.

Wound Healing and Transwell Assay

Tumor cells were seeded in six-well plates, scraped with a sterile 200-μl pipette tip, and cultured in serum-free medium, and the wound width was measured at 0 and 48 h, respectively. The migration and invasive abilities of CRC cells were determined *via* transwell assays after transfection with siRNAs. See **Supplementary Material** for a detailed description.

Colony Formation Assay

Equal numbers of transfected cells were inoculated into six-well plates at a density of 1,000 cells per well and incubated at 37°C for 2 weeks. Then, the cells were fixed with 4% paraformaldehyde for 15 min and stained with GIMSA for 10–30 min. Finally, the colonies were photographed and counted.

Cell Counting Kit-8 Proliferation Assay and 5-Ethynyl-20-deoxyuridine Incorporation Assay

Cell proliferation was determined by Cell Counting Kit-8 (Fu Heng, Shanghai, China), and the Cell-Light EdU Apollo567 *In Vitro* Kit (RiboBio, Guangzhou, China) was used to assess the proliferation of cells according to the manufacturer's instructions. For detailed steps, please refer to the **Supplementary Material**.

Statistical Analysis

The correlations between two variables were evaluated by Pearson correlation. The *Survival* package was utilized to perform Kaplan–Meier survival analysis, and the different significance was determined by the log-rank test. The ROC curves were plotted by the *pROC* package. Besides, the AUC was utilized to compare the accuracy for predicting the bevacizumab response of hub genes. Differences in key gene expression between the high and low groups were compared by independent-sample T-test or Wilcoxon rank-sum test. Multiple comparisons were conducted using Kruskal–Wallis tests. All statistical P values were two-sided, and $P < 0.05$ was defined as statistically significant. Adjusted P-value was employed using the Benjamini-Hochberg (BH) multiple-test correction. All data processing and plotting were finished in R 4.0.5 software.

RESULTS

Construction of Gene Co-Expression Modules

Firstly, three cohorts, namely, GES19860, GSE19862, and GSE72970, were combined into a meta-cohort and the batch effects were removed (**Figures 2A, B**). Afterward, the median absolute deviation (MAD) of the expression values for each gene was calculated, and the top 5,000 genes were taken for WGCNA after sorting in descending order. In the present study, a power of $\beta = 6$ was selected as the soft threshold to implement a scale-free network (**Figure 2C**) and the adjacency matrix was further transformed into a topological overlap matrix (**Figure S2A**). After calculating the signature genes of modules, the highly co-expressed genes were clustered into the same module by dynamic-tree cutting method (**Figure 2D**). Of note, the heatmap of the correlation for module traits showed that the purple, yellow, and cyan modules were tightly related to the response of bevacizumab in CRC (**Figure 2E**). The same results were also illustrated in the scatter plots of gene significance and module memberships (**Figures 2F–H**). Eventually, a total of 597 genes in purple, yellow, and cyan modules were defined as bevacizumab response-related genes.

GO and KEGG Enrichment Analyses

To explore the potential biological functions and potential molecular mechanisms of bevacizumab-related genes, GO and KEGG enrichment analyses were further employed. The result of GO analysis performed that bevacizumab-related genes were mainly enriched in DNA replication, chromosome segregation, cell-cycle checkpoint, and the regulation of cell-cycle phase transition pathways (**Figure 3A**). In parallel, the result of KEGG analysis exhibited that these genes were mostly enriched in the p53 signaling pathway, cell cycle, purine metabolism, and DNA replication pathways (**Figure 3B**). Taken together, GO and KEGG enrichment analyses indicated that bevacizumab-related genes might play an important role in the occurrence and development of tumors, as well as the proliferation and division of cancer cells.

ALOX12, a Hub Gene Tightly Associated With Bevacizumab Response and Prognosis

To further verify the correlation between bevacizumab-related genes and bevacizumab response in CRC, ROC curves and AUC were plotted in four cohorts (including GSE19860, GSE19862, GSE72970, and meta-cohort). Under the screening conditions of AUC >0.7, six genes (namely, *ALOX12*, *ASPM*, *ISL1*, *KIF14*, *SPIN4*, and *UHRF1*) were retained (**Figure 3C**). Patients were divided into high and low groups according to the median expression of these six genes, respectively. As illustrated in **Figure 3D**, the differences of bevacizumab response in the two groups were dramatically significant in the four external cohorts and our internal cohort, which indicated that ALOX12 was an accurate and stable biomarker in predicting the response to bevacizumab for CRC. Of note, in our in-house cohort, ALOX12 expression was significantly elevated in CRC compared with normal tissues, and it was further validated in IHC (**Figure S1**). Additionally, we explored the prognostic value of ALOX12. Kaplan–Meier analysis suggested that a high expression of ALOX12 predicted worse OS, RFS, and PFS (**Figures 3E**, **S2B**). Univariate and multivariate Cox regression analyses suggested that ALOX12 was not only dramatically

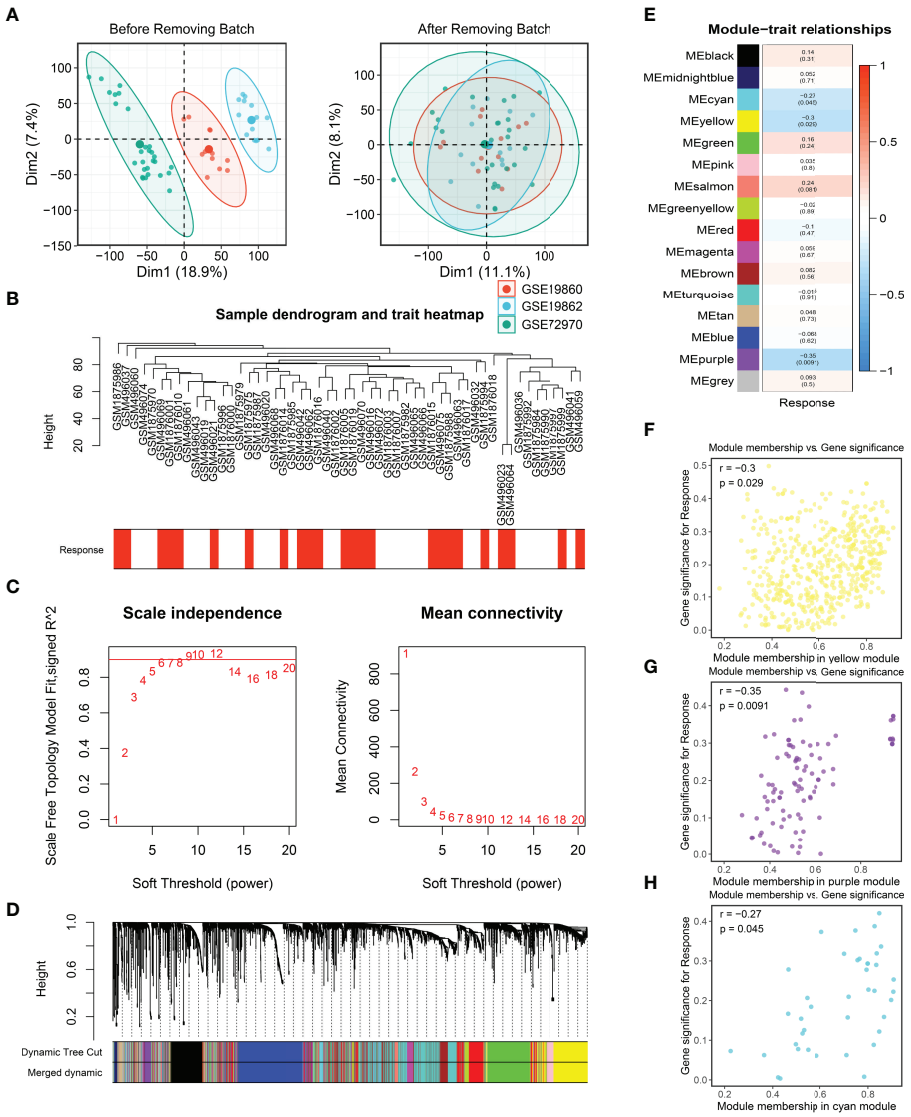

FIGURE 2 | Batch correction and the identification of bevacizumab-related genes. **(A, B)** Batch removing of GSE19860, GSE19862, and GSE72970 **(A)**, and the sample dendrogram and trait heatmap of the meta-cohort **(B)**. **(C)** Scale-free topology criterion of the co-expression network, and an SFT in review plot for choosing the power β for the unsigned weighted correlation network. **(D)** Cluster dendrogram of genes in the co-expression network. **(E)** The correlation between modules and bevacizumab response in CRC. **(F–H)** Scatter plots of bevacizumab-related genes.

significant in predicting OS, RFS, and PFS (**Figures S2C–G**) but also an independent prognostic factor for CRC patients after adjusting other clinical characteristics (**Figure 3F**).

Biological Function Analysis of ALOX12

To gain more insights into the potential functional characteristics and molecular mechanisms of ALOX12, GSEA and GSVA were employed, subsequently. As illustrated in **Figures 4A, B**, the top 20 pathways of GO and KEGG were selected separately according to the absolute value of normalized enrichment score (NES), from which we could observe that ALOX12 expression was tightly associated with tumor development, progression, metastasis, and immune-related pathways such as positive regulation of GTPase activity, small GTPase-mediated signal transduction, NOTCH signaling pathway, regulation of GTPase activity, ECM receptor interaction, and inositol phosphate metabolism pathways. As shown in **Figures 4C, D**, all these pathways were positively correlated with ALOX12 expression. Additionally, the GSVA result demonstrated that the vast majority of pathways in the hallmark gene set were significantly different between the high and low groups, indicating that ALOX12 expression was highly correlated with tumors (**Figure 4E**).

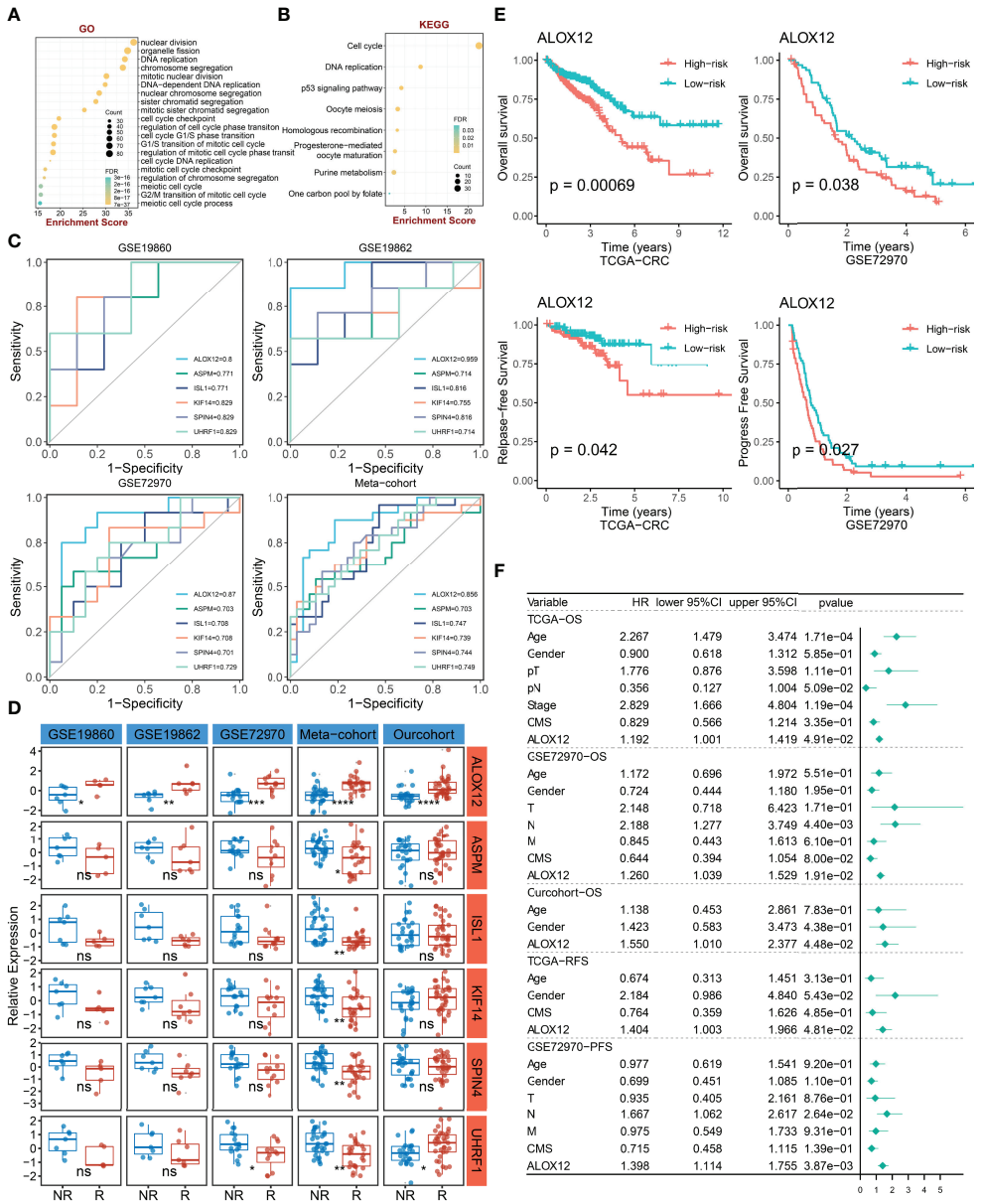

FIGURE 3 | The identification and prognostic value assessment of ALXO12. **(A, B)** GO **(A)** and KEGG **(B)** enrichment analysis results of bevacizumab-related genes. **(C)** Bevacizumab-related genes with AUC >0.70. **(D)** Validation of the correlation between six-hub genes and bevacizumab response in four external cohorts and our internal cohort. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. **(E)** Kaplan–Meier survival analysis of the high and low ALOX12 expression groups in TCGA-CRC and GSE72970. **(F)** Multivariate Cox regression analysis of OS, RFS, and PFS in TCGA-CRC, GSE72970, and our cohort. ns, no significance.

ALOX12 Promoted the Proliferation, Invasion, and Metastasis of CRC

To validate the biological function of ALOX12 in CRC cells, three special siRNAs were designed to knock down the expression of ALOX12 in HCT-116 and SW480 cell lines. As exhibited in **Figures 5A, B**, siRNA2 and siRNA3 efficiently reduced the ALOX12 expression in HCT-116 and SW480 cells. The growth curves of CCK8 assays demonstrated that down-regulation of ALOX12 suppressed the proliferation viability of HCT-116 and SW480 CRC cells (**Figures 5C, D**). Colony formation assays exhibited that the cell colony numbers of HCT-116 and SW480 cells were dramatically inhibited by the down-regulation of ALOX12 (**Figures 5E, F**). In parallel, 5-ethynyl-20-deoxyuridine (EdU) assays showed that knockdown of ALOX12 impaired the ratio of the positive cells (**Figures 5G–I**). Moreover, wound-healing assays suggested that knockdown of ALOX12 inhibited the migration of CRC cells (**Figures 6A–D**), and transwell assays, including migration and invasion assays,

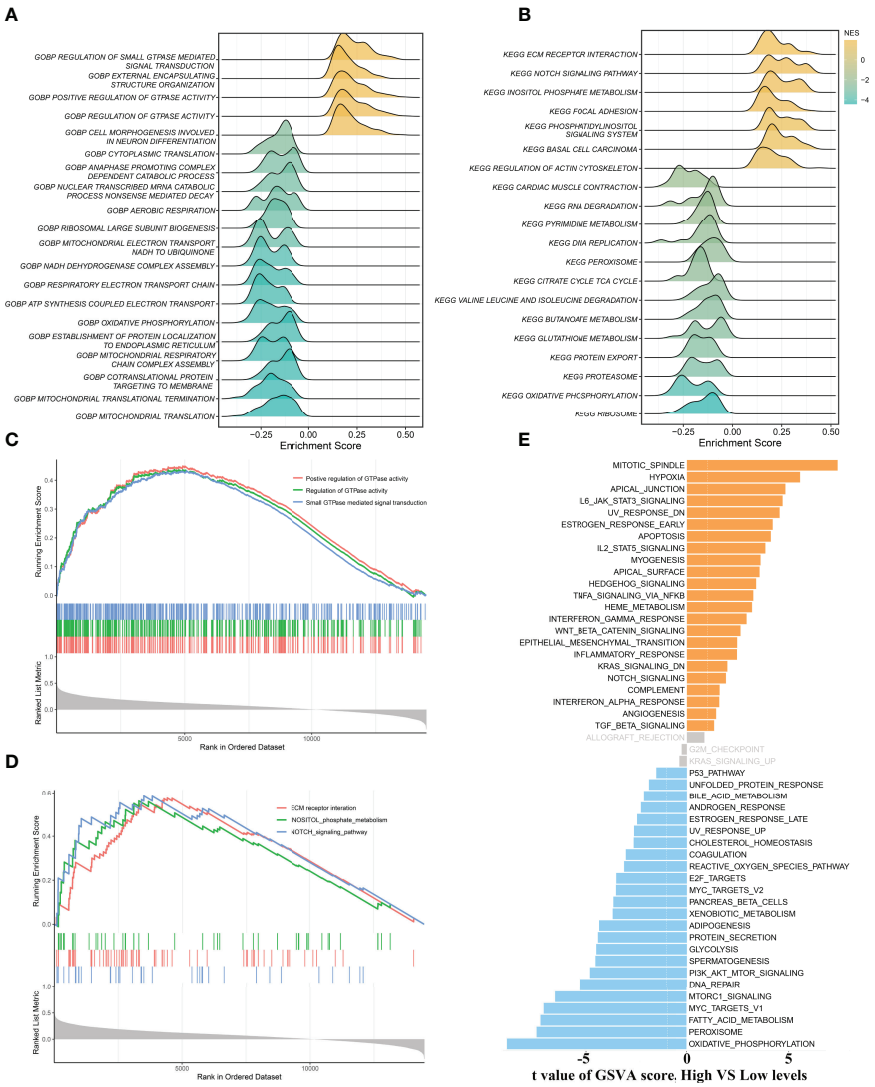

FIGURE 4 | Gene set enrichment analysis (GSEA) and gene set variation analysis (GSVA). **(A, B)** The results of GO **(A)** and KEGG **(B)** enrichment analysis by the GSEA algorithm. **(C, D)** The significantly enriched pathways are associated with ALOX12 expression. **(E)** The result of GSVA between high and low ALOX12 expression groups.

indicated that the migratory ability and invasive ability were reduced when ALOX12 was depleted (**Figures 6E–J**). Taken together, ALOX12 facilitated the proliferation, invasion, and metastasis of CRC.

Somatic Mutational Landscape With Regard to ALOX12

We further explored the mutational landscape of the top 30 frequently mutated genes (FMGs) (**Figure 7A**). Overall, seven FMGs exhibited a significantly higher mutational frequency between the high and low ALOX12 expression groups, including *APC*, *TTN*, *FAT4*, *OBSCN*, *DNAH11*, *RYR3*, and *MUC5B* (**Figure 7B**). Consistent with the mutation characteristics of these FMGs, the high-risk group also exhibited a generally superior burden including TMB ($P < 0.05$), single-nucleotide polymorphisms (SNPs, $P < 0.01$), and insertions and deletions (Indels, $P < 0.05$) (**Figure 7C**). Furthermore, we investigated the correlation between mutation status of the top 30 FMGs and ALOX12 expression. As illustrated in **Figure 7D**, *DNAH11* and *RYR3* were more likely to be mutated in CRC patients with high ALOX12 expression ($P < 0.05$). Besides, univariate and multivariate logistic regression revealed that a high expression of ALOX12 was not only tightly associated with RYR3 mutation but also remained an independent significance after adjusting for clinical characteristics such as age, gender, stage, and TMB (**Figure 7E**). Taken together, RYR3 mutation might drive the expression of ALOX12, and the higher ALOX12 expression predicted a superior mutational landscape.

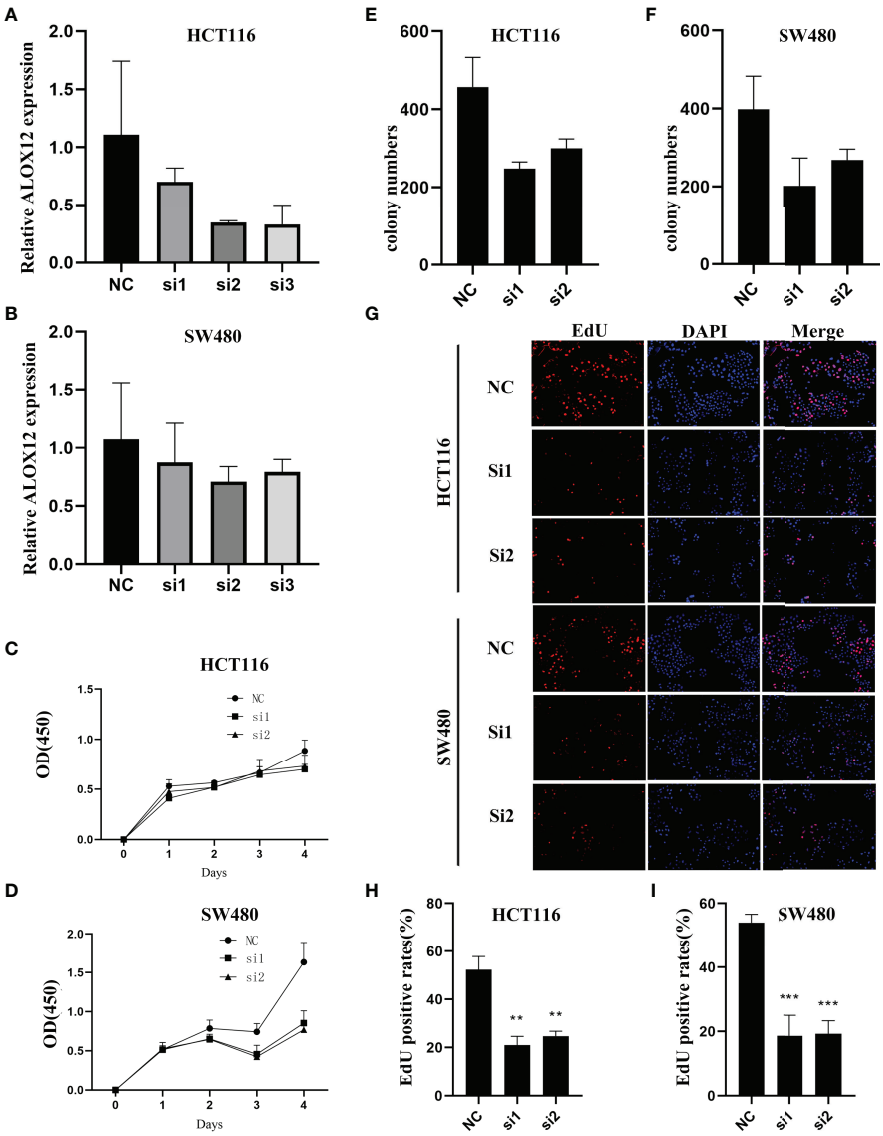

FIGURE 5 | External experiment validation of the correlation between ALOX12 expression and tumor cell proliferation. **(A, B)** Cell transfection of HCT-116 **(A)** and SW480 **(B)** CRC cell lines. **(C, D)** CCK-8 proliferation assay of HCT-116 **(C)** and SW480 **(D)** cells. **(E)** Colony formation assay of HCT-116 **(E)** and SW480 **(F)** cells. **(G–I)** 5-Ethynyl-20-deoxyuridine (EdU) incorporation assay in HCT-116 and SW480 CRC cell lines. **P < 0.01, ***P < 0.001.

Deletion of 17p12 Inhibited the Expression of ALOX12

Furthermore, we characterized the CNV status of the top 15 AMP and Homdel chromosome fragments between two ALOX12 expression groups (**Figure 7F**). As illustrated in **Figure 7G**, deletion of 17p-12, 15q15.2, 15q21.1, 4q35.1, and 4q22.1 displayed significant differences between the high- and low-risk groups. Univariate and multivariate logistic regression suggested that a low expression of ALOX12 not only predicted the deletion of 17p12 but also remained an independent significance after adjusting for clinical characteristics such as age, stage, gender, and FGL (**Figure 7H**). Taken together, the deletion of 17p12 inhibited the expression of ALOX12.

ALOX12 Expression Is Significantly Associated With Immune Infiltration

On the basis of GSEA and GSVA results, we observed that ALOX12 expression was tightly associated with tumor immune-related pathways. Therefore, the relative infiltration abundance of the 24 immune cell types was further calculated by the ssGSEA algorithm. As shown in **Figure 8A**, the infiltration abundance of immune cells exhibited a high correlation with ALOX12 expression, especially effective memory T (Tem) cell, natural killer (NK) cell, and T helper 2 (Th2) cell. Furthermore, the hierarchical clustering method was employed to classify the samples into three subtypes (high, medium, and low immune infiltration). Consistently, a high

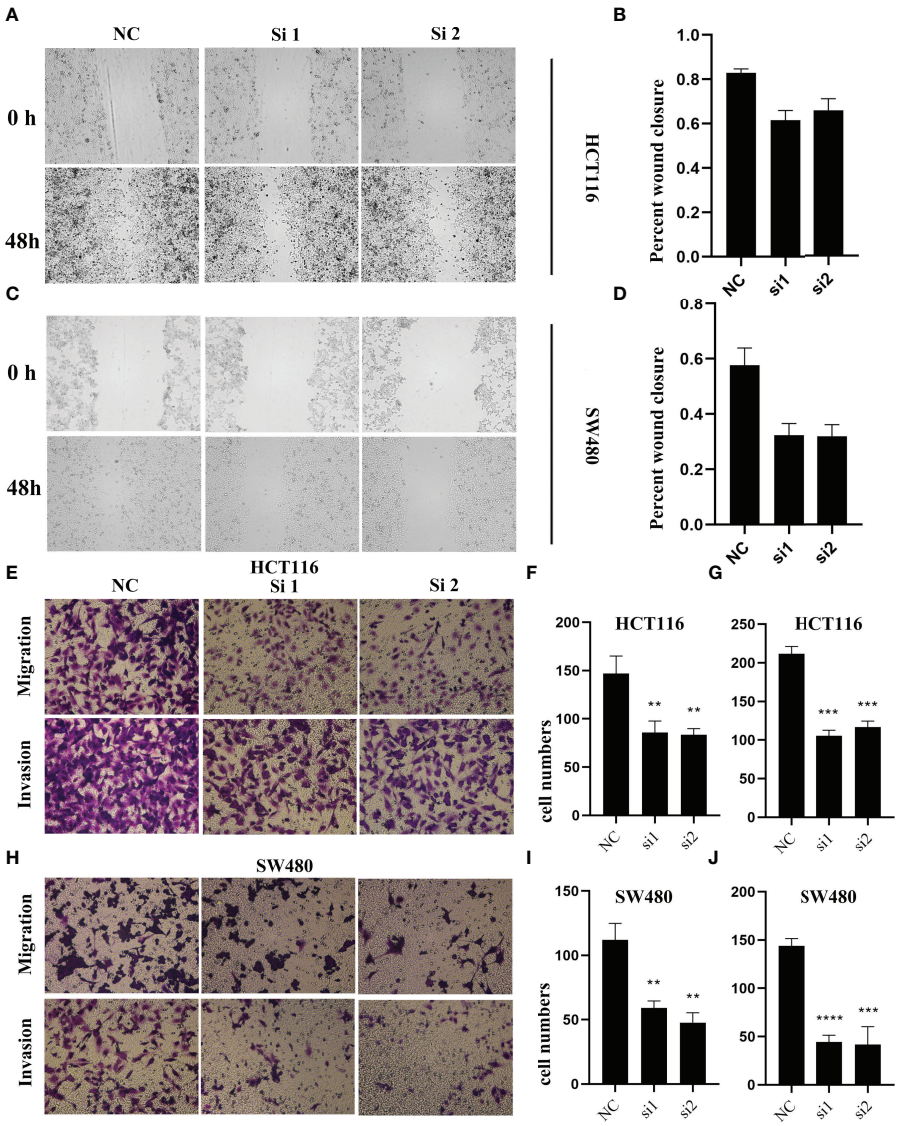

FIGURE 6 | The result of wound healing **(A–D)** and transwell assay **(E–J)** in HCT-116 and SW480 CRC cell lines. **P < 0.01, ***P < 0.001, ****P < 0.0001.

expression of ALOX12 revealed a higher abundance of immune cell infiltration, and ALOX12 expression was significantly different between the high immune infiltrating subtype and the medium and low immune infiltrating subtypes (**Figures 8B, C**). As illustrated in **Figures 8D, E**, most of the 27 immune checkpoints were significantly different between the high and low groups, including *CD276*, *CD70*, *ICOS*, *CTLA4*, *PDCD1* (*PD-1*), *CD274* (*PD-L1*), *PDCD1LG2*, *BTLA*, *CD27*, *CD40*, *CD40LG*, *HHLA2*, *TNFRSF18*, *TNFRSF4*, *ICOSLG*, *TNFRSF9*, *TNFSF14*, *LAG3*, *HAVCR2*, *ENTPD1*, and *NCR3* (all $P < 0.05$), which provided potential ICB therapeutic targets for CRC patients. Previous studies indicated that patients with a high expression of PD-1 and PD-L1 benefited more from nivolumab and pembrolizumab (2). Of note, the expressions of *PD-1*, *PD-L1*, and *CTLA4* were all significantly upregulated in the ALOX12 highly expressed group ($P < 0.0001$), revealing the encouraging application value of immune checkpoint blockade (ICB) in CRC patients with a high *ALOX12* expression.

High Expression of ALOX12 Suggested Higher ICB Clinical Benefit

TIDE indicated that the immunotherapy response rates of patients with high *ALOX12* expression were dramatically higher ($P < 0.001$; **Figure 8F**). In parallel, SubMap analysis performed that high group patients who responded to anti-CTLA-4 and anti-PD1 therapy exhibited high similarity ($P < 0.05$; **Figure S3A**). Both TIDE and Submap confirmed that high

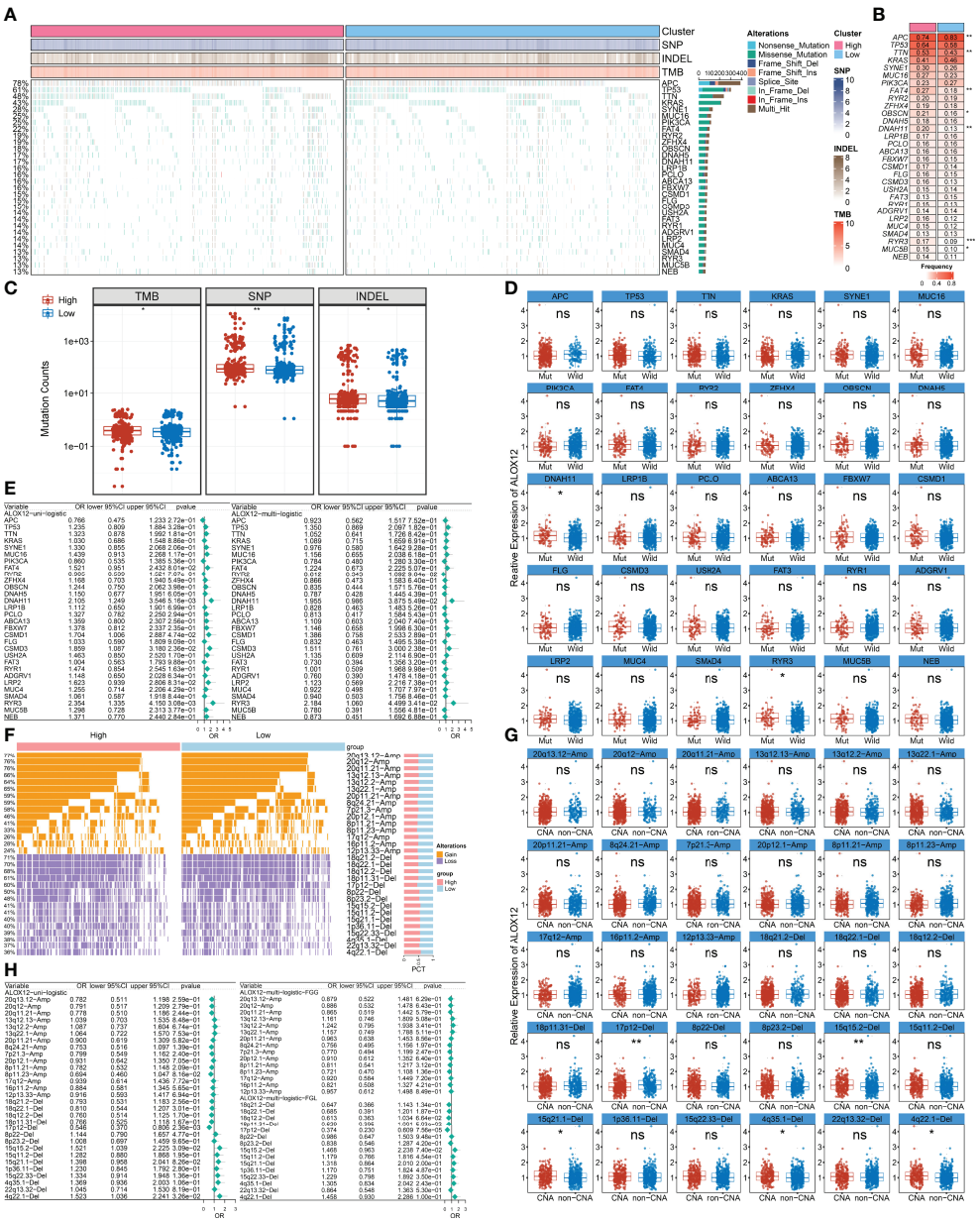

FIGURE 7 | Identification of ALOX12-related mutation and copy number variation driver targets. **(A)** The mutational landscape of the top 30 frequently mutated genes (FMGs). **(B)** The mutation frequency of 30 FMGs between the high and low ALOX12 expression groups. **(C)** Boxplot of TMB, SNP, and INDEL between the high- and low-risk groups. **(D)** Identification of ALOX12 expression-related mutations. **(E)** Determination of independent ALOX12 expression-related gene mutation through univariate and multivariate logistic regression analyses. **(F)** The CNV landscape of the top 15 AMP and Homdel chromosome fragments between two ALOX12 expression groups. **(G)** Identification of ALOX12 expression-related CNV chromosome segments. **(H)** Determination of independent ALOX12 expression-related CNV chromosome segment through univariate and multivariate logistic regression analyses. *P < 0.05, **P < 0.01, ***P < 0.001. ns, no significance.

ALOX12 expression group patients benefit more from immunotherapy, especially anti-CTLA-4 and anti-PD1 treatments.

CMap Analysis Determined Potential Compounds/Inhibitors for CRC

The candidate compounds of CRC that might target pathways related to ALOX12 were investigated. CMap, a systematic data-driven method to discover the relationships among chemicals, biological conditions, and genes, was further employed. As illustrated in **Figure S3B**, 15 potential CRC therapeutic agents that are highly associated with ALOX12 were finally observed.

DISCUSSION

CRC is a highly malignant cancer with dismal recurrence and mortality rates (34). Bevacizumab is an anti-angiogenic drug mainly

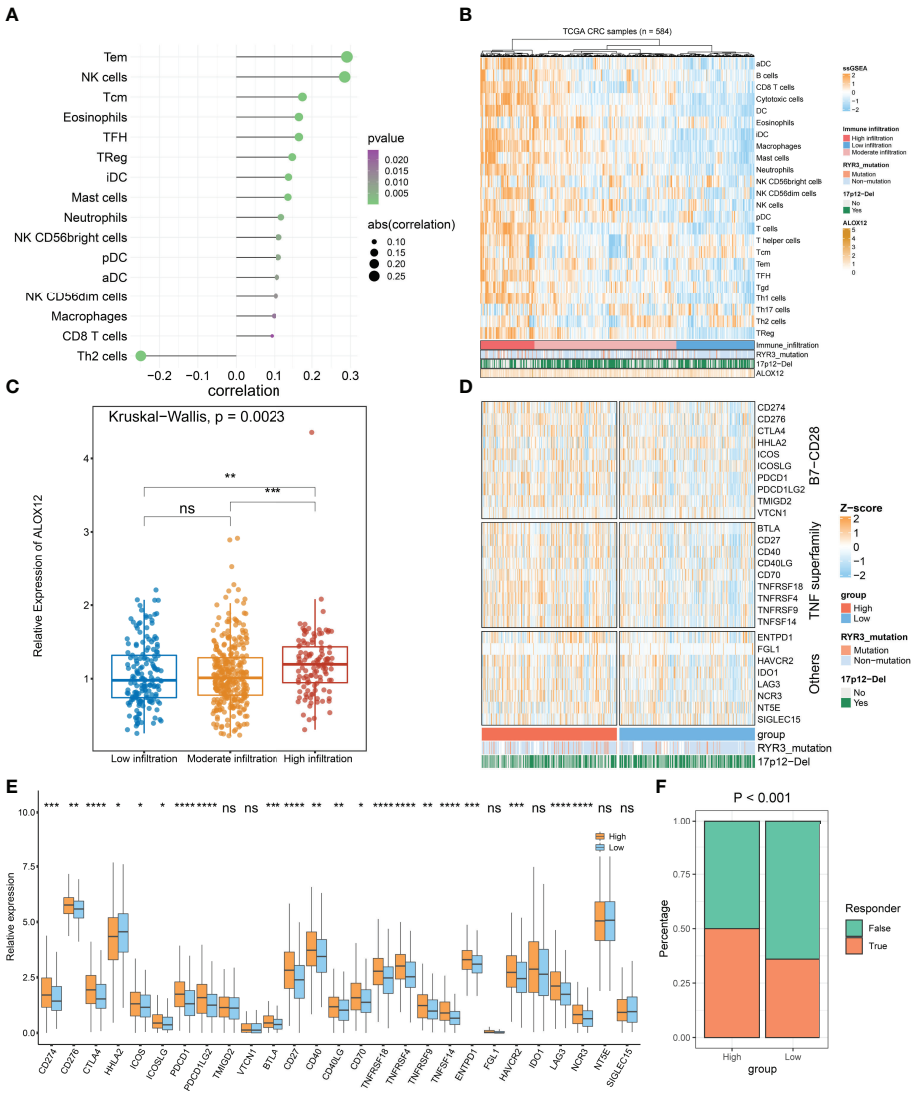

FIGURE 8 | Tumor immune microenvironment landscape, immune checkpoint profiles, and immunotherapy response prediction of ALOX12 in TCGA-CRC. **(A–C)** The correlation analysis between ALOX12 and 24-immune-cell infiltration abundance. **(D, E)** Heatmap and boxplot of 27 immune checkpoint profiles in high and low ALOX12 expression groups. **(F)** The result of the tumor immune dysfunction and exclusion (TIDE) method. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. ns, no significance..

applied in advanced mCRC. Past studies indicated that bevacizumab had obtained great benefits in combination with multiple treatment modalities such as chemotherapy, neoadjuvant chemotherapy, and immunotherapy (9). However, the response of CRC patients with different stages to bevacizumab was heterogeneous (35). Over the past years, immunotherapy has exhibited a huge sensation owing to its dramatic efficacy in the treatment of solid cancers. In 2017, ICI therapy was approved for the treatment of patients with advanced microsatellite instability (MSI-H) or DNA mismatch repair (dMMR) deficiency in CRC. Nonetheless, unlike dMMR/MSI-H CRC, immunotherapy performed limited benefits for other CRC patients (2). Considering the poor prognosis and the significant heterogeneity of bevacizumab response and immunotherapy in CRC, exploring a novel biomarker to comprehensively evaluate the prognostic and the response to bevacizumab and immunotherapy is of great significance.

To gain new insights into the mechanisms underlying bevacizumab response, we employed WGCNA to identify bevacizumab response-related modules. GO and KEGG enrichment analyses exhibited that most genes in these modules were enriched in cell proliferation and tumorigenesis-related pathways including DNA replication, P53 signaling pathway, cell cycle, and cell division pathways, indicating that the inhibition of cell proliferation processes might be a potential mechanism for bevacizumab to suppress the progression and migration of CRC. In

the present study, patients with a higher ALOX12 expression exhibited a better bevacizumab response. Zhonghua Zheng et al. reported that the expression of ALOX12 facilitated the proliferation of tumor cells (36), which was proved in the colony formation, CCK-8 proliferation, and EdU incorporation assays, and consistently with the results of GO and KEGG.

Elegant studies have revealed that ALOX12 encodes arachidonic acid 12-lipoxygenase and is widely expressed in various cell types. Due to the function of regulating cell migration, platelet aggregation, and tumor cell proliferation, ALOX12 was mainly associated with the occurrence and procession of diseases like atherosclerosis, thrombosis, and tumors (36). In our research, a high expression of ALOX12 revealed worse OS, RFS, and PFS in CRC, which was consistent with previous reports in kidney, breast, and prostate tumors (37). GSEA enrichment analysis suggested that ALOX12 was tightly associated with tumor occurrence and metastasis pathways such as ECM receptor interaction, NOTCH signaling pathway, pathways in cancer, and positive regulation of GTPase activity. Additionally, based on the median expression of ALOX12, patients were divided into high and low groups. GSVA demonstrated that most of the tumor-related pathways exhibited significant differences between the high and low ALOX12 expression groups, further validating the important potential functions of ALOX12 in tumors.

Generally, similar links exist between landscape diversity and landscape function (38). In the present study, gene mutation frequencies, SNPs, and indels were not identical in the mutant landscape of ALOX12, suggesting that there were differences between the high- and low-expression groups in gene levels. Of note, the mutation frequency of RYR3 was significantly frequent in the high-expression group, indicating that RYR3 mutation drives the expression of ALOX12. Lina Zhang and Zhen-Hao Liu et al. reported that RYR3 mutations were closely related to the prognosis and metastasis of breast and combined hepatocellular cholangiocarcinoma (CHC) (39, 40). From what we can speculate, high ALOX12 expression CRC patients tend to exhibit a worse prognosis and were more likely to undergo metastasis; subsequently, it was verified in wound healing and transwell assays. In parallel, our results indicated that the deletion of chromosome fragment 17p12 inhibited the expression of ALOX12. Studies by Han et al. and Kim et al. demonstrated that the absence of 17p12 in multivarious cancers such as breast and serous ovarian cancer promoted the resistance to neoadjuvant chemotherapy (41, 42). Given this, we hypothesize that patients with low ALOX12 expression in CRC are more likely to develop chemotherapy resistance, while it needs further clinical and experimental verification.

As is known to all, TMB was regarded as a sign of immunotherapy response in some tumors. High TMB normally indicated a better immunotherapy response (43, 44). In our research, patients with a high ALOX12 expression displayed a higher TMB. Additionally, we investigated the relationship between ALOX12 expression and immune cells and checkpoints. The results suggested that the higher the expression of ALOX12, the more abundant the infiltration of immune cells. Moreover, differences in ALOX12 expression between the high immune infiltration subtype and the moderate and low immune infiltration subtypes were dramatically significant. Previous studies confirmed that CTLA-4, PD-1, and PD-L1 as immune checkpoints could prevent the immune system from killing cancer cells by inhibiting the autoimmunity (45, 46). As we expected, patients with a high expression of ALOX12 illustrated higher levels of PD-1, PD-L1, and CTLA-4, indicating that high ALOX12 expression patients benefit more from ICB therapy. On the basis of the above results, SubMap and TIDE methods were further employed to predict the benefits of ICB treatment for CRC patients. Consistently, patients with a high ALOX12 expression indicated better ICB benefits. Last but not least, we investigated the potential drug targets for CRC patients, and 15 potential CRC therapeutic agents associated with ALOX12 were finally observed, which provided new insights into precision therapy for CRC patients.

Although ALOX12 is a promising comprehensive biomarker, some limitations should be acknowledged. Firstly, although we comprehensively searched public databases for bevacizumab treatment cohorts, the sample size is still limited, and future studies should be conducted in a larger sample cohort. Secondly, all the samples in our study were retrospective; future validation of ALOX12 should be conducted in prospective fresh samples. Thirdly, due to limited CRC recurrence data, we only explored the predictive performance of ALOX12 for recurrence in TCGA cohort, and the results suggest that ALOX12 is a promising CRC recurrence-predictive marker; however, future validation using more recurrence cohorts is necessary.

In summary, based on systematic and comprehensive bioinformatics analyses and experimental verification, we identified a stable and powerful biomarker, which filled the gap in this field to comprehensively predict the bevacizumab response, prognosis (OS, RFS, and PFS), and immunotherapy effects for CRC patients. In addition, high expression in tumor tissues makes ALOX12 easier to be detected, increasing its utility for clinical applications. GSEA, GSVA, and multi-omics data analysis demonstrated that high ALOX12 expression patients were not only tightly related to tumor development and metastasis but also possessed potential benefits for chemotherapy. In conclusion, our study provided a powerful biomarker for CRC patients, which exhibited a dramatic significance in clinical therapeutic regimen optimization, prognostic risk assessment, precision treatment, and the individualized treatment regimen formulation of CRC.

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

The studies involving human participants were reviewed and approved by the Ethics Committee Board of The First Affiliated

Hospital of Zhengzhou University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZL and SW contributed to the study design and data analysis. HX contributed to the manuscript writing. XH and JR contributed to the project oversight and manuscript revisiting. SW and XG collected the samples and generated the data. YR, QD, LL, JZ, and PL contributed to the manuscript revisiting. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.910582/full#supplementary-material

Supplementary Figure 1 | Immunohistochemical results of tumor and normal CRC tissues.

Supplementary Figure 2 | Topological overlap matrix and the prognostic analysis of ALOX12. **(A)** Topological overlap matrix of selected genes. **(B)** Kaplan-Meier survival analysis of ALOX12 in our in-house cohort. **(C–G)** Univariate Cox regression analysis of six genes with AUC>0.7 in TCGA-CRC, GSE72970, and our cohort.

Supplementary Figure 3 | The result of Submap algorithm **(A)** and Connectivity map analysis **(B)**.

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Efficacy and Safety of Neoadjuvant Monoimmunotherapy With PD-1 Inhibitor for dMMR/MSI-H Locally Advanced Colorectal Cancer: A Single-Center Real-World Study

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Objective: To explore the efficacy and safety of single-agent programmed cell death protein-1 (PD-1) inhibitor in the neoadjuvant treatment of patients with mismatch repair-deficient (dMMR) or microsatellite instability-high (MSI-H) locally advanced colorectal cancer (LACRC) through single-center large-sample analysis based on real-world data in China.

Methods: This study was a retrospective, single-center, case series study. 33 colorectal cancer (CRC) patients with clinical stage of T3~4N0~2M0 treated in Yunnan Cancer Hospital from June 2019 to June 2021 were analyzed retrospectively. Among them, 32 patients were dMMR or MSI-H or both dMMR and MSI-H, and one patient was both dMMR and microsatellite stability (MSS) (excluded in the final analysis). All 32 patients received neoadjuvant immunotherapy (nIT) with single-agent PD-1 inhibitor.

Results: Among the 32 patients, 8 (25%) were locally advanced rectal cancer (LARC) and 24 (75%) were locally advanced colon cancer (LACC); 4 (12.55%) were stage II and 28 (87.5%) were stage III. The median number of cycles of 32 patients with dMMR/MSI-H LACRC receiving nIT with single-agent PD-1 blockade was 6 (4~10), and the median number of cycles to achieve partial response (PR) was 3 (2~4). Among them, three LARC patients achieved clinical complete response (cCR) and adopted the watch-and-wait (W&W) strategy. The objective response rate (ORR) of the other 29 patients with radical surgery was 100% (29/29), the pathological response rate was 100% (29/29), the rate of major pathological response (MPR) was 86.2% (25/29), and the rate of pathological complete response (pCR) was 75.9% (22/29). The incidence of immune-related adverse events (irAEs) in 32 patients during nIT was 37.5% (12/32), while the incidence of irAEs in 22 patients with operation during adjuvant immunotherapy was 27.3% (6/22), all of which were grade 1~2. No grade 3 or above irAEs were occured. The median time from the last

nIT to surgery was 27 (16~42) days. There were no delayed radical resection due to irAEs in these patients. All 29 patients achieved R0 resection. The incidence of surgical-related adverse events (srAEs) in perioperative period was 10.3% (3/29).

Conclusions: Neoadjuvant monoimmunotherapy with PD-1 inhibitor has favorable ORR and pCR rate, and relatively low incidences of irAEs and srAEs for patients with dMMR/MSI-H LACRC, suggesting that this nIT regimen of single-agent PD-1 inhibitor is significantly effective and sufficiently safe.

Keywords: locally advanced colorectal cancer, neoadjuvant immunotherapy, programmed cell death protein-1 inhibitor, mismatch repair-deficient, microsatellite instability-high (MSI-H)

INTRODUCTION

The latest global cancer statistics displayed that colorectal cancer (CRC) is one of the most frequent malignancies, and that CRC ranked third in incidence rate and second in mortality rate (1). The great majority of CRC patients are in the stage of local progression upon diagnosis, which makes effective treatment more hard. Locally advanced colorectal cancer (LACRC) is defined as CRC stage II (clinical T3 to 4, N0) and stage III (any clinical T, N1 to 2). In recent years, advancements in standardized surgery and subsequent enhancements in neoadjuvant therapies have improved the survival and prognosis of LACRC patients. Nevertheless, surgical complications, and adverse events (AEs) caused by neoadjuvant chemotherapy (nCT) or neoadjuvant chemoradiotherapy (nCRT) remain significant and inevitable problems.

With the advent of the era of precision medicine, scientists have begun to explore the impact of microsatellite status on the tumor characteristics (2). Previous studies have found that mismatch repair-deficient (dMMR) or microsatellite instability-high (MSI-H) CRC accounts for 10% ~ 15% of all CRC, mostly colon cancer, and only 5% of rectal cancer (3). Among them, dMMR/MSI▪H metastatic CRC (mCRC) accounted for only 4%, while the proportion of dMMR/MSI▪H LACRC increased to 12% ~ 20% (4).

What is the efficacy of current neoadjuvant therapies for patients with MSI-H/dMMR LACRC? With regard to nCT for dMMR/MSI▪H LACRC, the FOxTROT study indicated that there was no tumor regression and no benefit in 2-year survival after nCT for dMMR locally advanced colon cancer (LACC) (5). Meanwhile, a retrospective analysis from Memorial Sloan Kettering Cancer Center demonstrated that almost 30% of dMMR locally advanced rectal cancer (LARC) treated with nCT exhibited disease progression (6).These two studies thus suggested that dMMR/MSI-H LACRC has obvious resistance to nCT. In terms of nCRT for dMMR/MSI▪H LARC, this study reported that the pathological complete response (pCR) rate of dMMR LARC after nCRT was 14% (6). Meanwhile, a retrospective study indicated that the tumor downstaging rate of dMMR LARC patients after nCRT was 70% (7). Besides, another study demonstrated that the pCR rate of dMMR LARC after nCRT was up to 27.6% (8). The above studies illustrated that dMMR/MSI-H LARC is sensitive to nCRT. Nevertheless, to some extent, nCRT can leads to post-surgical morbidities, such as anastomotic leakage and poor healing of the perineal wound, as well as long-term organ and function damage, such as urination and sexual dysfunction, as well as loss of anal sphincter function.

Fortunately, the emergence of neoadjuvant immunotherapy (nIT) brings new hope to these patients (9). The NICHE study published in *Nature Medicine* in 2020 has demonstrated that the pCR rate of 60% (12/20) in the treatment of dMMR/MSI-H stage I-III colon cancer with nivolumab combined with ipilimumab (10). This trial shows that immune checkpoint inhibitors (ICIs) may have great application space in the comprehensive treatment decision-making of non-mCRC in the future. Meanwhile, the PICC study published in *Lancet Gastroenterol Hepatol* in 2021 suggested that the pCR rate of initially resectable dMMR/MSI-H LACRC treated with treprizumab alone could reach 65% (11). This study has largely promoted the application of immunotherapy in the neoadjuvant setting for LACRC patients with dMMR/MSI-H.

Notwithstanding, data on nIT for LACRC are still scarce. Therefore, our center conducted a retrospective exploratory analysis to evaluate the safety and effectiveness of nIT with single-agent programmed cell death protein-1 (PD-1) inhibitor for dMMR/MSI-H LACRC.

MATERIALS AND METHODS

Patients Selection

This study is a retrospective, single center, case series study. The clinical data of 32 patients with clinical stage T3~4N0~2M0 dMMR/MSI-H CRC treated in Yunnan cancer hospital/The Third Affiliated Hospital of Kunming Medical University from June 2019 to June 2021 were collected excluding one patient with both dMMR and microsatellite stability (MSS). The baseline demographic and clinical characteristics of all patients with dMMR/MSI-H LACRC are indicated in **Table 1**. All data collected in this study have obtained informed consent. This study has received the full approval of the ethics committee of Yunnan cancer hospital/The Third Affiliated Hospital of Kunming Medical University.

TABLE 1 | Baseline demographic and clinical characteristics of patients with dMMR/MSI-H LACRC.

	nIT with PD-1 inhibitor group (n=32)
Age, years	44(23-62)
Sex	
Female	15/32 (46.88%)
Male	17/32 (53.13%)
ECOG performance status	
0	18/32 (56.25%)
1	14/32 (43.75%)
Gene detection of LS	9
LS associated gene mutation detected	3/9
MSH2 mutation	2
MLH1 mutation	1
LS associated gene mutation not detected	6/9
Suspected LS without genetic testing	5
Personal history of endometrial cancer	2/5
Family history of CRC	2/5
Family history of extra-intestinal malignancies	1/5
Previously received nCT	6/32 (18.75%)
Previously received nCRT	2/32 (6.25%)
Primary tumor location	
Ascending colon	7/32 (21.88%)
Hepatic flexure	4/32 (12.55%)
Transverse colon	4/32 (12.55%)
Splenic flexure	2/32 (6.25%)
Descending colon	2/32 (6.25%)
Sigmoid colon	2/32 (6.25%)
Rectosigmoid junction	3/32 (9.38%)
Rectum	8/32 (25%)
Clinical T stage	
T3	6/32 (18.75%)
T4	26/32 (81.25%)
Clinical N stage	
N0	4/32 (12.55%)
N1	5/32 (15.63%)
N2	23/32 (71.88%)
Clinical TNM stage	
II	4/32 (12.55%)
III	28/32 (87.5%)
Histological appearance	
Well differentiated	9/32 (28.13%)
Moderately differentiated	13/32 (40.63%)
Poorly differentiated	10/32 (31.25%)
Loss of expression of MMR proteins	
MLH1 only	2/26 (7.69%)
MSH2 only	5/26 (19.23%)
MSH6 only	1/26 (3.85%)
PMS2 only	4/26 (15.38%)
MLH1 and PMS2	10/26 (38.46%)
MSH2 and MSH6	3/26 (11.54%)
MSH2, MSH6 and PMS2	1/26 (3.65%)
Not tested	6 (18.75%)
MSI status	
MSI-H	17
Not tested	15
KRAS status	
Mutant type	6/17 (35.29%)
wild type	11/17 (64.71%)
NRAS status	
Mutant type	2/17 (11.76%)
wild type	15/17 (88.24%)
BRAF V600E status	
Mutant type	1/17 (5.88%)
wild type	16/17 (94.12%)

LACRC, Locally advanced colorectal cancer; LARC, Locally advanced rectal cancer; dMMR, Mismatch repair-deficient; MSI-H, Microsatellite instability-high; ECOG, Eastern cooperative oncology group; LS, Lynch syndrome; CRC, Colorectal cancer; nIT, neoadjuvant immunotherapy; nCT, neoadjuvant chemotherapy; nCRT, neoadjuvant chemoradiotherapy; TNM, Tumor Node Metastasis; MMR, mis-match repair; MSI, microsatellite instability.

One LARC patient who received nIT in this study was both dMMR and MSS, so the patient was not included in the baseline analysis.

Inclusion Criteria

1) Age from 18 to 75 years old;
2) Diagnosed with CRC by pathology;
3) The clinical stage of initial diagnosis by imaging is T3~4N0~2M0;
4) Confirmed as dMMR by immunohistochemistry (IHC) or MSI-H by polymerase chain reaction (PCR);
5) Eastern Cooperative Oncology Group (ECOG) physical status score ≤ 1 (12);
6) Treatment with neoadjuvant anti-PD-1 monotherapy (no manufacturer limited), regardless of whether nCRT or nCT has been received before;
7) Have not received radical surgery for this CRC before;
8) No history of biological therapy, immunotherapy or other experimental drug therapy;
9) Not accompanied by systemic infection requiring antibiotic treatment;
10) Not combined with immune system diseases.

Exclusion Criteria

1) One patient with discordant mis-match repair (MMR) and microsatellite instability (MSI) testing was excluded from the current analysis.

Data Collection

The data includes complete basic information of patients, serum carcinoembryonic antigen (CEA), colonoscopy, pathological biopsy, chest/abdomen/pelvic enhanced CT, pelvic high-resolution MRI, MMR proteins expression, and MSI status, etc. Efficacy evaluation and subsequent treatment approaches including surgery or watch-and-wait (W&W) strategy, and postoperative pathological outcomes including pTNM staging and tumor regression grade (TRG) grade were also recorded.

Treatment Methods

All 32 patients who met the inclusion and exclusion criteria received nIT with single-agent PD-1 inhibitor, of which 4 patients used

pembrolizumab, 9 patients used sintilimab and 19 patients treated with tiselizumab. Within the first day of each treatment cycle, they received nIT with intravenous drip of 200mg PD-1 inhibitor. The duration of each cycle of treatment was 3 weeks, regardless of how many courses of treatment were used (200 mg IV Q3W). Before using nIT, regardless of whether you have received nCT or nCRT. All patients underwent radical resection or W&W strategy. Of the 29 patients who underwent surgery, 22 received adjuvant immunotherapy with PD-1 blockade.

Observation Indicators and Evaluation Criteria

Evaluation Indicators of Imaging Efficacy After nIT

Objective response rate (ORR): The proportion of patients whose tumor volume shrinks to a predetermined value and can maintain the minimum time limit is the sum of the proportions of complete response (CR) and PR, that is, ORR=CR+PR. The efficacy of nIT were assessed by the Response Evaluation Criteria in Solid Tumors RECIST Version 1.1 (RECIST 1.1) (13).

Evaluation Indicators of Pathological Efficacy After nIT

Tumor Regression Grade

According to the TRG of National Comprehensive Cancer Network (NCCN) (14).

Pathological Complete Response

Defined as tumor regression induced by neoadjuvant therapy, there is no residual cancer cells or positive lymph nodes in pathology (pCR=TRG-0) (14).

Major Pathological Response

Considered as tumor regression induced by neoadjuvant therapy, with pathological residual tumor ≤ 10% (PCR = TRG-0 + TRG-1) (14).

Adverse Events During Immunotherapy

Refers to the AEs related to preoperative nIT and/or postoperative adjuvant immunotherapy that occurred from the beginning of nIT to the end of follow-up. Treatment-related adverse events were assessed by the Common Adverse Event Evaluation Criteria (CTCAE) version 5.0 published by the US Department of Health and Human Services (15). Among them, immune-related adverse events (irAEs) were evaluated according to literature standards (16).

Operation and Surgical-Related Adverse Events

The time from the end of nIT to surgery was defined as the time from the end of the last administration to surgery. Perioperative complications refer to complications directly or indirectly related to surgery that occurred from the day of surgery to 30 days after surgery. The grading of surgical complications was based on the Clavien-Dindo grading evaluation standard (17).

Follow Up Methods

Both patients who underwent surgery and those who adopted the W&W strategy were followed up every 3 months within 2 years, and every 6 months thereafter. During each follow-up, routine blood test, CEA, and enhanced CT examination of chest, abdomen and pelvis. were required for all patients. On this basis, patients underwent W&W strategy need to add the pelvic MRI, transrectal ultrasound and digital rectal examination. If there are suspicious lesions, colonoscopy and biopsy should be added. The last follow-up time was March 31, 2022.

Statistical Analysis

All data were processed using SPSS 24.0 statistical software. Measurement data were represented by M (range). Categorical variables were compared using the $\chi 2$ test or Fisher's exact test. The difference was statistically significant with $P<0.05$.

RESULTS

Characteristics of the Patients

The study profile of nIT in patients with LACRC is shown in **Figure 1**. One patient with both dMMR and MSS LARC was excluded from efficacy evaluation. There were 17 males and 15 females, with a median age of 44 (23–62) years. Of all patients, 8 cases were rectal cancer and 24 cases were colon cancer (7 cases in ascending colon, 4 cases in hepatic flexure of colon, 4 cases in transverse colon, 2 cases in splenic flexure of colon, 2 cases in descending colon, 2 cases in sigmoid colon and 3 cases at the junction of rectum and sigmoid colon). 6 cases were clinical T3 stage and 26 cases were clinical T4 stage. 4 cases were clinical N0 stage and 28 cases were clinical N+ stage. There were 4 cases of clinical TNM in stage II and 28 cases in stage III. 9 cases were well differentiated, 13 cases were moderately differentiated and 10 cases were poorly differentiated. 15 patients were diagnosed with dMMR by IHC. 6 patients were detected as MSI-H by PCR. Another 11 cases were detected as both dMMR and MSI-H by IHC and PCR.

Efficacy Evaluation of nIT With PD-1 Inhibitor

32 LACRC patients received neoadjuvant monoimmunotherapy with PD-1 blockade, of which 4 patients treated with pembrolizumab, 9 patients used sintilimab and 19 patients treated with tiselizumab. Among them, 2 LARC patients received nIT after the failure of nCRT and 6 LACC patients received nIT after the failure of nCT.

These 8 LACRC patients with second-line nIT were mainly because after the failure of first-line standard neoadjuvant therapies, our center detected their tumor tissues as dMMR by IHC or/and MSI-H by PCR, and developed a nIT regimen through multi-disciplinary team (MDT) discussion, which achieved significant ORR and pCR. Based on this, later, all newly diagnosed patients with CRC in our center were routinely tested for MMR or MSI status. If it is found to be dMMR or/and MSI-H, we began to try the exploration of first-line nIT. Therefore, the subsequent 24 LACRC patients were

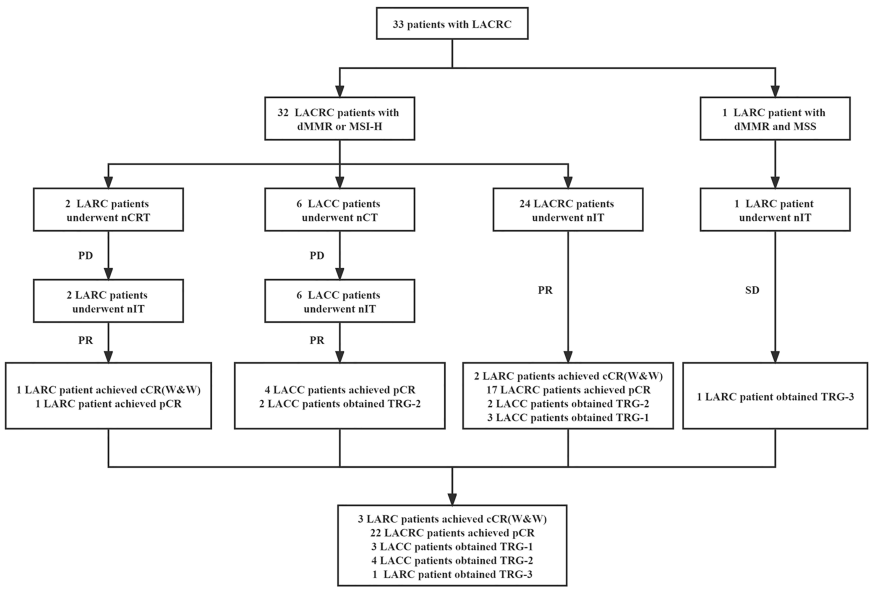

FIGURE 1 | Study profile of nIT in patients with LACRC. LACRC, Locally advanced colorectal cancer; LARC, Locally advanced rectal cancer; LACC, Locally advanced colon cancer; dMMR, Mismatch repair-deficient; MSI-H, Microsatellite instability-high; MSS, Microsatellite stability; nIT, Neoadjuvant immunotherapy; nCRT, Neoadjuvant chemoradiotherapy; nCT, Neoadjuvant chemotherapy; cCR, Clinical complete response; pCR, Pathological complete response; W&W, Watch-and-wait; TRG, Tumor regression grade.

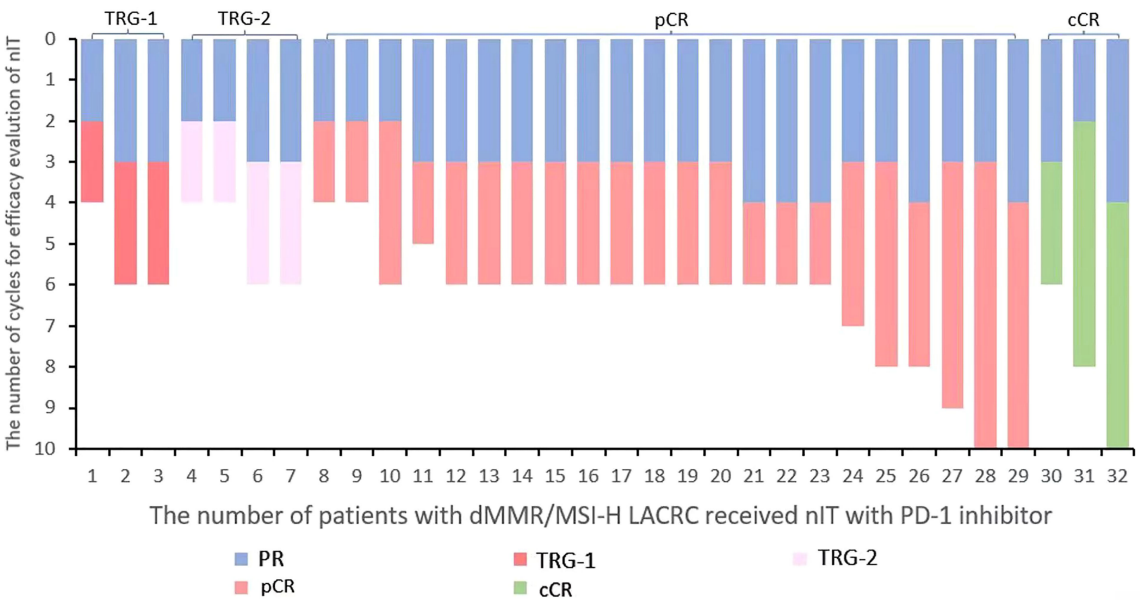

FIGURE 2 | Waterfall plot of efficacy evaluation of nIT in patients with dMMR/MSI-H LACRC. LACRC, Locally advanced colorectal cancer; dMMR, Mismatch repair-deficient; MSI-H, Microsatellite instability-high; nIT, Neoadjuvant immunotherapy; PD-1, Programmed cell death protein-1; PR, Partial response; pCR, Pathological complete response; cCR, Clinical complete response.

directly treated with first-line nIT when they were initially detected as dMMR/MSI-H.

The median number of cycles of 32 patients with dMMR/MSI-H LACRC receiving nIT with single-agent PD-1 inhibitor was 6 (4~10), that is, 18 weeks. The median number of cycles with efficacy evaluation reaching partial response (PR) was 3 (2~4) (**Figure 2**). Among 32 patients, three LARC patient achieved clinical complete response (cCR)

and adopted the W&W strategy. The ORR of the remaining 29 LACRC patients with surgery was 100% (29/29), the pathological response rate was 100% (29/29), the MPR rate was 86.2% (25/29), and the pCR rate was 75.9% (22/29) (**Table 2**).

Further subgroup analysis demonstrated that 25% (8/32) of LACRC patients with dMMR/MSI-H received nCT or nCRT and then changed to nIT after ineffective.

Among them, one LARC patient achieved cCR and adopted the W&W strategy. The ORR of the other seven LACRC patients was 100% (7/7), the pathological response rate was 100% (7/7), the MPR rate was 71.4% (5/7), and the pCR rate was 71.4% (5/7); while the remaining 75% (24/32) of patients chose anti-PD-1 monotherapy as the initial first-line neoadjuvant therapy. Among them, two patients with LARC achieved cCR and adopted the W&W strategy. The ORR of the other 22 patients with LACRC was 100% (22/22), the pathological response rate was 100% (22/22), the MPR rate was 90.9% (20/22), and the pCR rate was 77.3% (17/22) (**Table 2**).

IrAEs of nIT With PD-1 Inhibitor

The incidence of irAEs was 37.5% (12/32) during the nIT phase. Among them, the incidence of grade 1 irAEs was 31.25% (10/32), and grade 2 irAEs was 6.25% (2/32).

Meanwhile, after receiving nIT with single-agent PD-1 inhibitor, no grade 3 or above irAEs occurred in all patients. Furthermore, seven LACRC patients who achieved pCR after nIT and surgery did not receive postoperative adjuvant immunotherapy. While in the stage of adjuvant immunotherapy, the incidence of irAEs was 27.3% (6/22), all of which were grade 1 (**Table 3**).

Operation and srAEs After nIT With PD-1 Inhibitor

The median time from the last nIT to operation was 27 (16~42) days. All 29 patients underwent radical tumor resection. Among them, radical resection of rectal cancer was performed in 4 cases, radical resection of colon cancer in 24 cases, and resection of rectal cancer combined with other organs in 1 case. There were no delayed radical surgery due to irAEs in all patients. Intraoperative bleeding, postoperative bleeding and incision infection occurred in three patients (10.3%) respectively (**Table 3**). The other 26 patients had no srAEs such as intestinal obstruction, anastomotic bleeding, anastomotic stenosis and anastomotic fistula and so on.

IrAEs of Adjuvant Immunotherapy With PD-1 Inhibitor

Among the 29 patients who underwent surgery, 22 patients received adjuvant immunotherapy, while 7 patients did not received. The incidence of irAEs in these 22 patients during adjuvant immunotherapy was 27.3% (6/22) (**Table 3**). Whether during nIT or adjuvant immunotherapy, all irAEs were grade 1~2, mainly gastrointestinal reactions and skin adverse reactions. No irAEs of grade 3 or above were found. All irAEs recovered after symptomatic treatment during immunotherapy.

Follow Up Results

The last follow-up date was March 31, 2022. The median follow-up time of all 32 patients was 14 (3–28) months. No local recurrence or distant metastasis was found in all LACRC patients.

TABLE 2 | Pathological outcomes of dMMR/MSI-H LACRC patients treated with nIT and surgery.

	nIT with PD-1 inhibitor group (n=29)	First-line nIT with PD-1 inhibitor group (n=22)	Second-line nIT with PD-1 inhibitor group (n=7)
ORR	29/29 (100%)	22/22 (100%)	7/7 (100%)
Pathological response rate	29/29 (100%)	22/22 (100%)	7/7 (100%)
MPR rate	25/29 (86.2%)	20/22 (90.9%)	5/7 (71.4%)
pCR rate	22/29 (75.9%)	17/22 (77.3%)	5/7 (71.4%)
TRG			
0	22/29 (75.9%)	17/22 (77.3%)	5/7 (71.4%)
1	3/29 (10.3%)	3/22 (13.6%)	0
2	4/29 (13.8%)	2/22 (9.1)	2/7 (28.6%)
3	0	0	0
Pathological T stage			
ypT0	22/29 (75.9%)	17/22 (77.3%)	5/7 (71.4%)
ypT1	3/29 (10.3%)	3/22 (13.6%)	0
ypT2	3/29 (10.3%)	2/22 (9.1%)	1/7 (14.3%)
ypT3	1/29 (3.5%)	0	1/7 (14.3%)
Pathological N stage			
ypN0	29/29 (100%)	22/22 (100%)	7/7 (100%)
ypN1	0	0	0
Pathological TNM stage			
ypT0N0M0	22/29 (75.9%)	17/22 (77.3%)	5/7 (71.4%)
ypT1N0M0-I	3/29 (10.3%)	3/22 (13.6%)	0
ypT2N0M0-I	3/29 (10.3%)	2/22 (9.1%)	1/7 (14.3%)
ypT3N0M0-IIA	1/29 (3.5%)	0	1/7 (14.3%)

LACRC, Locally advanced colorectal cancer; dMMR, Mismatch repair-deficient; MSI-H, Microsatellite instability-high; nIT, Neoadjuvant immunotherapy; ORR, Objective response rate; MPR, Major pathological response; pCR, Pathological complete response; TRG, Tumor regression grade; TNM, Tumor Node Metastasis.

In our study, one LARC patient who received nIT was both dMMR and MSS, so the patient was not included in the pathological evaluation. Three patients with dMMR/MSI-H low LARC achieved cCR after nIT and adopted the WW strategy, so these three patients were also excluded from pathological evaluation.

TABLE 3 | Immune-related adverse events (irAEs) and surgical-related adverse events (srAEs).

	nIT and surgery group (n=29)		nIT and W&W group (n=3)	
	Grade 1	Grade 2	Grade 1	Grade 2
irAEs during the neoadjuvant phase				
Obstruction	1	1	0	0
Hyperthyroidism	1	0	0	0
Nausea	1	0	0	0
Fatigue	1	0	1	0
Aminotransferase increased	1	0	0	0
Abdominal pain	1	0	0	0
Pruritus or rash	0	1	0	0
Decreased appetite	1	0	0	0
Arthralgia or myalgia	1	0	0	0
Fever	0	0	1	0
Total	8/32 (25%)	2/32 (6.25%)	2/32(6.25%)	0
srAEs				
Incision infection	0	1		
Intraoperative haemorrhage	1	0		
Postoperative haemorrhage	1	0		
Total	2/29 (6.9%)	1/29 (3.4%)		
Surgery and adjuvant immunotherapy group (n=22)				
irAEs during the adjuvant phase				
Dry mouth	1	0		
Dizziness	1	0		
Nausea	1	0		
Somnipathy	1	0		
Decreased appetite	1	0		
Pruritus or rash	1	0		
Total	6/22 (27.3%)	0		

nIT, Neoadjuvant immunotherapy; W&W, Watch-and-wait; irAEs, Immune-related adverse events; srAEs, Surgical-related adverse events.
one LARC patient who received nIT in this study was both dMMR and MSS, so the patient was not included in the baseline analysis. Seven patients with LACRC who achieved pCR after nIT and surgery did not receive postoperative adjuvant immunotherapy.

DISCUSSION

Patients with LACRC have a higher risk of recurrence and metastasis. Neoadjuvant therapies can improve the prognosis to a certain extent. Notwithstanding, the sensitivity of dMMR/MSI-H LACRC to nCT or nCRT remains low. The emergence of immunotherapy has brought hope to these patients. Immunotherapy was listed as the top ten scientific progress by *Science* in 2013. Based on the study of KEYNOTE-016 in 2015, it is confirmed that dMMR/MSI-H is a biomarker for the efficacy of immunotherapy, thus ushering in a novel era of immunotherapy in the field of CRC, and opening a new journey of precision immunotherapy in the era of precision medicine (18). Based on the results of CHECKMATE-142 and KEYNOTE-177 trials, the FDA has successively approved nivolumab ± ipilimumab or pembrolizumab for the second-line and first-line treatment of dMMR/MSI-H mCRC (19–22).

Whether immunotherapy can be used as neoadjuvant therapy for LACRC has become a hot topic. The NICHE study has demonstrated shocking results, marking the opening of the door of nIT for mCRC, opening up a novel treatment approach for patients with dMMR/MSI-H LACRC (10). Meanwhile, the lastest PICC study is a driving research on the application of nIT in dMMR/MSI▪H LACRC (11). The NCCN guideline recommended universal screening for Lynch syndrome (LS) in CRC patients with dMMR/MSI-H (23). Subsequently, the NCCN guidelines (v1.2021) for colon cancer (24) and rectal cancer (25) recommend that all newly diagnosed CRC patients should be tested for MMR proteins expression (including MLH1, MSH2, MSH6 and PMS2) or MSI (including BAT25, BAT26, D5S346, D2S123 and D17S250) status. The guidelines also recommend nivolumab ± ipilimumab or pembrolizumab (preferred) as an option for preoperative neoadjuvant treatment of resectable dMMR/MSI▪H mCRC (24, 25). This is the first time the NCCN recommended an immunotherapy as a neoadjuvant therapy for CRC. In the newest NCCN guideline (v1.2022) for colon cancer (26) just released this year, an updated point is that the neoadjuvant therapy regimen of nivolumab ± ipilimumab or pembrolizumab is considered as an option for patients with dMMR/MSI▪H cT4b colon cancer.

Nevertheless, the safety and effectiveness of immunotherapy in the neoadjuvant setting for dMMR/MSI-H LACRC still need more clinical exploration and verification.

In our study, the median number of cycles of 32 patients receiving nIT with PD-1 inhibitor was 6 (4 ~ 10), and the median number of cycles with efficacy evaluation reaching partial response (PR) was 3 (2 ~ 4). One LARC patient with both dMMR and MSS was excluded from efficacy evaluation. Among 32 patients with dMMR/MSI-H LACRC, three LARC patients achieved cCR and adopted the W&W strategy. The ORR of the other 29 LACRC patients was 100% (29/29), the pathological response rate was 100% (29/29), the MPR rate was 86.2% (25/29), and the pCR rate was 75.9% (22/29). Subgroup analysis demonstrated that the pCR rate of 22 patients with first-line nIT

was 77.3% (17/22), while that of 7 patients with second-line nIT was 71.4% (5/7). No significant difference in pCR rate was found between the two nIT groups. It can be seen that nIT could achieved high rate of tumor down-staging and pCR for patients with dMMR/MSI-H LACRC, regardless of whether they have received nCT or nCRT before.

The main reason is that CRC patients with middle- and early-stage have relatively sound immune systems, and their tumor burdens are generally not as severe. These LACRC patients express many tumor neoantigens, and this can increase the activity of anti-tumor immune T cells, followed by dispersal throughout the body and removal of micro-metastases (27). After a multi-line treatment, the immune microenvironment and physical state of LACRC patients has varying degrees of dysfunction. Therefore, a better response could theoretically be obtained through the earlier application of immunotherapy.

Further comparative analysis indicated that 20 patients with dMMR stage I ~ III colon cancer included in NICHE study were treated with nivolumab [3 mg/kg (day1, day15)] combined with ipilimumab [1 mg/kg (day1)] for 4 weeks, and the pCR rate was 60.0% (12/20) (10), and 17 patients with dMMR/MSI-H LACRC included in PICC study received single-agent nIT with treprizumab (200mg, q3w) for 6 cycles (18 weeks), and the pCR rate was 64.7% (11/17) (11). We found that the pCR rate (75.9%) of our study was significantly higher than the reported data of the above two nIT trials (10, 11). Among the 29 patients treated with surgery in our study, the median number of preoperative treatment cycles of single-agent nIT with PD-1 inhibitor was also 6 (18 weeks), and the median time from the last nIT to operation was 27 (16 ~ 42) days. Thus, the high pCR rate in our study may be related to more cycles of preoperative immunotherapy, longer treatment intervals, and the tailing effect of immunotherapy. In addition, the patients selection may also be related.

With regard to adjuvant therapy in this study, of the 29 patients who underwent surgery, 22 with clinical stage III received only adjuvant immunotherapy without combined chemotherapy and the remaining 7 including 4 cases of clinical stage II and 3 cases of stage III did not received any adjuvant therapy including adjuvant immunotherapy or chemotherapy. The postoperative pathological results of these 7 patients achieved pCR. Among them, 4 patients were stage II, of which 1 received 6 cycles, 1 received 7 cycles and 2 received 8 cycles of nIT, and the remaining 3 patients were stage III, of which 1 received 9 cycles and 2 received 10 cycles of nIT. It can be seen that these 7 patients received a long cycle of nIT and achieved pCR, which are the main reason why they did not receive postoperative immunotherapy or chemotherapy. Among the 22 patients with clinical stage III, 4 patients obtained TRG-2 in pathological results, of which 2 received 4 cycles, and 2 received 6 cycles of nIT, 3 patients obtained TRG-1, of which 1 received 4 cycles, 2 received 6 cycles of nIT, and the remaining 15 patients achieved pCR, of which 2 received 4 cycles, 1 received 5 cycles and 12 received 6 cycles of nIT. It can be found that the cycle of these 22 patients receiving nIT is relatively short, and about 32% of the patients did not reach pCR, which are the main reason for them to receive adjuvant therapy.

Patients with dMMR/MSI-H pathological stage II CRC have good prognoses in general. The current guideline recommendations is that adjuvant chemotherapy is not needed for patients with low-risk stage II CRC. Meanwhile, patients with dMMR/MSI-H pathological stage III CRC who received adjuvant chemotherapy have better prognoses than MSS patients (28, 29). However, only adjuvant immunotherapy was chose for postoperative treatment regime in those 22 patients with clinical stage III, and no or not combined chemotherapy was chose. The reason is that preoperative nIT has achieved significant tumor regression and less irAEs. Actually, the principle of "effective drugs without changing drugs" is followed. Can adjuvant immunotherapy further improve the prognosis of dMMR/MSI-H LACRC patients? Several ongoing phase III prospective clinical trials are trying to answer this question. Among them, the ATOMIC study (NCT02912559) is a clinical study of atezilizumab combined with chemotherapy versus chemotherapy in the adjuvant treatment of dMMR stage III CRC, and the primary endpoint is disease-free survival(DFS). The POLEM trial (NCT03827044) is a multicenter phase III randomized clinical trials(RCTs) of avelumab combined with fluoropyrimidine in the adjuvant treatment of dMMR or POLE nucleic acid exonuclease mutations. Another Phase III clinical trial (NCT-03803553) will evaluate the efficacy of nivolumab in patients with MSI-H CRC after standard adjuvant chemotherapy. We look forward to the outcomes of the above and more postoperative adjuvant immunotherapy studies.

Meanwhile, three patients with dMMR/MSI-H low LARC achieved cCR after nIT and adopted the W&W strategy in our study. The last time they received immunotherapy was 6 months, 9 months and 12 months respectively, and no local recurrence or distant metastasis was found during follow-up. The breakthrough efficacy of nIT for many patients with dMMR/MSI-H LACRC provides colorectal oncologists with great hope, especially for patients with dMMR/MSI-H low LARC. The published studies manifested that nIT was associated with less risk of anal sphincter dysfunction, sexual dysfunction, abnormal fecal control, and bladder dysfunction than traditional nCT and nCRT (30, 31). From the extremely high pCR rate obtained by nIT and the characteristics of lasting benefits once immunotherapy is effective, it can be inferred that patients with dMMR/MSI-H low LARC who achieve cCR after nIT are ideal people to adopt the W&W strategy, but it is undeniable that long-term follow-up and high-level evidences are needed to support this viewpoint.

Additionally, the LACRC patients in our study were either determined by IHC detection of MMR proteins expression status or by PCR detection of MSI status. Remarkably, one LACC patient was identified as dMMR (PMS2 protein was missing) by IHC but confirmed to be MSS (five loci were not changed) by PCR testing, that is, the patient's MSI and MMR status were inconsistent. The reasons for the simultaneous detection of dMMR and MSS may be that the loss of some MMR protein is compensated by their function, or the tumor heterogeneity caused by the methylation of MLH1 promoter, which will

affects the judgment of results (32). Although this patient obtained the opportunity of radical resection after 6 cycles of neoadjuvant anti-PD-1 monotherapy, both the preoperative imaging and postoperative pathological results suggested poor tumor regression, and the patient was the only case with TRG of grade 3 in this study.Therefore, the efficacy of immunotherapy may be reduced when the MMR protein expression and MSI status are inconsistent. Studies have demonstrated that poor response to ICIs in several patients with dMMR may be related to mis-judgment of their MMR status (19, 33). Although the study have pointed out high sensitivity and specificity for MMR protein expression status detected by IHC and MSI status identified by PCR or next-generation sequencing (NGS), and the consistency of the three test results > 95% (34), we believe that for patients with dMMR/MSI▪H LACRC who are recommended for nIT, it is the most consistent with the principle of accurate diagnosis to determine both the status of MMR protein expression and MSI before receiving nIT. Interestingly, several clues can be found from our study and previous study (35), such as young patients (< 40 years old), huge tumor volume, typical lynch syndrome, including family history of CRC or endometrial cancer, which can indirectly assist in suggesting the high possibility of dMMR/MSI▪H.

The incidence of irAEs in 32 patients during nIT was 37.5% (12/32), while the incidence of irAEs in 22 patients during postoperative adjuvant immunotherapy was 27.3% (6/22), all of which were grade 1~2, mainly gastrointestinal reactions and skin adverse reactions. No patient had grade 3 or above irAEs, which proved that the nIT regimen with single-agent PD-1 blockade was relatively safe in general. Nonetheless, larger-sample prospective studies are warranted. Moreover, all irAEs in our study have been reported in other immunotherapy studies (10, 11, 35). In this study, 29 patients underwent radical surgery and achieved R0 resection. There was no delay in radical surgery due to irAEs in all cases. Only 10.3% (3/29) of the srAEs occurred, and recovered after symptomatic treatment. At last, no perioperative death and no local recurrence or distant metastasis was found in all patients by now. Consequently, we can deem that the early application of immunotherapy does not increase the additional risk of surgery.

In addition, our research team found a phenomenon, that is, when re-examinating the colonoscopy, it was found that the tumor regression of many patients was very significant, and even the tumor could not be seen by the naked eye. Meanwhile, compared with the colonoscopy before treatment, the intestinal cavity at the tumor here was so narrow that the mirror body was often unable to pass through. Furthermore, the serous surface contracture at the tumor of the postoperative specimen can be seen. When the intestinal tube is opened, the intestinal cavity contracture at the tumor is narrow, and the mucosal surface tissue is hard, but the submucosa and muscular tissue are thickened, the texture is soft, and a large amount of mucus or necrotic components can be seen. The reason for the change may be that the histological changes of intestinal wall after ICIs treatment include thickening of lamina propria, shortening of villi, infiltration of neutrophils and apoptosis of crypt glands in the epithelial layer, and there are few lymphocytes in the epithelial layer (36). Of course, these findings are anecdotes or subjective feelings, which are unlikely to be quantified as objective indicators at present.

Our study is a rare, relatively large-sample single-center, real-world study of neoadjuvant monoimmunotherapy in dMMR/MSI-H LACRC, covering initially unresectable or resectable stage II-III colon cancer or rectal cancer. Despite diverse treatment methods, such as various types of PD-1 inhibitors, and varying cycles of nIT, and a few patients received nCT or nCRT before nIT, all patients received single-agent anti-PD-1 antibody as nIT strategy, and achieved ideal tumor regression with low irAEs and srAEs. Of course, this study also has several limitations. First, there is a lack of long-term follow-up data on quality of life and survival. Second, this study is a single-center, retrospective, so the level of evidence-based medicine is not high enough. Third, the tumor characteristics of patients in this study were inconsistent with those reported in other large-sample study (37). For example, the proportion of BRAF mutations in this study was relatively low, while the proportion of isolated MSH2 loss was relatively high, about 20%. There were only 17 patients detected by PCR and 26 patients detected by IHC. Hence, there may be relatively few cases in our study, resulting in a certain degree of deviation in the proportion. Hence, we will continue to expand the sample size in order to make more convincing comparisons and draw more rigorous conclusions.

CONCLUSIONS

In conclusion, the nIT regimen of single-agent PD-1 inhibitor is sufficiently secure and remarkably effective for patients with dMMR/MSI-H LACRC. Large-scale, multi-center prospective RCTs are warranted to further verify the long-term efficacy and safety, optimal number of cycles and predictive biomarkers of nIT for LACRC. Meanwhile, it can be predicted that the W&W strategy is a promising treatment approach for managing patients with dMMR/MSI-H low LARC who achieve cCR after nIT and will help promote colorectal surgery into a new individualized and precise "non-invasive" era.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The ethics committee of Yunnan cancer hospital/ The Third Affiliated Hospital of Kunming Medical University, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Drafting the work and/or revising it critically: XZ, RY, and TW. Final approval of the version to be published: XZ, and YuL. All authors contributed to the article and approved the submitted version.

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Combination of phototherapy with immune checkpoint blockade: Theory and practice in cancer

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Immune checkpoint blockade (ICB) therapy has evolved as a revolutionized therapeutic modality to eradicate tumor cells by releasing the brake of the antitumor immune response. However, only a subset of patients could benefit from ICB treatment currently. Phototherapy usually includes photothermal therapy (PTT) and photodynamic therapy (PDT). PTT exerts a local therapeutic effect by using photothermal agents to generate heat upon laser irradiation. PDT utilizes irradiated photosensitizers with a laser to produce reactive oxygen species to kill the target cells. Both PTT and PDT can induce immunogenic cell death in tumors to activate antigen-presenting cells and promote T cell infiltration. Therefore, combining ICB treatment with PTT/PDT can enhance the antitumor immune response and prevent tumor metastases and recurrence. In this review, we summarized the mechanism of phototherapy in cancer immunotherapy and discussed the recent advances in the development of phototherapy combined with ICB therapy to treat malignant tumors. Moreover, we also outlined the significant progress of phototherapy combined with targeted therapy or chemotherapy to improve ICB in preclinical and clinical studies. Finally, we analyzed the current challenges of this novel combination treatment regimen. We believe that the next-generation technology breakthrough in cancer treatment may come from this combinational win-win strategy of photoimmunotherapy.

KEYWORDS

immune checkpoint blockade, photothermal therapy (PTT), photodynamic therapy (PDT), immunogenic cell death (ICD), tumor-infiltrating T cells, nanoparticles

Introduction

Over the past several years, with the recognition of tumor immune escape, several immune checkpoint molecules have been identified for cancer immunotherapy, such as PD-1: PD-L1 and CTLA-4: CD80/CD86 (1–3). Tumors escape those T cell based immune surveillance by crippling T cells' functionality *via* upregulating the expression of immune checkpoint molecules (4). Blocking the interaction of PD-1 with PD-L1 or CTLA4 with CD80/CD86 restores T cell function, restarting and amplifying the antitumor immune response. Thus, immune checkpoint inhibitors (ICIs) against CTLA4 and PD-1 and its ligand PD-L1, such as Ipilimumab, Pembrolizumab, Nivolumab, and Atezolizumab, have been used to treat the malignant tumors and significantly improve the survival of patients (5–8). Immune checkpoint blockade (ICB) therapy has become a routine treatment for more than 20 different indications (9–11), including non-small cell lung cancer (12, 13), colon cancer (14, 15), esophageal cancer (16, 17), melanoma (18), renal cell carcinoma (19), bladder or urothelial carcinoma (20), breast cancer (21).

Although ICB has held great promise for cancer treatment, the efficacy of ICB only benefits a minority of patients, which is due to the low response rates (22). For example, only approximately 20% of non-small cell lung cancer patients respond to ICB therapy (23). Moreover, the response rate of ICB alone is less than 40% in melanoma (24), less than 20% in hepatocellular carcinoma (25), and less than 10% in triple-negative breast cancer (26). This low therapeutic response rate accounts for many tumors that have evolved sophisticated strategies to evade immune surveillance. Generally speaking, there are four main reasons for a poor response to ICB: 1) tumor antigen deficiency, 2) insufficient infiltration of T lymphocytes, 3) defects in the tumor antigen processing and presentation mechanism, and 4) an immunosuppressive tumor microenvironment (TME) (27). In conclusion, insufficient T cell infiltration caused by the lack of tumor-specific antigens is the main reason for the failure of ICB therapy.

In recent years, phototherapy, especially nanoparticle-based photoimmunotherapy, has been recognized as an effective strategy for promoting T cell infiltration and improving the efficiency of ICB. Nanoparticle-based phototherapy, mainly including photothermal therapy (PTT) and photodynamic therapy (PDT), exhibits potent antitumor efficacy, minimal invasiveness, slight side effects, and immune regulator functions in tumor treatment (28, 29). During PTT, photothermal agents convert light energy into heat under near-infrared (NIR) light irradiation to kill tumor cells (30). PDT destroys tumor cells by exogenously reactive oxygen species (ROS) generated from light irradiated photosensitizers (31). In cancer treatment, both PTT and PDT can induce the immunogenic cell death (ICD) of tumor cells. ICD is characterized by the release of damage-associated molecular patterns (DAMPs), tumor-associated antigens (TAAs), neoantigens, and proinflammatory cytokines from dying tumor cells that activate tumor-specific immune responses, including activating immune effector cells, promoting T -cells infiltration, and enhancing the secretion of cytokines (32). In addition, photothermal and photodynamic agents can also be coloaded with immunostimulant, small molecular inhibitors, chemotherapeutic drugs, or immunoadjuvants for effective combination treatments. For example, albumin paclitaxel has been loaded into gold nanocage nanoparticles for enhanced antitumor efficiency and ICD levels (33). Importantly, PTT and PDT have been approved clinically by numerous regulatory agents to treat local tumors, such as melanoma, esophageal cancer, and lung cancer (34, 35). Based on the advantage of phototherapy in regulating antitumor immune responses and promoting T cell infiltration, phototherapy combined with ICB is considered a promising strategy for cancer treatment. Indeed, PTT and PDT have been combined with ICIs for the treatment of malignant tumors and metastatic tumors in preclinical and clinical settings, especially for those patients who lack tumor-infiltrating T cells.

Above all, combining nanoparticle-based phototherapy and ICB therapy can improve the treatment outcome and prevent tumor recurrence and metastasis by activating a specific antitumor immune response (Figure 1). Moreover, nanoparticle-based phototherapy is a promising strategy to coordinate the combination of ICB with other treatment strategies, such as targeted therapy and chemotherapy. Here, we summarized the mechanism of phototherapy in cancer immunotherapy and discussed the current progress of phototherapy combined with ICB therapy to treat malignant tumors, including melanoma, non-small cell lung cancer, esophageal cancer, etc., in pre-clinical and clinical settings. In addition, the progress of phototherapy combined with targeted therapy or chemotherapy to improve ICB has also been summarized. Finally, the current challenges, deficiencies, and future improvements of this novel combination treatment regimen have also been analyzed.

Phototherapy for cancer treatment and immune regulation

Phototherapies, including PTT and PDT, have been used to treat malignant tumors in the clinic for more than 40 years (35). Several tumor types, including melanoma, esophageal, lung tumors, hepatocellular carcinoma, glioma, etc., have shown good response rates to PTT and PDT (36). In addition to providing an elegant solution to ablate primary tumors, phototherapy also provides a localized source of tumor antigens and DAMPs, promoting antigen presentation and

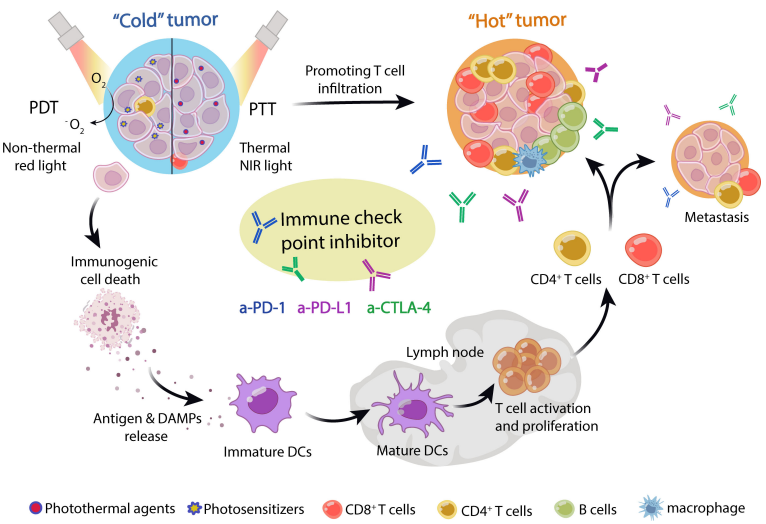

FIGURE 1
Schematic of phototherapy combined with ICB in cancer treatment. Nanoparticle-loaded photosensitizers or photothermal agents were efficiently enriched in tumor tissue. Upon laser irradiation, photosensitizers or photothermal agents generate reactive oxygen species (ROS) and heat energy in PTT and PDT, respectively, which induces immunogenic cell death (ICD) of tumors. The dying tumor cells released tumor neoantigens and DAMPs, which stimulated DC maturation and activated T cells. Then, the tumor-specific T cells were recruited into tumor niches and activated by ICIs, including anti-PD-1, anti-PD-L1, and anti-CTLA-4 antibody, which could effectively treat primary and metastatic tumors, as well as immune "cold" tumors. Thus, phototherapy can synergize with immune checkpoint blockade to treat tumors.

increasing systemic immunity to prevent tumor progression and metastasis. In fact, different types of phototherapies have different mechanisms of action: PTT can induce thermal tissue damage and achieve tumor thermal ablation without damaging normal cells and tissues, while PDT can induce chemical damage by generating ROS based on the local activation of photosensitizers in tumors. Therefore, in this section, we discuss the different mechanisms for anticancer immunotherapy treatment in PTT and PDT.

Role of PTT in cancer immunotherapy

PTT can take advantage of the photothermal effects of photothermal agents, obtaining energy from laser irradiation and converting it into heat. The absorption wavelength of photothermal agents is mostly in NIR-I window (750-1000 nm). At present, A small number of photothermal agents of NIR-II (1000-1500 nm) have been exploited. NIR-II light has better biological tissue penetration than red light (620-750 nm) and NIR-light (37). Once irradiated by light of a specific wavelength, the PTT agents absorb photon energy, migrating from the ground singlet state (S_0) to an excited singlet state (S_1). As the S_1 state PTT agents return to the S_0 state, they must undergo vibrational relaxation, a nonradiative form of decay that results in collisions between the excited photothermal agents and their surrounding molecules (38). This increased kinetic energy increases the surrounding microenvironment temperature (Figure 2A). When the temperature reaches 41°C, changes will happen in gene expression patterns, such as the generation of heat-shock proteins (HSP), to mitigate the effects of the initial thermal damage (39). Mild hyperthermia approximately 41-42° C can promote increased blood flow and improve the tumor's vascular permeability, enhancing the delivery of anticancer drugs to tumors (40). A further high temperature can cause tumor vessels to collapse, undergo necrosis, apoptosis, coagulation, and hypoxia. When the temperature of tumor tissue rises to 42°C, irreversible tissue damage will occur (41). For example, 42-46°C for 10 min will result in cell necrosis. In particularly, cell death is rapidly accelerated at 46-52°C owing to microvascular thrombosis and ischemia. When the temperature is above 60°C, protein denaturation happens, and the plasma membrane melts, leading to almost instant cell death (35).

In addition to directly killing tumor cells *via* hyperthermia, PTT also induces ICD in tumor cells, which releases TAAs and DAMPs to trigger the adaptive immune response and activate the tumor-specific immune response (42). Remarkably, PTT can provoke anticancer vaccine effects and produce long-term antitumor efficacy *in vivo*. DAMPs include high-mobility group box-1 (HMGB1), calreticulin (CRT), HSP90, HSP70, and adenosine triphosphate (ATP) (32). When thermal damage occurs, HMGB1 and ATP, serving as a "find me" signal for recruiting antigen-presenting cells (APCs), are released to the outside of the cells. CRT exposed on the cell membrane surface promotes phagocytosis by APCs as an "eat-me" signal (43). Moreover, HSPs can bind to TAAs to form HSP-antigen

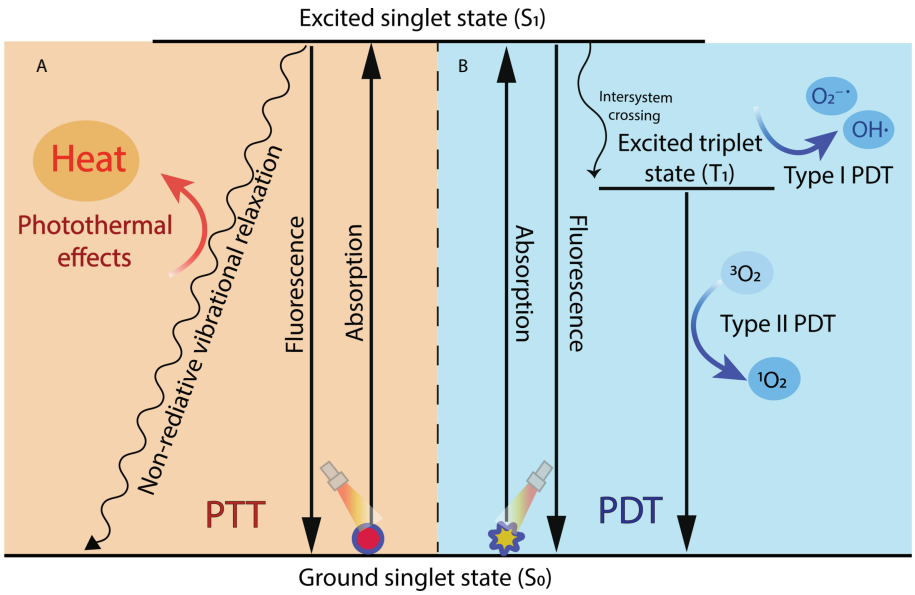

FIGURE 2
Mechanisms of photodynamic reaction and photothermal effect during PDT and PTT. **(A)** In PTT, when excited singlet state photothermal agents return to the ground state, they undergo nonradiative vibrational relaxation, increasing the surrounding microenvironment temperature and causing cell death. **(B)** In PDT, the excited singlet state of the photosensitizer transforms to a more stable excited T_1 through intersystem crossing and then finally generates ROS through type I reaction or type II reaction.

complexes, which can effectively activate APCs. Activated APCs migrate to lymphoid organs, where tumor antigens are presented to T cells, initiating a T cell-mediated immune response to eradicate cancer cells (44). These characteristics of PTT make it the ideal candidate for combined with immunotherapy. Photothermal immunotherapy is a new concept that combines PTT with immunotherapy. It can generate synergistic thermal immune effects, enhancing the control of primary tumors and metastases. ICD induced by PTT has been developed in combination with immunotherapies such as ICIs, immune adjuvants, and CAR T cell therapy to directly eliminate tumors and induce sustained antitumor immune effects. At present, photothermal immunotherapy has evolved from a concept to a promising clinical treatment for metastatic cancer.

Role of PDT in cancer immunotherapy

Unlike the direct tumor cell killing of PTT, PDT induces tumor cell apoptosis and necrosis by ROS generated from photosensitizers. PDT relies on photochemical reactions among three nontoxic components: photosensitizers, light, and oxygen dissolved in the cells. In general, photosensitizers used in PDT can be classified into three generations based on their evolution. The first generation of photosensitizers was constituted by naturally occurring porphyrins and their derivatives. Their phototherapy performance was limited by their red-light wavelength (about 630 nm) of excitation, which is difficult to reach deep tumors (45). The second-generation photosensitizers are synthetic compounds primarily based on porphyrins and chlorine structures. Compared with the first generation, the second-generation photosensitizers have more high purity, photosensitivity, tissue selectivity, and longer absorption wavelength in the visible-NIR (650-800 nm) (46). However, they still had poor water-solubility, body clearance rate, and low tumor selectivity. In recent years, third-generation photosensitizers are developed to solve the disadvantage of the second by utilizing chemical modification, nano-delivery system, or antibody conjugation (47). Another important trigger of PDT is laser irradiation. Upon laser irradiation, the electronic state of the photosensitizer is converted from the singlet primary energy state (S_0) to the unstable excited singlet state (S_1). Subsequently, part of the energy of the photosensitizer is radiated in the form of a quantum of fluorescence, transforming to a more stable excited triplet state (T_1). In addition, the T_1 state generates ROS through two mechanisms: type I and type II photodynamic reactions (48). In the type I pathway, T_1 directly reacts with endogenous cancerous substrates, such as cell membranes or biological macromolecules, and produces free radicals and anion radicals through a hydrogen or electron transfer. The radicals may further react with O_2 and water to generate ROS, including superoxide anions ($O_2^{-\cdot}$) and hydroxyl radicals ($OH^{\cdot}$). In the type II pathway, T_1 transforms the basic energetic state (the basic triplet state) O_2 into highly cytotoxic reactive singlet oxygen

($^1O_2$) by direct energy transfer. The generated singlet oxygen can oxidize macromolecular cellular components, such as nucleic acids, lipids, and proteins, which leads to cellular death by either apoptosis or necrosis (48–50) (Figure 2B).

PDT can kill tumor cells by generating ROS and activating an immune response against tumor cells. The different intracellular locations of photosensitizers will cause different types of ICD. For example, apoptosis is caused by mitochondria, the cell membrane destruction induces necrosis, and autophagy is provoked by lysosomes/endoplasmic reticulum damage (49). An acute inflammatory reaction and neoplasm infiltration by leukocytes can be led by local injuries and oxidative stress in tumor tissue induced by different types of PDT. In the tumor cells, damaged endothelial cells, and tumor stromal cells, PDT caused a rapid and massive release of proinflammatory mediators and cytokines, which participate in the recruitment of neutrophils and other myeloid cells. These proinflammatory mediators include arachidonic acid, cytokines, histamine, and the complement system. The cytokines include tumor necrosis factor, interleukin (IL)-1β, and IL-6. PDT also induces tumor ICD, which leads to the activation of antitumor immunity through danger signaling mechanisms caused by the activation of DAMPs. This process stimulates innate immunity, resulting in adaptive immune responses (51). In addition, ROS damage to vascular endothelial cells can activate clotting processes. Persistent tumor tissue hypoxia caused by the aggregation of platelets and the blockage of blood vessels leads to cell death (49). These properties of PDT support the combination of PDT with immunotherapy. Photodynamic immunotherapy has been used clinically in combination with immune stimulators and ICIs.

Mechanism and preclinical progress on phototherapy combined with ICB

ICIs exert excellent antitumor effects by restoring the cytotoxic function of tumor-specific T cells. However, infiltrating T cell is the prerequisite for effective anti-CTLA-4/PD-1 therapy, and not all tumors express ligands that bind to ICIs. For multiple cancers, the response rate of ICB only ranges from 10% to 40%, resulting in a large proportion of patients not benefiting from ICI treatment (52). In contrast, PTT or PDT can kill tumor cells through noninvasive apoptosis or ablation, promoting T cell infiltration into the tumor tissue. They have been introduced to T cell-based ICB therapy to enhance the systemic antitumor immune response. In addition, many current preclinical studies on multidrug combination therapy platforms have demonstrated the combinational treatment's potent inhibition of tumor growth, metastasis, and recurrence. Current approaches include ICB combined with PTT/PDT or further combined with targeted therapy, chemotherapy, and immune adjuvants based on ICB+PTT/PDT (Figure 3). However, the immune responses induced by PTT and PDT are complicated *in vivo*, and the exact molecular mechanism is not fully understood. Therefore, in this section, we will discuss the signaling pathway regulated by phototherapy and review the progress in preclinical research on the combination of ICB and phototherapy.

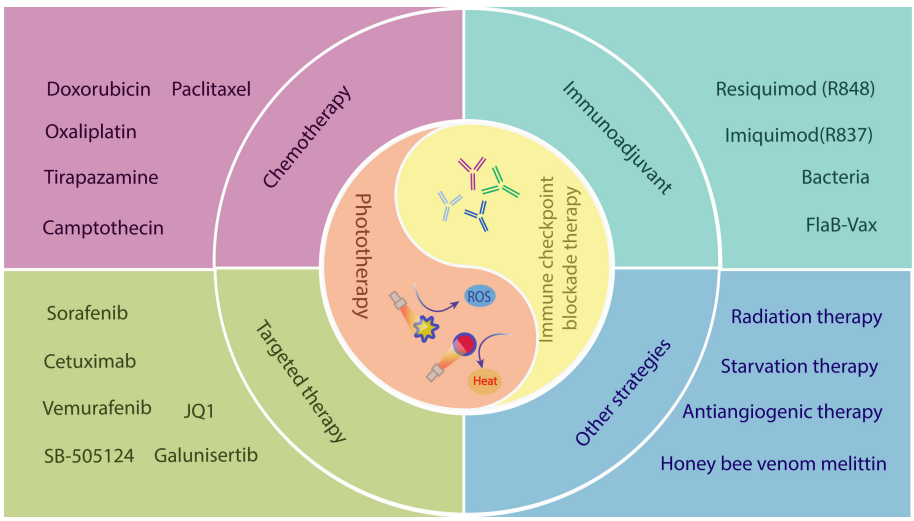

FIGURE 3
PTT/PDT combined with other treatment strategies to improve ICB therapy. PTT and PDT could be combined with different therapeutic modalities, including targeted therapy, chemotherapy, immune adjuvant therapy, starvation therapy, and antiangiogenic therapy to improve the antitumor immune response of ICB and inhibit tumor growth, metastasis, and recurrence.

The signaling pathway regulated by phototherapy and ICB

Besides inducing ICD of tumoral cells by releasing DAMPs to trigger the maturation of DCs and activation of $CD8^+$ T cells, phototherapy also regulates a variety of intracellular signal transduction pathways through generating ROS and heat (Figure 4). High temperatures can induce receptor interacting protein kinase (RIPK)-1, fas-associated death domain (FADD). And the upregulated RIPK1 and FADD in tumor cells can associate with caspase-8 to induce apoptosis (53–56). ROS promotes activation of death receptor pathways by inhibiting the production of Bcl-2/Bcl-xL and upregulating the expression of apoptotic-related proteins Bax. Meanwhile, the increased levels of ER stress and disruption of Ca^{2+} homeostasis induce intrinsic apoptosis (57). Generated heat and ROS by phototherapy can cause DNA damage, which leads to p53 activation (58). p53 is a tumor suppressor transcription factor that is known for its pro-apoptotic activity. Recently, an intriguing study demonstrates that PDT-induced p53 can re-educate tumor-associated macrophages (TAMs) to M1 antitumor phenotype, thereby stimulating antitumor T cell activation. p53 was positively correlated with M1-like macrophage makers (TNFα and IL1β) but was negatively correlated with M2-like macrophage markers (Arg1 and CCL16). In addition, p53 level was positively correlated with immune cells infiltration levels, including that B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and DCs. More interestingly, they discovered laser irradiation could induce p53 expression in a dose-dependent manner, providing new insight into phototherapy enhanced antitumor immune response (59). In addition, ROS-induced energy stress can strongly activate AMPK, and AMPK can upregulate autophagy by inhibiting mTORC1 activity (60). Therefore, phototherapy not only directly kills tumor cells but also improves the efficiency of ICIs by regulating the tumor immune environment.

Phototherapy improves the efficiency of ICB therapy

Given that phototherapy has proven to regulate antitumor immune response, the combination of PTT/PDT and ICB therapy has developed into a novel treatment regimen for

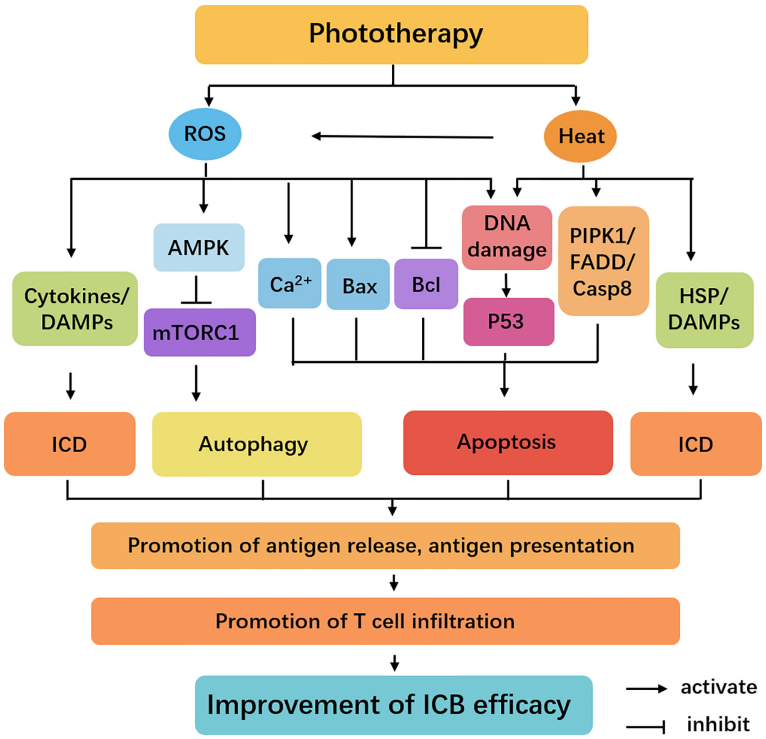

FIGURE 4
Cellular signaling pathways regulated by phototherapy and ICB. The irradiation triggers the activation of heat and ROS-mediated AMPK, Bax, Bcl, p53, and RIPK1 signaling. These pathways have different roles in the induction of apoptotic cell death and protective autophagy. Meanwhile, several DAMPs and cytokines play important roles in ICD. These dead cancer cells release antigens, promoting the tumor antigen processing and presentation and T cell infiltration to improve the efficiency of ICB.

cancer therapy. PTT/PDT selectively destroys cancer cells and induces ICD, initiating the local immune reaction by releasing antigens from dying cancer cells. Thus, combining phototherapy with ICB therapy may improve antitumor efficacy.

During the past years, PTT has been introduced to ICB to magnify the systemic antitumor immune response and applied to treat multiple cancers (Table 1) (99). For example, Ye and coworkers coated black phosphorus quantum dots with the surgically removed 4T1 tumor cell membrane to construct black phosphorus quantum dot nanovesicles (BPQD-CCNVs) as a kind of personalized photothermal vaccine (70). Under 808 nm laser irradiation, BPQD generates heat energy, which promotes GM-CSF and LPS continuously released from the nanoparticle. The released GM-CSF and LPS recruit and stimulate DCs, which activate the tumor-specific T cells to kill tumor cells. In addition, the rising temperature also upregulated

TABLE 1 Preclinical studies of phototherapy-ICB combined therapy or plus other therapeutics.

Phototherapy	Phototherapy agent	ICB	Combinationtherapy	Combinationtherapy agent	Cancermodel	Ref.
PDT	IRDye700	PD-1	alone	alone	4T1 Breast cancer	(61)
PDT	ZnP@pyro	PD-L1	alone	alone	4T1 Breast cancer	(62)
PDT	Fe-TBP	PD-L1	alone	alone	CT26 Colon cancer	(63)
PDT	ZnPc	CTLA-4	alone	alone	4T1 Breast cancer	(64)
PDT	PcN4	PD-L1	alone	alone	4T1 Breast cancer	(65)
PDT	Porphyrin	PD-L1	alone	alone	4T1 Breast cancer	(66)
PTT	SWNTs	CTLA-4	alone	alone	4T1 Breast cancer	(67)
PTT	PBNP	CTLA-4	alone	alone	Neuro2a Neuroblastoma	(68)
PTT	GNPs	PD-L1	alone	alone	HCC827 Lung cancer	(69)
PTT	BPQDs	PD-1	alone	alone	4T1 Breast cancer	(70)
PTT	CuS	PD-1	alone	alone	MDA-MB-231 Breast cancer	(71)
PDT	IRDye800CW	PD-L1	targeted therapy	cetuximab	CT26 Colon cancer	(72)
PTT	IR780	PD-1	targeted therapy	SB-505124	4T1 Breast cancer	(73)
PTT	AuNC	PD-1	targeted therapy	Vemurafenib	SMM103 Melanoma	(74)
PTT	PDMNs	PD-L1	targeted therapy	JQ1	4T1 Breast cancer	(75)
PTT	AuNC	PD-L1	targeted therapy	galunisertib	CT26 Colon cancer	(76)
PTT	PBNP	PD-L1	targeted therapy	sorafenib	HepG2 Hepatoma	(77)
PDT	Ce6	PD-L1	chemotherapy	paclitaxel	4T1 Breast cancer	(78)
PDT	Ce6	PD-1	chemotherapy	doxorubicin	4T1 Breast cancer	(79)
PDT	Pyrolipid	PD-L1	chemotherapy	oxaliplatin	MC38/CT26 Colon cancer	(80)
PDT	Ce6	PD-L1	chemotherapy	doxorubicin	4T1 Breast cancer	(81)
PDT	UCNPs-MOFs	PD-L1	chemotherapy	tirapazamine	CT26 Colon cancer	(82)
PDT	Ce6	PD-L1	chemotherapy	doxorubicin	4T1 Breast cancer	(83)
PDT	AuNp	PD-L1	chemotherapy	doxorubicin	CT26 Colon cancer	(84)
PTT	IRDye 800	PD-L1	chemotherapy	camptothecin	4T1 Breast cancer	(85)
PTT	Pd NP	PD-L1	chemotherapy	doxorubicin	CT26 Colon cancer	(42)
PTT	IR780	PD-L1	chemotherapy	oxaliplatin	CT26 Colon cancer	(86)
PTT	Ag prism	PD-1	chemotherapy	doxorubicin	4T1 Breast cancer	(87)
PDT	Ce6	CTLA-4	immune adjuvant	R837	4T1 Breast cancer	(88)
PDT	W-TBP	PD-L1	immune adjuvant	CpG	TUBO Breast cancer	(89)
PDT	PhA	PD-1	immune adjuvant	FlaB-Vax	B16-F10 Melanoma	(90)
PDT	UCNPs-Ce6	CTLA-4	immune adjuvant	R837	CT26 Colon cancer	(91)
PTT	HAuNS	PD-1	immune adjuvant	CpG	CT26 Colon cancer	(92)
PTT	Fe_3O_4 NPs	PD-L1	immune adjuvant	R837	4T1 Breast cancer	(93)
PTT	ICG	CTLA-4	immune adjuvant	R837	CT26 Colon cancer	(94)
PTT	CDs	PD-L1	immune adjuvant	R848	4T1 Breast cancer	(95)
PDT	IRDye700	PD-L1	antiangiogenic therapy	anti-CD276	4T1 Breast cancer	(96)
PDT	Ce6	PD-1	honey bee venom melittin	MLT peptide	4T1 Breast cancer	(97)
PTT+PDT	Cu_2MoS_4	CTLA-4	starvation therapy	GOx	U14 Cervical cancer	(98)

the expression of co-stimulatory molecules, such as CD80, CD86, MHC-I, and MHC-II molecules, further enhancing the ability to cross-present antigens and stimulate T cells. Moreover, administration of PD-1 antibody together with BPQD-CCNVs nanovesicles significantly promote $CD8^+$ T cell infiltration and eliminate surgical residual and lung metastatic tumors. This study demonstrates that photosensitive nanovesicles can be developed into individualized tumor vaccines and synergize with immunotherapy. Another work by Ran and coworkers reported a copper sulfide (CuS)-based nano-platform combined with PD-1 antibodies to obliterate primary tumors and inhibit metastatic tumors (71). Some photothermal agents, such as single-walled carbon nanotube (SWNT) and Prussian Blue (PB), can act as immunological adjuvants to promote the maturation of DCs and the production of antitumor cytokines (67). For example, in 2016, Fernandes et al. described a strategy of PBNP-based PTT with anti-CTLA-4 checkpoint inhibition for treating neuroblastoma (68). When combined with ICB, this photothermal agent with an immune-boosting effect could significantly inhibit metastatic tumor proliferation.

PDT can induce ICD by releasing CRT exposure and dying tumor cell debris, resulting in the activation of T cells to kill the residual tumor. Recently, Dr. Liu and his colleagues demonstrated that PDT synergistically promotes the antitumor efficacy of ICI (61). They developed a phthalocyanine dye-labeled probe to mediate PDT, significantly inhibiting tumor growth and T cells infiltration in a 4T1 mouse model. Based on PDT, PD-1 blockade also inhibited lung metastasis formation by agitating a systematic antitumor immune response. A ZnP@pyro PDT treatment was reported to sensitize tumors to PD-L1 antibody in a similar work from Lin et al. Their work also eradicated the primary 4T1 breast tumor and significantly prevented metastasis in the lung (62). Another physiologically self-degradable microneedle-assisted platform for combining PDT and anti-CTLA4 antibody can generate similar synergistic reinforcement outcomes while can reduce the side effects of treating breast cancer (64). Furthermore, since PDT can induce enhancement of hypoxia, Yoon et al. utilized the photosensitizer phthalocyanine derivatives (PcN4) to deliver hypoxia-activated prodrug (AQ4N), and create a more hypoxic TME for the activation of AQ4N. When combined with ICB therapy, it enables efficient abscopal responses and enhances antimetastatic effects in breast cancer treatment (65).

Downregulating the *de novo* expression of PD-L1 may be another new research direction for ICB therapy. Interestingly, nanomaterials of PTT can be used as gene silence carriers for PD-L1 due to their excellent modified performance. In the work of Cui and coworkers, they designed a new nanoplatform based on gold nanoprisms (GNPs) to carry PD-L1 siRNA. The platform not only functioned as a carrier for siRNA delivery to downregulate the PD-L1 expression but also served as the photothermal agent for PTT (69). In another work, Chen et al. synthesized a type of cationic flexible organic framework nanoparticle loaded with porphyrin of PDT and siRNA to mediate PD-L1 gene silencing to achieve an excellent antitumor effect, providing a basis for developing nanophotosensitizers and excellent gene carriers (66). In combination with ICB therapy, the nanomedicine of PTT and PDT not only achieved a superior effect in suppressing the growth of the primary tumor but also promoted long-lasting immune memory to inhibit tumor recurrence and metastasis.

Phototherapy combined with targeted therapy to improve ICB therapy

Targeted therapies inhibit the growth and progression of cancer cells by interfering with specific targeted molecules (100). Small-molecule inhibitors or monoclonal antibodies of targeted therapy, such as inhibitors for epidermal growth factor receptor (EGFR) (101), transforming growth factor (TGF)-β (102), BRAF (103), VEGF (104), and MYC (105), can produce impressive tumor responses in selected patients while having potentially fewer side effects. These inhibitors not only directly kill tumor cells but also activate the immune system through a variety of mechanisms, such as promoting tumor antigen processing and presentation, increasing intratumoral T cell infiltration, enhancing T cell function, and attenuating the immunosuppressive effect of the tumor microenvironment (106). For example, kinase inhibitor vemurafenib can enhance the induction of MHC Class I and Class II molecules by IFN-γ and IFNα2b, thereby enhancing antigen presentation and promoting antitumor immune response (107, 108). Since many of the targeted therapeutic agents can directly or indirectly modulate immune cell functions, combining ICB with targeted therapy has become a promising therapeutic strategy for malignant tumors. However, targeted drugs have some limitations: 1) poor aqueous solubility, 2) inhibition of normal cells that share targeted kinases, and 3) low bioavailability. Nanoparticle-based phototherapy loaded with targeted drugs can avoid these disadvantages. Moreover, this multidrug combination therapy can realize the dual advantages of PTT/PDT and targeted therapy, which is more conducive to immunotherapy (Table 1).

EGFR signaling pathway

EGFR, a transmembrane receptor, is one of the four closely related receptor tyrosine kinases and is involved in regulating cellular multiplication, survival, differentiation, metastasis, and plays a crucial role in the occurrence and immune escape of various malignant tumors. EGFR has historically served as the primary target for treating uncontrolled colorectal cancer growth. For example, Jin et al. reported a novel cerasome nanoparticle decorated with cetuximab, an anti-EGFR

antibody, IRDye800CW, and MRI contrast DOTA-Gd to enable *in vivo* tumor detection and PDT. The nanoparticles they designed possess significant potential for the dual-modality imaging-guided precise PDT of colorectal cancer due to its high ability to target tumors. Combining EGFR-targeted PDT with PD-L1 immunotherapy could achieve superior therapeutic efficacy without tumor recurrence (72).

TGF-β signaling pathway

TGF-β signal is involved in the proliferation, differentiation, adhesion, movement, and metabolism of tissue cells. Dysregulation of the TGF-β signaling pathway in TME is closely related to the blocking of T cell differentiation, the production of Treg subsets, and the restrained tumor-killing effect of CTLs, thus leading to a protumor immune environment (109). Suppose the TGF-β inhibitor is loaded into the nanosystem to specify delivery to tumor tissues. In that case, the inhibitor can reduce the generation of Treg cells and simultaneously avoid severe adverse reactions caused by nonselective systemic delivery. Recently, Li et al. coloaded TGF-β inhibitor (SB-505124) and photosensitizer (IR780) into nanoliposomes. PTT induced primary tumor ICD, allowing more CTL to infiltrate the tumor tissues and reducing Treg cells when incorporated with TGF-β inhibitor. In this work, PTT combined with PD-1/PD-L1 checkpoint blockade further unleashes T cells to attack 4T1 tumor cells (73). Yan et al. synthesized gold nanocages called GNC-Gal@CMaP functionalized with macrophage membrane and anti-PD-L1 antibody. They used GNC-Gal@CMaP load with galunisertib to improve synergistic PTT and immunotherapy against colorectal cancer (76). The nanocomposites designed can be selectively accumulated in the tumors, eliminate the primary tumor mass, and inhibit distant tumor growth *via* the abscopal effect.

MAPK signaling pathway

MAPK belongs to a large family of serine-threonine kinases, forming major cell-proliferation signaling pathways from the cell surface to the nucleus. The Ras/Raf/MAPK (MEK)/ERK pathway is the most critical signaling cascade among MAPK signal pathways (110). It plays an essential role in the survival and development of tumor cells. However, most patients treated with MAPK pathway inhibitors do not respond well to PD-1 immunotherapy (111). Using PTT/PDT nanomaterials can avoid this problem of insufficient tumor antigen presentation after MAPK pathway inhibitors resistance. A recent study by our team found that MAPK-targeted therapy impeded antitumor Tcell signatures in the tumor relapse phase by attenuating HSPs mediated antigen presentation. To address this problem, we developed a gold nanoparticle to load MAPK pathway inhibitor. Nanoparticle-mediated PTT and MAPK pathway targeted therapy can efficiently inhibit tumor cell growth and promote HSP expression, which promotes T cell infiltration and enhances the antitumor immune response in melanoma treatment. Based on PTT and MAPK pathway targeted therapy, PD-1 immunotherapy can efficiently convert immune "cold" tumors into "hot" ones and inhibit tumor growth. Our study revealed that gold particle mediated PTT can coordinate targeted therapy and ICB therapy, which provided a novel strategy for treating multifocal tumors and immune "cold" tumors (74).

Other signaling pathways

Currently, multikinase inhibitors that can simultaneously target more than one pathway have been approved for cancer treatment. Sorafenib (SF) is the first systemic therapy approved for hepatocellular carcinoma. It is a protein kinase inhibitor with activity against many protein kinases, including VEGFR, PDGFR, and RAF kinases (112). In recent work, Tian and colleagues designed hepatocellular carcinoma-targeted nanoparticles conjugated PB to load SF and combined with an anti-PD-L1 antibody to treat hepatocellular carcinoma. The combination treatment strategy effectively eliminates tumor cells at the primary site by nanoparticle-mediated SF targeted inhibition of photothermal effects. The antitumor effects produced by local treatment can be extended to the whole body and enable the establishment of long-term immunological memory, inhibiting tumor metastasis and recurrence (77). For another example, Lu et al. designed polydopamine nanoparticles (PDMNs) encapsulating JQ1 to treat triple-negative breast cancer. The JQ1-loaded PDMNs accumulated in the tumor tissue and released JQ1 in a self-degradable manner. The released JQ1 inhibits the growth of triple-negative breast cancer by inhibiting the BRD4-c-MYC axis and suppressing the expression of PD-L1, which facilitates the activation of T cells in tumor tissue. Meanwhile, PDMN transforms light energy into heat and ablates tumors upon laser irradiation (75).

Phototherapy combined with chemotherapy to improve ICB therapy

For many tumor types, chemotherapy still represents the therapy of choice. Some studies have shown that chemotherapy drugs such as doxorubicin (Dox), oxaliplatin (Oxa), and paclitaxel (PTX) can induce tumor cell death in an immunogenic manner, playing a synergistic antitumor effect with immunotherapy (113). However, the problems of low targeting, systemic toxicity, and poor water solubility of chemotherapy drugs are unavoidable. Nanoparticles of PTT/

PDT can not only be loaded with chemotherapy drugs to achieve targeted delivery but also be combined with chemotherapy to induce ICD and facilitate antitumor ICB immune therapy. Many studies have used nanotechnology to carry out triple phototherapy, chemotherapy, and immunotherapy treatments (Table 1) (80–82).

Oxa, a chemotherapeutic drug approved by FDA for the treatment of colorectal cancer, induces cell death by triggering apoptosis and stimulating CRT exposure (114). Lin and colleagues reported using nanoscale coordination polymer core-shell nanoparticles to carry Oxa in the core and a photosensitizer-lipid conjugate in the shell for effective chemotherapy and PDT to treat colon cancer. The result showed that chemotherapy and PDT synergized with ICB have the highest response rate compared to all controls in a bilateral colon carcinoma tumor model, with even induction of abscopal effects and induced cell death in distant tumors that were not irradiated (80). In another example, a tumor-targeting thermosensitive liposomal system carrying PD-L1 inhibitors, IR780, and Oxa, promoted antigen presentation and lymphocyte infiltration to enhance colon cancer immunotherapy (86). Their work proved that chemotherapy/PDT provides an efficient way to induce immunogenicity in the TME and enhance the antitumor immunity of anti-PD-L1 antibodies.

Dox-mediated ICD therapy has considerable potential in cancer treatment. However, undesirable drug delivery efficiency and unavoidable toxicity limit the ICD efficiency of Dox (115). Photothermal sensitive nanoparticles can avoid systemic toxicity and improve the ICD efficiency of chemotherapy. For example, Yang et al. developed a cascade chemo-photodynamic therapy (chemo-PDT) by loading Dox and Chlorine E6 (Ce6) into ROS-sensitive lipid-polymer hybrid nanoparticles. Under 660 nm laser irradiation, ROS were generated by the encapsulated Ce6, which works for cancer treatment and enhances intracellular DOX release. Based on this cascade combo regimen, administrating the PD-L1 antibody could efficiently inhibit primary tumor growth and ablate distant tumors (81). In addition, similar works are MMP2-responsive controlled-release systems for colon cancer therapy (42) and silver nano prisms core-shell nanoplatforms for breast cancer treatment (87). Combinational strategies like these can enhance the antitumor responses of ICIs against both primary and distant tumors.

PTX is a first-line anticancer chemotherapy agent. It can enhance immunotherapy efficacy by reversing immunosuppression in the TME (116). Combined PDT with PTX can improve the effects of a-PD-L1 even for treating tumors with low immunogenicity (117). Recently, Zhang et al. developed a chemo-PDT to enhance the therapeutic effect of PD-L1 immunotherapy by loading photosensitizer Ce6 and PTX into mesoporous silica nuclear nanoparticles. Nanoparticle-mediated chemo-PDT can induce the antitumor immune response and improve the therapeutic effect of PD-L1 blockade in primary and metastatic tumors (78). Camptothecin (CPT) possesses the most effective cytotoxicity for tumor cells in the S and G2 phases. For example, a polypyrrole-loaded CPT-conjugated HA nanoparticle (P@CH) was developed for tumor targeting and PDT combinational therapy. When combined with anti-PD-L1 immunotherapy, the primary tumor was completely depleted, and the lung metastasis were not observed. The result was much better than anti-PD-L1 antibody treatment, indicating that the combination could enhance the immunotherapy of ICIs (85).

Phototherapy combined with immunoadjuvants to improve ICB therapy

PTT and PDT are highly immunogenic treatments with the potential to recruit DCs to the TME by releasing tumor cell debris and TAAs. However, within the local TME, there are a variety of inhibitory immune cells and molecules, which are unfavorable for cancer immune treatment of combined PTT/PDT and ICB. Another strategy for enhancing immune reactions is exposure of DCs to toll-like receptor (TLR) agonists, such as imiquimod (R837), resiquimod (R848), and CpG. At present, some studies have reported loading PTT/PDT nanocarriers with immunoadjuvants and then combining them with ICB to induce a robust systemic antitumor immune response (Table 1).

R837 is a TLR-7 agonist that can promote DCs to phagocytize TAAs and mature, enhancing the activation and proliferation of antigen-specific lymphocytes in draining lymph nodes (118). Liu and his colleagues designed a light-triggered *in situ* gelation system containing a photosensitizer (88). Immune adjuvant R837 was further introduced into this system to trigger robust antitumor immune responses after PDT. With the help of the immune adjuvant, the hydrogel system could significantly enhance immune responses by multiround stimulation. Further combined with CTLA4 blockade offered the abscopal effect to inhibit the growth of distant tumors. It provided adequate long-term immune memory protection from a rechallenged tumor. Another multifunctional UCNP-based platform coloaded Ce6 and R837 onto polymer-coated UCNPs to treat colorectal cancer (91). The presence of those R837-containing nanoparticles as the adjuvant can promote strong antitumor immune responses. Efficiency was further promoted by the anti-CTLA-4 blockade to effectively eliminate both irradiated tumors and tumors grown on distant sites.

Fe_3O_4 NPs have excellent biocompatibility, nontoxicity, MRI and magnetic targeting capability. Yang et al. demonstrated an R837 nanodrug carrier based on Fe_3O_4 superparticles, which can directly destroy tumors and activate the immune system by inducing DC maturation and secretion of cytokines with the help of NIR irradiation and an external magnetic field. The combination with anti-PD-L1 therapy can eradicate primary tumors directly exposed to PTT, prevent lung/liver metastasis,

and inhibit the preexisting distant tumors after PTT (93). Recently, a kind of bone marrow mesenchymal stem cell (BMSCs) membrane-derived biomimetic nanovesicles attracts people's attention (119). Researchers generate biogenic nanovesicles by utilizing low immunogenic BMSCs to express anti-PD-L1 antibodies and OVA antigen. Then, photosensitizer indocyanine green (ICG) and immunoadjuvants R837 were loaded into the nanovesicles by ultrasound. The anti-PD-L1 antibodies expressed on the nanovesicles can specifically bind to PD-L1 ligands on tumor cells and guide the nanovesicles home into tumor tissues. Under laser irradiation, the photosensitizer ICG mediated photothermal therapy efficiently ablates primary tumor and remodels tumor immune microenvironment. Meanwhile, immunoadjuvants R837 and OVA antigens stimulate DCs to activate the body's immune response to residual tumors. In addition, Liu et al. developed a therapeutic strategy that coencapsulated indocyanine green (ICG) and R837 through oil-in-water emulsions to combine adjuvant nanoparticle-based PTT with ICB in colon cancer therapy (94).

Such strategies can offer stronger immunological memory effects to against tumors. R848 is another TLR-7 agonist. Li and his colleagues have designed using polydopamine (PDA) simultaneously loaded with R848 and carbon dots (CDs). The PTT effect of CDs triggered the release of R848, inducing a robust antitumor immune response. It can significantly potentiate the systemic therapeutic efficiency of PD-L1 therapy by activating both innate and adaptive immune systems in the body subsequently (95). Immunoadjuvant CpG can promote antigen presentation by DC maturation *via* binding to TLR-9 (89). You et al. coencapsulated hollow gold nanoshell and an anti-PD-1 peptide into nanoparticles. Their data demonstrated that perdurable PD-1 blocking combined with PTT and immune adjuvant could efficiently eradicate the primary cancer model CT26 tumor cells and inhibit the growth of metastatic tumors and their formation (92). The TLR5 agonist flagellin served as an excellent adjuvant to induce effective cell-mediated immunity. Rhee and his colleagues' work investigated the effect of combining PDT and TLR5 agonist flagellin-adjuvanted tumor-specific peptide vaccination (FlaB-Vax) on promoting PD-1 blockade-mediated melanoma suppression. The combination of an immunoadjuvant with PDT effectively induced a systemic and local response of peptide tumor antigen-specific IFNγ secretion and the accumulation of effector memory $CD8^+$ T cells, which further enhanced the PD-1 blockade therapeutic outcome in melanoma treatment (90).

Phototherapy combined with other strategies to improve ICB therapy

In addition to targeted therapy, chemotherapy, and immune adjuvants, some antitumor strategies, such as anti-vascular therapy, starvation therapy, etc., can also synergize with photoimmunotherapy to inhibit tumor growth (96, 98). Anti-vascular medicine can break vessel wall barriers and change the TME to compensate for conventional phototherapy and immunotherapy limitations (120). CD276 is a receptor that is overexpressed in various tumor cells and tumor vasculature but with limited expression in normal tissues (121). Therefore, Liu and colleagues conjugated photosensitizer IRDye700 with the Fab fragment of an anti-CD276 antibody to combine antiangiogenic therapy and PDT with PD-1/PD-L1 blockade to treat primary tumors and ablation of tumor metastases. There was a marked increase in PD-L1 expression in 4T1 tumors after CD276 targeted PDT, which improved the efficacy of immune checkpoint inhibition (96).

Starvation therapy is also a good treatment option for cancer therapy. It can effectively inhibit tumor growth by cutting off the nutrition supply as a considerable supply of nutrients is needed for the rapid proliferation of cancer cells (122). Glucose oxidase (GOx) is an ideal endogenous natural enzyme for tumor starvation therapy (123). Lin and his colleagues constructed a multifunctional cascade bioreactor by starvation therapy/PTT/PDT/ICB therapy to treat cervical cancer. This bioreactor is based on hollow mesoporous Cu2MoS4 loaded with GOx for synergetic cancer therapy (98). In contrast, this quadri-combination therapy would more effectively release TAAs and elicit more robust immune responses. Further combining anti-CTLA4 antibody effectively eradicated both primary and metastatic tumors.

Melittin (MLT), the main component of bee venom, acts as a nonselective cytolytic peptide (124, 125). Yang et al. developed an organic-inorganic nanocarrier to load with Ce6 and MLT, denoted Ce6/MLT@SAB, aiming to simultaneously improve PDT-mediated intracellular ROS production and ICD levels. The addition of the anti-PD1 antibody further augmented antitumor effects, generating increased numbers of $CD4^+$ and $CD8^+$ T cells in tumors with concomitant reduction of myeloid-derived suppressor cells (97).

The clinical application of phototherapy in cancer immunotherapy

Encouraging results from preclinical studies have prompted the clinical application of phototherapy in cancer immunotherapy. Phototherapy has been clinically or under clinical trials to treat solid tumors. Noninvasive phototherapy is very suitable for treating superficial cancers such as melanoma, osteosarcoma, squamous cell carcinoma, etc. Interstitial phototherapy, including laser interstitial thermal therapy (LITT) and interstitial photodynamic therapy (IPDT), can treat deep-seated tumors and avoid damage to healthy tissue by using the placement of interstitial laser fibers into tumors

(126–128). Further development of LITT and IPDT allows broadening the scope of phototherapy application to treat tumors, such as lung cancer, esophageal cancer, prostate cancer, breast cancer, head and neck cancer, bile duct cancer, bladder cancer, pancreatic cancer, cervical cancer, brain cancer, etc. Phototherapy has considerable potential to be used in combination with ICB to treat a wide range of tumor types. Related clinical studies have shown that phototherapy combined with ICB therapy can reduce primary tumors, control untreated metastases, and prolong the survival of patients. This photoimmunotherapy has been used to treat patients with late-stage cancer who have failed other feasible treatment modalities.

Lung cancers

Lung cancer remains the leading cause of cancer death in the latest global cancer statistics, with an estimated 18% of cancer-related deaths (129). Currently, phototherapy is increasingly being used to treat various forms of lung cancer, such as early-stage, advanced, or metastatic non-small cell lung cancer, multiple primary lung cancers, and small cell lung cancer (SCLC) (130). FDA approved Porfimer sodium (Photofrin) for the treatment of palliation of patients with esophageal cancer, treatment of microinvasive endobronchial non-small-cell lung cancer, and reduction of obstruction and palliation of symptoms in patients with wholly or partially obstructing endobronchial non-small cell lung cancer (131). Talaporfin is a second-generation photosensitizer approved in Japan (132). It can be clinically used in PDT for early-stage lung cancer, primary malignant brain tumors, and locally remnant recurrent esophageal cancer. Phototherapies are also attractive options for treating malignant pleural mesothelioma (MPM) (133).

In April 2021, a phase I clinical trial was initiated at Roswell Park Cancer Institute to evaluate PDT's ability to amplify the response to immunotherapy in patients with non-small cell lung cancer with pleural disease (NCT number: NCT04836429). Sixteen patients are expected to be enrolled to receive porfimer sodium IV over 3-5 minutes 24-48 hours prior to standard of care VATS. This trial evaluates the side effects of intraoperative PDT with porfimer sodium in enhancing the response to an ICI drug. The incidence of serious adverse events (SAEs) was determined by recording the occurrence of SAEs during the first 28 days of poststudy-related immunotherapy. In addition, patients were followed for progression-free survival (PFS), overall survival (OS), changes in the immune phenotype of peripheral blood $CD8^+$ T cells, and changes in the platelet-to-lymphocyte ratio within a time frame of two years. The study is expected to be completed in December 2023 (Table 2).

Head and neck cancer

Head and neck cancer is the seventh most common type of cancer worldwide. It comprises a diverse group of tumors affecting the upper aerodigestive tract (136). Several multi-institutional phase II clinical trials of PDT have demonstrated its efficacy in treating early oropharyngeal primary and recurrent cancers and the palliative treatment of refractory head and neck cancers. Patients with early-stage cancers or early recurrences in the oral cavity and larynx respond well to PDT (137). In July 2015, a phase I clinical trial was initiated to evaluate the safety and antitumor activity of RM-1929 in patients with terminal head-and-neck cancer (NCT number: NCT02422979). RM-1929 is a chemical conjugate of the dye IR700 with an EGFR receptor-targeting antibody. Composed of a silica core and a gold shell, "AuroShell" NPs have also been used for pilot clinical trials in PTT applications to treat head and neck cancer (NCT00848042) (Table 2).

A successful treatment option combined the ICI nivolumab with redaporfin-mediated PDT to treat a patient with head and

TABLE 2 Clinical trials of combination of phototherapy and ICB therapy in malignant tumors.

Clinical trail	Phase	Year	Cancer type	Phototherapy	Phototherapy agent	ICB	ICB agent
NCT04836429	Phase I	2021	Non-small cell lung cancer with pleural disease	PDT	Porfimer sodium	PD-1	ICIs
Case Report (134)	Clinical Case Report	2018	Head and neck squamous cell carcinoma (HNSCC)	PDT	Redaporfin	PD-1	Nivolumab
NCT03727061	Phase I/2	2018	Head and Neck Cancer	PDT	Porfimer sodium	PD-1	Nivolumab/ Pembrolizumab
NCT04305795	Phase I/2	2020	Head and Neck Cancer	PIT	ASP-1929	PD-1	Pembrolizumab/ Cemiplimab
NCT05220748	Phase I	2022	Head and Neck Cancer	PIT	ASP-1929	PD-1	Pembrolizumab
NCT05265013	Phase II	2022	Head and Neck Cancer	PIT	ASP-1929	PD-1	Pembrolizumab
Case Report (135)	Clinical Case Report	2017	Melanoma	LIT	Laser	CTLA-4	Ipilimumab

neck squamous cell carcinoma (HNSCC) in 2018. The patient had failed numerous prior therapies, including surgery, radiotherapy, and multiple lines of systemic treatment. PDT destructed all visible tumors with Redaporfin, and the combination of an ICI immunotherapy promoted a complete sustained response (134) (Table 2).

In addition, several early-phase clinical trials combining phototherapy with ICB have been initiated. A phase 1/2 (NCT number: NCT03727061) study of 82 patients was launched in 2018 to study how I-PDT works with standard care of cetuximab, nivolumab, or pembrolizumab. The study is expected to be completed in July 2023. Asp-1929 is also a photoimmunotherapy agent with a similar structure to RM-1929, and its current clinical trials are focused on head and neck cancer. In 74 patients with recurrent or metastatic head and neck and squamous cell cancer or advanced or metastatic cutaneous squamous cell carcinoma, an open-label study using ASP-1929 photoimmunotherapy combined with anti-PD1 therapy was launched in 2020 (NCT number: NCT04305795) (Table 2). The estimated study completion date for this trial is June 2024. In addition, two clinical studies started this year. One is to use RM-1995 with pembrolizumab in 36 patients with advanced cutaneous squamous cell carcinoma (cuSCC) or head and neck squamous cell carcinoma (HNSCC) to evaluate the safety and efficacy (NCT number: NCT05220748). Another study combined ASP-1929 with pembrolizumab in 33 patients with locoregional recurrent squamous cell carcinoma of the head and neck, with or without metastases (NCT number: NCT05265013) (Table 2).

Skin cancer

Skin cancers are generally classified into melanoma skin cancer (MSC) and non-melanoma skin cancer (NMSC) (138). Laser immunotherapy (LIT) is a promising modality that combines local, selective PTT with immunological therapy to treat metastatic melanoma (139). Non-melanoma skin cancer or precancerous cutaneous lesions, including basal cell and squamous cell skin cancers and actinic keratosis, are essential indications for PDT. Tens of millions of patients are likely to have been treated by PTT/PDT worldwide to date (140, 141).

LIT induces long-term antitumor immunity by enhancing antigen uptake and presentation, leading to an enhanced response to ICIs such as ipilimumab. In one exciting example, one patient with advanced (stage IV) melanoma used the combination of LIT and ipilimumab. The patient received treatment with imiquimod and an 805 nm diode laser on the target sites for three months. After three months, all treated cutaneous melanomas in the head and neck cleared completely. Then the patient was treated with ipilimumab. After ipilimumab treatment, all lung tumor nodules entirely resolved (Table 2) (135).

Esophageal cancer

Esophagectomy carries a high risk of postoperative complications and mortality. Esophageal cancer was responsible for one in every 18 cancer deaths in 2020 global cancer statistics (129). Thus, PDT is considerably appealing as a locally minimally invasive treatment. PDT has been approved as a curative treatment for superficial esophageal squamous cell carcinoma (ESCC) in Japan and approved for dysplastic Barrett's esophagus and palliative treatment for symptomatic obstructive esophageal cancer in the US (142). In recent years, PDT has regained popularity due to the invention of second-generation PDT using talaporfin sodium and diode laser. The efficacy and safety of PDT as a salvage treatment for patients with local failure after chemoradiotherapy (CRT) have been demonstrated in several clinical trials (143).

Prostate cancer

Prostate cancer was the second most frequent cancer in 2020. There are primarily four photosensitizers, including Tibofen, Motexafin Lutetium (MLU), Vitibofen, and Padeliporfin, used in clinical PDT for prostate cancer (144). PDT using various photosensitizers for the focal ablation of prostate tumors has been tested in clinic (145). Recently, AuroShells, which are tiny silica spheres with a thin outer shell of gold, were developed for PTT treatment of patients with prostate cancer in a clinical pilot study. Fifteen men aged 50-79 years with low to medium-risk localized prostate cancer were treated with PTT. There were no detectable signs of cancer in 86.7% (13/15) of patients within one year (146). "AuroShell" NPs have been used in pilot clinical trials to treat men with low- or intermediate-risk prostate cancer (NCT04240639).

Breast cancer

Female breast cancer has now surpassed lung cancer as the leading cause of global cancer incidence, representing 11.7% of cancer cases (129). In recent years, phototherapy has made significant progress in breast cancer research. LITT has been explored to treat benign breast tumors (NCT00807924). A multicenter clinical trial was designed to determine the efficacy and outcome of percutaneous laser ablation (PLA) in treating invasive ductal breast carcinoma (IDC). In this trial, 51 (84%) of 61 patients had complete tumor ablation confirmed by pathology analysis (147). In a clinical trial in Peru, ten breast cancer (stage III or stage IV) patients considered out of other available treatment options were enrolled in a photothermal immunotherapy clinical trial, using ICG as the photo agent and GC immunoadjuvant. In 8 patients available for evaluation, the

overall 3-year survival was significant. These patients only had a life expectancy of 3-6 months prior to the study (148).

Other indications

In addition to the indications mentioned above, PDT has also been tested clinically for dozens of other cancer indications, including bladder cancer (149), brain cancer (150, 151), cholangiocarcinoma (152, 153), pancreatic cancer (154), and gynecological cancers (155). Bladder cancer was the first indication for which porfimer sodium was approved in Canada in 1993. TLD-1433 is the first ruthenium (II)-based photosensitizer for photosensitizer to enter human clinical PDT trials to treat nonmuscle invasive bladder cancer. (NCT03945162). The FDA approved oral ALA for the fluorescence-guided resection (FGR) of high-grade gliomas in 2017 (150).

Conclusions

There is growing evidence that nanotechnology-based phototherapy can improve ICB treatment efficacy by regulating the anti-tumor immune response. In addition to mediating PTT and PDT for cancer therapy, photosensitive nanocarriers also serve as drug carriers to deliver small molecular inhibitors, chemotherapeutic drugs, or immunomodulators into tumor tissues to treat tumors or regulate tumor microenvironment. Therefore, combing ICBs with nanotechnology-based phototherapy is one of the most promising strategies in anticancer therapy.

In PTT/PDT, the photothermal/photosensitizer agents convert the light energy to heat and elevate the temperature of tumors to the cytotoxic level or generate cytotoxic ROS to ablate tumor cells. There is no doubt that nanoparticle-based PTT/PDT combined with ICB therapy has produced impressive preclinical results. However, most of these studies are still at the laboratory stage and face multiple challenges that limit their full synergistic potential with cancer immunotherapy. One major challenge is the limited penetration depth of red and NIR light for deep-seated tumors. As a result, phototherapy is commonly used in the treating specific skin carcinoma and superficial cancers. To solve this problem, interstitial and NIR-II phototherapy were developed to circumvent these barriers. Recently, MR-guided LITT, as a minimally invasive treatment modality, has been used to treat primary and metastatic brain tumors (156, 157). In addition, light in NIR-II has a higher upper limit of radiation and greater tissue tolerance than in the NIR-I window. NIR-II treatment can trigger more homogeneous and deeper immunogenic cancer cell death in solid tumors (158). In recent, a NIR-II SWNT modified by a novel immunoadjuvant, glycated chitosan (GC), was used by Chen and colleagues to treat metastatic mammary tumors in mice. After intratumoral administration of SWNT-GC, they used a 1064 nm laser to irradiate the primary tumors to achieve local ablation through PTT. Combination with anti-CTLA-4 antibody produced systemic antitumor immunity that inhibited lung metastasis and prolonged the survival time of the treated animals (159). We believe that with more research into this field, the current library of phototherapy agents will certainly be expanded and a "blockbuster" agent to solve this problem is just around the corner. It is also worth mentioning that the FDA has published particular guidance for nanomedicines until recently, as they are categorized under complex products with multiple components (160, 161). Although nanoparticles of phototherapy are typically better agents, caution should be exercised when selecting them, as there are some questions still remain on their safety and toxicity profiles. Consequently, it is necessary to develop the targeting ability of phototherapy agents to increase the accumulation of drugs in tumor tissue or residual tumor cells. The current targeting strategies use high-affinity ligands or targeting moieties on the surface of the nanocarriers that bind to specific overexpressed receptors on the target cancer cells (162). Standard ligands or targeting moieties include antibodies or antibody fragments, aptamers, carbohydrates, human transferrin protein, peptides, and vitamins such as folate (163, 164).

Improving photothermal conversion efficiency (PCE) is essential to facilitate therapeutic performance during PTT should be considered as well. An ideal photothermal agent would have a high PCE without absorption disturbance from the chromophores in biological tissue. Photothermal agents with high PCE have been developed with the advancement of PTT research (165–167). In recent years, many efforts have been made to improve PCE (168). Reducing the fluorescence emission and inhibiting the singlet to triplet state intersystem crossing (ISC) reaction are valuable strategies for improving heat generation (169). In addition, reducing tumor heat tolerance is another strategy to address this problem. For example, the tolerated thermal ranges were reduced in hypoxia, suggesting that the combination of PDT and PTT may be a good choice (170). Lastly, strong oxygen dependence in PDT is another main challenge in ablating tumor cells. Rapid tumor growth and insufficient blood supply lead to a hypoxic microenvironment in tumors (171). Moreover, local oxygen consumption of PDT also aggravates tumor hypoxia, which can seriously affect the efficacy of PDT. Multifunctional delivery strategies have been proposed to overcome this problem, such as oxygen-replenishing strategies (using oxygen carriers and generators to deliver oxygen into tumors), downregulation of oxygen consumption, and O_2-independent strategies (subcellular organelle-targeted and O_2-independent PDT), and hypoxia utilization (combinations of hypoxia-responsive chemotherapeutic drugs) (172–174).

Combining ICB with the assistance of phototherapy could greatly improve cancer treatment effects as described in this review. With the development of biomedical and optical technologies, reduction of costs, and in-depth study of biological mechanisms, more novel, and innovative photosensitive agents will continue to be developed to solve the above problems. Phototherapy combined with ICB therapy is under investigation in large-cohort clinical trials and has the potential to move forward as next-generation technology for cancer treatment. The path of clinical transformation of the combination therapy strategy of "PTT/PDT + ICB" has begun.

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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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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Characteristics of the immune microenvironment and their clinical significance in non-small cell lung cancer patients with ALK-rearranged mutation

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Background: Although immune checkpoint inhibitors (ICIs) are one of the most important treatments for advanced-stage non-small-cell lung cancer (NSCLC), NSCLC patients with ALK-rearranged usually don't obtain a clinical benefit. The reason may be related to the unique tumor microenvironment (TME). We evaluated the characteristics of immune biomarkers of the TME and their prognostic value in ALK-rearranged NSCLC.

Methods: Tumor samples from patients with ALK-rearranged (N = 39) and EGFR- (N = 40)/KRAS- (N = 30) mutated NSCLC were collected. Immunohistochemistry (IHC) was used to assess the expression of 9 tumor immune markers as well as 6 immune markers of tumor-infiltrating cells. To research the TME of ALK-rearranged NSCLC, EGFR/KRAS-positive patients were used as controls. Furthermore, the correlation between the efficacy and prognosis of patients with advanced-stage (IIIC-IV) ALK rearrangements treated with targeted drugs was analyzed in terms of the TME.

Results: The proportion of PD-L1+ tumors was lower in ALK-positive NSCLC than in KRAS-positive NSCLC. Besides, the proportion of T cells expressing TIM-3-CD8+ (15.38%), CTLA4-CD8+ (12.82%), LAG3-CD8+ (33.33%) and PD-1-CD8+ (2.56%) in ALK-positive NSCLC was lower than that in EGFR/KRAS-positive NSCLC. The expression of CD3, CD8 T cells and CD20 B cells was lower in ALK-positive NSCLC than in KRAS-positive NSCLC ($p < 0.0001$, < 0.005, and < 0.001, respectively). Nevertheless, the level of CD4 helper T cells was higher in ALK-positive NSCLC than in EGFR/KRAS-positive NSCLC ($p < 0.0001$ and $p < 0.05$, respectively). The repression of TIM3 was higher in ALK-positive NSCLC than in KRAS-positive NSCLC ($p < 0.001$). In addition, our data showed that high expression of PD-L1 (HR = 0.177, 95% CI 0.038–0.852, p = 0.027) and CTLA4 (HR = 0.196, 95% CI 0.041–0.947, p = 0.043) was related to

lower OS in advanced-stage ALK- rearranged NSCLC patients treated with ALK tyrosine kinase inhibitors (TKIs).

Conclusions: Immunosuppressive status was characteristic of the TME in patients with ALK-positive NSCLC compared with EGFR/KRAS-positive NSCLC. High expression of PD-L1 and CTLA4 was an adverse prognostic factor in advanced-stage ALK-rearranged NSCLC patients treated with ALK-TKIs. Immunotherapy for ALK-rearranged patients requires further exploration and validation by clinical trials.

KEYWORDS

ALK-rearranged, EGFR mutation, KRAS mutation, tumor microenvironment (TME), NSCLC

Introduction

Cancer-related deaths are most commonly associated with lung cancer, which is a major global health problem. Non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. The prevalence of anaplastic lymphoma kinase (ALK) translocation positivity is 3-5% (1, 2). A previous study showed that ALK tyrosine kinase inhibitors (TKIs), such as crizotinib or alectinib, improved the prognosis of NSCLC with ALK rearrangements (3, 4).

Although targeted therapies are efficient in the context of oncogenic driver mutations, resistance and tumor recurrence inevitably develop (5). Immune checkpoint inhibitors (ICIs) are a new standard of care for blocking the programmed death-1 (PD-1)/programmed death ligand 1 (PD-L1) axis (6–8). Recently, immunotherapies have been considered an extremely promising therapeutic measure for lung cancer and propelled the field of oncotherapy into a new era. PD-L1 expression in tumor cells is associated with improved clinical outcomes of PD-1 pathway blockade in NSCLC patients (9, 10). Previous trials have reported that the maximum response rate of ICI is usually 20% and that the overall survival (OS) benefit was good in unselected NSCLC patients (11–14). Numerous clinical trials have shown that ICI treatment alone or even in combination with chemotherapy results in significantly longer overall survival (OS) or progression-free survival (PFS) in NSCLC patients with or without high PD-L1 expression (15–19). In contrast, a retrospective analysis found that NSCLC patients harboring EGFR mutations or ALK rearrangements are associated with low responses to PD-1/PD-L1 inhibitors (20). A previous study reported that the response of ALK-positive NSCLC patients treated with ICIs was limited (21). Likewise, the Checkmate 012 trial, a multicohort clinical trial, demonstrated that first-line nivolumab monotherapy or in combination with standard therapies showed no meaningful activity in NSCLC with EGFR mutations (22). Furthermore, numeral clinical trials combining ICIs and TKI resulted in remarkable toxicity without a signal of improved activity above that of the TKI (23–25). Furthermore, previous study had shown that ICIs inhibited more effectively the tumor progression in NSCLC patients with KRAS mutations compared with ALK rearrangements (26).

In addition to PD-1 or PD-L1 immune checkpoints, other immune checkpoints are gradually being identified. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is negative for T-cell activation. Neoadjuvant therapy in combination with CTLA4 and PD-1 inhibitors has significant benefits in NSCLC (27). Similarly, lymphocyte-activation gene 3 (LAG-3) and mucin-domain containing-3 (TIM-3) are expressed in various kinds of immune cells and transmit inhibitory immune signals (28–30). Combining LAG-3 inhibition with PD-1 blockade can enhance antitumor immunity (28). A previous study reported that TIM-3 positivity was significantly associated with worse prognosis in lung adenocarcinoma (31). T-cell immunoglobulin and ITIM domain (TIGIT), expressed on NK cells, CD8+ T cells and so on, inhibits the antitumor immune responses mediated by T cells and NK cells (32). Moreover, tumor microenvironment (TME) is the environment of all kinds of tumour–immune cells interactions. Spatial profiling technologies are powerful tool to analyze immune cell typing, immune activation and therapeutic options (33, 34).

Abbreviations: TME, tumor microenvironmen; ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; KRAS, kirsten rat sarcoma viral oncogene; ORR, objectives response rates; OS, overall survival; TKIs, tyrosine kinase inhibitor; ICIs, Immune checkpoint inhibitors; TILs, tumor-infiltrating lymphocytes; IHC, immunohistochemistry; SD, standard deviation.

Likewise, co-location of immuno-biomarkers can be used as prognostic indicators (35).

To date, it is still unclear why patients with ALK-positive NSCLC do not benefit from ICIs. Therefore, it is of extreme importance to perform this study to research the TME of these NSCLC patients.

Materials and methods

Patient cohorts

A total of 103 NSCLC patients from Tianjin Medical University General Hospital and 6 patients from Tianjin Medical University Cancer Institute and Hospital were enrolled in this study by reviewing medical records between 2014 and 2021. Patients were included in our study if the following criteria were met: 1) confirmed non-small-cell lung cancer according to the eighth edition of the TNM classification of the Union for International Cancer Control; and 2) underwent molecular testing of genetic mutations and was found to be EGFR-, KRAS- or ALK-positive. To obtain a more reasonable dataset for this study, patients were also excluded if they received chemotherapy, targeted therapy or radiotherapy before diagnosis. Because the research was a retrospective chart and specimen review, no personally identifiable information was included.

To achieve our objectives, the expression of immune checkpoint molecules (PD-L1, PD-1, LAG3, TIM3, CTLA4, TIGIT, and OX40) and TILs (T cells (CD3+), cytotoxic T cells (CD8+, Granzyme B), macrophages (CD68+), regulatory T cells (FOXP3+, CD4+), NK cells (CD56+), and B lymphocytes (CD20+)) was evaluated by IHC. The flow chart is shown in Figure 1. The clinicopathological information of the patients in the study was collected from clinical records and pathology reports. Overall survival (OS) was defined as the time from confirmed diagnosis or treatment initiation to the date of the last follow-up or death due to any cause. If patients had not progressed or died at the time of analysis, they were censored on the date of the last assessment. Survival associations were also assessed for categorical variables using the Kaplan–Meier method, with the log rank test to assess significance. The data were updated as of April 2022. Detailed characteristics of the study cases are presented in Table 1. All tissue specimens were used after approval from the Ethics Committee of Tianjin Medical University General Hospital (Ethical No. IRB2021-WZ-055) according to the Declaration of Helsinki. All patients who were enrolled in the study signed informed consent.

Immunohistochemical staining and scoring

The tumor tissues from core biopsy or resected samples were prepared in a tissue microarray format by a professional pathologist. Each of the immunological biomarkers was stained by Immunohistochemical (IHC), respectively, in the continuous pathological sections of formalin-fixed, paraffin-embedded tissue microarray (TMA). The 4 μm TMA was baked at 70°C for 50 minutes, deparaffinized in xylene baths two times for 20 min (Solarbio, China) and rehydrated in graded alcohol baths. Slides were washed three times in phosphate-buffered saline (PBS). Next, antigen retrieval was performed in a microwave oven (Midea, China) with Citrate Antigen Retrieval Solution (Beyotime, China) pH 6.0 for 15 min at 100°C and 10 min at 30°C. The slides were washed as described above. After blocking endogenous peroxidase (ZSGB-BIO, China) for 15 minutes, the slides

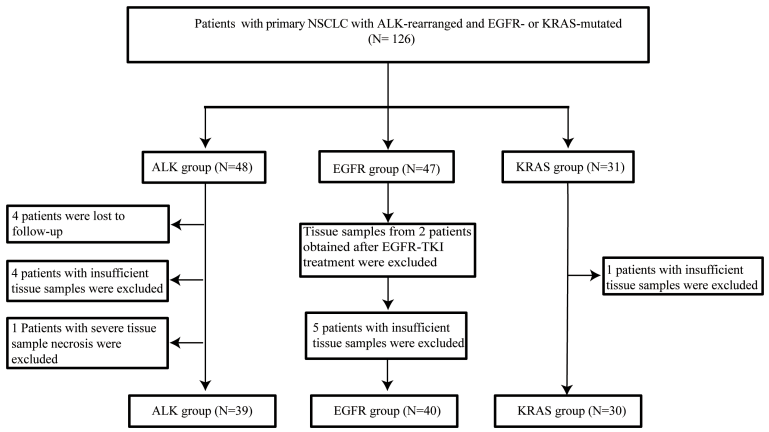

FIGURE 1
Flow chart for inclusion or exclusion of patients and specimens.

TABLE 1 Clinical and pathological characteristics of ALK-/EGFR-/KRAS-positive NSCLC.

Mutation subtype	ALK-rearranged	EGFR-positive	KRAS-positive	P
Number	39	40	30	
Age: (mean ± SD, years)	56.00 ± 11.13	60.05 ± 7.88	65.93 ± 9.22	0.221
< 65	28 (71.79%)	28 (70.00%)	16 (53.33%)	
≥ 65	11 (28.21%)	12 (30.00%)	14 (46.67%)	
Gender				0.081
Male	17 (43.59%)	11 (27.50%)	16 (53.33%)	
Female	22 (56.41%)	29 (72.50%)	14 (46.67%)	
Smoking status:				< 0.001
Current or ever	8 (20.51%)	8 (20.00%)	17 (56.67%)	
Never	31 (79.49%)	32 (80.00%)	13 (43.33%)	
Tumor stage*:				< 0.001
I-IIIB	12 (30.77%)	27 (67.50%)	22 (73.33%)	
IIIC-IV	27 (69.23%)	13 (32.50%)	8 (26.67%)	
Histology type				0.531
Adenocarcinoma	38 (97.44%)	40 (100%)	29 (96.67%)	
Non-adenocarcinoma	1 (2.56%)	0 (0%)	1 (3.33%)	
Distant metastasis				0.397
Yes	11 (28.21%)	7 (17.50%)	5 (16.67%)	
No	28 (71.79%)	33 (82.50%)	25 (83.33%)	
ALK TKIs treatment#				
Yes	33 (84.62%)	NA	NA	
No	6 (15.38%)	NA	NA	

*AJCC 8th edition.
#ALK TKIs include alectinib or crizotinib.
NA, Not Available.

were incubated with goat serum (ZSGB-BIO, China) for 30 minutes at room temperature. Then, the slides were incubated with the primary antibodies overnight at 4°C in a humidified chamber. Detailed information on the primary antibodies is presented in Supplementary Table 1. After washing three times in PBS, the slides were incubated with biotin-labeled goat anti-rabbit IgG polymer (ZSGB-BIO, China) and horseradish peroxidase-conjugated streptavidin (ZSGB-BIO, China) in sequence for 30 minutes and visualized by a 3,3′-diaminobenzidine (DAB) (ZSGB-BIO, China) stain system under a microscope. The slides were counterstained in a hematoxylin dye vat for 30-40 seconds. The slides were washed in PBS and then rehydrated in graded alcohol (70% to 100%) and xylene baths before applying coverslips.

The process of pathological assessment was performed in this way. Firstly, the tumor tissue was assessed and prepared by a professional pathologist, followed by evaluation and scoring with an image-based analysis. Briefly, all stained slides were scanned by a panoramic scanner (Pannoramic MIDI, 3DHISTECH, Hungary) and CaseViewer2.4 software (3DHISTECH, Ltd.). Subsequently, the images were scored automatically by Aipathwell software (Servicebio, Wuhan, China). Finally, quality control of pathological tissues was carried out independent by another professional pathologist. Folds and tears, impurities, stain smudges, tumor necrosis or non-tumor in pathological tissue sections before scanning analysis were all excluded in this study. Some parameters, including positive cells density and the rate of positive cells in the tumor compartment were determined in the tumor compartment (36).

Statistical analysis

The Kruskal–Wallis U test was used to compare differences between multiple groups. We used the chi-square test to compare differences between categorical variables. Time-dependent receiver operating characteristic (ROC) curves were drawn to identify the optimal cutoff threshold for the high expression of different biomarkers (including PD-L1, PD-1 and CD8). Kaplan–Meier (KM) survival curves and the log-rank test were used to assess the significant differences. The P value was based on a two-sided hypothesis, and a P value < 0.05 was considered statistically significant. ROC analyses and chi-square test were conducted with SPSS 24.0 (SPSS, Chicago, IL, USA). All analyses and survival curves were made with GraphPad Prism (V.8.0.1, La Jolla, CA, USA) and R software (version 4.1.3).

Results

Characteristics of immune checkpoint markers in NSCLC with EGFR mutation, KRAS mutation or ALK rearrangement

Among the 109 patients with NSCLC, 39 patients had ALK rearrangements, 40 patients had EGFR mutations, and 30 patients were positive for KRAS mutations. All these patients had tissue slides for PD-L1 staining. Different biomarkers of tissue samples were stained by IHC examination (Supplementary Figures 1-3).

In the ALK-rearranged group, the proportions of PD-L1 in the TME were 0 (≥ 50%), 79.49% (1-49%) and 20.51% (< 1%) (Figure 2A). In the EGFR-positive group, the proportions were 0 (≥ 50%), 97.50% (1-49%) and 2.50% (< 1%) (Figure 2A). For the KRAS group, the proportions were 13.33% (≥ 50%), 86.67% (1-49%) and 0 (< 1%) (Figure 2A).

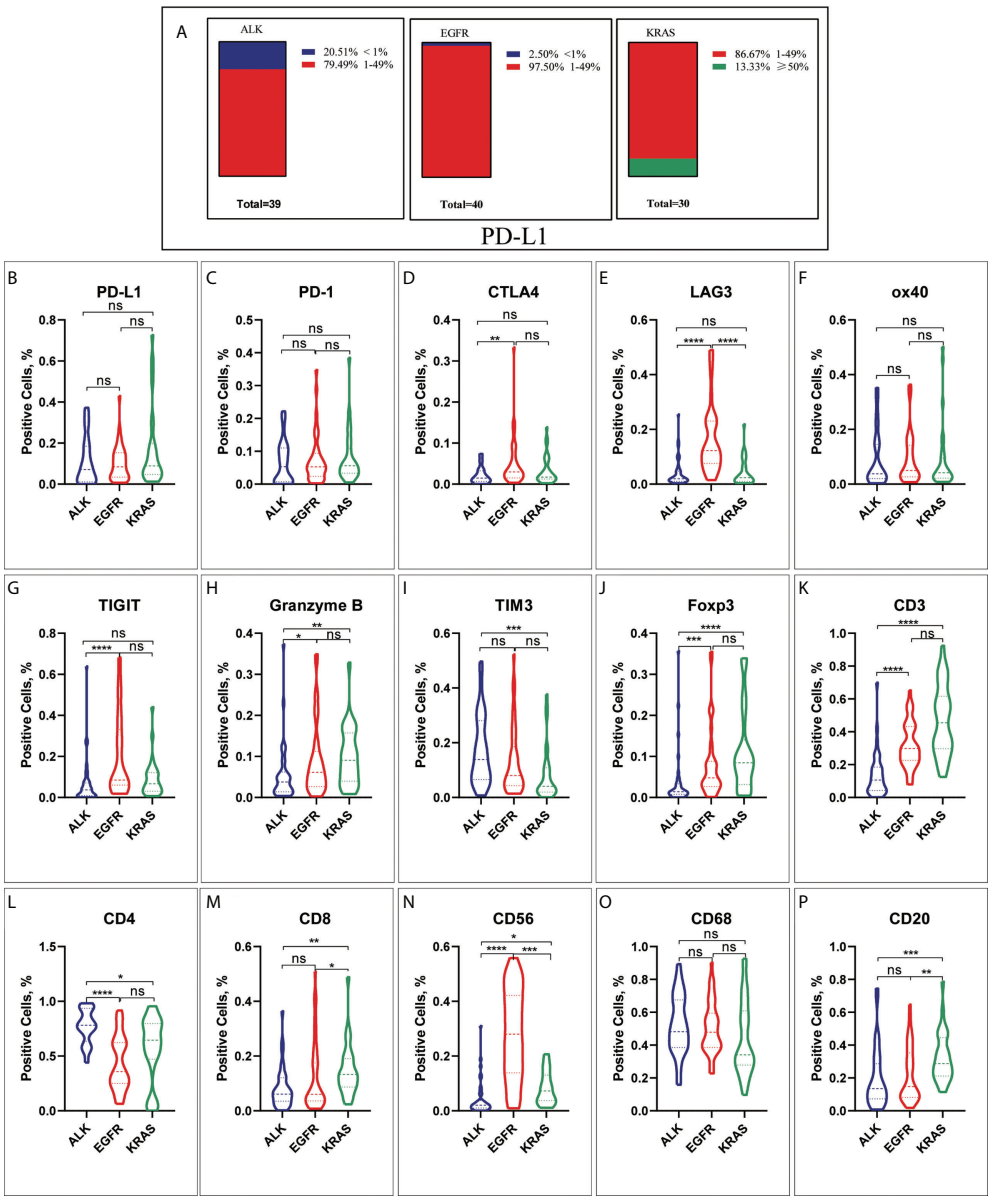

FIGURE 2
The characteristic of different immune checkpoints in ALK-positive, EGFR-positive and KRAS-positive NSCLC. **(A)** Frequency distribution of PD-L1 expression in ALK-rearranged, EGFR-positive and KRAS-positive NSCLC. **(B)** PD-L1, **(C)** PD-1, **(D)**CTLA4, **(E)** LAG3, **(F)** ox40, **(G)** TIGIT, **(H)** Granzyme B, **(I)** TIM3, **(J)** Foxp3, **(K)** CD3, **(L)** CD4, **(M)** CD8, **(N)** CD56, **(O)** CD68, **(P)** CD20. *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.0001. ns, No significant.

This indicates that the percentage of PD-L1 ≥ 50% in ALK- or EGFR-positive NSCLC patients was lower, and in KRAS-positive NSCLC patients, it was higher. In addition, we compared the expression of PD-L1 in the three groups according to different smoking statuses. We found that the expression of PD-L1 in EGFR-positive patients with a history of smoking was higher than that in patients without a history of smoking ($p < 0.05$, Supplement Figure 4). The expression of PD-L1 in KRAS mutation patients with stage IIIC-IV disease and distant metastasis was higher (all $p < 0.05$, Supplemental Figures 5, 6).

We investigated nine immune markers (PD-L1, PD-1, TIM3, LAG3, CTLA4, TIGIT, OX40, Granzyme B and Foxp3) and analyzed their expression in TME compartments. Although the expression of PD-1, PD-L1 and OX40 was not significantly different in ALK+, EGFR+ and KRAS+ NSCLC (all $p > 0.05$, Figures 2B, C, F), we discovered that patients with EGFR- and KRAS-positive NSCLC were more likely to have higher expression levels of granzyme B and Foxp3 than patients with ALK-positive NSCLC (all $p < 0.05$ Figures 2H, J). Patients with ALK rearrangements had lower expression levels of CTLA4, LAG3 and TIGIT in the TME than patients with EGFR mutations (all $p < 0.05$, Figures 2D, E, G). In contrast, the data verified that TIM3 expression was significantly increased in patients with ALK-positive NSCLC compared to patients with KRAS-positive NSCLC ($p < 0.05$, Figure 2I).

Characteristics of immune cell infiltration in the TME

The distribution of different TILs was analyzed in specimens from ALK-, EGFR- or KRAS-positive NSCLC patients by IHC examination. The statistical results showed that the proportions of CD3+, CD8+ and CD20+ TILs were significantly different between the KRAS+ and ALK+ and EGFR+ NSCLC groups, and the proportion was higher in KRAS+ NSCLC patients (all $p < 0.05$, Figures 2K, M, P). Conversely, the expression of CD4+ T cells was higher in the ALK-positive NSCLC group than in the KRAS- and EGFR-positive groups ($p < 0.05$, Figure 2L). As shown in Figure 2N, there were more EGFR+ patients with CD56+ NK cells than ALK+ and KRAS+ patients ($p < 0.05$). Nevertheless, there were no significant differences in CD 68+ macrophages between the ALK+, EGFR+ and KRAS+ NSCLC groups (all $p > 0.05$, Figure 2O).

A previous study defined exhausted T cells as CD8+PD-1+, CD8+LAG3+, CD8+TIM-3+ or CD8+CTLA4+ and non-exhausted T cells as CD8+PD-1–LAG-3–TIM-3– (37–41). Therefore, to explore the function of T cells in the TME, we performed further research. In the ALK-rearranged group, the proportions of PD-1 CD8 T cells in the TME were 43.59% (PD-1+/CD8+), 23.08% (PD-1-/CD8-), 30.77% (PD-1+/CD8-), and 2.56% (PD-1-/CD8+) (Figure 3A). In the EGFR+ group, the percentage of exhausted T-cell infiltration in the TME was 35.00% (PD-1+/CD8+), 15.00% (PD-1-/CD8-), 12.50%

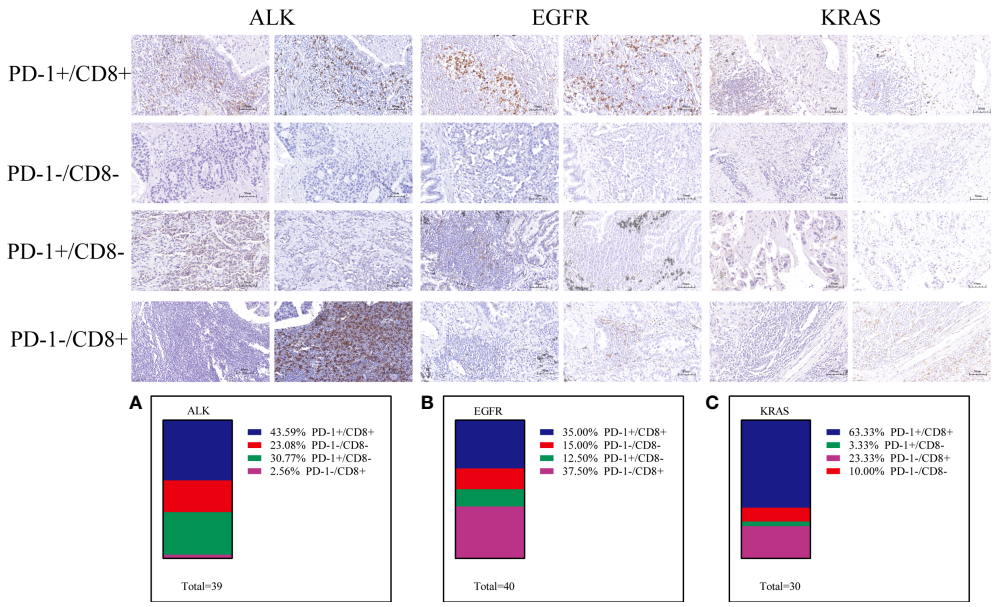

FIGURE 3
Differential proportion of PD-1+/CD8+, PD-1-/CD8-, PD-1+/CD8-and PD1-/CD8+ in ALK-positive **(A)**, EGFR-positive **(B)** and KRAS-positive **(C)** NSCLC. Low (-) and High (+) expression.

(PD-1+/CD8-), and 37.50% (PD-1-/CD8+) (Figure 3B). In the KRAS-positive group, the proportions were 63.33%, 10.00%, 3.33% and 23.33%, respectively (Figure 3C). Likewise, we further researched the distribution of LAG-3 CD8 T cells in the TME. The proportions were 12.82% (LAG-3+/CD8+), 46.15% (LAG-3-/CD8-), 7.69% (LAG-3+/CD8-), and 33.33% (LAG-3-/CD8+) for ALK-positive NSCLC (Figure 4A). For EGFR-positive NSCLC, the proportions were 22.50% (LAG-3+/CD8+), 17.50% (LAG-3-/CD8-), 10.00% (LAG-3+/CD8-), and 50.00% (LAG-3-/CD8+) (Figure 4B). In the KRAS-positive group, the proportions were 16.67% (LAG-3+/CD8+), 10.00% (LAG-3-/CD8-), 3.33% (LAG-3+/CD8-), and 70.00% (LAG-3-/CD8+) (Figure 4C). We next studied the effect of TIM3 CD8 T cells on the three groups of patients and found that the proportions were 30.77% (TIM3+/CD8+), 48.72% (TIM3-/CD8-), 5.13% (TIM3+/CD8-), and 15.38% (TIM3-/CD8+) in the ALK-positive group (Figure 5A). For EGFR-positive NSCLC, the proportions were 40.00% (TIM3+/CD8+), 20.00% (TIM3-/CD8-), 7.50% (TIM3+/CD8-), and 32.50% (TIM3-/CD8+) (Figure 5B). In the KRAS-positive group, the proportions were 53.33%, 10.00%, 3.33% and 33.33%, respectively (Figure 5C). Similarly, the proportions of ALK-rearranged NSCLC were 33.33% (CTLA4+/CD8+), 35.90% (CTLA4-/CD8-), 17.95% (CTLA4+/CD8-), and 12.82% (CTLA4-/CD8+) (Figure 6A). For the EGFR group, the proportions of ALK-rearranged NSCLC were 52.50% (CTLA4+/CD8+), 7.50% (CTLA4-/CD8-), 20.00% (CTLA4+/CD8-), and 20.00% (CTLA4-/CD8+) (Figure 6B). In the KRAS-positive group, the proportions were 50.00%, 6.67%, 6.67% and 36.67%, respectively (Figure 6C).

Overall survival of ALK-rearranged NSCLC based on TME subtypes

To explore the clinical efficacy of TKIs in patients with advanced ALK-positive NSCLC, we performed survival analysis. As shown in Table 1, 27 out of 33 ALK-rearranged NSCLC patients who received TKIs were advanced stage (IIIC-IV). The baseline clinical characteristics of these patients are presented in Supplementary Table 2 and Supplementary Table 3. Patients whose tumor samples showed higher PD-L1 expression levels in the tumor cells had a shorter overall survival (OS) than those with lower PD-L1 expression levels (HR = 0.177 [95% CI 0.038–0.825], p = 0.027; Figure 7A). Furthermore, we analyzed correlations between the expression of other immunomarkers and OS in the study. The group of patients whose tumor samples showed low CTLA4 expression showed a significant increase in OS compared to the group with high CTLA4 expression (HR = 0.196 [95% CI 0.041–0.947], p = 0.043; Figure 7B).

Discussion

A previous study indicated that NSCLCs harboring ALK rearrangements are associated with low ORRs to PD-1/PD-L1

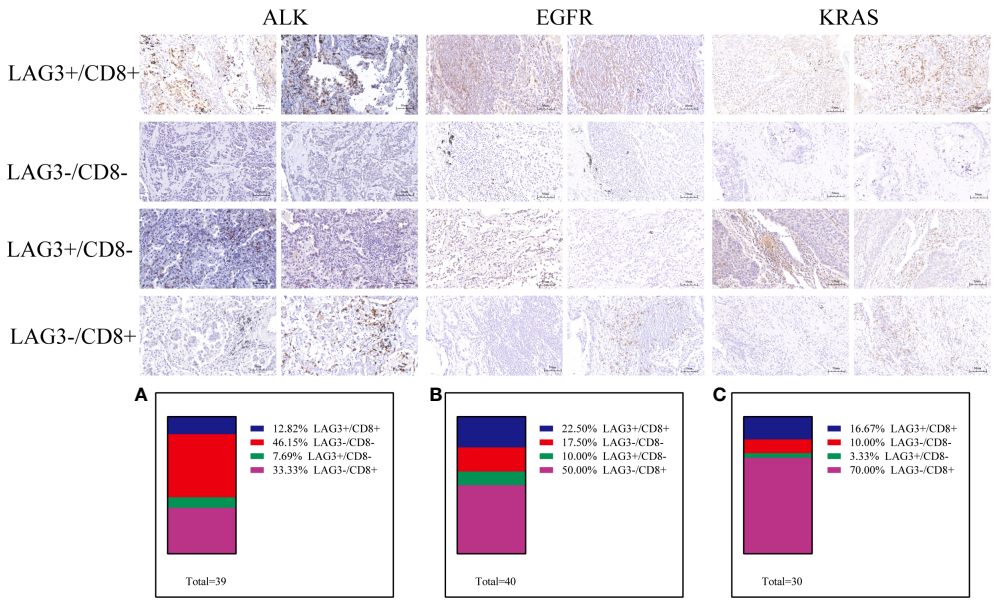

FIGURE 4
Differential proportion of LAG3+/CD8+, LAG3-/CD8-, LAG3+/CD8-and LAG3-/CD8+ in ALK-positive **(A)**, EGFR-positive **(B)** and KRAS-positive **(C)** NSCLC. Low (-) and High (+) expression.

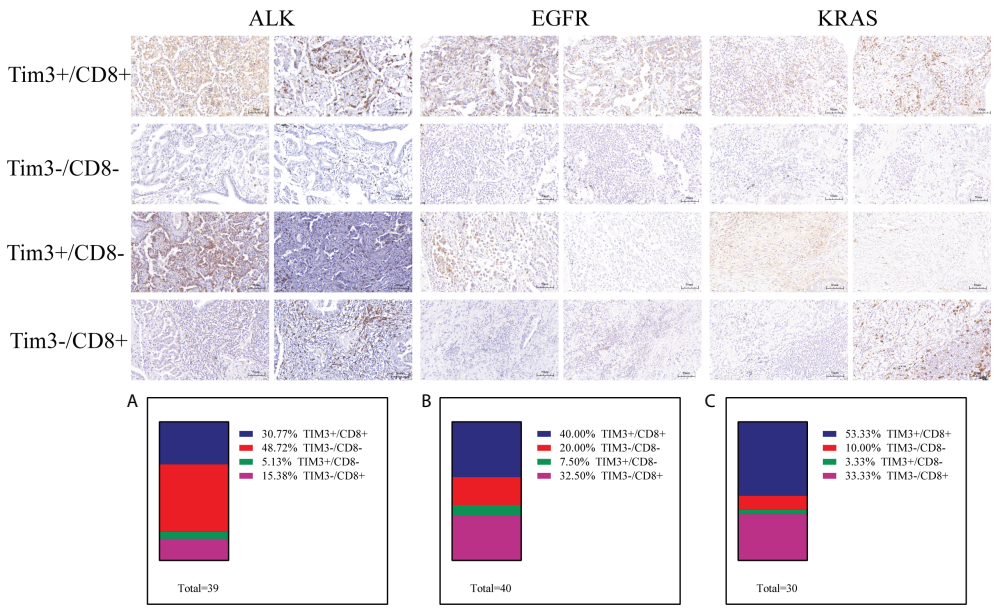

FIGURE 5
Differential proportion of TIM3+/CD8+, TIM3-/CD8-, TIM3+/CD8-and PTIM3-/CD8+ in ALK-positive **(A)**, EGFR-positive **(B)** and KRAS-positive **(C)** NSCLC. Low (-) and High (+) expression.

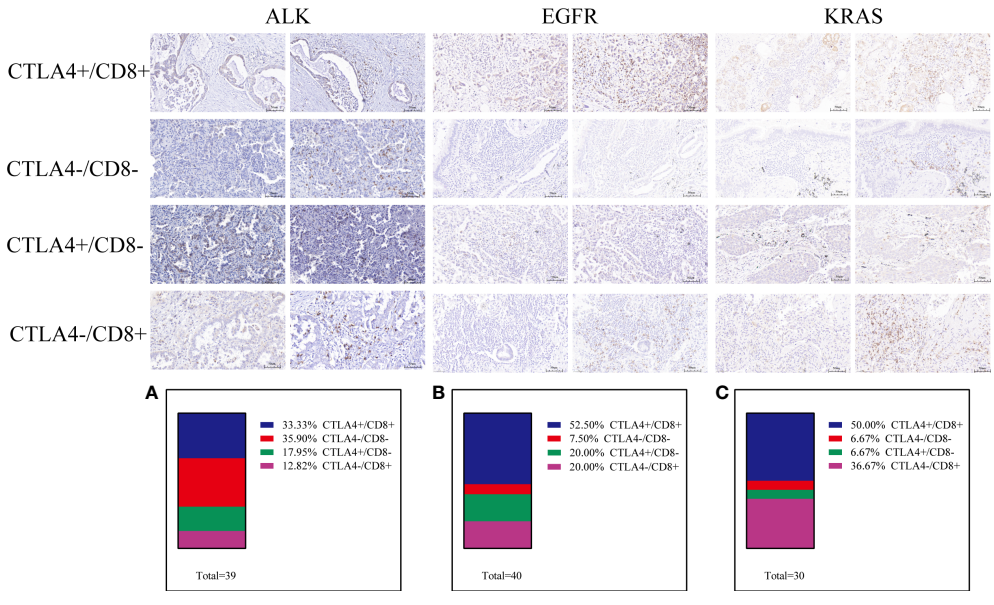

FIGURE 6
Differential proportion of CTLA4+/CD8+, CTLA4-/CD8-, CTLA4+/CD8- and CTLA4-/CD8+ in ALK-positive **(A)**, EGFR-positive **(B)** and KRAS-positive **(C)** NSCLC. Low (-) and high expression (+).

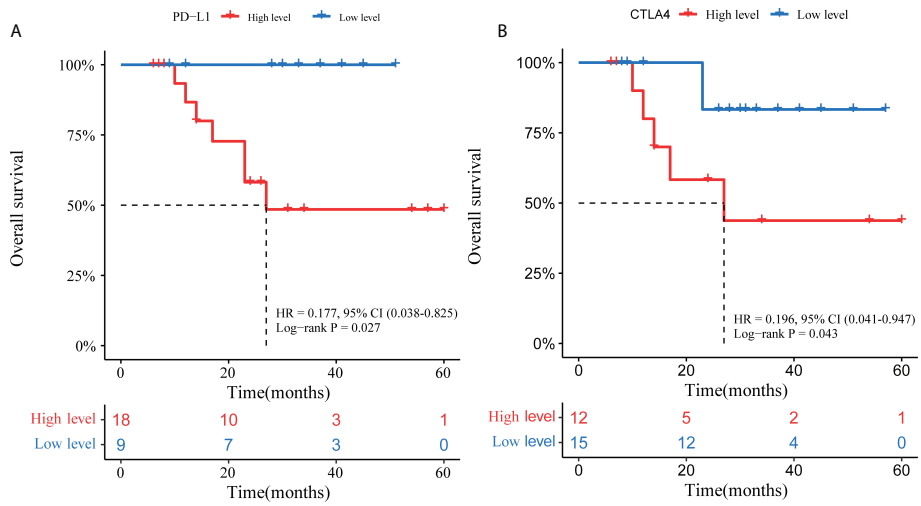

FIGURE 7
Overall survival Kaplan-Meier curves by PD-L1 **(A)** and CTLA4 **(B)** expression.

inhibitors (20), but the specific background of the TME is still unclear. In this regard, we evaluated the different lymphocytic infiltration statuses, expression of immune markers and clinical significance in 109 patients with ALK rearrangements and EGFR/KRAS positivity. This study, to our knowledge, is the largest real-world study which investigates the TME of Chinese patients with ALK rearrangements to date.

Our study found that there was a significant difference in the TMEs of patients with different driver gene positivity statuses. PD-L1≥50% expression was more obvious in KRAS-positive patients than in ALK-/EGFR-positive patients, and the number of PD-L1-positive patients was greater than the number of PD-L1-negative patients in the ALK+ group. A previous study illustrated that high PD-L1 expression was significantly associated with ALK-/EGFR-negative NSCLC (42). Our results showed KRAS-mutant patients had relatively higher rates of concurrent PD-1 expression and CD8+ TILs than ALK-/EGFR-mutant patients. The proportion of non-exhausted CD8+PD-1- T cells in KRAS-mutated NSCLC was higher than that in ALK -mutated NSCLC. Immunological competent cell infiltration was associated with antitumor activity. Preclinical studies have shown that PD-1 inhibitors improve the survival of mice with EGFR-driven lung cancer by enhancing T-cell function (43). Chang Gon Kim et al. showed that patients with PD-1high CD8+ TILs (PD-1-high expressers) exhibited characteristics associated with a favorable anti-PD-1 response compared with those without these lymphocytes (non-PD-1-high expressers) (44). Mechanistically, a low proportion of PD-1+ and CD8+ TILs co-expressed may underlie the low response rates to PD-1/PD-L1 inhibitors in EGFR-/ALK-positive NSCLC patients. Dysfunctional CD8+ TILs expressing inhibitory receptors facilitate tumor immune escape. Our data showed that the proportion of non-exhausted T cells expressing Tim-3-CD8+, CTLA4-CD8+ and PD-1-CD8+ in ALK-positive NSCLC was lower than that in EGFR/KRAS-positive NSCLC. Thus, the TME in patients with ALK-positive NSCLC may be inhibited.

In addition, a study showed that the TME of EGFR-mutant NSCLC was immunosuppressive (45). Previous studies reported that a high proportion of tumor-infiltrating lymphocytes (TILs), particularly those with cytotoxic functions, such as CD8+ and natural killer cells, are correlated with a favorable prognosis in various cancers (46, 47). Our data illustrated that ALK-mutated NSCLC patients had a lower population of activated immune markers, such as CD3, CD8, Granzyme B, and CD20, and a higher expression of immunosuppressive markers, such as TIM3, than KRAS-mutated NSCLC patients. Previous studies showed a similar trend. A previous study showed that TIM3 is a marker of highly suppressive tissue-resident Tregs that play an important role in shaping the antitumor immune response (29). A retrospective analysis showed that NSCLCs with EGFR mutations or ALK rearrangements generally lack T-cell infiltration (20). Nevertheless, in ALK-positive NSCLC patients, the levels of CD4+ helper T cells were significantly higher than those in EGFR-/KRAS-positive patients. Although the TME of ALK-positive patients was different from that of EGFR-/KRAS-positive patients, there was not a total absence of immune infiltration. Our study findings were supported by the work of Jan Budczies et al., who showed that the specific immunosuppressive characteristics of ALK- and EGFR-positive lung adenocarcinoma suggest further clinical evaluation of immune modulators as partners of ICBs in such tumors (48).

To date, no research has performed an overall survival analysis in ALK-positive patients treated with TKI based on PD-L1 expression. Several studies only evaluated the immune landscape with a few markers, such as PD-L1, PD-1, CD3, and CD8 (20, 48, 49). Our results showed that high PD-L1 expression was associated with lower OS than low PD-L1 expression. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is expressed on the membrane of T cells and inhibits T-cell activation. The immune checkpoint receptor CTLA4 plays a crucial role in negatively regulating function in TME. In our study, in ALK-positive patients treated with TKI, those with low expression of CTLA4 had longer OS, which showed that CTLA4 may have a negative prognostic impact. A clinical trial showed that targeted EGFR or ALK therapy combined with ipilimumab could notably improve PFS and OS (50).

There are still some limitations in our study. First, based on the nature of this retrospective study, some selection biases were inevitable. Second, immunohistochemical assessment based on artificial intelligence is unable to fully distinguish between tumor nests and tumor stroma. Third, we only enrolled patients from a single institution. Other multicenter studies with larger patient cohorts may address these limitations. Lastly, spatial transcriptomics was not performed in this study due to limited tissue specimens.

In summary, the TME of patients with ALK-positive NSCLC was immunosuppressive compared with that of patients with EGFR/KRAS mutations. High expression of PD-L1 and CTLA4 was an adverse prognostic factor in ALK-rearranged NSCLC patients treated with ALK-TKIs. Immunotherapy for ALK-rearranged patients requires further exploration and validation by clinical trials.

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

All tissue specimens were used after approval from the Ethics Committee of Tianjin Medical University General Hospital (Ethical No. IRB2021-WZ-055). The patients/participants provided their written informed consent to participate in this study. 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

BZ contributed to write the manuscript. BZ, JZ and SZ performed the IHC. BZ, HZ, HW, JH, LY and NZ performed data selection and data analysis. BZ and HZ contributed to the follow-up of patients. LZ and XX contributed to methodology. ZS and SX contributed to the conceptualization, writing—review and editing, supervision and design of the research. 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/fimmu.2022.974581/full#supplementary-material

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Association between immune-related adverse events and the efficacy of PD-1 inhibitors in advanced esophageal cancer

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Introduction: Recent developments in immune checkpoint inhibitors (ICIs) have improved the treatment outcomes of esophageal cancer (EC); however, it may initiate immune-related adverse events (irAEs) in some patients. The ICIs' therapeutic efficacy is associated with irAEs in patients with non-small cell lung cancer or renal cell carcinoma, although this association is unknown in EC. The purpose of this study was to explore the association between irAEs and the efficacy of programmed death 1 (PD-1) inhibitors in EC patients.

Patients and methods: This study included patients with advanced EC treated with PD-1 inhibitors. The patients were divided into two groups according to the occurrence of irAEs. Afterward, the efficacy was compared between the irAE-negative and irAE-positive groups, and we analyzed the predictive factors of irAEs and survival.

Results: Overall, 295 patients were included in this study. Baseline characteristics were balanced in the irAE-negative and irAE-positive groups. In total, 143 (48.47%) patients experienced irAEs. The most frequent irAEs were anemia (49, 16.61%), hyperthyroidism (45, 15.25%), and pneumonitis (44, 14.92%). In total, 33 (11.19%) patients had grade ≥ 3 irAEs and pneumonitis have 15 (5.08%). No grade 5 adverse events were observed. A total of 52 (17.63%) and 91 (30.85%) patients had single and multiple irAEs, respectively. Compared with patients without irAEs, those with irAEs had significantly higher objective response rate (ORR) (37.76% vs. 25.00%, $p = 0.018$) and disease control rate (DCR) (92.31% vs. 83.55%, $p = 0.022$). Univariate Cox analyses indicated the significant association between irAEs and improved median progression-free survival (PFS) (10.27 vs. 6.2 months, $p < 0.001$) and overall survival (OS) (15.4 vs. 9.2 months, $p < 0.001$). In multivariate analyses, irAEs were independently associated with longer PFS ($p = 0.011$) and OS ($p = 0.002$). Moreover, multivariate analysis revealed that cycles > 8, radiation, as well as antiangiogenic therapy were strongly associated with irAEs development ($p < 0.001$, $p = 0.002$, and $p = 0.025$, respectively).

Conclusion: In advanced EC, patients with irAEs showed markedly better efficacy in ORR, DCR, PFS, and OS compared with patients without irAEs.

KEYWORDS

esophageal cancer, immune-related adverse events, PD-1 inhibitors, immune checkpoint inhibitors, prognostic marker

Introduction

Esophageal cancer (EC) ranks seventh in the incidence of cancer and the sixth most frequent cause of cancer-related death worldwide (1). However, conventional radiotherapy and chemotherapy have limited efficacy and cause serious adverse effects for EC patients. Recently, immune checkpoint inhibitors (ICIs) have become an essential and promising therapy for advanced EC (2). Programmed death 1 (PD-1) and programmed death-ligand 1 (PD-L1) are immune checkpoints, whose inhibitors have been found to trigger T lymphocytes, inhibit the growth of cancer cells, and improve survival in cancer patients (3, 4). Until recently, nivolumab, pembrolizumab, and camrelizumab plus chemotherapy, which have resulted in a more prolonged overall survival (OS) and progression-free survival (PFS) compared to cytotoxic chemotherapy, are recommended as first-line treatment for advanced EC patients (5–8).

However, it has to be noticed that ICIs can cause immune-related adverse events (irAEs), which may occur in any organ system and may be permanent or even life-threatening. IrAEs might impair quality of life or even lead to death (9). The incidence rate of irAEs of any grade has been reported to be 66% with PD-1/L1 inhibitors, and combination therapy can increase the risk of irAEs in patients with multiple solid tumor types (10–13). Although the mechanism of irAEs is unclear, a potential mechanism might be that ICIs enhance systemic T-cell activity resulting in the loss of immune tolerance in individual organs, which causes irAEs (14). Some retrospective studies have claimed that the occurrence of irAEs is associated with better treatment response or prognosis, such as objective response rate (ORR), disease control rate (DCR), PFS, and OS, in renal cell carcinoma and non-small cell lung cancer (NSCLC) (15–19). However, currently, reliable data regarding the relationship between irAEs and prognosis in patients with advanced EC treated with PD-1 inhibitors are insufficient.

In this study, we aimed to investigate the potential association between irAEs and outcomes of PD-1 inhibitors and identify factors related to the outcomes of PD-1 inhibitors treatment in patients with advanced EC.

Methods

Patients

Patients with histologically confirmed EC who had been treated with ICIs therapy at least two doses between January 2018 and August 2021 at Shandong Cancer Hospital and Institute, Shandong First Medical University were included in this study. Patients who had previously received ICIs were excluded. We reviewed the medical records and the following patient characteristics prior to initiation of ICIs treatment: age, sex, Eastern Cooperative Oncology Group Performance Status (ECOG PS), stage, histology, history of surgery, metastatic sites, immunotherapy line, and lactate dehydrogenase (LDH) level. The PS at the initiation of ICIs therapy was evaluated by the ECOG PS scale. The disease stage was evaluated on the basis of the American Joint Committee on Cancer VIII staging system.

Clinical assessments were performed by the Response Evaluation Criteria in Solid Tumors version 1.1 criteria at baseline and every 2-3 courses (every two months). The best overall response was defined as the best response achieved after the initiation of PD-1 inhibitors. Data regarding irAEs were collected from clinical notes, hospitalization records, and laboratory values. All irAEs were graded by the senior doctors according to the Common Terminology Criteria for Adverse Events version 5.0. Multiple irAEs were defined as irAEs of ≥ 2.

All patients were divided into an irAE-positive group (with irAEs) and an irAE-negative group (without irAEs) based on the occurrence of irAEs. Differences in efficacy were analyzed between the irAE-positive and irAE-negative groups.

Statistical analysis

Categorical data were analyzed based on the chi-squared test, and Student's t-test was performed to analyze quantitative data. Survival data were evaluated with both Kaplan-Meier and log-rank tests. Logistic regression analyses were used to determine whether data were associated with irAEs. Univariate and multivariate comparisons of PFS and OS were performed

using Cox proportional hazards regression models. A two-tailed $p < 0.05$ was considered statistically significant. Statistical analyses were performed with IBM SPSS 26.0.

Results

Patient characteristics

This study included 295 patients. The median age was 60 (range, 36–84) years, the majority were male (259, 87.8%), and 210 (71.2%) patients received at least one prior systemic treatment. 23 were treated with immunotherapy alone, 95 with immunotherapy combined with chemotherapy, 148 with immunotherapy combined with radiotherapy, and 29 with immunotherapy combined with antiangiogenic therapy.

There were no significant differences in baseline characteristics between patients with and without irAEs (Supplementary Table 1).

Incidence of immune-related adverse events

In total, 143 (48.47%) patients experienced irAEs. The most frequent irAEs were anemia (49, 16.61%), hyperthyroidism (45, 15.25%), and pneumonitis (44, 14.92%). In total, 33 (11.19%) patients experienced ≥ 3 grade irAEs, with the most frequent being pneumonitis (15, 5.08%). It was not observed that grade 5 adverse events related to immunotherapy. A total of 52 (17.63%) and 91 (30.85%) patients had single and multiple irAEs, respectively. Twenty-three of the patients who experienced irAEs were treated with glucocorticoid for serious irAEs, and 10 patients with endocrine irAEs required hormonal replacement therapy. The details of irAEs are described in Table 1.

Association between irAEs and the efficacy of PD-1 inhibitors

Patients who presented with irAEs had better ORR (37.76% vs. 25.00%, $p = 0.018$) and DCR (92.31% vs. 83.55%, $p = 0.022$) than those without irAEs, as shown in Figure 1 and Table 2.

Patients in irAE-positive group had higher median PFS compared with patients in the irAE-negative group (10.27 months vs. 6.2 months; hazard ratio [HR], 0.509; 95% confidence interval [CI], 0.374–0.694; $p < 0.001$) (Figure 2A). Patients in the irAE-positive group had higher median OS compared with those in the irAE-negative group (15.4 months vs. 9.2 months; HR, 0.420; 95% CI, 0.301–0.585; $p < 0.001$) (Figure 2B).

Interestingly, even in patients who received ICIs for ≤ 8 cycles (n = 221), the median PFS and OS were significantly longer in the irAE-positive group (n = 92) than in the irAE-negative group (n = 129) (PFS: 5.7 months vs. 4.6 months; HR, 0.682; 95% CI, 0.488–0.953; $p = 0.024$; OS: 11.2 months vs. 7.1 months; HR, 0.585; 95% CI, 0.413–0.829; $p = 0.002$).

When analyzing survival outcomes based on the number of irAEs, patients who presented with a single irAE (n = 52) had a significantly longer PFS and OS compared to those with ≥ 2 irAEs (n = 91) or who did not experience irAEs (n = 152) (PFS:

TABLE 1 Immune-related adverse events according to category and grade.

Category	Total N (%)	Grade 1-2 N (%)	Grade 3-4 N (%)
Any	143 (48.47)	110 (37.29)	33 (11.19)
Anemia	49 (16.61)	44 (14.92)	5 (1.69)
Hyper/hypothyroidism	45 (15.25)	43 (14.58)	2 (0.68)
Pneumonitis	43 (14.58)	28 (9.49)	15 (5.08)
Cardiovascular toxicities	35 (11.86)	32 (10.85)	3 (1.02)
Fatigue	23 (7.80)	22 (7.46)	1 (0.34)
Thrombocytopenia	22 (7.46)	20 (6.80)	2 (0.68)
Fever	17 (5.76)	17 (5.76)	0 (0)
Elevated transaminase	16 (5.42)	13 (4.41)	3 (1.02)
Anorexia	15 (5.08)	1 (0.34)	0 (0)
Reactive cutaneous capillary endothelial proliferation	13 (4.41)	12 (4.07)	1 (0.34)
Nausea/vomiting	10 (3.39)	10 (3.39)	0 (0)
Rash	5 (1.69)	4 (1.36)	1 (0.34)
Pruritus	5 (1.69)	5 (1.69)	0 (0)
Arthralgia/Myalgia	4 (1.36)	4 (1.36)	0 (0)
Diarrhea/colitis	1 (0.34)	1 (0.34)	0 (0)

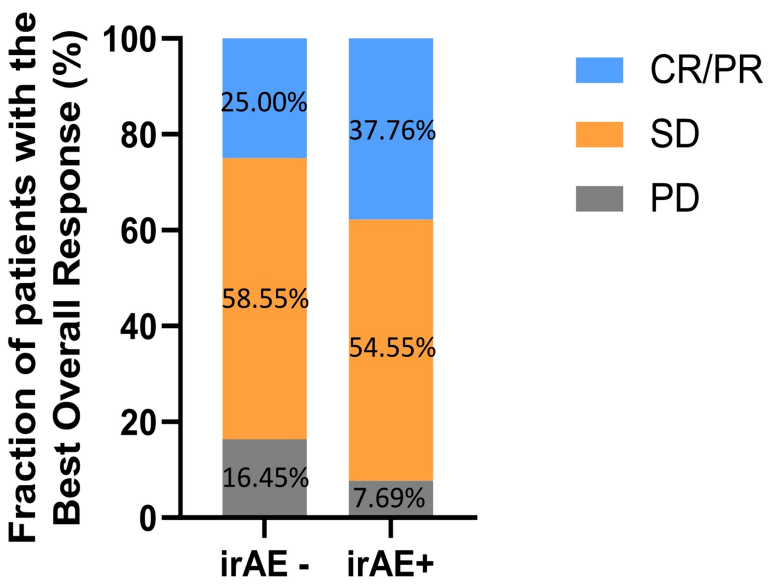

FIGURE 1
The fraction of patients with the best overall response in patients with or without irAEs. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; irAEs, immune-related adverse events.

12.1 vs. 8.5 vs. 6.2 months, p < 0.001; OS: 23.9 vs. 12.9 vs. 9.2 months, p < 0.001) (Figures 3A, B).

Among the various irAEs, no significant association between pneumonia or skin et al. irAEs and survival was observed in our study.

Univariate and multivariate cox analyses of PFS and OS

Univariate analysis revealed that ECOG PS ≥ 2, number of organs with metastases ≥ 2, cycles ≤ 8, LDH level > the upper limit of normal (ULN), and without irAEs were significantly associated with shorter PFS. Multivariate analysis demonstrated that ECOG PS ≥ 2, cycles ≤ 8, LDH level > the ULN, and no irAEs were independent factors for worse PFS (Table 3).

Univariate analysis showed that ECOG PS ≥ 2, immunotherapy line ≥ 2nd, cycles ≤ 8, no radiation, LDH > the ULN, and no irAEs were associated with poor OS. Multivariate analysis of OS showed that ECOG PS ≥ 2, immunotherapy line ≥ 2nd, cycles ≤ 8, no radiation, and no irAEs were independent poor prognostic factors (Table 4).

Age, sex, history of surgery, history of smoking, and history of alcohol consumption were not associated with PFS or OS (Supplementary Table 2).

Prognostic factors predicting irAEs

Patients who received PD-1 inhibitors for > 8 cycles, combination radiation, or antiangiogenic therapy during immunotherapy were found to have an increased risk of irAEs by univariate and multivariate analyses. No significant associations between irAEs and age, sex, ECOG PS, therapy line, or a number of organs with metastases and LDH level were observed (Table 5).

TABLE 2 Best overall response during PD-1 inhibitors.

	All patients	irAE - group	irAE + group	p value
Total	295	152	143	
CR/PR	92	38	54	
SD	167	89	78	
PD	36	25	11	
ORR, %	31.19	25.00	37.76	**0.018***
DCR, %	87.80	83.55	92.31	**0.022***

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate; DCR, disease control rate; irAEs, immune-related adverse events. *p<0.05.

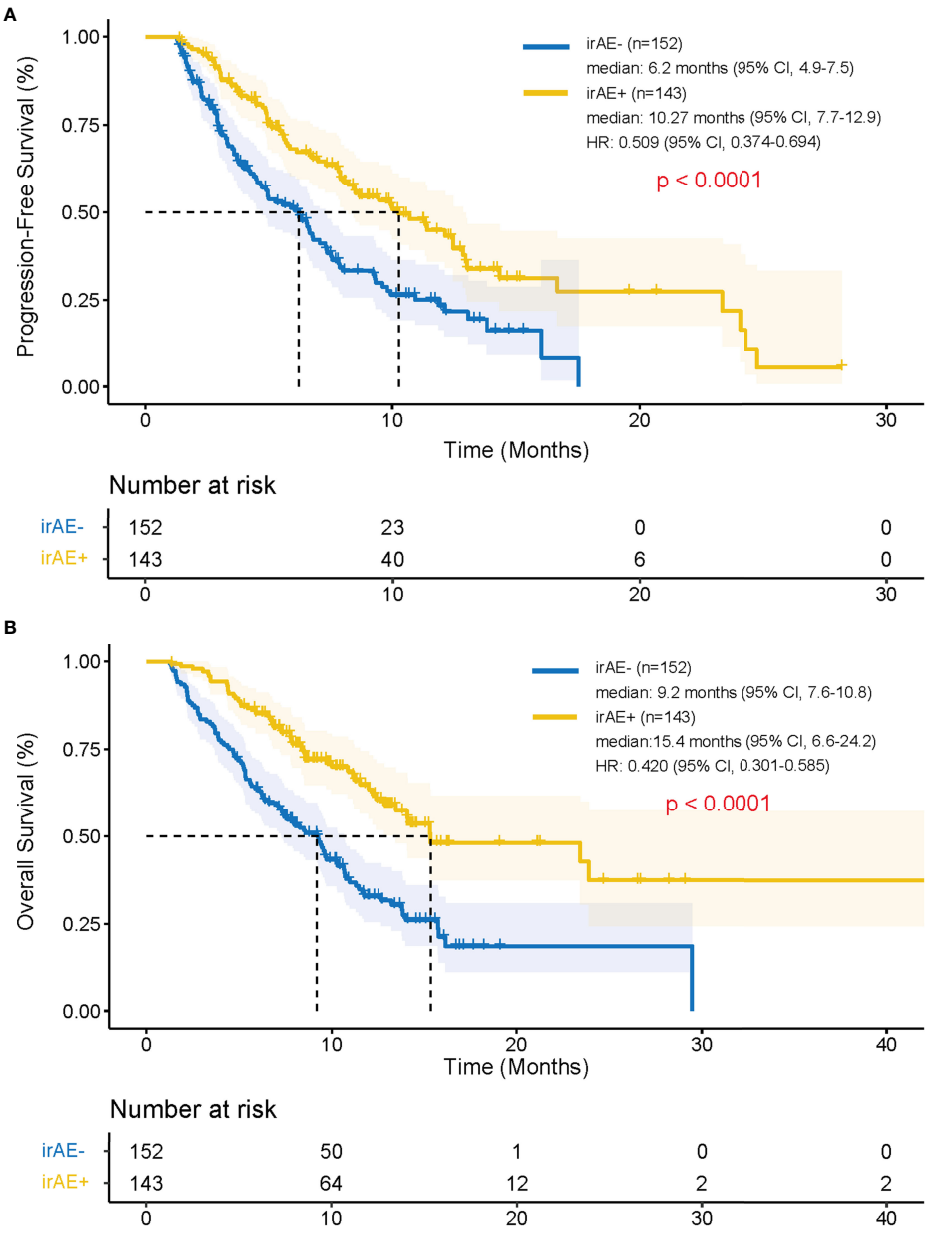

FIGURE 2
Progression-free survival and overall survival after the treatment of PD-1 inhibitors depending on the development of irAEs using Kaplan-Meier method. Kaplan–Meier curves for **(A)** progression-free survival and **(B)** overall survival in patients with or without irAE. irAEs, immune-related adverse events; HR, hazard ratio; CI, confidence interval.

Discussion

ICIs have shown superior therapeutic efficacy and prognosis in patients with EC. However, ICIs treatment is frequently accompanied by irAEs. It is unclear whether the development of irAEs is related to the better outcome of ICIs in patients with EC. To our knowledge, this study demonstrates that patients with irAEs had superior outcomes from PD-1 inhibitors, including higher ORR and DCR, and better PFS and OS in patients with advanced EC.

A significant association has been reported with increased survival after ICIs treatment for irAEs in gastrointestinal cancer (20) or NSCLC (16, 17). However, whether the occurrence of irAEs indicates a superior response and survival outcomes in melanoma patients remains contentious (21, 22). This indicates that the differences in the association between irAEs and ICIs

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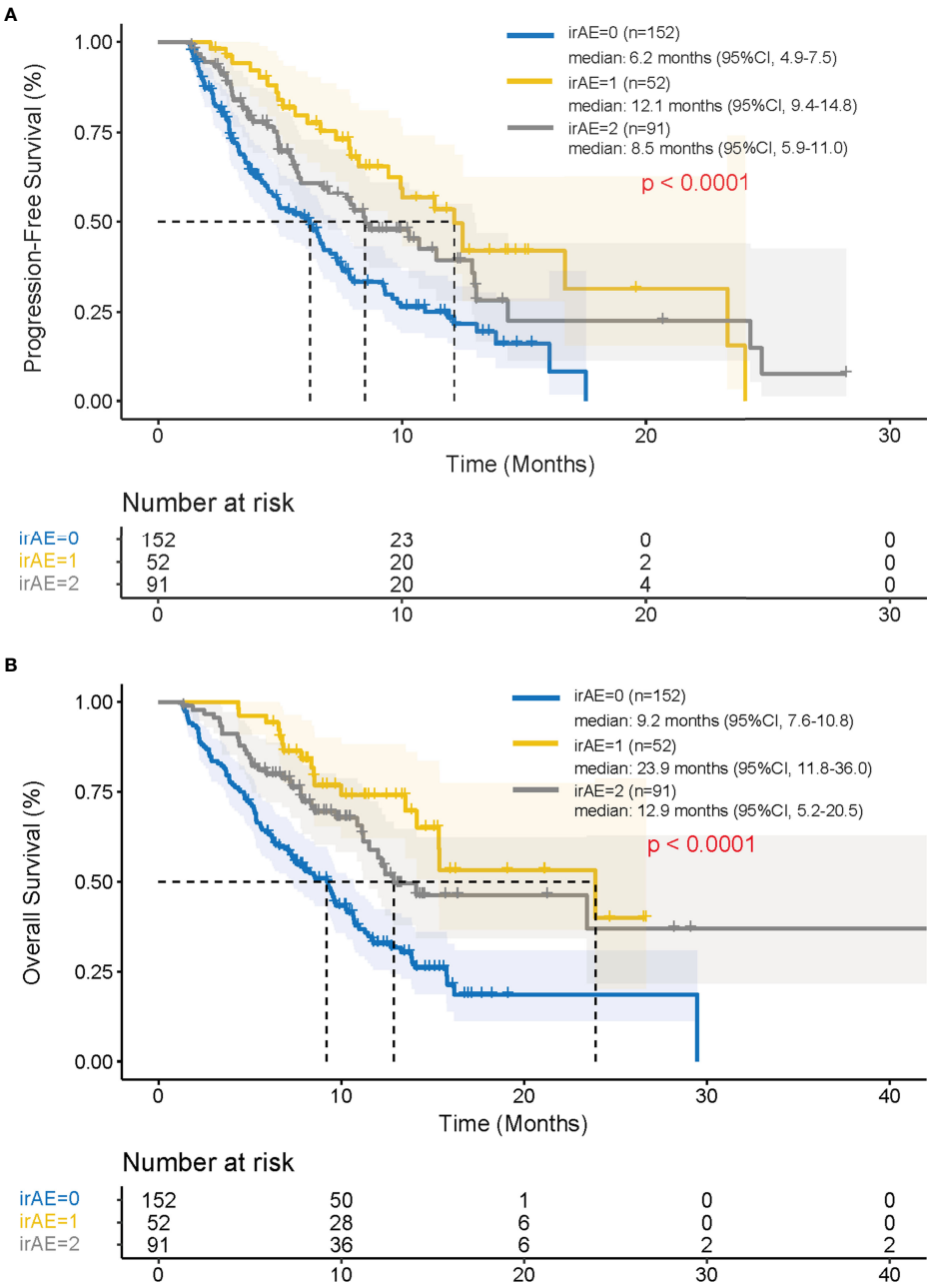

FIGURE 3
Progression-free survival and overall survival after the initiation of PD-1 inhibitors depending on the number of irAEs using Kaplan-Meier method. Kaplan–Meier curves for **(A)** progression-free survival and **(B)** overall survival in patients with ≥2 irAEs compared with those with one irAE and none irAEs. irAEs, immune-related adverse events; CI, confidence interval.

treatment may differ in different tumors. Thus, this study evaluated the association between irAEs and the clinical outcomes of PD-1 inhibitors treated in advanced EC. Although the actual pathophysiology of irAEs has not been completely elucidated, various mechanisms have been reported to explain the development of irAEs. IrAEs may be triggered by antigens commonly shared by tumors and normal tissues, which then release T cells to attack these two tissues, producing both response and toxicity. In a prospective study of 73 NSCLC patients who received PD-1 inhibitors, TCR clonotype analysis was made on four patients with skin irAEs, and common T-cell clones were found to exist in both the skin and tumor in four patients (23). Another study showed that pre-existing organ-specific antigen exposure may be responsible for the irAEs from ICIs (4, 24).

TABLE 3 Results of univariate and multivariate analyses showing factors affecting the progression-free survival.

Variables	Univariate analysis		Multivariate analysis	
	HR for PFS (95% CI)	p value	HR for PFS (95% CI)	p value
ECOG PS				
<2	1.750 (1.296-2.362)	**<0.001***	1.607 (1.170-2.206)	**0.003***
≥2				
Number of organs with Metastases				
<2	1.408 (1.028-1.928)	**0.033***	1.366 (0.975-1.913)	0.070
≥2				
Immunotherapy line				
1st	1.270 (0.911-1.771)	0.159		
≥2nd				
Cycles				
≤8	0.177 (0.115-0.273)	**<0.001***	0.196 (0.125-0.307)	**<0.001***
>8				
Radiation				
No	0.720 (0.514-1.007)	0.055		
Yes				
LDH level				
≤ULN	1.440 (1.006-2.063)	**0.046***	1.416 (0.965-2.079)	0.076
>ULN				
Group				
irAE(-)	0.509 (0.374-0.694)	**<0.001***	0.655 (0.474-0.907)	**0.011***
irAE(+)				

ECOG PS, Eastern Cooperative Oncology Group Performance Status; HR, hazard ratio; CI, confidence interval; LDH, lactate dehydrogenase; ULN, upper limit of normal; irAEs, immune-related adverse events; PFS, progression-free survival. *p<0.05.

The potential risk factors, along with supporting evidence, include potential germline genetic factors, autoimmune diseases, radiotherapy, chemotherapy, targeted therapy, and preexisting autoantibodies (25, 26). In the present study, combination treatment with ICIs and radiation or antiangiogenic therapy were risk factors for irAEs.

Mounting evidence indicates that concomitant use of chemotherapy, targeted therapy, or radiotherapy with ICIs enhances efficacy, but leads to the risk of augmented treatment toxicities (26–28). In addition to inducing immunogenic cell death and priming and activation of naive T cells (29), radiotherapy can also produce immunogenic damage to nontumor cells and increase immune cell infiltration, leading to increased irAEs when combined with ICIs. Antiangiogenic therapies can induce the upregulation of PD-L1 in endothelial and tumor cells, resulting in an increased risk of irAEs when combined with ICIs (30). In contrast, the finding of > 8 PD-1 inhibitors > 8 cycles was also related to the high risk of irAEs in this study. Prolonged ICIs administration may result in a higher incidence of irAEs. With the increase in ICIs treatment cycles and doses, the enhanced activity of T lymphocytes and high levels of cytokines and inflammatory factors lead to side effects in normal tissues (31). Multi-institutional randomized controlled trials are required to identify predictive biomarkers of irAEs.

Several retrospective studies found that patients with multiple irAEs have better survival outcomes than in those with single or no irAEs (16, 32, 33), which may be explained by the development of multiple irAEs reflecting the immune system effectively targeting several organs and sustaining antitumor responses. However, those studies were mostly restricted to a handful of patients included, and the mechanisms of this association have yet to be identified. Interestingly, our study showed that patients with a single irAE had longer PFS and OS than those with multiple irAEs and no irAEs. In total, 29 (9.83%) patients had ≥ 3 grade irAEs among patients with multiple irAEs. The result of patients with multiple irAEs having a worse prognosis than those with single irAEs may be attributed to the serious adverse events that improve the danger of death and neutralize the efficacy of ICIs. Thus, additional studies are needed to elucidate the association between irAEs and ICIs efficacy (34).

This study has several limitations. Most importantly, this was a retrospective investigation, and there was an unavoidable bias in the selection of patients and potential confounding factors. Second, the mechanisms of irAEs were unclear; thus, further studies are required to illustrate the related results. Considering these limitations, we should carefully interpret the current results and conduct prospective studies to verify the findings of the association between irAEs and ICIs efficacy.

TABLE 4 Results of univariate and multivariate analyses showing factors affecting the overall survival.

Variables	Univariate analysis		Multivariate analysis	
	HR for OS (95% CI)	p value	HR for OS (95% CI)	p value
ECOG PS				
<2	1.703 (1.243-2.335)	**0.001***	1.470 (1.056-2.046)	**0.022***
≥2				
Number of organs with Metastases				
<2	1.034 (0.739-1.448)	0.845		
≥2				
Immunotherapy line				
1st	2.052 (1.390-3.030)	**<0.001***	1.755 (1.179-2.612)	**0.006***
≥2nd				
Cycles				
≤8	0.170 (0.098-0.295)	**<0.001***	0.186 (0.104-0.333)	**<0.001***
>8				
Radiation				
No	0.571 (0.397-0.821)	**0.002***	0.668 (0.451-0.988)	**0.043***
Yes				
LDH level				
≤ULN	1.471 (1.026-2.111)	**0.036***	1.339 (0.927-1.934)	0.120
>ULN				
Group				
irAE(-)	0.420 (0.301-0.585)	**<0.001***	0.565 (0.397-0.806)	**0.002***
irAE(+)				

ECOG PS, Eastern Cooperative Oncology Group Performance Status; HR, hazard ratio; CI, confidence interval; LDH, lactate dehydrogenase; ULN, upper limit of normal; irAEs, immune-related adverse events; OS, overall survival. *p<0.05.

TABLE 5 Results of univariate and multivariate analyses showing factors affecting the irAEs.

Variables	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Age (>60)	1.443 (0.913-2.283)	0.117		
Sex (female)	1.217 (0.605-2.446)	0.582		
ECOG PS (≥ 2)	0.658 (0.410-1.056)	0.083		
Therapy line (≥ 2)	0.681 (0.410-1.130)	0.137		
Number of organs with metastases (≥ 2)	0.741 (0.457-1.201)	0.224		
Cycles>8	3.109 (1.776-5.444)	**<0.001***	3.062 (1.726-5.432)	**<0.001***
Radiation	2.121 (1.288-3.492)	**0.003***	2.332 (1.382-3.936)	**0.002***
Antiangiogenic therapy	1.934 (1.007-3.711)	**0.047***	2.189 (1.101-4.353)	**0.025***
LDH level (>ULN)	0.763 (0.440-1.325)	0.337		

ECOG PS, Eastern Cooperative Oncology Group Performance Status; OR, odds ratio; CI, confidence interval; LDH, lactate dehydrogenase; ULN, upper limit of normal. *p<0.05.

Conclusion

The occurrence of irAEs predicts better survival outcomes, including patients receiving PD-1 inhibitors ≤ 8 cycles, in advanced EC. We believe that the development of irAEs can potentially be an effective and promising marker of survival in advanced EC.

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

This study was reviewed and approved by institutional review board of the Shandong Cancer Hospital and Institute. Written informed consent was obtained from all participants for their participation in this study.

Author contributions

Data collection, WQ and YD. Manuscript preparation, data analysis, WQ, LY, BF and BZ. Visualization, YD, BL and LW. Project administration, LW. Funding acquisition, LW. 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/fimmu.2022.931429/full#supplementary-material

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Advancing immune checkpoint blockade in colorectal cancer therapy with nanotechnology

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Immune checkpoint blockade (ICB) has gained unparalleled success in the treatment of colorectal cancer (CRC). However, undesired side effects, unsatisfactory response rates, tumor metastasis, and drug resistance still hinder the further application of ICB therapy against CRC. Advancing ICB with nanotechnology can be game-changing. With the development of immuno-oncology and nanomaterials, various nanoplatforms have been fabricated to enhance the efficacy of ICB in CRC treatment. Herein, this review systematically summarizes these recent nano-strategies according to their mechanisms. Despite their diverse and complex designs, these nanoplatforms have four main mechanisms in enhancing ICB: 1) targeting immune checkpoint inhibitors (ICIs) to tumor foci, 2) increasing tumor immunogenicity, 3) remodeling tumor microenvironment, and 4) pre-sensitizing immune systems. Importantly, advantages of nanotechnology in CRC, such as innovating the mode-of-actions of ICB, modulating intestinal microbiome, and integrating the whole process of antigen presentation, are highlighted in this review. In general, this review describes the latest applications of nanotechnology for CRC immunotherapy, and may shed light on the future design of ICB platforms.

KEYWORDS

colorectal cancer, immune checkpoint inhibitors, nanotechnology, drug delivery, tumor microenvironment

1 Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide (1, 2). CRC induced 0.94 million deaths all over the world in 2020 (3). Moreover, more than 3.0 million new CRC cases are predicted in 2040 (2–4). To date, the standard treatment for CRC patients still remains surgical resection, but one-third of them are suffering from post-operative diseases. Challenges in the treatment of CRC are the formation of distant metastasis and the development of drug resistance (4). CRC

gradually shows no response to traditional chemotherapeutics, thus novel therapies are urgently needed. Recently, therapy strategies that harness the host immune system against CRC seem to be beneficial for patients, especially those with high mutations (5, 6).

Tumor cells utilize immune checkpoint pathways to dampen T cell activation and evade attack by tumor-specific T cells (7). Immune checkpoint inhibitors (ICIs) competitively bind to checkpoint molecules and block the checkpoint-mediated suppression of the immune system (8). Monoclonal antibodies against checkpoint molecules such as programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1) and cytotoxic T lymphocyte antigen 4 (CTLA-4) have yielded unprecedent success in CRC patients (9, 10). Some small-molecule compounds that directly inhibit PD-1/PD-L1 interaction (11, 12) and its regulatory proteins, such as bromodomain and extra-terminal domain (BET) (13, 14) and Src homology 2 domain containing protein tyrosine phosphatase (SHP2) (15, 16), as well as inhibitors of other immune checkpoints (CD47, CTLA-4, V-domain Ig suppressor of T-cell activation (VISTA)) (17, 18), are also under pre-clinical investigations. However, only highly mutated CRC patients (about 15% of total cases) that are mismatch repair deficient (dMMR) or exhibit high levels of microsatellite instability (MSI-H) can benefit from ICIs. In contrast, majority of CRC patients which are mismatch-repair-proficient (pMMR) or microsatellite instability-low (MSI-L) show negligible response to ICIs. Low tumor mutation and lack of immune cell infiltration are hypothesized as underlying mechanisms in these tumors (19–21). To date, nanotechnology provides powerful devices to detect, diagnose, and treat cancer (22, 23), and is considered as a potential strategy to reverse the immune resistance of CRC (24).

Compared with conventional chemotherapeutics, nanomedicines not only exhibit superior tumoricidal ability and less side effects, but also present the potential to enhance immune checkpoint blockage (ICB) therapies (25) (1): By advancing the delivery of ICIs to CRC tumor sites, nanotechnology can directly enhance checkpoint blockade. During blood circulation, ICIs, especially small molecules, are difficult to accumulate in tumor beds. Macrophage-mediated phagocytosis system attenuates the delivery efficiency of ICIs (13). Even after entering CRC tissues, the amphiphilic cell membranes, lysosome degradation, and subcellular barriers (such as nuclear membranes, mitochondrial membranes, and endoplasmic reticulum membranes) also hinder the efficacy of ICIs (26). On the contrary, micro and nanosized particles can target to tumor tissue passively *via* the leaky tumor vasculature or actively *via* binding to receptors on tumor cell surface (27). Some systems can even deliver ICIs to certain subcellular compartments (28) (2). By regulating the cell death pathways, nanotechnology can transform immunologically tolerant cell corpse into immunogenic tumor vaccines, therefore amplifying ICIs efficacy (29, 30). Different from apoptosis, some engineered nano-systems can induce immunogenic cell death (ICD). In contrast to immune escape, tumor cells undergoing ICD will recruit antigen presenting cells (APCs), accelerate immune cells maturation, and initiate tumor antigen specific immune response *via* releasing various cytokines (31). Some other types of cell death such as ferroptosis, pyroptosis, and necroptosis might also be beneficial for increasing tumor immunogenicity (32–34) (3). By reprogramming immune-suppressive tumor microenvironment (TME), nanotechnology can revive the functions of ICIs (35–37). In order to respond to ICB, sufficient tumoricidal immune cell infiltration is necessary (38). In the progress of tumorigenesis, CRC tissues constantly release chemokines, cytokines, and exosomes as systemic factors to remold extracellular matrix (ECM) and recruit immunosuppressive cells, creating an immune desert milieu, which also terms as "cold" tumors (39, 40). Nanoparticles (NPs) can delivery agents that cut off the immune suppressive pathways, reversing malignant hallmarks in TME and reducing tumor-reside immunosuppressive cells (35, 41) (4). By facilitating the immune response against tumor-exclusive antigen pulses, NPs can potently provoke antigen specific immunity against CRC. As prophylactic or therapeutic interventions, NPs co-deliver antigens and immune-boosting adjuvants to host, pre-sensitizing immune system and systematically generating cytotoxic $CD8^+$ T lymphocytes (CTLs) (42, 43). These four mechanisms are schematically illustrated in Figure 1.

In this review, we summarize the progress of nano technology applied to ICB-based CRC treatment in recent three years according to their underlying mechanisms. Particular advantages of nanotechnology in CRC immuno therapy, such as innovating the mode-of-actions of ICB, modulating intestinal microbiome, and integrating the whole process of antigen presentation, are highlighted. This review is expected to clarify the cross-interactions among drugs, materials, and organisms in CRC immunotherapy, and further improve the future design of ICB nanoplatforms.

2 Nanotechnology facilitates immune checkpoint blockade therapy

2.1 Targeting immune checkpoint inhibitors to tumor foci

Although ICIs have shown considerable clinical potency in prolonging survival of patients (44), growing evidences indicate that systemic administration of checkpoint blockade antibodies such as anti-PD-1 antibodies (αPD-1), anti-PD-L1 antibodies (αPD-L1), and anti-CTLA-4 antibodies (αCTLA-4) may cause undesirable autoimmune and inflammatory responses, such as

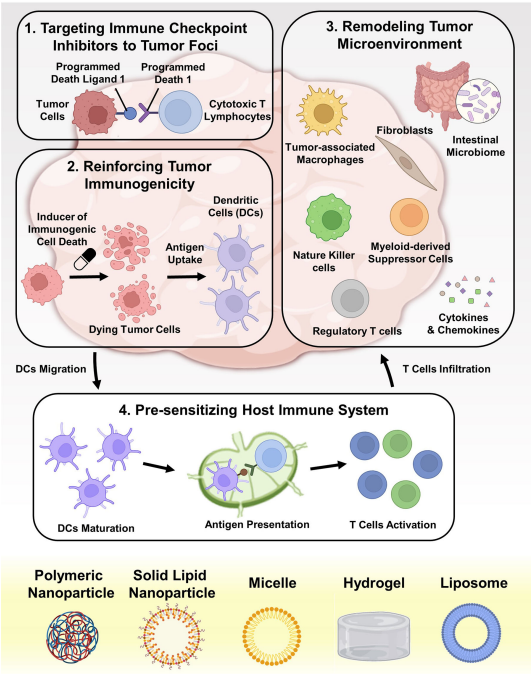

FIGURE 1
Schematic depiction of advancing ICB in CRC therapy with nanotechnology. The underlying mechanisms can be divided into four aspects: (i) targeted delivering various ICIs (such as antibodies, small molecules, peptides, and SiRNAs) into tumor foci; (ii) reinforcing the immunogenicity of dying tumor cells by cytotoxic agents and other drugs; (iii) remolding the immunosuppressive TME, including eliminating immunosuppressive factors and depleting immunosuppressive cells; (iv) pre-sensitizing the host immune system by delivering tumor vaccines and adjuvants to APCs.

colitis, dermatitis, and hypophysitis (45–47). Once happened, these unbearable adverse effects would seriously weaken therapeutic outcomes, or even fail the whole treatment (48, 49). Nanotechnology offers an attractive approach to bypass these side effects. Antibodies can be conjugated on or encapsulated in natural/artificial drug carriers, therefore avoiding antibody exposure in blood circulation (50, 51). In addition, nanosized drug delivery systems (DDSs) can passively/actively accumulate in solid tumors post systemic administration. Some DDSs can even be locally applied within tumor tissues (52, 53). Collectively, these formulations would remarkably elevate the selectivity of ICIs to tumors.

Checkpoint antibody-loaded NPs have been extensively studied. Early in 2010, Hellstrom et al. leverage functionalized mesoporous silica (FMS) to entrap αCTLA-4 (54–56). High drug loading and sustained drug release were achieved *via* adjusting the pore size of FMS, which minimize the risk of autoimmunologic toxicity (56). In addition to serving as drug reservoirs, antibody-conjugated NPs play significant roles in tumor theranostics. In colon tumor-bearing mice models, Popovtzer et al. demonstrated that the accumulation level of αPD-L1 conjugated gold NPs in tumors is an important parameter to predict the response of ICB therapy (57). Kang et al. attached methoxy poly (ethylene glycol) (MePEG) and chlorin e6 (Ce6) to Atezolizumab, a PD-L1 antibody, with a cathepsin B responsive linker (58). This immune checkpoint inhibitor nanocomposites (ICI NCs) avoided the ICI exposure in normal tissues, and exhibited tumor-activated fluorescence imaging (FI) and photodynamic therapy (PDT) on murine colon tumor #26 (CT26) tumor xenografts. Schneck et al. developed immuno-switch NPs that modified with αPD-L1 and anti 4-1BB antibodies on their surface (59). These dual-targeting NPs exhibited prolonged tumor retention than soluble free antibodies. After administration, immuno-switch NPs inhibited PD-L1 signal in tumor cells, and concurrently activated 4-1BB signal in $CD8^+$ T cells, activating immune response against murine colon carcinoma 38 (MC38) in a two-pronged pathway.

Solid tumors exhibited higher vascular density than normal tissues, and the wall of blood capillaries are highly leaky. Therefore, blood-circulating macromolecules (above 40 kDa) and NPs tend to extravasate and retain in tumor tissues (60). Moreover, the lymphatic drainage system is dysfunctional in tumors, which prevents the clearance of intra-tumoral NPs (61). This phenomenon is termed as the enhanced permeability and retention (EPR) effect. In general, the tumor accumulation efficiency depends on the blood circulation time of NPs and the tumor volume (62). NPs with prolonged blood circulation as well

as decreased clearance by liver and kidney have more opportunity to be transported into tumor capillaries. And larger tumors have more disorganized vasculatures for NPs accumulation. Although this effect has been widely-acknowledged in mice models, its contribution to drug delivery in human is still controversial (62). The carrier with active tumor-homing capability is a better choice for ICI delivery. Wang et al. developed platelets as the carrier for αPD-L1 delivery (63, 64). Platelets have inflammation-targeting ability, and can secret various chemokines to boost T cell immunity, which is very favorable for delivering αPD-L1 into residual microtumors. Platelets binding with αPD-L1 (P-αPD-L1) aggregated in tumor tissue, turning into platelet-derived microparticles (PMPs) for tumor-specific antibody release (64). Treatment of P-αPD-L1 effectively prevented tumor metastasis and recurrence in incomplete tumor resection and thermal ablation (TA) models (Figure 2A).

Rather than systemic delivery, local application of antibodies within tumoral and peritumoral regions is an excellent approach to obviate the overactivation of the immune system (66). Melief et al. formulated αCTLA-4 into a water in oil emulsion composed of Montanide ISA-51 for subcutaneous (*s.c.*) injection in the tumor area (67, 68). This sustained-release platform had similar therapeutic consequences with systemic administration, but the dosage was only one-eighth of intravenous injection (*i.v.*), which contrastingly decreased antibody titers in serum and improved therapeutic safety. Similarly, Hubbell et al. prepared peptide-functionalized ICB antibodies for peritumoral injection (69, 70). A peptide derived from placenta growth factor-2 (PlGF-$2_{123-144}$) showed super affinity with ECM. They showed the conjugation of PlGF-$2_{123-144}$ elevated the tissue retention and decreased the plasma concentration of therapeutic antibodies (αPD-L1 and αCTLA-4), reducing the risk of systemic adverse effects, such as autoimmune diabetes. PlGF-$2_{123-144}$ functionalized antibodies facilitated the infiltration of $CD8^+$ and $CD4^+$ T cells into tumors, resulting in delayed growth of primary and distance tumors. Gu et al. reported a microneedle (MN) patch for the transdermal delivery of αPD-1. Glucose oxidase (GOx) and αPD-1 were co-encapsulated into pH-sensitive dextran NPs, and these NPs were further loaded into hyaluronic acid MN arrays (65). GOx catalyzed the conversion of blood glucose to gluconic acid, forming a local acidic milieu for the self-disintegration of dextran NPs, leading to a sustained release of αPD-1 for three days. This simple and biocompatible platform could also be applied to co-deliver αPD-1 and αCTLA-4, achieving synergic antitumor effects (Figure 2B).

As the biological drugs, administration of antibodies may lead to infusion reactions and anti-drug antibodies (71, 72).

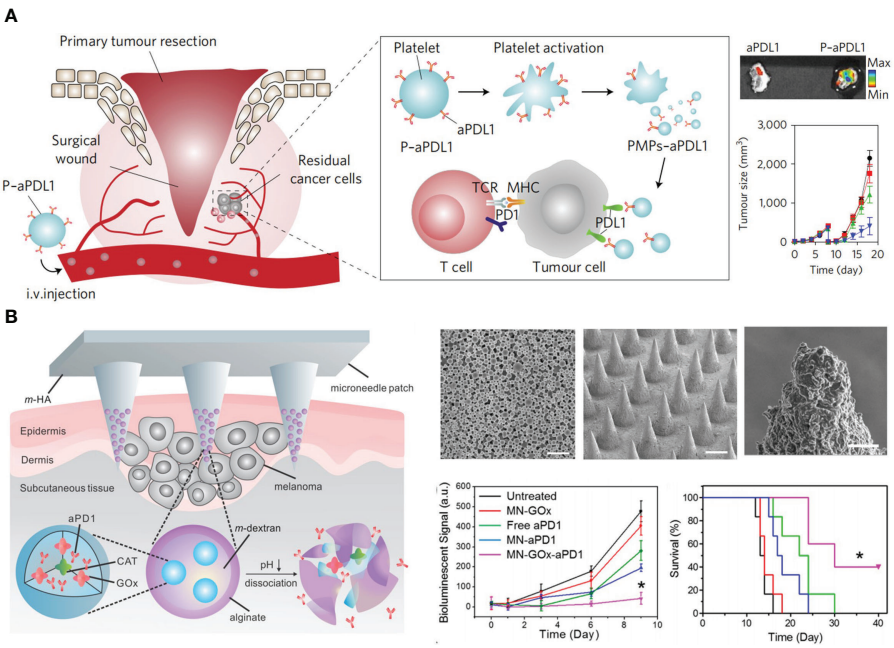

FIGURE 2
Nanotechnology targets ICIs to tumor foci to advance ICB in CRC therapy. **(A)** Left: schematic depiction of delivering αPD-L1 (aPDL1) to postsurgical tumor bed by platelets (P-aPDL1). Right top: P-aPDL1 sustainedly accumulated in tumor tissues. Right bottom: P-aPDL1 (blue curve) effectively inhibited tumor growth as compared with PBS-treated group (black curve). Reprinted with permission from reference (64). **(B)**. Left: schematic depiction of the composition of αPD-1 loaded microneedle (MN-GOx-aPD1). Right top: scanning electron microscope (SEM) images of αPD-1 loaded nanoparticles, microneedle patch, and magnified microneedle apex (from left to right). Right bottom: MN-GOx-aPD1 significantly inhibited tumor growth and prolonged mice survival. Reprinted with permission from reference (65). *$p<0.05$ versus untreated.

Peptide-based ICIs, especially antagonists against PD-1 and PD-L1, are more preferable options. In comparison with antibodies, peptide drugs have deeper tumor penetration due to their low molecule weight (Mw). Given the lacking of Fc fragments, peptides exhibited lower immunogenicity and better safety. Moreover, peptide ICIs are more stable in structure, and cost less in manufacture, storage, and drug administration (73, 74). Even though, peptide ICIs have weaknesses including insufficient affinity, short body circulation, and a lack of tumor selectivity, which requires to be solved by suitable delivery systems. Kim et al. designed ferritin nanocages (PpNF) that displayed PD-L1- binding peptide (CLQKTPKQC) with multivalency on their surface (75). PpNF specifically accumulated in tumor tissues, and restored the antitumor activities of T cells. Notably, PpNF loaded with doxorubicin (DOX) had better tumor inhibition effect than αPD-L1 in CT26 tumor models (75). Huang et al. synthesized a liner polymer-drug conjugate of PD-L1 antagonistic peptide MSP (CPLGVRGSGQYASYHCWCWRDPGRSGGSK) (76). This polymer (P-MSP-DMA) intertwined with a mitochondria-targeted polymer-drug conjugate (P-D-R8MTS) *via* electrostatic interaction to form a nanocomplex (SNV). SNV specifically dissociated in tumors in response to the charge reversal of dimethylmaleic anhydride (DMA) group triggered by acidic TME. This nanoplatform integrated PD-L1 blockade with mitochondria-targeted induction of ICD, resulting in considerable inhibition of tumor growth and metastasis (76). NPs incorporated with PD-L1-binding peptides can also be combined with photothermal therapy (PTT), which was exemplified by Zhang et al. and You et al. (50, 52). Zhang et al. conjugated a PD-L1 antagonistic peptide (NYSKPTDRQYHF) on the surface of IR780-loaded NPs (aNP@IR780) (50). And You et al. co-encapsulated aN anti-PD-1 peptide ($(SNTSESF)_2$ KFRVTQLAPKQIKE-NH_2) and the hollow gold nanoshell (HAuNS) into NPs (AA@PN) (52). Both strategies simultaneously triggered tumor ablation and blocked PD-1/PD-L1 interaction between tumor cells and T cells, exhibiting an abscopal effect to suppress distant tumor growth in a bilateral CT26 tumor model.

Besides peptides, nuclei acid-based therapeutics against checkpoint molecules is another therapeutic alternative for ICB therapy. Wang et al. used poly (ethylene glycol)-block-poly (d,l-lactide) (PEG-PLA) and *N*-bis(2-hydroxyethly)-*N*-methyl-*N*-(2-cholesteryloxycarbonyl aminoethyl) ammonium bromide (BHEM-Chol) to encapsulate CTLA-4 siRNA (77). The prepared NPs ($NP_{siCTLA-4}$) were capable to deliver siRNA cargos to both $CD8^+$ and $CD4^+$ T cells *in vivo*, facilitating their activation and proliferation. Ahn et al. prepared poly (lactic-co-glycolic acid) (PLGA) NPs to co-loading PD-1 siRNA and PD-L1 siRNA (siRNA@PLGA) (78). In the MC38 tumor model, they found the concurrent silencing of PD-1 and PD-L1 by siRNA@PLGA had better antitumor effect than single silencing of each one. Han et al. reported a nanoplatform with a novel PD-L1 binding aptamer, PL1 (51). In their design, PL1 single-stranded oligonucleotides were hybridized with folic acid (FA) and siRNA against proprotein convertase subtilisin-kexin type 9 (PCSK9) to obtain DNA tetrahedral nanoparticles (TDN-FA/PL1/Pcsk9-siRNA). TDN-FA/PL1/Pcsk9-siRNA were guided to CT26 CRC cells by FA, ensuring the synergy between PD-L1 blockade and Pcsk9 downregulation.

More than merely serving as a carrier for ICIs delivery and a platform for combinatory tumor immunotherapy, nanotechnology provides an opportunity to innovate the mode-of-action of ICB therapeutics. For example, Yang et al. leveraged lysosome-mediated receptor degradation to realize a durable PD-L1 downregulation. PD-L1 peptide antagonists (PPA, NYSKPTDRQYHF) were conjugated to the linear polymer composed of *N*-2 hydroxypropyl methacrylamide (HPMA) (79). By this way, PPA were transformed into a multivalent polymer-peptide antagonist against (MPPA). MPPA could gather and crosslink PD-L1 on tumor cell surface, biasing their trafficking to lysosome degradation and preventing their recycling to cell surface. This polymer-assisted receptor crosslinking strategy produced a long-lasting elimination of PD-L1 checkpoint, and strongly facilitated polymer-epirubicin (EPI) conjugate (KT-1) mediated chemo-immunotherapy. Nanotechnology may foster the druggability of ICI-like agents (79). Huang et al. designed a engineered PD-L1 trap as a novel ICB protein (53). In order to address the side toxicities of systemic PD-L1 blockade, the coding plasmid of PD-L1 trap was encapsulated in lipid-protamine-DNA nanoparticles (LPD). This system specifically distributed in tumor tissues, enabling the local production of the PD-L1 trap. In a CT26 murine colon tumor model, this strategy not only improved the tolerance of ICB therapy without inducing Th17 cells accumulation in spleen, but also achieved potent PD-L1 inhibition to potentiate oxaliplatin (OXA)-mediated chemotherapy (53).

2.2 Reinforcing tumor immunogenicity

In CRC, highly immunogenic tumors showed relatively good response to ICB-based immunotherapy (1, 80, 81). In contrast, the effect of ICIs in tumors with low immunogenicity requires further improvement (82). Recently, ICD has been reported to transform originally immunotolerant cell debris into immunogenic vaccines (83). ICD induced by anthracyclines was first reported by Guido Kroemer et al. in 2007 (84). Different from immune tolerant apoptosis, ICD can provoke the immune system to generate response against antigens from dead tumor cells, which is also known as "bystander effect" (85). Briefly, tumor cells undergoing ICD expose calreticulin (CRT) on the outer leaflet of the cell membrane, secreting adenosine triphosphate (ATP) and releasing high mobility group box 1 (HMGB1) into extracellular microenvironment (29, 30, 86).

These markers facilitate APCs recruitment, antigen engulfment and presentation during immune initiation (87).

DOX is often administrated along with other chemotherapeutics to elevate efficacy. Although DOX is found to facilitate effector T cells infiltration and synergize with ICIs *via* ICD induction, applications of free DOX are still hindered by cardiac toxicity and unsatisfactory tumor accumulation (29). Jeffrey A. Hubbell et al. reported a collagen-binding serum albumin platform for advanced colon carcinoma therapy (88). Serum albumin (SA) based carrier can passively deliver drug to tumor sites *via* the extravasation through pathological vasculature. To further endow SA active targeting capacity, a collagen-binding domain (CBD) was fused recombinantly to give CBD-SA. Lastly, DOX was loaded to CBD-SA *via* a pH-sensitive linker (DOX-CBD-SA). Surprisingly, when combing with αPD-1, a complete eradication of MC38 colon carcinoma was observed. To understand the underlying mechanism, T cells and natural killer (NK) cells in treated tumors were extracted. The numbers of $CD8^+$ T cells, $CD4^+$ T cells and NK cells per unit tumor mass increased after DOX-CBD-SA treatment. The increased tumor-infiltrating lymphocytes subsequently potentiated therapeutic efficiency of immune-checkpoint blockade. Generally, DOX-CBD-SA can potently kill tumor cells and simultaneously stimulate host antitumor immunity, decreasing adverse events. To further improve the immunogenicity, subcellular level targeting strategy was considered. Mitochondria is one of the most important organelles and serves as the source of damage associated molecular patterns (DMAPs) such as ATP, heat shock protein 70 (HSP70), and HSP90. The released DMAPs facilitate the presentation of tumor-associated antigens. Zhan et al. engineered a mitochondria-targeted polymeric nanoparticle (R848@cRGD-PDCS) (89). Under near-infrared irradiation exposure, mitochondria were destroyed by photothermal-mediated hyperthermia, causing the release of tumor-associated antigens and DMAPs. αPD-L1 therapy showed limited inhibitory effects in tumor growth, but the combination with R848@cRGD-PDCS (under irradiation) exhibited favorable ability to eradicate primary tumors and prevent metastasis (Figure 3A).

Chemotherapeutics (such as OXA and DOX) and PDT were reported to induce ICD synergistically. Lin et al. proposed a core-shell nanoscale coordination polymer (NCP@pyrolipid) which not only directly eliminated tumor cells but also promoted the checkpoint blockade immunotherapy (91). In the study, OXA in the core and the photosensitizer pyropheophorbide-lipid conjugate (pyrolipid) in the shell synergistically eradicate cancer cells, resulting in robust ICD and subsequent abscopal effects. Moreover, after integrating PD-L1 blockade with NCP@pyrolipid, tumor regression was observed in both light-irradiated primary tumors and distant tumors without light-irradiation, indicating that a potent tumor-specific immunity was evoked. The authors observed increased portions of antigen-specific CTLs in the CRC-bearing mice injected with NCP@pyrolipid (with irradiation) plus αPD-L1. The immunogenic environment induced by both OXA and PDT remarkably enhanced PD-L1 therapy *via* spurring systemic antitumor immune response. Yu et al. reported an prodrug-based polymeric nanoparticle to realize optimal administration of OXA combining with PTT (92). Besides, fluorescence -guided PTT can further enhance tumor immunogenicity and release drug in a spatiotemporally controllable way. A donor-spacer-acceptor-space-donor (D-S-A-S-D) type fluorophore was farther inserted to improve immunogenicity and amplify the efficacy of αPD-L1 therapy. This combinatory chemo/photothermal therapy with PD-L1 blockade (PBOXA@TQTCD+L-αPDL1) was tested *in vivo*. Results revealed that the combinatory therapy not only inhibit tumor growth much more potently than αPD-L1 therapy alone, but also improve the survival rate of mice with tumor. When it comes to the tumor recurrence inhibition, the central memory $CD8^+$ T cells (T_{CM}) in spleen representing long-term immune memory was analyzed. T_{CM} ratio in the combinatory therapy was at least two times higher than that of αPD-L1 alone, indicating the activation of a long-term immune surveillance against tumor recurrence. This combinatorial therapy might enlighten clinical CRC management.

However, the PDT or PTT is a localized therapy and restricted by light penetration. Alternatively, Lin et al. reported another tactic that using reactive oxygen species (ROS) based chemotherapeutic to induce potent ICD and synergize with OXA. The author engineered self-assembled coordination polymer nanoparticles (OxPt/DHA) loading OXA in the core and ROS-generating dihydroartemisinin (DHA) in the shell for CRC treatment (93). In a tumor rechallenge experiment, mice vaccinated with OxPt/DHA-treated cells showed a potent immune resistance against live MC38 cells and no tumor formation was observed. The efficacy of OxPt/DHA combining with α-PD-L1 blockade therapy was tested in tumor models of CT26 and MC38 on immunocompetent BALB/c and C57BL/6 mice, separately. In both CT26 and MC38 models, the α-PD-L1 therapy alone failed to control tumor growth. In the contrary, all of the tumors treated with OxPt/DHA plus α-PD-L1 regressed and ultimately disappeared on days 40~50. Until 120 days, no recurrence was found. Results revealed that OxPt/DHA is a potential clinical candidate to synergize with ICIs.

Besides inducing ICD *in situ* in tumor sites to enhance immunogenicity and amplify immune checkpoint blockade (ICB) therapy, immunogenically dying tumors cells themselves can also be transformed into a powerful platform for cancer vaccination. Moon et al. have manufactured dying tumor cells surface-modified with adjuvant-contained NPs (94). Results revealed that dying tumor cells undergoing ICD could be further filled with adjuvant nano-depots to successfully initiate antigen cross-presentation by dendritic cells and activate potent antigen-specific $CD8\alpha^+$ T cells in mice model bearing CRC. Additionally, the combinatory regimen using this whole tumor-cell vaccination and immune checkpoint inhibition resulted in a complete tumor eradication in about 78% of mice inoculated with CT26. A long-term immunity was also observed, indicating

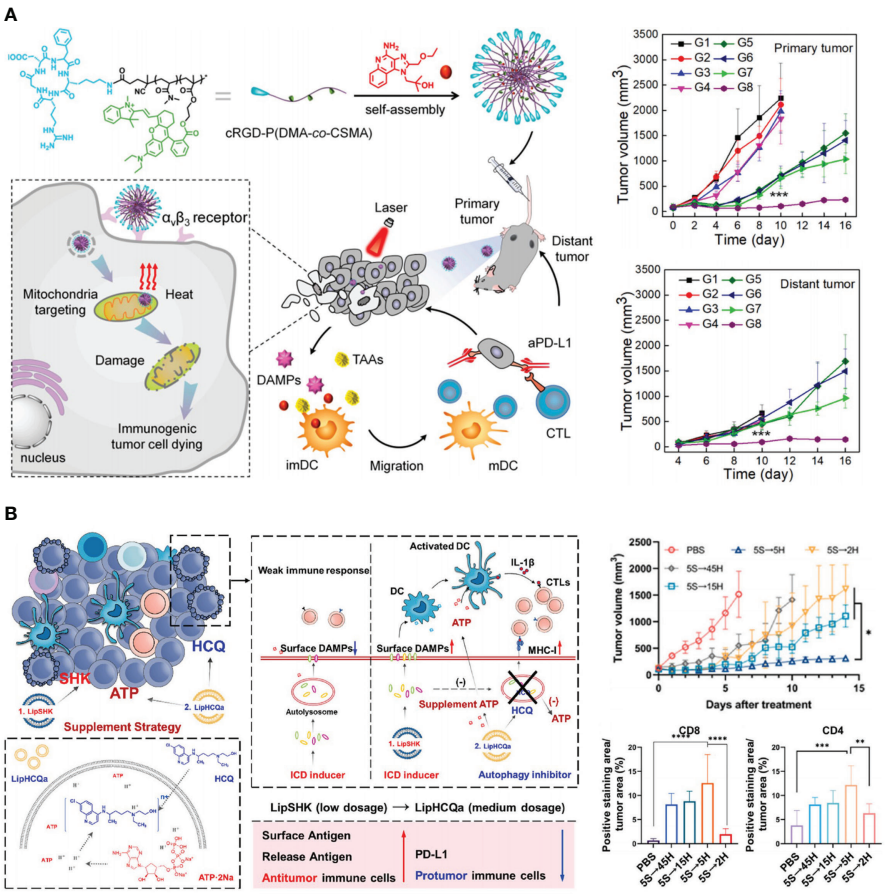

FIGURE 3
Nanotechnology reinforces tumor immunogenicity to advance ICB in CRC therapy. **(A)** Left: schematic depiction of mitochondria-targeted and photo-activated nanoparticles (R848@cRGD-PDCS) that triggered ICD to potentiate ICB therapy. Right: R848@cRGD-PDCS (G8) inhibited the growth of both primary tumor and distant tumor in combination with αPD-L1. Reprinted with permission from reference (89). ***p<0.001 versus G1. **(B)** Left: schematic depiction of the mechanism of recovering tumor immunogenicity by autophagy inhibition. Right: impacts of different autophagy inhibition therapies on tumor growth and the percentage of $CD8^+$ and $CD4^+$ T cells. Reprinted with permission from reference (90). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

the potential to prevent tumor recurrence. This strategy might shed light on "personalized" therapy which is tailored according host's own tumor cells. The inflammatory microenvironment after surgery and residual tumor "seeds" were responsible for post-operative metastasis. To solve this dilemma, Li et al. embedded autologous cancer cells succumbing to ICD and anti-inflammatory drug dexamethasone in hydrogel, the hydrogel could be injected into a resection site, in which it was rapidly solidified and gradually degraded (95). The dying cells provided a whole array of tumor-associated antigens and became highly immunogenic vaccines which enabled antigen specific immunization. After combining with αPD-L1 therapy, a complete tumor regression was observed, which might be attributed to their complementary functions to evocate and unleash tumoricidal T cells. This strategy provides a novel option for inhibiting metastasis after surgery.

Autophagy refers to the process by which cells degrade their constituents by autophagosomes. Autophagy is necessary in sustaining and modulating cell homeostasis. In addition, autophagy facilitates the release of ATP from lysosome in ICD inducing, promoting antitumor immune response. However, autophagy can destroy tumor-associated antigens, therefore attenuating antitumor immunity. To overcome this difficulty, Wang et al. designed a liposome named as LipHCQa which encapsulated shikonin (ICD inducer), hydroxychloroquine (autophagy inhibitor), and ATP for the treatment of colon cancer (90). This compensatory liposome showed enhanced immune infiltration when compared with shikonin loaded liposome alone, indicating the importance of blocking autophagy on ICB amplification (Figure 3B).

Specific series of intracellular suicide process was named as programmed cell death (96, 97). In the past few decades, apoptosis

had been assumed as the sole modality of programmed cell death (84, 98). Recently, several other pathways of programmed cell death were identified, such as ferroptosis and pyroptosis (99, 100). These particular cell death pathways might be used to enhance the immunogenicity of tumor cells and synergize with ICB (101). Since firstly proposed in 2012 by Stockwell and co-workers, ferroptosis has attracted numerous attentions in the field of oncology and biochemistry (102, 103). Ferroptosis is an iron and ROS dependent dell death. Cells undergoing ferroptosis showed increased lipid peroxidation products and ROS that is derived from iron metabolism. Han et al. designed core-shell nanoparticles (ZnP@DHA/Pyro-Fe) loaded with a cholesterol derivative of dihydroartemisinin and pyropheophorbide-iron (Pyro-Fe) to potentiate CRC immunotherapy *via* inducing ferroptosis. ZnP@DHA/Pyro-Fe treated cancer cells showed increased DAMPs release and result into intra-tumoral immune cell infiltration (104). Further combination with αPD-L1 checkpoint blockade led to better therapeutic effect. Different from caspase-dependent apoptosis, necroptosis is featured by expanded cell volume, organelle swelling, cell membrane fracture, and leaking of intracellular components. Nowadays, mixed-lineage kinase domain like protein (MLKL), receptor interacting protein-1 (RIPK1), and RIPK3 pathways were thought to be essential in tumor necrosis factor-α (TNF-α) mediated necroptosis. Compared with poorly immunogenic apoptosis failing to activating antitumor immunity, necrosis showed great potential in priming immune response due to the increase immunogenicity (105). Sun et al. prepared dimethyl fumarate loaded star-PCL-azo-mPEG (sPCEG-azo) polymeric micelles (106). The micelles are colon-targeted and induce necroptosis in colon cancer cells *via* a mechanism characterized with increased ROS. The elevated ROS generation result in immunogenicity and contribute to antitumor immunity, which may further augment ICB. Another necroptosis-inducible nanoparticle was reported by Park et al. The nanobubbles (NBs) contains Ce6 as the sonosensitizer and perfluoropentane as the gas precursor (107). After ultrasound exposure, NBs could disintegrate plasma membrane and lead to damage-associated molecular patterns release, inducing acoustic cavitation mediated necroptosis and ROS-mediated tumor regression. The NBs promoted antitumor immunity *via* accelerating dendritic cells maturation and $CD8^+$ T cells activation. Further combinatory regimen including PD-L1 blockade plus NBs even led to complete eradication of primary CT26 tumor and metastasis.

2.3 Remodeling tumor microenvironment

TME is an intricate milieu including tumor and immune cells, bacteria, as well as multiple soluble signal mediators (108, 109). All of them contribute to the distinct physiological characteristics (hypoxia, acidity, inflammation, and immune escape) of TME. Mounting evidence reveal that the heterogeneity of TME is an important factor for the low responsiveness of ICB therapies, and reversing immunosuppressive TME is very promising to overcome ICI resistance (110). Considering the close interaction between components in TME, nanoplatforms that counteract these suppressors in multi-pronged ways are very promising (36, 111, 112). Nanoplatforms that potentiated ICB against CRC by modulating TME are summarized in Table 1.

2.3.1 Hypoxia

Hypoxia reduced the therapeutic efficiency of ICIs in many aspects. It increased the expression of PD-L1 and CTLA-4 *via* hypoxia inducible factor-1α (HIF-1α) pathway, and weakened antigen presentation of APCs (124, 125). You et al. adopted three strategies to alleviate hypoxia (113): (i) directly deliver oxygen into tumors by a perfluorocarbon-loaded liposome (PFC@lipo); (ii) directly deliver oxygen into tumors by a hemoglobin-loaded liposome (Hb@lipo); and (iii) indirectly inhibit HIF-1α in tumors by a small molecular inhibitor PX-478. They found systematic administration of Hb@lipo was the most ideal strategy to combine with αPD-1 for the treatment of murine triple-negative breast cancer (4T1) and CT26 tumors. Due to the hypoxia milieu, tumor cells are glycolytic and produce plenty of lactate as the metabolite, resulting in anergy of tumor-infiltrated immune cells. Dhar et al. developed a mitochondria-targeted NPs (T-Mito-DCA-NPs) for the delivery of dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase 1 (PDK1) (126). This formulation selectively inhibited tumoral glycolysis without affecting immune cells. As a result, T-Mito-DCA-NPs significantly elevated the intra-tumoral infiltration of $CD8^+$ and $CD4^+$ T cells and downregulated the expression of checkpoint molecules including PD-1, CTLA-4, LAG3, and Tim3 on their surface. Combination of mitochondria-targeted DCA and αPD-1 effectively improved the infiltration of $CD8^+$ and $CD4^+$ T cells in CT26 tumors.

2.3.2 Inflammation

Inflammation is an important hallmark in TME that promote tumorigenesis, expansion, metastasis, and immune escape (127). Tumor cells highly expressed cyclooxygenase-2 (COX-2) and secreted a large amount of prostaglandin E2 (PGE2) to recruit myeloid-derived suppressor cells (MDSCs) (128, 129). MDSCs generate several immunosuppressive cytokines, such as ROS, transforming growth factor-β (TGF-β), and interleukin 10 (IL-10), leading to failure of ICB therapy (130–132). Chen et al. synthesized a self-assembled polymeric prodrug (P3C-Asp) of aspirin, a classical non-steroidal anti-inflammatory drug (NSAID) (114). P3C-Asp released aspirin in response to high ROS level in tumor tissues, decreasing PGE2 secretion and reversing tumor immunosuppression. Combination therapy of P3C-Asp+αPD-L1 eradicated CT26 tumors in 100% of mice.

TABLE 1 Summary of nanoplatforms that remolds TME to advance ICB in CRC therapy.

Target	Nanoplatform	Route of administration	Mechanism of TME remolding	Reference
Hypoxia	perfluorocarbon-loaded liposome (PFC@lipo), hemoglobin-loaded liposome (Hb@lipo), hypoxia inducible factor-1α (HIF-1α) inhibitor (PX-478)	Liposomes: *i.v.* Free drug: *i.p.*	PFC@lipo and Hb@lipo loaded and delivered oxygen to tumor, PX-478 inhibit the hypoxia signal pathway	(113)
cyclooxygenase-2 (COX-2)	Self-assembled polymeric prodrug of aspirin (P3C-Asp)	*i.v.*	P3C-Asp released aspirin in response to high ROS level and specifically inhibited COX-2 in TME	(114)
Intestinal microbiome	lipid–protamine–DNA nanoparticles (LPD) that loaded the plasmid encoding lipopolysaccharide (LPS)-binding protein	*i.v.*	The nanoparticles accumulated in tumor, and then expressed protein which depleted LPS in CRC tissues	(115)
	M13 bacteriophage coated with silver nanoparticles (M13@Ag)	*p.o.*	The M13 specifically bound with *Fusobacterium nucleatum* (*Fn*) and then Ag nanoparticles eliminated *Fn*	(116)
Indoleamine 2,3-dioxygenase 1 (IDO-1)	Boolean logic prodrug nanoparticles (BLPNs) incorporated with IDO-1 inhibitor NLG919 and photosensitizer pheophorbide a (PPa)	*i.v.*	BLPNs released NLG919 in response to the high glutathione (GSH) level in tumor cells, which inhibited the metabolism of tryptophan	(117)
	Cationic lipid-assisted nanoparticles loaded with siRNA of IDO-1 ($CLAN_{siIDO1}$) and oxaliplatin (OXA)	*i.v.*	$CLAN_{siIDO1}$ accumulated in both tumor tissues and tumor-draining lymph nodes (TDLNs), downregulating IDO-1 that upregulated after OXA treatment in these two tissues	(118)
phosphatidylserine (PS) externalization	Annexin A5-modified and neoantigen-loaded nanoparticles (AnnV_PLGA(Nbea)_NPs) and cisplatin	AnnV_PLGA (Nbea)_NPs: *i.v.* Cisplatin: *i.p.*	The surface Annexin A5 of AnnV_PLGA(Nbea)_NPs blocked the immunosuppressive effects of PS on dying tumor cells treated by cisplatin	(119)
Tumor-associated macrophages (TAMs)	Pexidartinib-loaded nanoparticles (PLX-NPs) and αPD-L1 conjugated platelets (P-aPD-1)	*s.c.* after incorporation together into hydrogel or *i.v.* separately	Pexidartinib blocked the colony-stimulating factor 1 receptors (CSF1R) on TAM surface and depleted TAM	(120)
	Red blood cell (RBC) membrane-coated *Porphyromonas gingivalis* (*cmPg*)	Intra-tumoral injection	*Pg* promoted the polarization of TAM towards anti-tumoral M1 phenotype	(121)
Signal pathways and bacteria	polymeric metformin (Polymet)	*p.o.*	Polymet activated adenosine 5'-monophosphate activated protein kinase (AMPK) pathway, inhibited mammalian target of rapamycin (mTOR) pathway, and increased anti-tumoral *Lactobacillus* in CRC tissues	(122)
Hypoxia, IDO-1, and myeloid-derived suppressor cells (MDSCs)	MnO_2 mineralized nanocage co-encapsulated with IDO-1 SiRNA and gemcitabine (GEM), the surface of nanocage was modified with antibody against PD-L1/CD47	*i.v.*	MnO_2 catalyzed the generation of oxygen in TME, inhibited HIF-1α and promoted M1 macrophage polarization; SiRNA silenced IDO-1 and suppressed regulatory T cells (Tregs); GEM eliminated MDSC	(123)

i.v., intravenous injection; i.p., intraperitoneal injection; p.o., oral administration; s.c., subcutaneous injection.

2.3.3 Intestinal microbiome

Different from other cancers, the progress of CRC is closely associated with intestinal microbiome. The species and abundance of intestinal microbiome highly affected the balance in GI track, contributing to several gut diseases, such as colitis, fibrosis and CRC (133). *Fusobacterium nucleatum*, *Bacteroides fragilis*, and *Escherichia coli* are the main pathogenic bacteria in gut that promote tumor progression and hinder the responsiveness of CRC to αPD-L1 therapy (134–136). Bacteria colonized in cancerous GI tracts produced massive endotoxin, also known as lipopolysaccharide (LPS). LPS accelerated the growth and liver metastasis of colorectal tumors *via* Toll-like receptor 4 (TLR4) and nuclear factor kappa-B (NF-κB) pathway (137–139). Moreover, chemotherapeutic agents are able to disrupt the mucus barrier in gastrointestinal (GI) track, facilitating the colonization and invasion of gut bacteria (140). Huang et al. designed an LPS-binding fusion protein as the trap to deplete LPS in orthotopic CRC tissues (115). For tumor selectivity, they encapsulated the coding sequence of LPS trap protein into lipid–protamine–DNA nanoparticles (LPD). This system passively accumulated in CT26 tumors, enabling LPS trap protein expressed within malignant tissues. They found LPS trap treatment elevated the infiltration of T cells, which favored ICB therapy. Combinatory treatment of the LPS trap and αPD-L1 not only retarded the growth of orthotopic CT26-FL3 tumors but also inhibited their spontaneous liver metastasis. Zhang et al. identified *Fusobacterium nucleatum* (*Fn*) as pro-tumoral gut bacteria that restricted T cell infiltration and enriched MDSCs in CRC tissues (116). They screened a *Fn*-binding M13 bacteriophage by phage display technology and modified antibacterial silver nanoparticles (AgNP) on its surface. The obtained M13@Ag could specifically eliminate *Fn* in GI track to reduce MDSCs in TME, and facilitate antigen presentation due to its intrinsic immunogenicity. In orthotopic CRC models,

combination therapy of M13@Ag and αPD-1 significantly improved the overall survival of mice (Figure 4A).

2.3.4 IDO-1

Indoleamine 2,3-dioxygenase 1 (IDO-1) is highly overexpressed on tumor cells (141, 142). It is a rate-limiting enzyme in the kynurenine pathway that convert tryptophan to kynurenine (143). The accumulation of kynurenine in TME contributed to dendritic cells (DCs) deactivation, CTL apoptosis, and regulatory T cells (Tregs) increment (144, 145). Due to its key role in tumor immunosuppression, IDO-1 is termed as the "metabolic immune checkpoint" (118). Inhibiting IDO-1 have been demonstrated to potentiate ICD-based chemo/photo-immunotherapy and PD-1/PD-L1 blockade therapy, but the therapeutic consequence is strongly relied on well-designed delivery strategies. Yu et al. developed boolean logic prodrug nanoparticles (BLPNs) that logically gated by matrix metalloproteinase (MMP), acid, and glutathione (GSH) to release photosensitizer pheophorbide a (PPa) and IDO-1 inhibitor NLG919 to treat CT26 tumors (117). PPa induced ICD to trigger T cell response, which was further amplified by NLG919. Wang et al. accomplished the concurrent inhibition of

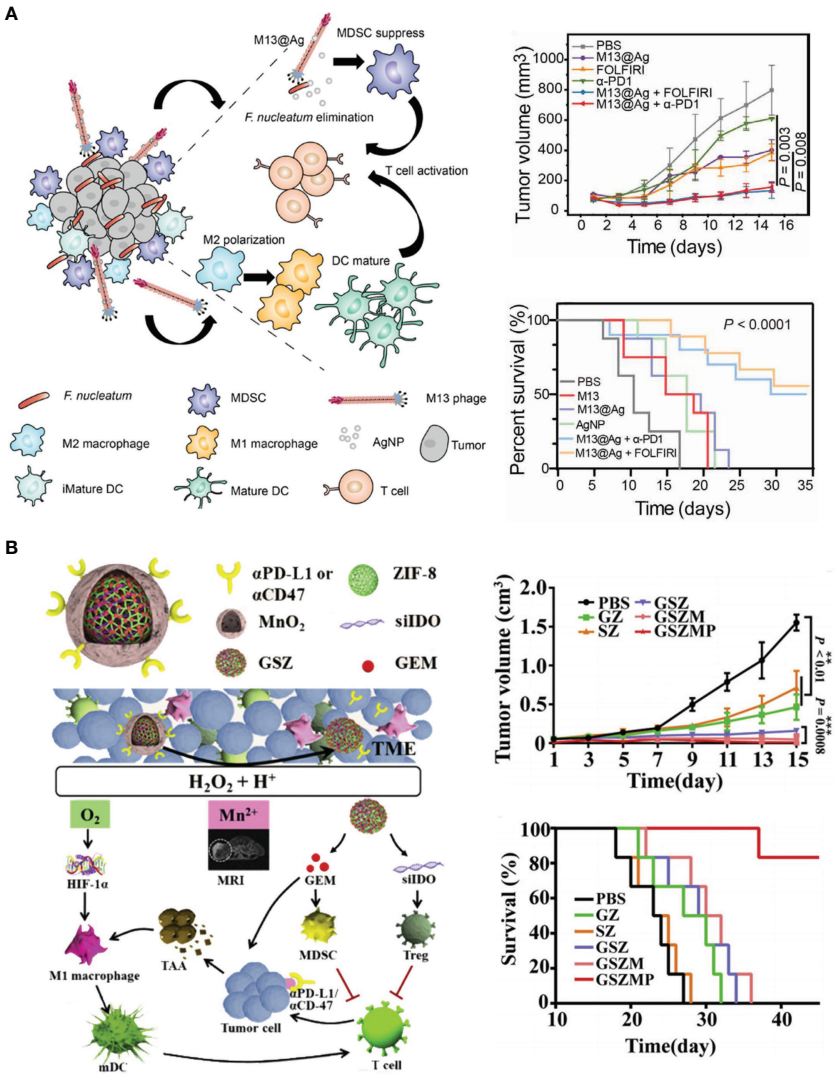

FIGURE 4
Nanotechnology remolds TME to advance ICB in CRC therapy. **(A)** Left: schematic depiction of engineered bacteriophage (M13@Ag) that regulated intestinal microbiome to modulate TME against CRC. Right: M13@Ag significantly inhibited tumor growth and prolonged mice survival in combination with αPD-1. Reprinted with permission from reference (116). **(B)** Left: schematic depiction of the composition of versatile nano-modulator (GSZMP) and its mechanism in potentiating ICB therapy. Right: GSZMP potently inhibited tumor growth and prolonged mice survival. Reprinted with permission from reference (123).

IDO-1 in both tumors and tumor-draining lymph nodes (TDLNs) by siRNA-loaded cationic lipid-assisted nanoparticles ($CLAN_{siIDO1}$) (118). They found OXA treatment aggravated IDO-1 overexpression in tumors and TDLNs, which was in accordance with other immune checkpoints like PD-L1 and CTLA-4. $CLAN_{siIDO1}$ accumulated in TDLNs and tumors, downregulated IDO-1 in both tissues, and improved tumor inhibition by OXA in the CT26 colon cancer model.

2.3.5 PS externalization

Chemotherapy triggers phosphatidylserine (PS) externalization on the surface of tumor cells undergoing apoptosis (146, 147). In line with canonical immune checkpoints, the exposed PS consolidated the immunosuppressive TME, restricted the phagocytosis of APCs and upregulated the expression of PD-L1 (148, 149). Recently, Park et al. developed annexin A5-labeled NPs (AnnV_PLGA_NPs) as the inhibitor to this innate immune checkpoint. Mutant neoantigen peptides (Nbea, PAPRAVLT GHDHEIVCVSVCAELGLVI) were loaded in NPs (AnnV_PLGA (Nbea)_NPs) to elicit antigen-specific antitumor immunity (119). In company with cisplatin (Cis)-mediated chemotherapy, AnnV_PLGA(Nbea)_NPs spurred the infiltration of immune-activate cells and the secretion of pro-inflammatory cytokines, while depleting immune-suppressive MDSCs and Tregs and decreased the production of anti-inflammatory cytokines at the same time. The immunostimulatory effect of AnnV_PLGA(Nbea) _NPs can be amplified by αPD-L1, and the triple-therapy of Cis + AnnV_PLGA(Nbea)_NPs + αPD-L1 resulted in noticeable rejection of CT26 tumor growth.

2.3.6 TAMs

Tumor-associated macrophages (TAMs) represented a large population in intra-tumoral immunosuppressive cells (150). Generally, TAM can be divided into anti-tumoral M1 phenotype and pro-tumoral M2 phenotype *via* their different markers. M1 macrophages not only killed tumors in an innate manner, but also presented tumor antigen to T cells and activated adaptive tumor immunity (151). M2 macrophages are the major TAMs in immunosuppressive tumors, such as triple-negative breast cancer (TNBC) and CRC (152). They affected the function of tumor and immune cells in TME by secreting vascular endothelial growth factor (VEGF), IL-10, TGF-β, and arginase-1 (153). There are three paradigms for targeting TAMs: (i) inhibiting TAMs recruitment, (ii) depleting pre-existing TAMs, and (iii) re-educating TAMs from pro-tumoral M2 macrophages to anti-tumoral M1 phenotype (151, 152). Results of Hu et al. revealed that locally depleting TAMs in postsurgical tumor beds by Pexidartinib-loaded nanoparticles (PLX-NPs) created an appreciable condition for the local and systemic PD-1 blockade therapy (120). Pexidartinib eliminated TAMs by blocking colony-stimulating factor 1 receptors (CSF1R) on TAM surface. Alginate hydrogel incorporated with PLX-NP (PLX-NP@Gel) decreased $F4/80^+$ macrophages and increased IFN^+CD8^+ CTLS in tumor beds. αPD-1 conjugated platelets (P-aPD-1) were co-encapsulated into hydrogel for local implantation (PLX-NP-P-aPD-1@Gel) or systematically injected (PLX-NP- @Gel+P-aPD-1). Both regimes considerably inhibited post-surgery tumor recurrence in murine melanoma (B16F10), CT26, and 4T1 tumor models. Conventional TAM-modulating strategies employed small molecules, peptide, antibodies, and nuclei acids, while Zhou et al. reported a bacteria-based approach to repolarize TAMs (121). They prepared a red blood cell (RBC) membrane-coated formulation of *Porphyromonas gingivalis* (*cmPg*). *Porphyromonas gingivalis* (*Pg*) not only promoted the conversion of TAMs to M1 phenotype, but also secreted melanin for tumor PTT. With the help of PD-L1, *cmPg* retarded the growth of primary and secondary CT26 colon tumors.

2.3.7 Multiple targets

Because of the intricate crosstalk between various immune cells, it is plausible to manipulate versatile targets by one nanoplatform. Huang et al. developed an orally delivered polymeric metformin (Polymet) that notably reinforced αPD-L1 therapy (122). The underlying mechanism of Polymet involved reprograming the immunosuppressive TME *via* adenosine 5′-monophosphate activated protein kinase (AMPK) pathway and mammalian target of rapamycin (mTOR) pathway, as well as lifting the abundance of anti-tumoral *Lactobacillus* in CRC tissues. In comparison with this single-mode therapy, it is more preferable to design multi-modular nanodrugs that counteracted several immune suppressors in TME. To this end, Jiang et al. developed a versatile nano-modulator, GSZMP. SiRNA targeting IDO-1 (siIDO) and gemcitabine (GEM) were co-encapsulated in a nanocage composed of Zinc 2-methylimidazole (ZIF-8) metal organic frameworks (MOFs) (123). The surface of drug-loaded nanocage was further tattooed with MnO_2 mineralization and electrostatically modified with αPD-L1 or anti-CD47 antibody (αCD47) for the treatment of TNBC and colon adenocarcinoma (COAD), respectively. MnO_2 catalytically generated O_2 to alleviate hypoxia in TME, which promoted the repolarization of TAMs into M1 phenotype. GEM selectively depleted MDSCs, and siIDO inhibited the activity of Tregs. Overall, GSZMP reversed the "cold" TME in a multi-pronged pathway, which effectively potentiate ICB therapy (Figure 4B).

2.4 Pre-sensitizing host immune system

Tumor vaccines exhibit unique prophylactic effect against tumorigenesis and have showed combinatory therapeutic effect with immune checkpoint therapies (154–156). The aim of them

is to pre-sensitize the immune system before tumor expansion and generate sufficient antigen-specific T cells, which creates a condition for subsequent invigoration of these T cells (157). Advantages of tumor vaccines can be summarized as follows: (i) tumor vaccines greatly decrease the risk of tumorigenesis; (ii) tumor vaccines induce systemic antitumor immunity that is able to attack undetectable tumor foci and metastasis (158); (iii) tumor vaccines elicit durable immune surveillance that against tumor recurrence for a long time (159); (iv) tumor vaccines can be personalized by using autologous components, which is more favorable to address the mutation of tumor antigens (158, 160). Nowadays, several tumor-exclusive neoantigens have been identified for vaccine design, but their immunostimulatory efficiency are still limited in *in vivo* studies (161). Codelivery with adjuvants will potently enhance T-cell-spurring by tumor vaccines, wherein the contrasting different drug properties between antigen and adjuvant should be concerned (159, 162). Most tumor-specific antigens are water-soluble macromolecules, such as peptides, proteins, and long chain ribonucleic acids. TLR agonists are a class of well-studied adjuvants with multiple drug forms: polyinosinic-polycytidylic acid (poly I:C, double-stranded RNA analogue, TLR3 agonist), monophosphoryl lipid A (MPLA, lipid, TLR4 agonist), Resiquimod (R848, hydrophobic small molecule, TLR7/8 agonist), and cytosine phosphate guanidine (CpG, oligodeoxynucleotides, TLR9 agonist) (163, 164). Therefore, it is highly challenging to synchronize the pharmacokinetics of antigens and adjuvants in their codelivery.

Chen et al. synthesized a bi-adjuvant neoantigen nanovaccine (banNV) that co-loaded peptide neoantigen Adpgk and two adjuvants R848 and CpG in one nanoplatform (165). Because of the activation of two TLR pathways, immunization with bi-adjuvant banNV elicited stronger T cell response than single adjuvant vaccines. As a result of immune activation, PD-1 was profoundly upregulated on Adpgk-specific $CD8^+$ T cells, which led to the incomplete MC38 tumor regression after banNV treatments. Coordination withαPD-1 significantly improved the therapeutic outcomes of this bi-adjuvant vaccine therapy (Figure 5A). Lee et al. demonstrated the synergism of PD-L1 blockade with DC vaccine (168). They developed an immunoadjuvant nanocomplex (PSPEI-PIC) consisted of polysorbitol-co-PEI (PSPEI) polymer and poly(I:C). PSPEI-PIC assisted DC vaccines to activate tumor-specific T cells, but undesirably increased PD-L1 expression in tumor beds. Accordingly, combination of PDPEI-PIC, DC vaccine, and αPD-L1 achieved considerable therapeutic efficacy on MC38 tumor model.

Co-delivery of cytotoxic agents and immune adjuvants provides another template for tumor immunization. Chemical drugs and photosensitizers that can trigger ICD are usually used as cytotoxic agents in these nanomedicines, such as DOX, OXA, and Ce6 (169, 170). After administration, tumor cells succumbed to ICD inducers and released plenty of whole-cell antigens. These *in-situ* generated tumor antigens were immediately captured and presented by APCs with the help of adjuvant, such as imiquimod (R837, TLR7 agonist). It should be noted that cytotoxic agents worked within tumor tissues but immune adjuvants stimulated APCs in TDLNs, raising a paradox in drug delivery (41, 171). To simultaneously fulfill the site-of-actions of cytotoxic agents and adjuvants, Chen et al. developed a programmable immune activation nanomedicine (PIAN) (166). PIAN initially accumulated in tumor tissues, releasing Pt (IV) compounds (PPCD) in response to ROS for tumor killing and antigen release. Concurrently, CpG-loaded dendrimers (CpG/PAMAM) also released, capturing antigen and then transferred into TDLNs to facilitate antigen presentation. PIAN resulted in strong antitumor immune response, and completely cured 40% of colorectal tumor bearing mice in combination with PD-L1 blockade (Figure 5B).

Except for co-delivery with tumor antigens or antigen inducers, a sole-delivery of TLR agonists also functioned in tumor beds and exhibited synergistic effect with checkpoint inhibitors. Researches from Liu et al. revealed that intratumoral injection of NPs loaded with R837 or MPLA (PLGA-R837 or PLGA-MPLA) promoted DC maturation after surgery or TA of tumors, while consolidated the immunosuppressive TME (42). The anti-CTLA4 antibody (αCTLA4) were employed to inhibit Tregs. Triple therapy of TA, PLGA-R837 and αCTLA4 exerted abscopal effect to eradicate the secondary CT26 tumors and saved 100% mice from death. After primary tumor ablation, immune memory against CT26 tumors was able to lasted for 80 days. Zhang et al. prepared platelet membrane-coated nanoparticles (PNP-R848) to locally deliver R848 into tumors (172). They found the coating of platelet membrane prolonged tumor retention, improving the binding and uptake of NPs by tumor-resided immune cells. Treatment with PNP-R848 thoroughly eliminated MC38 murine colorectal adenocarcinoma and triggered a long-term immune memory that allowed mice to reject tumor rechallenge for twice.

In addition to TLR agonists, some materials derived from bacteria and virus intrinsically have adjuvant-like effects (164). Moreover, their particulate properties enable drug encapsulation. For example, Steinmetz et al. combined cowpea mosaic virus (CPMV) with αPD-1 or agonistic OX40-specific antibodies (αOX40) to combat several immunocompetent tumor models (173, 174). CPMV upregulated PD-1 on $CD4^+$ and $CD8^+$ effector T cells and OX40 on Tregs, which sensitized tumors to OX40 agonists and PD-1 inhibitors. In the CT26 colon cancer model, combination of αOX40 and CPMV realized better therapeutic outcomes than αPD-1+CPMV. Similar results were obtained in ovarian tumor and B16F10 melanoma models. Sun et al. are devoted to exploit *Salmonella* Typhimurium as the drug carrier for tumor immunotherapy (167, 175, 176). *Salmonella* has intrinsic tumor-homing capability, and it can release several pathogen-associated molecular patterns (PAMPs) such as flagellin and LPS to stimulate immune cells *via* TLR pathways (175). Many strategies have been developed to

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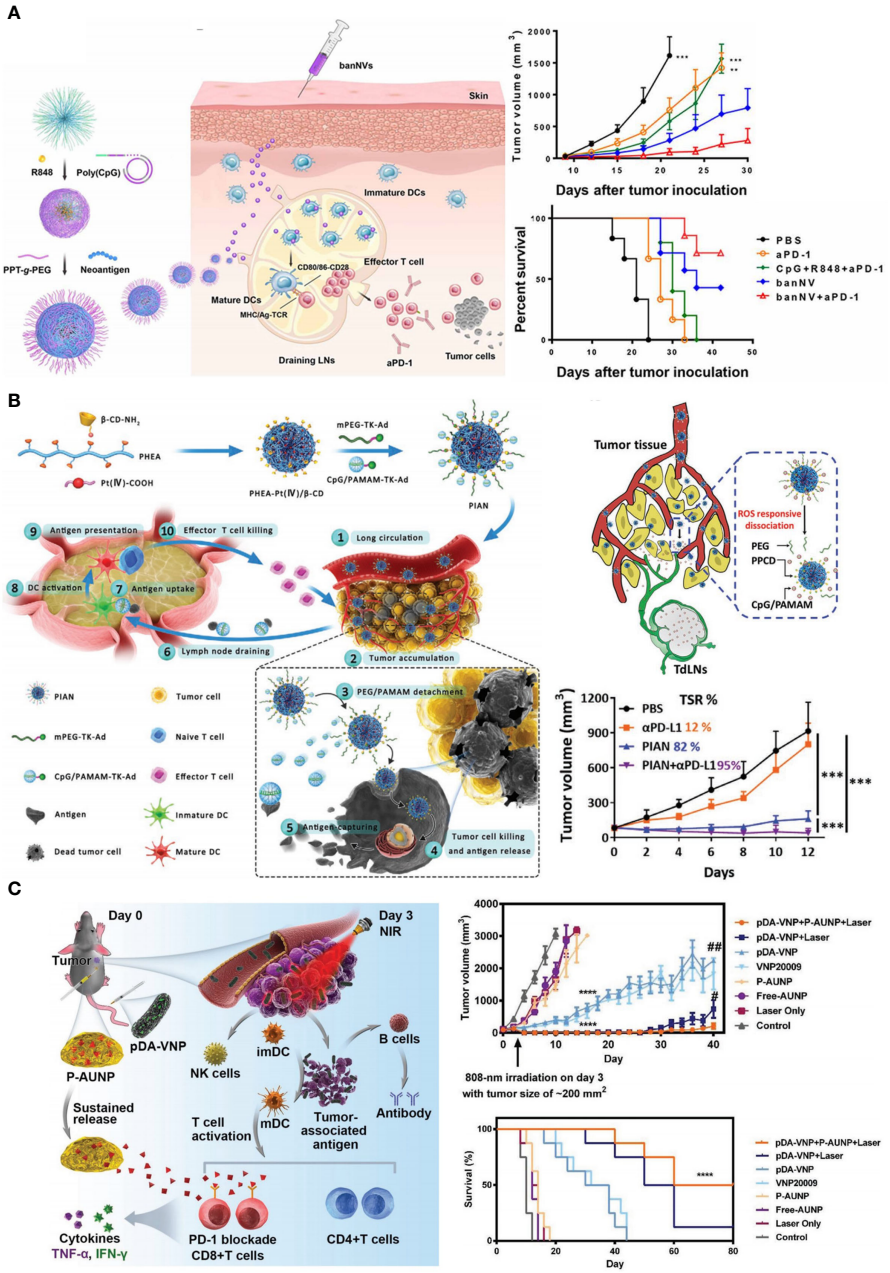

FIGURE 5
Nanotechnology pre-sensitizes host immune system to advance ICB in CRC therapy. **(A)** Left: schematic depiction of bi-adjuvant nano-vaccine (banNV) that sensitized antitumor T cells. Right: banNV remarkably facilitated αPD-1 in tumor growth inhibition and survival improvement. Reprinted with permission from reference (165). $^{**}p<0.01$, $^{***}p<0.001$ versus banNV+αPD-1. **(B)** Left: schematic depiction of the programmable immune activation nanomedicine (PIAN) that generated tumor antigens *in situ* and transported these antigens to TDLNs. Right bottom: the combination of PIAN and αPD-L1 achieved considerable tumor inhibition with a tumor suppression rate (TSR) of 95%. Reprinted with permission from reference (166). $^{***}p<0.001$. **(C)** Left: schematic depiction of the combination therapy of anti-PD-1 peptide depot and bacteria-based PTT. Right: the combination therapy notably inhibited tumor growth and prolonged mice survival. Reprinted with permission from reference (167). $^{****}p<0.0001$ versus Control. $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus pDA-VNP+P-AUNP+Laser.

engineer *Salmonella* as a nanocarrier, including genetic modulation, surface modification with therapeutic agents or NPs (Figure 5C) (167), as well as extracting their bacterial outer membrane vesicles (OMVs) as the adjuvant to coat nano-vaccines (177, 178). These pathogen-mimicking strategies offer a simple method to achieve colocalization of antigen and adjuvant in drug delivery, and strongly correlate with the peptide-based local PD-1 blockade therapy.

3 Conclusions and perspectives

Although ICB-based immunotherapy has revolutionized CRC treatment, challenges such as side effects, tumor metastasis, low response rate, and therapy resistance remain in clinic. Application of nanotechnology might be ground-breaking. This review systematically discusses the current strategies that utilize nanotechnology to potentiate CRC therapy in combination with ICIs, wherein four main mechanisms are involved, including increasing delivery efficiency of ICIs, reinforcing tumor immunogenicity, reprogramming TME, and directly initiating immunity. In coming decades, we hope to witness the progress of more advanced CRC immunotherapies. Further researches are required to establish regimens that can benefit more CRC patients.

Author contributions

ZL, YX, and YZ searched literatures and drafted the manuscript. ZL and XK designed the topic and revised 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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The role of immune profile in predicting outcomes in cancer patients treated with immunotherapy

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Background: Despite the efficacy of immunotherapy, only a small percentage of patients achieves a long-term benefit in terms of overall survival. The aim of this study was to define an immune profile predicting the response to immune checkpoint inhibitors (ICIs).

Methods: Patients with advanced solid tumors, who underwent ICI treatment were enrolled in this prospective study. Blood samples were collected at the baseline. Thirteen soluble immune checkpoints, 3 soluble adhesion molecules, 5 chemokines and 11 cytokines were analyzed. The results were associated with oncological outcomes.

Results: Regardless of tumor type, patients with values of sTIM3, IFNα, IFNγ, IL1β, IL1α, IL12p70, MIP1β, IL13, sCD28, sGITR, sPDL1, IL10 and TNFα below the median had longer overall survival ($p<0.05$). By using cluster analysis and grouping the patients according to the trend of the molecules, two clusters were found. Cluster A had a significantly higher mean progression free survival (Cluster A=11.9 months vs Cluster B=3.5 months, $p<0.01$), a higher percentage of disease stability (Cluster A=34.5% vs. Cluster B=0%, $p<0.05$) and a lower percentage of disease progression (Cluster A=55.2% vs. Cluster B = 94.4%, $p=0.04$).

Conclusion: The combined evaluation of soluble molecules, rather than a single circulating factor, may be more suitable to represent the fitness of the immune system status in each patient and could allow to identify two different prognostic and predictive outcome profiles.

KEYWORDS

immunotherapy, tumor biomarker, cytokines, chemokines, soluble immune check-points

1 Background

Immune checkpoint inhibitors (ICIs), a class of drugs targeting the inhibitory immune checkpoint receptors, have revolutionized clinical practice in oncology, demonstrating a significant improvement in progression-free survival (PFS) and overall survival (OS) in many types of cancer (1). The ability of immune cells to recognize, kill and control tumor cells has a strong impact in tumor progression (2). On the other hand, tumor immune-evasion mechanisms are mainly responsible for determining the failure of therapeutic strategies (3). Several studies have demonstrated that, in a portion of patients, ICIs could overcome tumor immune evasion, inducing a durable immune response against tumors (4). Thus, immunotherapy has become the standard of care for several cancer including advanced melanoma (5), non-small cell lung cancer (NSCLC) (6, 7), metastatic renal cell carcinoma (RCC) (8) and locally advanced and metastatic head and neck squamous cell carcinoma (HNSCC) (9). Even in uveal melanoma (UM), although considered a different clinical and biological entity from cutaneous melanoma, immunotherapy has become an important first line option (10). Nevertheless, some patients fail to respond to ICIs or become resistant during treatment. Early detection of intrinsically resistant patients is a crucial issue in clinical practice, as it could prevent immunotherapy failure (11–13). New, robust data are required to develop and validate molecular and genetic predictive biomarkers of ICIs resistance. In recent years, research focused on sampling soluble immune checkpoint (sIC), circulating molecules of adhesion, as well as cytokines and chemokines (14, 15).

Tumor cells employ several mechanisms to escape the control of the immune system. Among these processes, tumor microenvironment associated soluble factors and/or surface-bound molecules are mostly responsible for dysfunctional activity of the immune system (16). Recent results suggest that the concentration of these sICs is lower in patients benefitting from immunotherapy, with a potential role in predicting time to treatment failure (14, 17).

Soluble programmed death-ligand 1 (PD-L1) can inhibit the activation of either infiltrating or circulating T cells by means of PD-1/PD-L1 pathway (18, 19). CD-137, released as soluble form, negatively regulates the activation of T cells, blocking the interaction between T cells and antigen presenting cells (APCs) (20). These soluble factors, produced by alternative splicing or through proteolytic shedding of extracellular region of the cellular membrane can impede efficacy of ICI antibodies acting as decoy from the drug.

In this study a large spectrum of circulating molecules was analyzed, including soluble immune check-points, cytokines/chemokines and adhesion molecules, in patients with advanced/metastatic solid tumors before anti-PD-1 treatment. Focus was put on the differences in immune systems at baseline, trying to create a soluble immune profile (SIP) which could preemptively identify immunotherapy responder or non-responder patients.

2 Materials and methods

2.1 Clinical data

This prospective, multicentric study included patients with advanced or metastatic solid tumors including NSCLC, UM, RCC and HNSCC, who started immunotherapy between January 2017 and December 2020. Patients aged 18 years or older were included, with histologically confirmed solid tumors with advanced and/or metastatic disease, eligible for immunotherapy. Patients with Eastern Cooperative Oncology Group performance status (ECOG PS) ≤ 2 with adequate bone marrow, renal and liver function, fit for immunotherapy and able to provide a signed informed consent were included. Patients with ECOG PS >2 and patients with absolute contraindications to immunotherapy were excluded from the study. Baseline staging was performed according to the TNM system (AJCC 8th edition), with contrast-enhanced computed tomography (CT) and contrast-enhanced magnetic resonance imaging (MRI) based on clinical judgement. Age, sex, baseline,

ECOG PS, previous treatments received and tumor histology data were collected.

ICI treatment was administered according to the standard schedule approved for each primary tumor and line of treatment. Nivolumab was administered at the standard dose of 240 mg intravenously at 2-weeks interval and pembrolizumab at the standard dose of 200 mg intravenously at 3-weeks interval. Imaging assessment was performed after 12 weeks, or earlier in case of evident clinical disease progression. Tumor response was assessed using immune-related Response Evaluation Criteria in Solid Tumors (i-RECIST) and classified as complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD).

Progression free survival (PFS) was defined as the time from the first administration of ICIs until the first progression or in-treatment death. Overall survival (OS) was defined as the time from patient registration, or treatment commencement, to death from any cause or last follow up available.

Data were collected anonymously into a specific database. Protocol approval from Local Ethics Committee was obtained [CE 4421].

2.2 Samples collection

Peripheral blood samples were drawn from 81 patients with advanced/metastatic solid tumors before starting immunotherapy with anti-PD-1 agents (Nivolumab or Pembrolizumab). Peripheral blood samples were collected at baseline (T0) in red top collection tubes to allow blood to clot. After centrifugation at 1,500 x g for 10 minutes, serum samples were collected and stored at - 80°C until use. Immunomonitoring analyses were performed evaluating soluble circulating molecules.

2.3 Circulating soluble molecules

The immune profile was studied as an ensemble of 11 inflammatory cytokines, 5 chemokines, 3 soluble adhesion molecules and 13 soluble immune checkpoint molecules (Table 1) through a multiplex assay using the ProcartaPlex Human Inflammation Panel (catalog number EPX200-12185-901) and the Human Immuno-Oncology Checkpoint 14-plex ProcartaPlex Panel 1 (catalog number EPX14A-15803-901) (eBioscience) and evaluating the following circulating immune molecules: sE-Selectin; ICAM-1/CD54; IFN alpha; IFN gamma; IL-1 alpha; IL-1 beta; IL-4; IL-6; IL-8; IL-10; IL-12p70; IL-13; IL-17A/CTLA-8; IP-10/CXCL10; MCP-1/CCL2; MIP-1alpha/CCL3; MIP-1 beta/CCL4; sP-Selectin; TNF alpha, CD137, CTLA4, PD1, PDL1, PDL2, TIM3, LAG3, GITR, HVEM, BTLA, CD80, CD27 and CD28. For each patient, an amount of 50 µl of serum was used and added to a 96 well plate together with a mixture of magnetic beads coated with an antibody, according to the manufacturer's instructions. After that, a biotinylated detection antibody was added to the plate and then bound to Phycoerythrin (PE)-conjugated streptavidin. Samples were measured in single using the Luminex 200 platform (BioPlex, Bio-Rad). Data, expressed in pg/ml, were analyzed using Bio-Plex Manager Software. Subsequently to the evaluation two soluble molecules, i.e. IDO and GM-CSF, were excluded from the analysis. GM-CSF was not considered because the instrument didn't detect its serum value for the majority of patients. The exclusion of IDO, on the other hand, is due to poor reliability of the multiplex method, infact its activity evaluation is preferentially performed by high-performance liquid chromatography tandem mass spectrometry, in which is evaluated the concentration of kynurenine and tryptophan.

2.4 Statistical analysis

Statistical evaluation was performed using the statistical package SPSS Release Version 21.0 (SPSS Inc., Chicago, IL). Statistical significance cut-off level was set for $p < 0.05$. All tests of significance were two tailed. Continuous data were shown as means and categorical data were shown as frequencies (percentiles). Differences between continuous data were evaluated using the nonparametric Mann–Whitney U-test. In univariate analysis, the non-parametric Mann-Whitney U-test (two groups) was first used to compare soluble molecules continuous values in subjects with a given type of cancer; then, each variable of interest was dichotomized (as under the respective median or above the median) to study the OS or PFS in the two groups thus obtained. In addition, each variable of interest was dichotomized (as below or above the median value) to study the proportion of subjects with OS < or >12Mo in the two groups thus obtained. Categorical variables were compared between groups using the Chi squared test. Pearson's Chi squared test or Fisher's exact test (used for two-by-two contingency tables with less than 50 cases) were used to assess if paired observations on two variables, expressed in a contingency table were independent of each other. Multiple logistic regression was performed for the clinical variables with dichotomous scores to investigate whether associations between OS and soluble immune checkpoint were present after simultaneously adjusting for other variables of interest. Separate modelling was performed for each condition including all molecules, in addition to sex and age. P values <0.05 were considered significant. Since survival and prognosis varies widely by primary tumor type, 12 months was used as the cut-off value to assess the association between molecule concentration and survival, as it is similar to the median OS of the study population. Moreover, this value could be suitable, in our opinion, in discriminating the slice of patients primarily resistant to immunotherapy (21–23).

A time to event analysis was performed using non-parametric Kaplan-Meier (KM) product limit survival estimates, and differences between KM survival curves were analyzed using the Mantel-

TABLE 1 Soluble immune molecules: Characteristics and function.

Soluble molecules	Class of molecules	Cell source	Ligands	Main function	Type of action
sCD137	sIC	PBMCs	CD137L	Inhibits CD137/CD137L binding	Inhibitory
sPD1	sIC	PBMCs	PDL1/PDL2	Blocks PD1/PDL1 interactions	Activatory
sPDL1	sIC	Mature DCs	PD1	Binds PD1 and inhibits T cells response	Inhibitory
sPDL2	sIC	Tumor exosomes, alternatively activated macrophages	PD1	Unknown	Unknown
sCTLA4	sIC	Monocytes, immature DCs, regulatory T cells	CD80/CD86	Inhibits T cell responses	Inhibitory
sTIM3	sIC	Activated lympocytes	Tim3-L	Unknown	Unknown
sLAG3	sIC	Activated and exhausted $CD4^+$, $CD8^+$ T cells, regulatory T cells	Unknown	Unknown	Unknown
sGITR	sIC	Macrophages and regulatory T cells	GITRL	Unknown	Unknown
sCD27	sIC	Activated lymphocytes	CD70	Unknown	Unknown
sCD28	sIC	T cells	CD80/CD86	Inhibits T cells activity and counteracts anti-PD1 activity	Inhibitory
sBTLA	IC sIC	T cells, B cells, dendritic cells and myeloid cell	HVEM	Unknown	Unknown
sHVEM	sIC	T cells, B cells, natural killer cells, monocytes, neutrophils and dendritic cells		Unknown	Unknown
sCD80	sIC	Unstimulated monocytes and B cells	CTLA4/CD28	Unknown	Unknown
sICAM-1	Molecoles of adhesion	B and T lymphocytes Endothelial cells	LFA-1	binding the transmembrane receptor, antagonises leukocyte recruitment	Inhibitory
sE-selectin	Molecules of adhesion	Endhotelial cells	Carbohydrate ligands on tumor cells, sialyl Lewis-X	-Enhance angiogenis - Upregulation of ICAM-1 on tumor cells	unknown
sP-selectin	Molecules of adhesion	Endhotelial cells	PSGL-1, sialyl Lewis-X	-leukocyte recruitment -metastatisation -masking of tumor cells by binding to platelets	immune evasion
MCP1	chemokine	macrophage monocytes	CCR2 CCR4	-leucocyte recruitment	proinflammatory
MIP1 α **MIP1 β**	chemokine	Macrophages Hematopoietic cells	CCR1 CCR5	-granulocyte degranulation -production of pro-inflammatory cytokines -Promote cronic inflammation	proinflammatory
IP10	chemokine	Monocytes Endotelial cells fibroblast	CXCR3	Leucocytes recruitment	proinflammatory
INFα	Cytokine	DC Macrophages NK cells Macrophages Endothelial cells Fibroblasts	INFαR1/2	-NK activation -Cells B proliferation -Possible suppression of -Treg cells -Antiviral activity -Enhances MHC expression	proinflammatory immune-activation
INFϒ	Cytokine	Lymphocytes T (th1) CD8 and NK	INFγR1/2	-activation of macrophages -activation of Th1 responses -potential antigen presentation to T lymphocytes -induces apoptosis of tumor cells and reduces VEGF -increases expression of IDO	immunoactivating/ possible immunosuppressive activity)
TNFa	Cytokine	Macrophages NK T cells	TNFR1 TNFR2	-pro-inflammatory activity -stimulates cell proliferation and survival	Immune-activation/ pro-inflammatory

(Continued)

TABLE 1 Continued

Soluble molecules	Class of molecules	Cell source	Ligands	Main function	Type of action
				-induction of apoptosis -implicated in resistance to antiPD1 drugs	
IL1α	Cytokine	DC Macrophages Neutrophils Endothelial cells fibroblast	IL1R1 IL1R2	-production of acute phase proteins -stimulates TNFa pathway -implicated in fever, sepsis and inflammation	Immune activation Pro-inflammatory
IL1β	Cytokine	DC Macrophages Neutrophils Endothelial cells fibroblast	IL1R1	-production of acute phase protein -implicated in fever -induces differantiation of lymphocytes Th17	Immune activation Pro-inflammatory
IL4	Cytokine	T cells Mast cells	IL4-Rα	-activation of Th2 immune response -cell growth/activation	Pro-inflammatory
IL6	Cytokine	Macrophages Endothelial cells T cells	IL6Rα	-B lymphocyte proliferation and antibody response -production of prostaglandins and acute phase proteins -antagonises Treg -anti-inflammatory action through inhibition of TNFa and induction of IL10	Pro-inflammary/anti-inflammatory
IL8	Chemokine	Macrophages Endothelial cells Platelets	CXCR1 CXCR2	- chemotaxis -powers phagocytosis -ability to mediate infiltration of MDSCs into the tumor environment	Immuneactivation/ Immune-evasion
IL10	Cytokine	Macrophages Treg cells B cells Mast cells Th2 Tcells	IL10Rα IL10Rβ	-downregulation of Th1 cytokines -inhibits CD4 T cell activity -suppresses expression of costimulatory molecules -increases survival of B lymphocytes -blocks secretion of proinflammatory cytokines	Antinflammatory/ Possible immunostimulating anti-tumor activity
IL12p70	Cytokine	Macrophages DC	IL12Rb1 IL12Rb2	-activation of Th1 responses -powers CD8 and NK T-cell activity -Increases INFa production by T cells -suppresses Treg proliferation and angiogenesis	Immune activation
IL13	Cytokyne	T CD4 Cells CD8 cells NK Eosinophils Mast cells	IL13Rα1 IL13Rα2	-involved in Th2 immune responses -potential expression of adhesion molecules on endothelial cells -activation of magrophages and production of TGFb	Proinflammatory
IL17A	Cytokine	Lymphocytes TCD4 Th17	IL17Rα	-induces IL6 and chemokines production - promotes recruitment of MDSCs into the tumor bed	Proinflammatory

DC, dendritic cells; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; IP, interferon induced protein; pd-l1, programmed death ligand 1; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; TIM3, T-cell immunoglobulin domain and mucin domain 3, LAG3, lymphocyte Activating 3; B- and T-lymphocyte attenuator; HVEM, Her-pesvirus entry mediator; ICAM-1, Intercellular Adhesion Molecule 1.

Haenszel log-rank test. Relatedness of soluble molecules was tested by applying unsupervised Eisen's hierarchical cluster methods (24) to the data set, encompassing immune molecules across all samples and using as agglomeration rule the average linkage clustering as implemented in the Genesis soft-ware (25). Unsupervised clustering involved the sorting of both soluble ICs and cytokines/chemokines/adhesion molecules values. The soluble molecules tree was computed on the basis of a full data set and the distances between samples were computed by using Pearson correlation as similarity measures. Each square in the heat-map represents the higher value (red), equal value (black) or lower level (green) of signal of any given test-ed soluble molecule for each tested subject.

The color intensity of every single square in the heatmap is directly associated with the measured concentration in pg/ml. Interpretation of the heat-map generated by the software could be performed either visually, where clustering distinct soluble factors tends to give more homogeneous areas, or by taking into consideration the higher or lower level of dendrograms on the patient side of the graph.

3 Results

3.1 Patients

Eighty-one metastatic patients treated with anti PD-1 agent were enrolled in this study: 22 patients with UM, 10 patients with RCC, 13 with HNSCC, and 36 with NSCLC. Baseline clinical–pathological characteristics of patients are summarized in Table 2. All 10 patients in the RCC group had clear cell carcinoma and all 13 HNSCCs had squamous histology. Fifty-one patients were male (63%), 30 patients were female (37%). The mean age was 51 ± 9 years. All patients were treated with anti-PD-1 agents (nivolumab and pembrolizumab): 25 patients in a first-line, 56 patients in a second- or subsequent-line setting.

3.2 Outcomes

Median OS was 27.4 ± 25:2 months in UM, 49.2 ± 20.7 months in RCC, 18.5 ± 11.5 months in HNSCC, and 24.8 ± 24 months in NSCLC. Median OS was significantly lower in HNSCC than in the RCC group ($p<0.05$). Median PFS was 9 ± 10.8 months in UM, 17.6 ± 16.23 months in RCC, 4.9 ± 5 months in HNSCC, and 12.6 ± 14.6 months in NSCLC.

3.3 Soluble profile by type of cancer

3.3.1 Serum value of sICs

Mean values of each sIC in UM, RCC, HNSCC and NSCLC are shown in Table S1. There is a wide heterogeneity of soluble ICs serum levels between cancers. The same table shows moreover the statistically significant differences between sICs value means in the tumor subgroups. In NSCLC, sCD27 had the highest values. A similar trend was noted for sCD137, sHVEM and sLAG3 levels. sHVEM values were higher in RCC when compared to UM and HNSCC. On the other hand, RCC showed the lowest levels of sPDL2, which had its greater values sampled in HNSCC and NSCLC patients. HNSCC patients had the highest values of sCD80 and sCTLA4, and the lowest levels of sPDL1.

3.3.2 Serum value of soluble adhesion molecules

Table S2 shows the main value of each soluble adhesion molecule and the statistically significant differences in the comparison between pairs of tumor subgroups. The highest sICAM-1 values were found in the HNSCC group and their lowest ones in the UM group. Similarly, the highest sP-selectin values were found in HNSCC patients, and RCC showed lower values when compared to HNSCC and NSCLC. The sE-selectin value was higher in HNSCC and NSCLC when compared to UM and RCC groups.

3.3.3 Serum value of cytokines/chemokines

Table S3 shows the mean serum value of cytokines and chemokines in each cancer subgroup with a highlight on their statistically significant differences. Cytokines and chemokines levels were the highest in NSCLC patients, except for IL17A values. Mean value of IL17A was significantly higher in HNSCC compared to other types of cancer. In UM IP10 had the lowest values compared to all the other types of cancer, even though lower values of the other cytokines and chemokines were noted, when compared to HNSCC and NSCLC. Lower concentrations of IFNγ, MCP1, MIP1 β and TNFα were found in RCC than in HNSCC and NSCLC. IL10 values were found to be lower in the HNSCC group than in RCC and NSCLC.

TABLE 2 Clinicopathological characteristics.

Parameter	N (%)	UM	RCC	HNSCC	NSCLC
Total	81	22	10	13	36
Age years(mean, range)	51 ± 9	67 ± 10	56 ± 10	63 ± 9	65 ± 9
Gender					
Male	51 (63%)	11	8	9	25
Female	30 (37%)	11	2	4	11
• Pembrolizumab	25	22	–	–	3
• Nivolumab	56	–	10	13	33
• I line	25	22	–	–	3
• II/subsequent line	56	–	10	13	33

UM, uveal melanoma; RCC, renal cell carcinoma; HNSCC, head and neck; squamous cell carcinoma; NSCLC, non small cell lung cancer.

3.4 Soluble molecules and oncological outcomes

3.4.1 Differences between patients with OS below and above 12 months

There are significant differences in the mean values of several cytokines and chemokines (IFNα, IFNγ, IL10, IL12p70, IL13, IL1α, IL1 β, IL4, IL8, MCP1, MIP1α, MIP1β and TNFα) between the OS < 12 months group and the OS > 12 months group (Table S4). The concentrations of all of these soluble factors were significantly lower in patients with OS longer than 12 months. Multiple logistic regression analysis, considering simultaneously all the molecules studied together with age and sex, showed a significant relationship between OS and IL1α levels (p: 0.037 ORa= 0.151, 95% CI= 0.025-0.893).

3.4.2 Multiple Soluble ICs and cytokines/chemokines correlation with OS and PFS

Each soluble factor was dichotomized based on the median value found (Figure 1 and Table S5). After Kaplan-Meier evaluation, significantly longer OS was found in patients with low levels of sCD28, sGITR, sPDL1, sTIM3, IFNα, IFNγ, IL1β, IL10, IL1α, IL12p70, IL13, MIP1β and TNFα. Furthermore, each soluble factor was also dichotomized based on the median value found in relation to PFS (Figure 2 and Table S6). Significantly longer PFS in patients with low levels of sCD28, sGITR, sPDL1, IL10 and IL13 were found.

3.5 Comprehensive prognostic and predictive immune profile

Unsupervised hierarchical cluster analysis, performed for those patients with all available soluble evaluation, identified two distinct groups of patients (Cluster A and B) based on soluble molecules serum levels, prior to ICI therapy (Figure 3). The distribution of cancer types varied between the two clusters. Cluster A included 9 UMs, 6 RCCs, 12 NSCLCs and 2 HNSCCs cases, while cluster B included 7 UMs, 2 RCCs, 2 NSCLCs and 10 HNSCCs. No significant differences were shown between the two clusters for rate of patients in the II-line setting (68.9% cluster A and 66.6% cluster B, p=0.251) and platinum-refractory patients (48.2% in Cluster A vs. 57.1% in cluster B, p=0.535). The first group represented patients with high concentration of the soluble checkpoints sTIM3, sPDL2, sCD27, sCD28 and adhesion molecules. The second group, in addition to the remaining soluble ICs (sPD1, sPDL1, sCD137, sCD80, sCTLA4, sGITR, sHVEM, sBTLA, sLAG3), showed an increase of the values of all the cytokines and chemokines. Patients in cluster B showed a significantly shorter PFS (3.5 months vs. 11.9 months in cluster A, p < 0.01), as shown by the Kaplan Meier curves in Figure 4. At the same time, PD was found in 94.4% of cluster B vs. 55.2% of cluster A patients (p = 0.04). Consequently, risk of PD was about 7 times higher in cluster B patients than in cluster A ones throughout anti-PD-1 treatment (Odd Ratio = 6.9, 95% C.I.(1.34-35.52)). Accordingly, SD was observed more often in Cluster A patients (34.5%) than in Cluster B ones (0%, OR = 0.1, 95% C.I.(0.01-0.91), p<0.05) (Figure 3).

4 Discussion

The challenge of immuno-oncology is the identification of new therapeutic strategies to overcome resistance to immunotherapy. Soluble immune profiles (SIP), resulting from the combined evaluation of circulating checkpoints, adhesion and inflammatory molecules (cytokines and chemokines) could be considered as a portrait of the immune system fitness of a patient, which may interfere or affect the response to treatment with ICIs. This study highlighted that given the variability of immune status, the analysis of circulating factors could provide meaningful prognostic and predictive information.

Mean basal values of soluble molecules differed according to tumor histology, suggesting that these differences may reflect a different organ-dependent immunity. In the examined patient cohort, NSCLC was characterized by a high expression of sICs, such as sCD27, sCD137, sHVEM and sLAG3, and by higher values of circulating cytokines and chemokines. On the other hand, HNSCCs presented the highest values of sPDL2, sCD80, sCTLA4, soluble adhesion molecules such as sICAM-1, sP-selectin and sE-selectin, while UMs showed the lowest values of cytokines and chemokines compared to NSCLCs and HNSCCs. Pre-treatment levels of several circulating molecules, regardless of tumor type, were associated with OS and PFS. Longer OS was reported in patients with low levels of sCD28, sGITR, sPDL1, sTIM3, IFNα, IFNγ, IL1β, IL10, IL1α, IL12p70, IL13, MIP1β and TNFα. Patients with OS of less than 12 months had significantly higher levels of multiple cytokines and chemokines: IFN α, IFNγ, IL1α, IL1β, IL10, IL12p70, IL13, TNFα and IL4.

In the landscape of soluble immune biomarkers, sICIs seem to be particularly promising, even though their predictive and prognostic meaning is still unclear and their role seems to depend on histology and on the setting of disease (13–15, 26).

In particular, sLAG3 (higher in NSCLC compared to UM and RCC) could be considered a marker of Th1 activation and DCs maturation, while high levels of sCTLA4 are associated with worse prognosis in patients affected by HNSCC, NSCLC, RCC and in colorectal cancer (CRC) (13, 14, 26–29). Furthermore, sPDL1 could contribute to the immune evasion mechanism, treatment resistance, and worse prognosis as well as sTIM3 values below the median (7972 pg/ml), reflecting the role attributed to their transmembrane form when expressed by tumor cells (18, 28–37).

Considering other soluble molecules and in accordance to the available literature, this study seems to show an association between IL10, IL13, IL1α, IL1β, TNFα and longer OS and PFS in several diseases (38–42). While the role of IFNγ in cancer is still

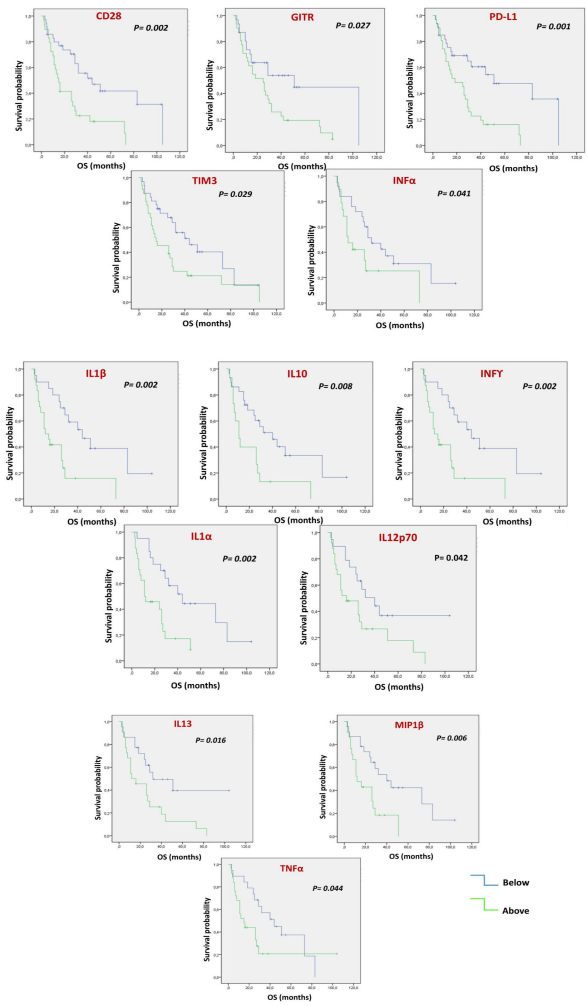

FIGURE 1
Multiple Soluble ICs and cytokines/chemokines are correlated with OS. Each value of soluble factor, regardless of cancer type, was dichotomized as under the median or above the median. Kaplan-Meier evaluation showed that low values of soluble CD28, GITR, PDL1, TIM3, INFα, INFγ, IL1β, IL10, IL1α, IIL12p70, IL13, MIP1β and TNFα were associated with better OS ($p<0.05$).

controversial, this cytokine could exploit both anti-tumor and pro-tumor activities as well (43, 44). Lower IFNγ values at the baseline were reported in patients with squamous esophageal carcinoma responding to immunotherapies and in patients with RCC responding to the anti-VEGFR TKI (28, 39).

On the other hand, concentrations of IL4, MIP1α/β, IP10 and IL8 are significantly higher in subjects with lower OS, probably because they are involved in the processes of Th1 lymphocyte inhibition, induction of M2 differentiation in macrophages and in metastatic liver spreading (41, 44–50). The chaotic pattern of circulating cytokines can be interpreted through the identification of a 'cytokines signature', in which it is not the single cytokine which acquires a predictive value for response to immunotherapy, but the specific combination of several cytokines. This innovative approach has recently been explored in the literature with promising results (51–53).

This portrait of the immune system emphasizes the complexity of molecules and solubility interactions and the difficulty of interpreting it, highlighting the need of an immunological comprehensive profile rather than the evaluation of individual markers. This study focused on the analysis of a soluble immune profile in relation to cancer outcomes. Two distinct groups of cancer patients were identified by means of cluster analysis, which take into account the pattern of soluble molecules detected at the baseline (Figure 5).

The first group was identified as the soluble immune profile (SIP) one which benefits the most, in terms of PFS and response, from immunotherapy (Cluster A). It seems probable that in this

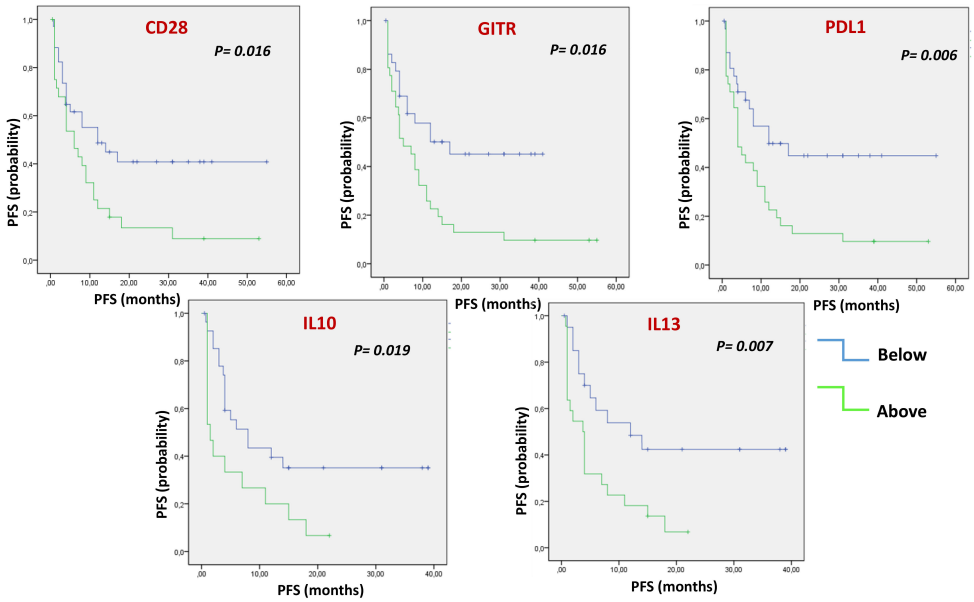

FIGURE 2
Multiple Soluble ICs and cytokines/chemokines are correlated with PFS. Kaplan-Meier evaluation showed that, dichotomizing values of soluble factors under or above the median, low levels of soluble CD28, GITR, PDL1, IL10 and IL13, were associated with longer PFS (p<0.05).

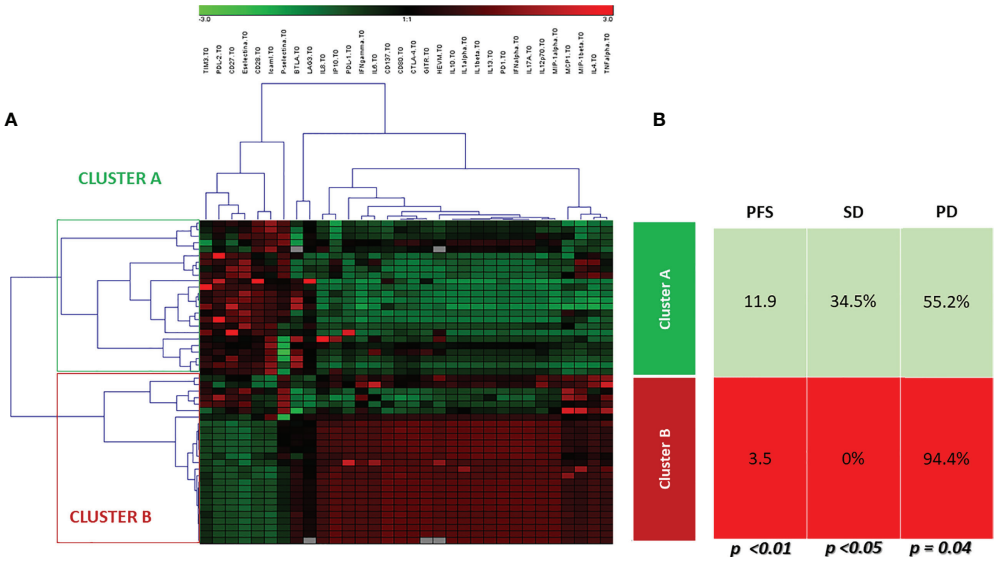

FIGURE 3
Unsupervised hierarchical cluster analysis. **(A)** The heat-map of cluster analysis. Soluble molecule tested are listed in the top of the figure. The unsupervised hierarchical cluster analysis identified 2 distinct clusters of patients based on the soluble immune profile associated with a different oncological outcome: Cluster A (green box) and Cluster B (red box). The color intensity of every single square in the heat-map is directly associated with the measured concentration in pg/ml. Each square in heat-map represents the higher value (red), equal value (black) or lower level (green) of signal of any given tested soluble molecules for each tested patient, **(B)** Oncological outcomes were reported for each cluster. Cluster A was associated with longer PFS and higher SD rate than Cluster B (11.9 months vs. 3.5 months, and 34.5% vs.0, respectively).

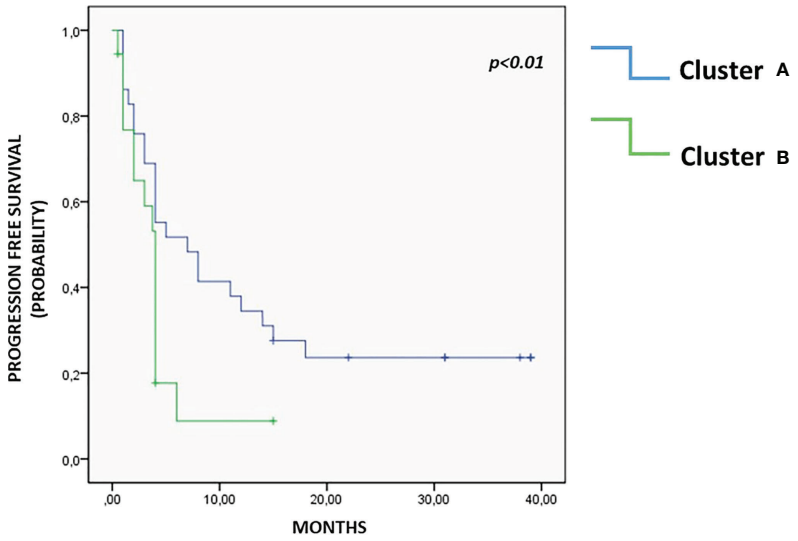

FIGURE 4
Progression free survival. As highlighted in Kaplan Meier curves, patients in Cluster A showed a significantly longer PFS than patients in Cluster B, 11.9 months vs. 3.5 months, p<0.01.

cluster the effect of the elevated value of the soluble checkpoints CD27 and CD80, which drive the differentiation of T-cells into memory cells, is preponderant (54). Thus, in this group the inhibitory activity of TIM3 and PD-L2 receptors is overcome by the cooperation of immune activating pathways and by immunotherapy (55).

In the second group (Cluster B), in addition to the numerical supremacy of inhibitory immune checkpoints, a high pro-inflammatory state is evident. The overexpression of the inhibitory PD-1/PD-L1, BTLA/HVEM, CTLA-4, LAG3 checkpoints axis, associated with the concurrent overelevation of cytokines and chemokines could play a decisive role in reducing the benefits obtainable with immunotherapy (55–59). Cytokines and chemokines have contrasting roles in promoting tumor immunity, inflammation, and response to immunotherapy. The presence of high levels of cytokines/chemokines in cluster B suggests an inflammatory state not capable of eliciting an active antitumor immune response. We know, to date, that elevated levels of some cytokines, such as IL6, are associated with worse outcomes to immunotherapy. However, in the inflammatory picture described in Cluster B, it is difficult to say which cytokine determines the pathway activation with dominant effect (60). The profound dysregulation of immune mechanisms, results in a hostile environment for the proper functioning of therapy with anti-PD-1 monoclonal antibodies. This soluble profile correlates to a significantly shorter PFS (3.5 versus 11.9 months).

The soluble profile varies widely by primary tumor type, as evidenced by their distribution in the two clusters. Cluster A collects mainly RCCs and NSCLCs, whereas Cluster B is dominated by HNSCCs. This finding confirms that important mechanisms of immunosuppression are involved in tumor progression of HNSCC, which could limit the efficacy of immunotherapy outside of combination strategies (61). All HNSCC patients included in the study were platinum refractory, representing a patient population achieving lower response rates to immunotherapy than the ones with tumor histotypes (62). Therefore, it is not surprising that the majority of patients with platinum refractory HNSCC had an unfavorable immune profile and fell into cluster B. However, it is relevant to note that through cluster analysis it was possible to identify a common immunological profile in non-responder patients. Presumably any patient falling into cluster B will have an unfavorable immune fitness and a tumor with an immunological behavior much more similar to that of a platinum-refractory HNSCC than to what would be expected on the basis of cancer type. The two clusters appear to be homogeneous in terms of rate of patients in the II-line and platinum-refractory settings, although the rate of platinum-refractory patients in cluster A is lower than cluster B (48.2 vs 57.1%, respectively). Recently, the possible immunomodulatory effect of chemotherapies has been studied to define the rationale of new combination strategies. Chemotherapeutic agents have different immunologic effects that could influence the response to immunotherapy. Cisplatin seems to be able to increase the activation and proliferation of T cells and their cytotoxic activity (63). In addition, recent *in vitro* and *in vivo* experiments have shown that cisplatin can enhance tumor immunogenicity by increasing MHC I cell surface expression, but, at the same time, it can induce up-regulation of PD-L1 in human and mouse ovarian cancer cell lines (64). Therefore, the role of prior treatments should be studied specifically and on larger, homogeneous populations in order to define the effect they may

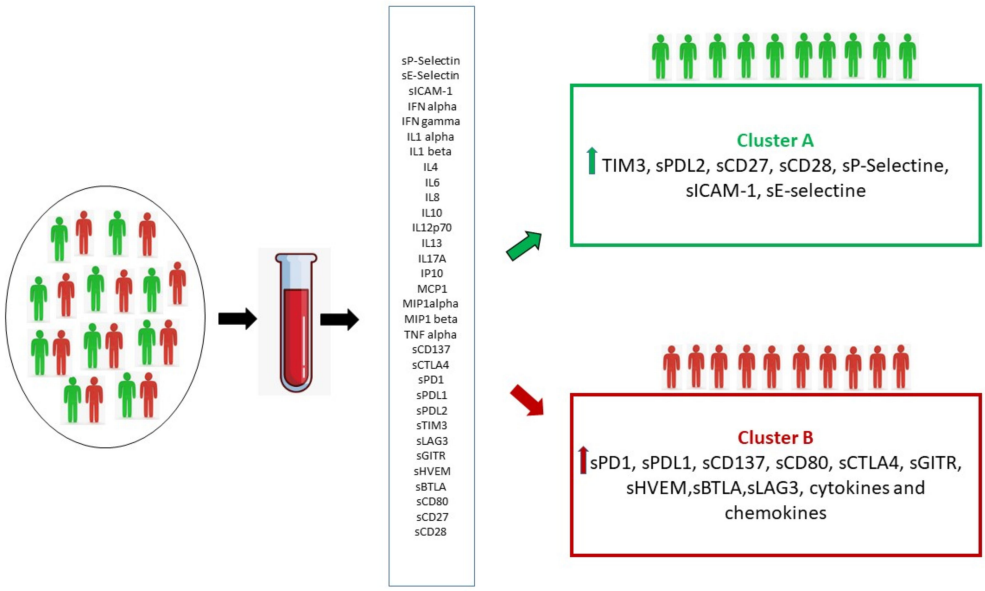

FIGURE 5
By means of cluster analysis, the circulating immune profile detected at baseline made it possible to identify two distinct groups of patients (Cluster A and B) characterized by the activation of different immune pathways resulting in two distinct clinical outcomes.

have on the soluble immune profile and outcomes to immunotherapy. Otherwise, RCCs, which are more represented in cluster A, had better outcomes, especially in terms of OS. In our population, RCCs had a mOS of 49.2 ± 20.7 months, higher than survival rates reported in the Checkmate 025 trial (65). However, in this series RCC patients had a favorable MSKCC risk. In addition, most of our patients with RCC had less than 2 sites of metastasis. Escudier et al. tried to investigate which baseline clinical factor was associated with better OS with nivolumab. In patients with 1 site of metastasis at baseline and a favorable MSKCC risk, OS was not reached at a median follow up of 22 months (66). In our series mUM patients are similarly distributed between clusters A and B. To date, no data are available from controlled clinical trials regarding immunotherapy in uveal melanoma, which in clinical practice is commonly treated in a similar fashion as cutaneous melanoma. However, recent prospective data have shown that the small proportion of patients who respond achieves significant disease control. Therefore, it is crucial to identify predictive factors for response (67).

The main limitation of this study is due to the small sample of patients involved and the heterogeneity of the population in terms of primary tumors, treatment line and patient prognosis. However, it provides important insights which should direct further investigation in a larger patient population. Surely this study could most likely be considered as a hypothesis generator, which should be validated on a more homogeneous population in terms of both histotype and treatment setting.

5 Conclusions

In conclusion, this study highlights: 1) a significant variability of immune status biomarkers in each patient; 2) an organ dependent immunity; 3) a significant association between multiple soluble ICs, cytokines/chemokines and outcome regardless of tumor type; 4) two soluble immune profiles, resulting from the combination of several circulating molecules, significantly associated with both treatment response and PFS. A predictive biomarker profile of oncological outcomes represents an urgent yet unmet need for a rational treatment of each patient based on their own immune features.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics statement

The studies involving human participants were reviewed and approved by Ethical Committee no. 4421, "Sapienza University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceptualization, AB, SM and PM; Data curation, AC, AC; SS and SP; Formal analysis, AS; Investigation, IGZ, ADF and MN; Resources, IGZ, ADF, MN; Supervision, ST, EC, MN, PM; Writing – original draft, GP, AB and SM; Writing – review & editing, BC, SA, ER, GS, GT. All authors contributed to the article and approved the submitted version.

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Conflict of interest

PM has/had a consultant/advisory role for BMS, Roche, Genentech, MSD, Novartis, Amgen, Merck Serono, Pierre Fabre, and Incyte.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.974087/full#supplementary-material

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Predictive biomarkers of colon cancer immunotherapy: Present and future

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Immunotherapy has revolutionized colon cancer treatment. Immune checkpoint inhibitors (ICIs) have shown clinical benefits for colon cancer patients, especially those with high microsatellite instability (MSI-H). In 2020, the US Food and Drug Administration (FDA)-approved ICI pembrolizumab as the first-line treatment for metastatic MSI-H colon cancer patients. Additionally, neoadjuvant immunotherapy has presented efficacy in treating early-stage colon cancer patients. Although MSI has been thought of as an effective predictive biomarker for colon cancer immunotherapy, only a small proportion of colon cancer patients were MSI-H, and certain colon cancer patients with MSI-H presented intrinsic or acquired resistance to immunotherapy. Thus, further search for predictive biomarkers to stratify patients is meaningful in colon cancer immunotherapy. Except for MSI, other biomarkers, such as PD-L1 expression level, tumor mutation burden (TMB), tumor-infiltrating lymphocytes (TILs), certain gut microbiota, ctDNA, and circulating immune cells were also proposed to be correlated with patient survival and ICI efficacy in some colon cancer clinical studies. Moreover, developing new diagnostic techniques helps identify accurate predictive biomarkers for colon cancer immunotherapy. In this review, we outline the reported predictive biomarkers in colon cancer immunotherapy and further discuss the prospects of technological changes for biomarker development in colon cancer immunotherapy.

KEYWORDS

biomarker, immune checkpoint inhibitor (ICI), colon cancer, predictive, immunotherapy

Introduction

Colon cancer is currently one of the malignant tumors with a high incidence and death rate worldwide (1). Traditionally, the main therapeutic strategies in colon cancer include surgery, chemotherapy, and targeted therapy. Among these therapy methods, surgery is applicable to early-stage patients with lesions confined to the colon, while

approximately 20% of colon cancer patients have distant metastases at the time of diagnosis and miss the opportunity for surgery (2). Chemotherapy is the main treatment option for metastatic colon cancer patients. In addition, depending on the RAS gene mutational status and tumor location, corresponding targeted agents, such as bevacizumab or cetuximab, were combined to enhance the anti-tumor effect of chemotherapy agents. Even so, the patients' prognosis is still dismal (3). Recently, immunotherapy has revolutionized colon cancer treatment. In the clinical study, pembrolizumab, an anti-programmed cell death 1 (PD-1) agent, resulted in significant improvements in progression-free survival (16.5 vs. 8.2 months) and fewer adverse events than chemotherapy as a first-line treatment in patients with microsatellite instability-high (MSI-H) or mismatch repair-deficient (dMMR) metastatic colorectal cancer (mCRC) (4). This promising result led to the US Food and Drug Administration's (FDA) approval of pembrolizumab as the first-line treatment for MSI-H mCRC patients. Moreover, double ICI combination, anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) agent, and anti-PD-1 agent combination therapy presented promising anti-tumor efficacy in MSI-H/dMMR mCRC (5, 6). Furthermore, neoadjuvant immunotherapy also presented promising efficacy in treating early-stage MSI-H/dMMR colon cancer patients (7).

Currently, MSI-H/dMMR is the only well-recognized biomarker that can be used to guide the immunotherapy of colon cancer. However, the mechanism of why MSI-H/dMMR can be used as a biomarker for colon cancer immunotherapy is still not clarified (8). The possible mechanism was thought to be that MSI-H/dMMR may be correlated with a higher mutational load, which leads to neoantigen formation and activation of the body's immunity (9, 10). The efficiency of MSI as the biomarker for cancer immunotherapy is relatively low. The reported objective response rate (ORR) in MSI-H/dMMR colon cancer patients varies between 30%-70% (4, 5, 11–14). Additionally, colon cancer patients with MSI-H/dMMR are not in a high proportion of the total number of colon cancer patients. Only approximately 20% of colon cancer patients are MSI-H (8); whereas, in stage IV colon cancer patients, MSI-H is less than 5% (15). In addition, a small percentage of patients with microsatellite stable (MSS) or proficient MMR (pMMR) can benefit from immunotherapy, while a significant proportion of MSI-H/dMMR patients demonstrate intrinsic or acquired resistance to immunotherapy (16–19). These results suggest that more precise biomarkers are needed to stratify colon cancer patients that could benefit from immunotherapy.

Other potential predictive biomarkers which are proposed in colon cancer immunotherapy include PD-L1 expression level, tumor mutation burden (TMB), tumor-infiltrating lymphocytes (TILs), gut microbiota, ctDNA, and circulating immune cells (20–24). In addition, relevant indicators that reflect the tumor microenvironment (TME) have also been proposed for use as biomarkers for immunotherapy in colon cancer (25). Immunoscore and consensus molecular subtypes (CMS) based on immune cells and molecular typing have implications for clinical management and are predictive of prognosis and treatment response in patients with colon cancer (26, 27). New techniques, such as multiplex immunohistochemistry (mIHC) and single-cell RNA sequencing, could provide a more comprehensive evaluation of the TME and genetic heterogeneity in colon cancer. This will help find precise biomarkers for screening and efficacy assessment of colon cancer immunotherapy beneficiary populations (28, 29). In this review, we comprehensively summarize the reported biomarkers of colon cancer immunotherapy and further discuss the prospects of technological changes for biomarker development in colon cancer immunotherapy.

MSI and dMMR as the predictive biomarker for colon cancer immunotherapy

Microsatellites (MS) are short tandem repeats (STRs) in the human genome that are composed of several short and repetitive DNA sequences. Microsatellite instability (MSI) refers to the failure of the DNA mismatch repair mechanism during the DNA replication process, which results in length changes in MS (8). MSI was first identified in hereditary nonpolyposis colorectal cancer syndrome, known as Lynch syndrome (30). Subsequently, multiple types of malignant tumor patients were found to present with MSI (31–35). MSI is an indicator of tumor prognosis and treatment response (36). According to the status of MMR, tumors can be classified as dMMR and pMMR. IHC is the main method to test the MMR status. Tumors with loss of expression of MMR genes, including MutS homolog 2 (MSH2), MutL homolog 1 (MLH1), MutS homolog 6 (MSH6), or postmeiotic segregation increased by 2 (PMS2), were defined as dMMR; otherwise, they were defined as pMMR. In addition, according to the mutation frequency of MS, tumors can be termed MSI-H, low-frequency MSI (MSI-L), and MSS. Polymerase chain reaction (PCR) is the main method used to assess the frequency of MSI mutations. Due to the respective limitations of these two methods, a combination test of both IHC and PCR is usually required to evaluate the status of MS (37). In general, MSI-H is equivalent to dMMR.

Approximately 20% of colon cancer patients are MSI-H/dMMR (8). Except for approximately 3% of MSI-H/dMMR colon cancer patients who were hereditary with Lynch syndrome, most MSI-H/dMMR colon cancer patients are sporadic. The mechanisms of MSI in hereditary and sporadic MSI-H/dMMR patients are different (8). MSI-H/dMMR colon cancer patients all present unique clinicopathological features that correlate with patient prognosis and treatment response. For example, colon cancer patients with MSI-H/dMMR were

reported to have a better prognosis than those with MSI-L/pMMR (38, 39). Colon cancer patients with MSI-H/dMMR did not benefit from fluorouracil-based therapy (40). In particular, there is now considerable clinical evidence that MSI-H/dMMR colon cancer patients present a high ORR to ICIs. The relationship between MSI and colon cancer immunotherapy response was initially found in a phase I clinical trial. In this study, the safety and tolerability of the anti-PD-1 antibody BMS-936558 were evaluated in treatment-refractory solid tumor patients. A post-operative recurrent colon cancer patient who reported a durable complete response after therapy was MSI-H (41). Then, the KEYNOTE-016 trial was conducted to identify the role of MMR status as a biomarker for predicting the clinical benefit of ICI treatment. In this study, progressive metastatic carcinoma patients with or without dMMR were treated with the PD-1 antibody pembrolizumab. In the cohort of patients with dMMR colorectal adenocarcinomas, the reported ORR was 40% versus 0% in the cohort of pMMR patients (11). A similar result was observed in another clinical study, KEYNOTE164. In this study, the antitumor activity of pembrolizumab was tested in previously treated and metastatic MSI-H/dMMR colorectal cancer (CRC) patients. The reported ORR was 33% (14). In addition, in CheckMate 142, another PD-1 agent called nivolumab also induced durable responses and disease control in pretreated dMMR/MSI-H metastatic CRC patients. At a median follow-up of 12.0 months, 31.1% of patients achieved an investigator-assessed objective response (13). Based on these results, the FDA approved ICIs (pembrolizumab and nivolumab) for the treatment of MSI-H/dMMR metastatic CRC patients in 2017. Then, in KEYNOTE-177, the efficacy of pembrolizumab was compared with chemotherapy as first-line therapy for MSI-H/dMMR advanced or metastatic CRC. After a median follow-up of 32.4 months, pembrolizumab therapy demonstrated superiority over chemotherapy in terms of median progression-free survival (16.5 months vs. 8.2 months). ORR was observed in 43.8% of the patients in the pembrolizumab group versus 33.1% in the chemotherapy group (4). This result prompted the FDA to approve pembrolizumab as the first-line treatment for metastatic CRC patients with MSI-H. In addition, in the GERCOR NIPICOL phase II study, an impressive DCR was observed in MSI/dMMR mCRC patients treated with anti-PD-1 inhibitor nivolumab combined with anti-CTLA-4 inhibitor ipilimumab (6). Recently, the preliminary results of nivolumab plus low-dose ipilimumab as the first-line therapy cohort from the CheckMate 142 study were reported. This double ICI combination demonstrated a durable clinical benefit as a first-line treatment for MSI-H/dMMR mCRC. The ORR is 69% in the combination group (5).

However, although MSI-H/dMMR has a promising prospect as the biomarker for colon cancer immunotherapy, the reported ORRs in MSI-H mCRC patients vary from 30%-70% (5, 6, 11–14); this implies that a certain number of MSI-H mCRC patients also do not benefit from immunotherapy. In contrast, a small subset of MSS colon cancer patients responded to immunotherapy (42). The diagnostic mistake caused by the test method is one of the reasons for this phenomenon (18). The current methods could be used for detecting MSI, including IHC, PCR, and next-generation sequencing (NGS). By using IHC to detect the expression of four MMR genes (MLH1, MSH2, MSH6, and PMS2) in the tumor cell nucleus, the presence of one or more negative proteins was defined as dMMR; otherwise, they were defined as pMMR. The advantage of IHC lies in that it is easy to perform and allows direct identification of the MMR gene status. The disadvantages of IHC are its subjectivity and lack of a uniform standard (43). PCR was based on comparing DNA extracted from tumor tissue and normal tissue to detect MSI status. In 1997, the National Cancer Institute (NCI) first formalized the guidelines for PCR testing of MSI, which contained 2 single nucleotide repeat sites (BAT-25 and BAT-26) and 3 dinucleotide repeat sites (D2S123, D5S346, and D17S250) (44). Subsequent studies identified the limitations of this criterion and improved the criteria for PCR detection of MSI. Currently, the five poly-A panel (BAT-25, BAT-26, NR-21, NR-24, and NR-27) is the usually recommended panel for MSI-PCR tests. MSI at more than two loci out of five is defined as MSI-high (MSI-H); MSI at one of five loci is defined as MSI-low (MSI-L); and no instability at any of the loci is defined as MSS (45). PCR is the currently accepted gold standard for the detection of MSI, with high accuracy and standardization. The disadvantage of PCR for MSI detection is that it cannot directly determine abnormal proteins. In addition, considering the difference in MSI incidences among different ethnicities, although there are standardized recommendations, the selection of the appropriate panel for different populations is also essential for the detection of MSI PCR tests (46). NGS is the third alternative method for MSI measurement, which determines MSI by directly measuring the length of altered MS. The NGS method does not require normal tissue as a control, requires lower sample quality, and is more compatible than PCR. NGS can simultaneously provide information on MSI loci, MMR gene status, and information on other gene statuses. In addition, NGS can detect MSI status by peripheral blood samples, unlike traditional PCR methods. However, what limit the clinical promotions and uses of NGS are the high cost, complexity of the data analysis process, and lack of uniform evaluation criteria (47).

Furthermore, reasonable direct evidence for MSI-H/dMMR as a biomarker for immunotherapy is still lacking. Existing studies suggest that the main reason for the response of MSI-H patients to immunotherapy is that MSI may cause the production of new antigens, leading to the recruitment of immune cells and the release of proinflammatory factors. A higher TMB and infiltration of TILs were found in patients with MSI-H/dMMR (48). However, TMB and MSI do not always match perfectly (49). Immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and T-regulatory cells (Tregs),

were also found in MSI-H/dMMR patients, which means that a more comprehensive biomarker portfolio is needed for immunotherapy efficacy prediction (50).

TMB as the predictive biomarker for colon cancer immunotherapy

TMB is another potential effective biomarker in the field of tumor immunotherapy. TMB is usually defined as the total number of somatic mutations detected per million bases (muts/Mb). It was thought that a high TMB (TMB-H) status was related to more tumor neoantigens, and more tumor neoantigens presented on the surface of tumor cells may be recognized by immune cells and activate the body immune system to kill the tumor (51). In KEYNOTE-158, pembrolizumab was tested in several types of advanced solid tumor patients but did not include colon cancer patients. The results show that patients with TMB-H status (TMB≥10 muts/Mb) presented higher objective responses to pembrolizumab. The ORRs were reported to be 29% in the TMB-H group versus 6% in the non-TMB-H group (52). Based on this result, in June 2020, the FDA approved the use of pembrolizumab for unresectable or metastatic solid tumor patients with TMB-H status. With a cutoff value of 10 muts/Mb, higher response rates to immunotherapy were confirmed in melanoma and non-small cell lung cancer (NSCLC) patients with TMB-H (53, 54).

In colon cancer, TMB was found to be potentially correlated with patient survival. It was reported that colon cancer patients with high TMB (TMB≥8 muts/Mb) presented longer OS than those with low TMB (55). Additionally, TMB was reported as an additional predictive biomarker for MSI in metastatic CRC. Among MSI-H mCRC patients, patients with TMB-H (the TMB cutoff point was defined between 37 and 41 mutations/Mb) have shown a better prognosis than those with TMB-L after receiving immunotherapy (18). However, the independent application of TMB for the prediction of immunotherapy response in colon cancer is still controversial. In the KEYNOTE 177 trial, the limits of TMB as a predictor of the response of CRC to anti-PD1 immunotherapy were observed (56). However, in the Canadian cancer trials group CO.26 study, elevated plasma TMB levels (≥28 muts/Mb) showed a predictable response to anti-PD-L1 agent durvalumab and anti-CTLA4 agent tremelimumab combination therapy in MSS colon cancer patients (57).

One of the greatest obstacles causing this controversy is the difficulty in defining the TMB cutoff value. Nonetheless, the cutoff of 10 muts/Mb presented a relatively good sensitivity in the prediction of immunotherapy in NSCLC and melanoma, but this cutoff value cannot be generalized for different tumor types (58). Currently, there is no uniform TMB cutoff value for colon cancer. In a recently reported study, TMB≥16 mut/Mb was proposed as the optimal threshold for ICI atezolizumab monotherapy in advanced solid tumor types. In patients with TMB ≥16 mut/Mb, durable clinical activity was observed, and particularly high response rates (70%) were reported in CRC patients, including both MSI-H and MSI-L (59). Nonetheless, this finding still awaits further validation by prospective studies. In addition, TMB is proposed as a predictive biomarker for immunotherapy because it may represent a useful estimation of tumor neoantigens. However, not all neoantigens presented on the cell surface are immunogenic. Only mutations resulting in higher 'quality' antigens can induce an antitumor immune response (60, 61). This may explain why TMB cannot be used as an independent marker of the effectiveness of tumor immunotherapy. Furthermore, the testing method hinders the clinical application of TMB. TMB was initially performed using whole-exome sequencing (WES), and this technology is complex and costly. Currently, NGS has been used in the clinic as a substitute for WES; however, there are different algorithms for WES-based and NGS-panel-based methods. In addition, the advantage of TMB as the biomarker for immunotherapy is that TMB can be not only obtained from tumor tissue but also detected by peripheral blood. Therefore, in some studies, tissue TMB was substituted with plasma TMB when unavailable. But there are differences in the criteria for assessing TMB in blood samples versus tissue samples, which cause inconsistencies among different studies and interfere with TMB standardization (62).

In summary, although several pieces of evidence indicate that TMB cannot be used as an independent predictive biomarker in colon cancer immunotherapy, it is still valuable in immunotherapy efficacy prediction, especially when sufficient evidence is obtained for a valid TMB cutoff value in colon cancer. TMB can be used as an important complementary biomarker, such as when combined with MSI, and for identifying other significant gene mutations; it was found, that some MSS colon cancer patients with high TMB had polymerase epsilon (POLE) mutation, and they responded well to immunotherapy (63).

The value of molecular subtype and consensus molecular subtype as predictive biomarkers in colon cancer immunotherapy

Colon cancer is a heterogeneous disease. Colon cancer patients usually present different molecular subtypes. Some molecular subtypes, such as RAS and BRAF mutations, have already been used to guide the treatment and prognostic assessment of colon cancer (64). Among these molecular subtypes, the encoded DNA POLE and delta 1 (POLD1) mutation has attracted much attention due to its potential association with immunotherapy response. POLE/POLD1 play an important role in proofreading and ensuring the fidelity of

DNA replication. The somatic or germline mutations in POLE and POLD1 lead to defects in DNA repair and, consequently, to tumorigenesis (65). In colon cancer, about 7.4% of patients harbor POLE or POLD1 mutations, and most of this population was MSS or MSI-L (66). In 2019, Wang and his colleagues, through analyzing medical data of 47,721 patients with various cancer types with POLE/POLD1 mutations, proposed that POLE/POLD1 mutations are promising potential predictive biomarkers for positive ICI outcomes (66). However, in a previous clinical study, which enrolled three CRC patients with POLE mutations, three of the patients did not show an achieved response to anti-PD-L1 inhibitor avelumab (67). In a recent study, where anti-PD-L1 inhibitor durvalumab monotherapy was used to treat previously treated MSI-H/dMMR or POLE-mutated mCRC patients, the results showed that POLE mutation mCRC patients had a clinical response to durvalumab, only those with exonuclease domain mutation (68). Furthermore, there is a limited role of other molecular mutations as biomarkers in predicting the response of colon cancer to immunotherapy; this includes KRAS mutation, a common molecular subtype in colon cancer. Although KRAS mutation is proposed to modulate tumor immunity (69), its biomarker value in colon cancer immunotherapy was found to be weak. According to Lal et al., KRAS mutation is proposed to be associated with suppressed cytotoxic immunity in CRC, and the extent of the effect is modulated by consensus molecular subtype (CMS) (70).

The CRC Subtyping Consortium, based on the gene expression of the tumor, proposed four CMSs for CRC using transcriptomics in 2015. The CMS classification included CMS1, CMS2, CMS3, and CMS4. CMS1 is categorized as MSI immune and presents with strong immune activation; CMS2 is categorized as canonical and is characterized by chromosomal instability as well as WNT and MYC signaling activation; CMS3 is categorized as metabolic and is associated with metabolic dysregulation; CMS4 is categorized as mesenchymal and is associated with prominent transforming growth factor β (TGF-β) activation, stromal invasion, and angiogenesis (71). CMS classification can be used in guiding colon cancer treatment strategies and predicting patient prognosis (72–74). The correlation between CMS and tumor immune characteristics has been proposed in several studies (27, 75). In 2016, Becht E et al. integrated the CMS classification with the TME of CRC. Their results show that the good prognosis of CMS1 is related to the overexpression of cytotoxic lymphocytes. In contrast, the poor prognosis of CMS4 is related to a high density of fibroblasts, which produce chemokines and cytokines, resulting in inflammatory and immunosuppressive TMEs. The CMS2 and CMS3 groups presented an intermediate prognosis, exhibiting low immune and inflammatory signatures (75). In a recent study, Hu et al, based on data from multiple databases and using algorithms, further analyzed the molecular characteristics of colon cancer CMS and their immunotherapy responses. Their results indicate that CMS1 patients present a higher positive response to immunotherapy among the four CMS subtypes due to immune infiltration and activation. TILs were significantly higher in the CMS1 subtype than in the other three subtypes. In contrast, CMS4 patients may not respond well to immunotherapy, due to the high Treg and NK-cell infiltration found in the CMS4 subtype (27). Meanwhile, in Chida K and his colleagues' study, transcriptomic profiling for MSI-H/dMMR gastrointestinal tumors was performed to determine the predictors of response to PD-1 blockade. The results show among 13 CRC patients, the reported ORR for CMS1 was 100%, for CMS4 was 16.7%, but for CMS2 and CMS3 all were 0%. This study indicates that CMS classification may serve as a predictive biomarker for colon cancer immunotherapy (76).

However, existing CMS classification still has certain limits. CMS classification relies on transcriptome analysis of the entire tumor, which has inherent limitations such as stromal confounding and the presence of varied cell-type mixtures. Moreover, the differences in cancer cells and other stromal cells (e.g., immune cells, fibroblasts, and vascular cells) are masked and indistinguishable (77, 78). There is transcriptomic intratumor heterogeneity in CMS classification, which may impact its accuracy (79). To solve this, Joanito et al, using single-cell and bulk transcriptome sequencing, identified two epithelial tumor cells and refined the CMS classification of colon cancer. The refined CMS classification includes intrinsic epithelial subtype, MSI status, and fibrosis. By this classification, a specific subtype of MSS was identified. They proved that despite a lower TMB, iCMS3_MSS tumors are more similar to MSI-H colon cancers, and this refined classification may provide new clues for screening the population benefiting from immunotherapy in colon cancer (80). Recently, Khaliq et al. also refined CRC classification and clinical stratification through a single-cell atlas; they proved that distinct cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAMs) are sufficient to explain CMS predictive ability and, based on these cellular phenotypes, could stratify CRC patient prognosis with greater precision (81).

In conclusion, existing molecular subtypes and CMS may not be suitable for stratifying colon cancer patients for immunotherapy. The molecular subtypes and CMS help define the molecular and immunological characteristics of colon cancer, which contribute to the precise therapy of colon cancer. As research progresses, the understanding of the molecules subtype of colon cancer patients continues to improve and a more precise molecular subtype of colon cancer may be recognized, and a more precise CMS classification may be refined, which will further contribute to colon cancer immunotherapy.

TILs, immunescore, and PD-L1 expression as predictive biomarkers for colon cancer immunotherapy

TME plays an essential role in tumorigenesis, development, and immune escape (82). Including immune cells, other components of the TME can influence the immune state and response to immunotherapy in tumors (83). TILs are core components of immune cells involved in tumor immunity. TIL is a global term for a variety of lymphocytes in the TME, including T cells, B cells, and NK cells. Several studies have proven that TILs play prominent roles in malignant tumor development and progression and have been proposed as predictive biomarkers for patient prognosis (84–86). The relationship between TILs and colon cancer patient prognosis was first reported in 1998. In this study, CD8+ T cells infiltrated within cancer cell nests were observed to be a prognostic factor in human CRC (87). A series of studies then reported the role of TILs in the prognosis of patients with colon cancer (88–91).

In several clinical trials of colon cancer immunotherapy, TILs showed potential in being used as predictive biomarkers for immune response. In an analysis study of the KEYNOTE 177 trial, colon cancer patients' response to immunotherapy was found to be not associated with TMB, but rather with TILs. Immunotherapy-responsive CRC patients were found rich in CD-8+PD-1+ T cells (56). In Loupakis F et al' study, they proposed that there was a significant positive correlation between high TMB and the number of TILs in the ICI-responsive MSI-H mCRC patients (92). In the pilot clinical trial of perioperative durvalumab combined with tremelimumab for treating resectable CRC liver metastases, the treatment induced activation of CD8+ and CD4+ T cells, and an increase in B-cell density was correlated with patients' prolonged relapse-free survival (93). In the study of neoadjuvant immunotherapy for early-stage colon cancer patients, CD8+PD-1+ T cell infiltration was a predictive biomarker of response in pMMR patients (7). For MSS mCRC patients, higher CD8+ TIL density at baseline was associated with a greater likelihood of benefit from immunotherapy treatment and activated TILs are considered as the biomarker of effective immune induction (94). And according to Kuang C et al's study, immune modulation may result from treatment with azacitidine, chemotherapy refractory mCRC patients with higher CD8+TIL density at baseline are more likely to benefit from the combination therapy of pembrolizumab and azacitidine combination (95). In addition to CD8+T cells, the role of CD3+T cells as the predictive biomarker in colon cancer immunotherapy has also been reported in some studies (96, 97). In Turksma et al's study, they found that high numbers of pre-existing stromal CD3+ T cells are own positive predictive value in adjuvant immunotherapy treatment for MSS colon cancer patients (96). In Chakrabarti S. et al's study, higher CD3+ and CD8+ T-cell densities were associated with higher ORR in dMMR mCRC patients treated with pembrolizumab (97).

CD3+ T cells and CD8+ T cells are two important types of TILs that represent the total T cells and cytotoxic T cell subsets, respectively. In 2018, the Society for Immunotherapy of Cancer proposed using Immunoscore (IS) to estimate the risk of recurrence in colon cancer patients, and their findings proved the powerful role of IS in CRC recurrence risk assessment (98). IS is based on the quantification of CD3+ T cells and CD8+ T cells at the tumor center and at the invasive margin using IHC. A scoring system ranging from IS0 (I0) to IS4 (I4) and high IS was associated with prolonged survival in CRC (99). In Mlecnik et al's study, IS is proposed to play a bigger role in predicting CRC patient survival than MSI (100). However, IS as the predictive biomarker for colon cancer immunotherapy has certain limitations. First, IS assays are mainly performed by IHC, which is a semiquantitative test, and the results are susceptible to subjectivity. Second, the IS test requires simultaneous assessment of lymphocytes in the center and margin of the tumor, which is difficult to achieve in metastatic tumors. In addition, intratumoral heterogeneity can also affect the accuracy of IS. For example, heterogeneity of T cells was observed in the primary tumor and hepatic metastases of CRC patients (97). Finally, current studies have shown that the effectiveness of tumor immunotherapy is influenced by the immune landscape rather than by a single immune cell (63, 101–103). Except for CD8+T cells, other immune cells, such as Treg cells, NK cells, DC cells, and B cells, are also closely related to the immune response of tumors (104, 105). Recently, a special lymph node structure, tertiary lymphoid structure (TLS), was also proposed as a biomarker in cancer prognosis and response to immunotherapy (106). The effect of TLS on the prognosis of colon cancer has now been demonstrated in several studies (107–110). But the role of TLS in colon cancer immunotherapy still needs to be verified. In addition, the phenotypic profiles and subsets of TILs were also found to affect the patient's response to ICI and have the potential to be biomarkers of immunotherapy (96, 111–113). For example, a CD39 subgroup of CD8+ T cells was reported in colorectal and lung tumors, the absence of CD39 in CD8+ TILs causes them to act as bystanders that lack an immune response (112). A similar phenomenon has also been observed in B cells; it was found that B cells with CD86 expression were enriched in tumors with increased numbers of TLSs, induced specific T-cell responses, and enhanced the antitumor effect of ICI (114). Epigenetic alterations of TILs, such as DNA methylation, are also involved in the colon cancer immune response. According to Zou et al's study, the DNA methylation-based signature of CD8+ TILs was related to the immune response and prognosis of CRC patients (22). Thus, further screening TIL subgroups and studying the immune landscape of colon cancer are key to improving the accuracy of screening for beneficial ICI populations.

PD-L1 is another important indicator for TME. Tumor cells induce tumor immune escape by upregulating PD-L1 expression, which binds to PD-1 on the surface of T cells, causing T-cell deactivation. ICIs can reactivate the body's antitumor immunity by blocking the binding of PD-1 and PD-

L1 (115). Thus, in theory, the higher level of PD-L1 expression in tumor tissues, the better the response to ICI treatment. PD-L1 expression is postulated as a predictive biomarker of immunotherapy response in some solid tumors, such as NSCLC, melanoma, and renal cell cancer (116–118). Positive PD-L1 expression (with a cut-off value of 10%) is reported in more than half of colon cancer patients (119, 120). Although a high PD-L1 expression is associated with a better prognosis in colon cancer patients (20, 121–123). The current clinical data suggest that PD-L1 expression alone cannot be used to precisely predict immunotherapy response in colon cancer (Table 1). Several factors limit PD-L1 expression as a biomarker for colon cancer immunotherapy response: First, there is intratumoral heterogeneity of PD-L1 expression (135), which makes assessing tumor PD-L1 expression level. Second, PD-L1 expression is dynamic, and treatment modalities can affect the expression level of PD-L1. PD-L1 expression varies widely between tumor types and presents a significant intrapatient heterogeneity with a frequent discordance between primary tumors and metastases. Third, the test method also affects the assessment of PD-L1 expression. IHC is now widely used in clinical practice to detect PD-L1 expression in tumor tissues. However, this method is difficult to quantify, the consistency of detecting PD-L1 expression levels between different platforms is poor, and there is still lack of a standardized testing criteria (136). Lastly, tumor cells and immune cells can both express PD-L1. Thus, the predictive effects of PD-L1 expression by tumor cells and PD-L1 expression by lymphocytes on immunotherapy need to be clarified separately. This is illustrated when PD-L1-expressing tumor cells were reported to be a marker of poor prognosis; in contrast, PD-L1-expressing TILs were a marker of good prognosis (137).

Nonetheless, PD-L1 expression still has value in immunotherapy for colon cancer patients. The PD-L1 expression level is an important indicator of the immune status of cancer patients (138–140), and the immune status indicates the tumor response to immunotherapy. PD-L1 combined with other immune indicators demonstrated a promising predictive role in colon cancer immunotherapy. Such as, Llosa et al. proposed the incorporation of histopathologic characteristics (percentage of extracellular mucin) and PD-L1 expression at the invasive front to generate a composite score (CPM score). The CPM score has the potential of discriminating mCRC patients who exhibited clinical benefits from pembrolizumab (124). Additionally, using multiplex immunohistochemistry (mIHC), multiple immune indicators combined with PD-L1 expression can be analyzed simultaneously as well as report TME in various solid tumors, including colon cancer (28, 141, 142).

Therefore, TME immune landscape is significantly related to tumor immunotherapy response. It is not sufficient to evaluate tumor response to immunotherapy by a single index only, such as PD-L1 expression or the number of TILs. A more comprehensively quantified TME immune landscape is necessary for the prediction of colon cancer immunotherapy response.

Other gene signatures of the TME as predictive biomarkers for colon cancer immunotherapy

With the development of gene sequencing technology, several TME-related gene signatures have been proposed as predictive biomarkers for colon cancer immunotherapy. Previously, Ravensbergen et al, using bioinformatics approaches, proved that combined assessment of the tumor-stroma ratio and TILs could be used as a response prediction biomarker of ICI therapy in colon cancer (143). This result reveals the role of the tumor stoma in the response to tumor immunotherapy in patients with colon cancer. CAFs are the main cell type within the tumor stroma, and they are also thought to be an available indicator for assessing the response to immunotherapy. CAFs can interact with tumor cells and TILs *via* the secretion of various cytokines and chemokines, shaping an immunosuppressive TME and helping tumor cell immune evasion. In addition, CAFs play a significant role in constituting the inflammatory TME of colon cancer (144). Some studies have proven that CAF-derived gene signatures can determine prognosis in colon cancer patients (29, 145, 146). In the area of immunotherapy, it was proven that CAFs promote the upregulation of PD-L1 expression in CRC (147). CAFs have an impact on the prognosis of CRC patients by inhibiting the immune response; thus, patients with higher CAF levels were more prone to be unresponsive to immunotherapy (29, 148). Additionally, among the CMSs, CMS4 is typically characterized by infiltration of adjacent tumor tissues by CAFs and transforming growth factor β (TGF-β) signaling activation, and this subtype presented insensitivity to immunotherapy. Recently, the refinement of CMS through single-cell characterization based on specific CAF subtypes presented the potential role of identifying immunotherapy responses in CRC patients (81). Thus, further study of CAF gene signatures may contribute to the precise stratification of immunotherapy efficacy for colon cancer.

TME metabolic characteristics also influence patients' response to immunotherapy. Hypoxia is one of the metabolic characteristics of the tumor TME. Hypoxia can play an essential role not only in tumor proliferation, apoptosis, angiogenesis, invasion, and metastasis but also in immune evasion. Hypoxia and the related acidic TME greatly impair the functions of TILs, while alleviating hypoxia could improve the efficacy of ICIs (149). Transcriptomic profiling of MSI-H/dMMR gastrointestinal tumors showed that hypoxia-related signaling pathways were upregulated in ICI nonresponders (76). Recently, several studies proposed that

TABLE 1 Summary of biomarkers for colon cancer immunotherapy in reported clinical trials.

Biomarker	Tumor type	Patients' number	Immunotherapy agent	Association with clinical outcome	Tissue type for bio-marker assessment	NCT	Ref
MSI	MSI-H-dMMR mCRC	307	pembrolizumab(anti-PD-1)	dMMR/MSI-H was positive with patients' clinical outcome	tumor tissue	NCT02563002 (KEYNOTE-177)	(4)
MSI	MSI-H/dMMR mCRC	57	nivolumab(anti-PD-1) +ipilimumab(anti-CTLA-4)	dMMR/MSI-H was positive with patients' clinical outcome	tumor tissue	NCT03350126	(6)
MSI	advanced dMMR solid tumors	86	pembrolizumab(anti-PD-1)	dMMR/MSI-H was positive with patients' clinical outcome	tumor tissue and blood	NCT01876511 (KEYNOTE-016)	(11)
MSI	MSI-H/dMMR mCRC	124	pembrolizumab(anti-PD-1)	dMMR/MSI-H was positive with patients' clinical outcome	tumor tissue	NCT02460198 (Keynote164)	(14)
MSI	dMMR/MSI-H CRC	119	nivolumab(anti-PD-1) +low-dose ipilimumab (anti-CTLA-4)	dMMR/MSI-H was positive with patients' clinical outcome	tumor tissue	NCT02060188 (CheckMate 142)	(5)
TMB	MSI-H mCRC	22	PD-1/PD-L1 inhibitors	The optimal predictive cut-point for TMB was estimated between 37 and 41 mutations/Mb.	tumor tissue	NA	(18)
TMB/TIL	CRC	29	pembrolizumab(anti-PD-1)/nivolumab (anti-PD-1)	Patients' response to immunotherapy not associated with TMB, but with TILs.	tumor tissue	NCT02563002 (Keynote177)	(56)
bTMB	advanced CRC	179	durvalumab(anti-PD-L1)+tremelimumab (anti-CTLA-4)	Patients who were MSS with plasma TMB of 28 variants per megabase or more had the greatest OS benefit.	blood	NCT02870920	(57)
MSI/POLE mutation	MSI-H/POLE mutation mCRC	33	avelumab(anti-PD-L1)	Avelumab displayed antitumor activity with manageable toxicity in patients with previously treated mCRC harboring dMMR/MSI-H. Further clinical studies with larger sample sizes are necessary to evaluate the activity of ICIs and its association with sites in POLE-mutated CRC.	tumor tissue	NCT0315-0706	(67)
MSI-H/ dMMR or POLE EDM	previously treated MSI-H/ dMMR or POLE-mutated metastatic or unresectable CRC	33	durvalumab(anti-PD-L1)	Durvalumab showed promising clinical activity with encouraging response rates and satisfactory survival outcomes in mCRC patients with MSI-H/dMMR or POLE exonuclease domain mutation (EDM). In patients with POLE-mutated mCRC, clinical response to durvalumab may be restricted to those with EDM.	tumor tissue	NCT03435107	(68)
CMS	MSI-H/dMMR gastrointestinal tumors	CRC (n=13)	anti-PD-1 inhibitor	The ORR was 100%,0%,0%,and 16.7% for CMS1, CMS2, CMS3, and CMS4, respectively. Several transcriptomic features,including CMS classification and related genes, were associated with response to PD-1 blockade in MSI-H/dMMR gastrointestinal tumors.	tumor tissue	NA	(76)
TIL	early-stage colon cancer	40	nivolumab(anti-PD-1) +ipilimumab(anti-CTLA-4)	CD8+PD-1+ T cell infiltration was predictive of response in pMMR tumors.	tumor tissue	NCT03026140	(7)
TIL and TMB	MSI-H mCRC	85	ICI	A significant correlation between higher TMB and increased number of TILs was shown. A significantly higher activity and better PFS and OS with ICI in MSI-H mCRC were reported in cases with high number of TILs.	tumor tissue	NA	(92)
TIL	resectable pMMR mCRC	24	perioperative durvalumab(anti-PDL1)+tremelimumab (anti-CTLA-4)	An increase in B-cell transcriptome signature and B cell density was present in post-treatment samples from patients with prolonged RFS.	tumor tissue	NCT02754856	(93)
TIL	MSS mCRC	29	durvalumab(anti-PD-L1) + trametinib (MEKi)	The response rate in the first stage of the study did not meet efficacy criteria to proceed to the second stage. TIL was related with clinical outcome.	tumor tissue	NCT03428126	(94)

(Continued)

TABLE 1 Continued

Biomarker	Tumor type	Patients' number	Immunotherapy agent	Association with clinical outcome	Tissue type for bio-marker assessment	NCT	Ref
TIL	chemotherapy refractory mCRC	30	pembrolizumab(anti-PD-1)+azacitidine (DNA methyltransferase inhibitor)	Higher CD8+ TIL density at baseline was associated with greater likelihood of benefit from treatment.	tumor tissue	NCT02260440	(95)
TIL	MSS colon cancer	106	adjuvant active specific immunotherapy(ASI)	High numbers of pre-existing stromal CD3 positive T cells are of positive predictive value in adjuvant ASI treatment.	tumor tissue	NA	(96)
CPM score (composite PD-L1 and mucin)	advanced mCRC	26	pembrolizumab(anti-PD-1)	The CPM score discriminated patients who exhibited clinical benefit from those patients with progressive disease.	tumor tissue	NCT01876511	(124)
gut microbiome	advanced-stage GI cancer	CRC (n=19)	anti–PD-1/PD-L1 immunotherapy	An elevation of the Prevotella/Bacteroides ratio in patients, with a preferred response to anti–PD-1/PD-L1 treatment.	fecal sample	NA	(125)
gut microbiome	mCRC(97.4% MSS)	33	regorafenib +toripalimab(anti-PD-1)	Gut microbiome analysis of the baseline fecal samples shows significantly increased relative abundance and positive detection rate of Fusobacterium in non-responders than responders.	fecal sample	NA	(126)
gut microbiome	RAS wild-type mCRC	14	cetuximab + avelumab (anti-PD-L1)	Agathobacter M104/1 and Blautia SR1/5 expression were associated with PFS.	fecal sample	NCT04561336	(127)
ctDNA	Refractory MSS mCRC	18	regorafenib +nivolumab/ pembrolizumab	ctDNA may represent a powerful tool for predicting early therapeutic efficacy of immunotherapy in the MSS CRC population.	blood	NA	(128)
ctDNA/ NLR	RAS wild type mCRC	77	cetuximab+avelumab (anti-PD-L1)	Plasma ctDNA analysis before treatment may allow selection of patients who could benefit. Baseline NLR <3 significantly correlated with improved survival and may represent a potential predictive biomarker of cetuximab plus avelumab rechallenge activity in ctDNA RAS/BRAF WT patients.	blood	NCT04561336	(129)
circulating immune cells	refractory pMMR mCRC	24	durvalumab(anti-PD-L1)+tremelimumab (anti-CTLA4) +concurrent radiotherapy	Increased circulating CD8+ T lymphocyte activation, differentiation, and proliferation in patients with objective response	blood	NCT03122509	(130)
circulating immune cells	MSS mCRC	10	mFOLFOX6 +bevacizumab+CEA-targeted vaccine + avelumab(anti-PD-L1) (SOC+IO)	SOC+IO generated multifunctional MUC1- and brachyury-specific CD4+/CD8+ T cells despite concurrent chemotherapy.	blood	NCT03050814	(131)
circulating immune cells	dMMR/MSI-H CRC	41	anti-PD-1 inhibitor (nivolumab, pembrolizumab, triprizumab, toripalimab, and camrelizumab)	The ratio of CD4+/CD8+ and the frequency of CD4+ Tcell might be crucial independent biomarkers within dMMR mCRC to better identify patients for anti-PD-1 immunotherapy.	blood	NA	(132)
circulating immune cells	mCRC	24	pembrolizumab(anti-PD-1)+modified FOLFOX6	Baseline levels and changes in circulating MDSC and Treg subsets are not associated with RECIST response or mPFS.	tumor tissue and blood	NCT02375672	(133)
NLR	unresectable CEA+ liver mCRC	6	CART	NLR variations and associated cytokine changes may be useful surrogates of response to CAR-T.	blood	NCT01373047	(134)

CRC, colorectal cancer; MSI, microsatellite instability; TMB, total mutation burden; TILs, tumor-infiltrating lymphocytes; CMS, consensus molecular subtype; PD-L1, programmed cell death-ligand 1; CTLA4, Cytotoxic T Lymphocyte antigen 4; ctDNA, circulating tumor DNA; NLR, neutrophil-lymphocyte ratio; CART, Chimeric antigen receptor T cell therapy. NA, not available.

hypoxia-related genes can be classified as predicting immune cell infiltration and prognosis of colon cancer patients (25, 28, 150). These genes provide potential therapeutic targets for immunotherapy as well as prognostic biomarkers for colon cancer patients. In addition to hypoxia, ferroptosis-related gene signatures are another study hot spot in the TME-related prognostic assessment of immunotherapy for colon cancer. Several studies support that ferroptosis plays a vital role in tumor immunotherapy and TME regulation, and ferroptosis-related gene signatures were proposed as potential targets for tumor immunotherapy and patient prognosis (151–153). In recent studies, several ferroptosis-related gene signatures were proposed for the prediction of prognosis and immunotherapy response in colon cancer patients (154–156). These findings further confirm the relevance of ferroptosis to the immune microenvironment and prognosis of colon cancer. Therefore, further understanding of the metabolic characteristics of the TME and the search for metabolism-related gene signatures are valuable for the identification of new biomarkers of colon cancer immunotherapy.

Furthermore, the inflammatory microenvironment of colon cancer induces immune-related genetic alterations, and inflammatory-related genes affect the response of patients to immunotherapy. Wang et al. explored the relationship between inflammation-related genes and the immune TME in CRC. Eight prognostic genes (CX3CL1, CCL22, SERPINE1, LTB4R, XCL1, GAL, TIMP1, ADIPOQ, and CRH) were identified and used to construct a risk-scoring model. The results of this study show that the inflammatory response has a direct impact on CRC patient prognosis and immune infiltration. Thus, further classifying inflammatory response-related genes may help find predictive biomarkers for immunotherapy in colon cancer (157).

Through analysis by transgenomic techniques, some TME-related gene signatures were proposed for use as biomarkers for colon cancer immunotherapy. However, most of these studies were derived from bioinformatic analyses of databases; further validation of these genes in large prospective clinical studies is necessary.

The potential role of certain gut microbiota as the predictive biomarker for colon cancer immunotherapy

The gut microbiota is another hot topic in the current field of immunotherapy. Several studies have proposed that gut microbiota are involved in tumor formation and progression and correlate with patient therapy response in solid cancers (158–164). It has been established that tumor patients have distinct microbiota compared with healthy subjects (165, 166). In addition, compared to patients that did not respond to immunotherapy, a unique intestinal microbiome was found in cancer patients that did respond to immunotherapy (167). Increasing evidence indicates that transplanting the gut microbiome of immunotherapy responders can activate immune cells and make immunotherapy nonresponders respond to immunotherapy (168–171). Therefore, the gut microbiome could be a promising therapeutic target as well as a predictive biomarker in cancer immunotherapy.

Colon cancer presents with an altered state of gut microbiota, which is known as dysbiosis (165). The gut microbiome plays a significant role in the formation of the inflammatory microenvironment during the development of colon cancer. Gut microbes can interact with TILs and influence the tumor immune microenvironment and host sensitivity in favor of immunotherapy in colon cancer (172–174). Recently, certain gut microbes have been proposed as promising predictive biomarkers of colon cancer immunotherapy (23). In 2020, Peng and his colleagues recruited advanced-stage GI cancer patients receiving anti-PD-1/PD-L1 treatment and collected their fecal samples. By comparing the gut microbes of patients before and after treatment, they found an elevation of the Prevotella/Bacteroides ratio in patients with a preferred response to immunotherapy (175). In another phase Ib/II clinical trial of regorafenib plus toripalimab treatment for mCRC, gut microbiome analysis presented a significantly increased relative abundance and positive detection rate of Fusobacterium in nonresponders compared to responders (125). In a recent clinical study, Agathobacter and Blautia species were proposed as potential biomarkers of outcome in mCRC and NSCLC patients treated with cetuximab and avelumab (126). However, the evidence related to the role of gut microbiome as a prognostic marker of colon cancer immunotherapy is still lacking, and further investigations are still required to consider the gut microbiome as a predictive biomarker for the immunotherapy response in colon cancer.

The testing method of gut microbiome detection also needs to be optimized and unified. Current methods for testing the gut microbiome are mainly stool-based genetic tests. The two commonly used methods are PCR-based 16S amplicon sequencing and macrogenome sequencing. PCR-based sequencing of 16S amplicons is relatively less costly, however this method is limited to the genus level and can easily miss microbiomes with low expression levels. In contrast, macrogenomic sequencing has several potential advantages over 16S amplicon sequencing. Macrogenomic sequencing can extend gut microbiome taxonomic resolution to the species level and can also provide information on metabolic pathways of the microbiome. However, the cost of macrogenomic sequencing is relatively high, and the interpretation of analysis results is complex (127). Measurement differences may exist between the two methods due to differences in stool sample collection, storage, and handling, as well as nucleic acid extraction protocols and data analysis methods. Therefore, to make better use of gut microbiota as the predictive biomarker for immunotherapy of colon cancer, it is also necessary to further optimize the testing methods and standardize the testing criteria.

Peripheral blood biomarkers in colon cancer immunotherapy

CtDNA is the most used peripheral blood biomarker. Previously, ctDNA already presented high sensitivity in colon cancer early diagnosis, recurrence detection, and treatment outcome prediction (176, 177). CtDNA is derived from apoptotic and necrotic tumor cells that release their fragmented DNA into circulation. Information on genetic variation could be detected through ctDNA test (178). In 2017, Cabel et al. proposed a proof-of-concept study, they enrolled patients with NSCLC, uveal melanoma, or MSI CRC who were treated by nivolumab or pembrolizumab monotherapy, their results demonstrated that quantitative ctDNA monitoring can be used as a valuable tool to assess tumor patients' response to anti-PD-1 agents (179). Several studies also focused on the predictive value of ctDNA in colon cancer immunotherapy. Wang et al. proposed ctDNA can be used as a powerful tool for predicting MSS CRC patients' response to regorafenib and nivolumab combination therapy (180). Gong et al. through four cases illustrated that ctDNA can be used as a dynamic predictive biomarker for colon cancer immunotherapy (128). In the CAVE trial, cetuximab and avelumab combination therapy were tested in RAS wild-type mCRC. In this study, patients' KRAS, NRAS, BRAF, and EGFR-S492R mutation was analyzed through ctDNA, and the result show patients with RAS/BRAF WT ctDNA presented with better mOS and mPFS compared to patients with mutated ctDNA. These findings presented the potential role of ctDNA for colon cancer immunotherapy (181). However, the reports of ctDNA in the immunotherapy of colon cancer are still limited and ctDNA's predictive role in immunotherapy needs to be verified by larger clinical studies. In addition, the following questions also need to be paid attention to in the further study of ctDNA (129, 182). First, the concentration of ctDNA in blood is relatively low. Thus, the sensitivity of the detection method is strictly required. Second, ctDNA is vulnerable to a variety of factors, such as trauma, which is a crucial factor affecting the determination of ctDNA. Therefore, strict avoidance of interfering factors is important for accurate measurement of ctDNA. Third, ctDNA is dynamically changing, thus the ctDNA results from different studies sampled at different points in time are difficult to unify and quantify, and there is yet to be a uniform detection standard for ctDNA. Furthermore, the testing method for blood ctDNA still needs to be optimized. Digital PCR (dPCR), amplification refractory mutation system (ARMS), and NGS are currently the main available methods for the detection of ctDNA. Each method has its strengths and limitations. dPCR with a low cost and relatively high sensitivity is the most used method for ctDNA detection. But the limitations of the dPCR are low throughput and the inability to detect unknown mutations. ARMS is moderate in cost, simple to operate, and can sequentially detect multiple mutations in a single gene, but it is not as sensitive as dPCR. The advantages of the NGS method lie mainly in the high throughput and sequencing of unknown mutations, but its economic cost is relatively high. Moreover, some study proposed the optimization of ctDNA detection (183). With the optimization of detection methods and the uniformization of standards, the value of ctDNA in immunotherapy of colon cancer will be better demonstrated.

In addition to ctDNA, various immune cells can also be tested in the peripheral blood, including T cells, B cells, NK cells, and myeloid cells (184). These circulating immune cells are proposed as predictive biomarkers for therapeutic response and clinical benefit of ICIs in solid cancer patients (185, 186). However, based on the existing study, the role of circulating immune cells in colon cancer immunotherapy is controversial. In a phase II study of durvalumab and tremelimumab with concurrent radiotherapy for pMMR mCRC patients, an increase in circulating CD8+T lymphocyte activation was observed in patients with an objective response. However, this combination of radiotherapy plus ICI did not meet the study endpoint criteria (187). In another study of mFOLFOX6 combined bevacizumab alone or with AdCEA vaccine combined avelumab immunotherapy for untreated mCRC, combination therapy generated brachyury-specific CD4+/CD8+T cells but did not improve patients' PFS (130). In Cheng et al's study, the peripheral blood of dMMR mCRC patients receiving anti-PD-1 immunotherapy was analyzed, the results show that the ratio of CD4+/CD8+ in peripheral blood and the frequency of CD4+ T cells are promising predictive biomarkers for dMMR mCRC patients responding to immunotherapy (131). According to Herting CJ et al's study, the baseline levels and changes in circulating immunosuppressive myeloid and T cell subsets were not associated with advanced CRC patients' response to pembrolizumab combined modified FOLFOX6 therapy (132). Moreover, according to Clouthier et al's study, they found that the immune biomarkers were significantly varied between the blood and tissue (131). The reasons for this phenomenon are mainly related to the small sample size included in the existing studies and, similar to the reports of ctDNA, the different sampling times and analysis methods can also have an impact on the results. Test methods for the detection of various immune cell subsets including multi-color fluorescence flow cytometry, mass cytometry, and NGS, are still developing, and evaluation criteria need to be normalized (133). In addition, a larger population-based cohort study is necessary to further test the value of circulating tumor cells in immunotherapy of colon cancer.

Inflammation also plays an essential role in colon cancer tumorigenesis and influences patients' immunotherapy response (188). Lymphocytes and neutrophils are two common indicators of the inflammatory state of the body in the peripheral blood (189). Some studies reported the predictive role of neutrophil-lymphocyte ratio (NLR) in colon cancer immunotherapy (134, 190, 191). In Saied et al's study, they proposed that NLR changes correlated with CEA+ liver metastases CRC patients' early responses to chimeric antigen receptor-modified T-cell (CAR-T) hepatic artery infusions

(HAI) variations. Increased NLR levels were proven to be associated with poor responses following CAR-T HAI (190). The final results of the CAVE trial show that a baseline NLR <3 significantly correlated with improved survival of ctDNA RAS/BRAF WT patients after cetuximab plus avelumab therapy (191). Furthermore, through a retrospective study, Corti et al. proposed a blood-based biomarker, Pan-Immune-Inflammation Value which integrates neutrophil, platelet, monocyte, and lymphocyte counts, as a strong predictor of outcomes in MSI-H mCRC patients receiving ICIs (192). However, based on the limited number of studies conducted so far, the role of peripheral inflammatory cell-related involvement (e.g., NLR) in the immunotherapy of colon cancer remains to be further demonstrated.

In summary, tissue-based predictive biomarkers are more accurate and closely related to TME. However, there are still some challenges in some clinical situations. Tissue biopsies are invasive, and may be difficult to obtain in advanced and metastatic patients. In addition, intratumoral heterogeneity is prevalent among tumor tissues. Multipoint sampling is necessary to obtain more accurate results, but it is difficult to achieve in clinical settings, as it requires invasive procedures for the patient (193). Under such conditions, liquid biopsies through peripheral blood provide a method with minimally invasive, reproducible sampling and dynamically observe changes in indicators. The variety of information that can be acquired through liquid biopsy includes inflammatory cells, ctDNA, circulating immune cells, cytokines, and so on. Several peripheral blood biomarkers are now being proposed as predictive biomarkers for colon cancer immunotherapy response in existing clinical studies (Table 1). The development of high-throughput sequencing technology provides a deeper and broader view of peripheral blood biomarkers. However, since various multiplexed assays are employed for peripheral blood analyses, these assay protocols and their reporting methods need to be standardized, and additional studies will also be needed on the sampling time points, sensitivity, and specificity of each assay for clinical applicability. In further clinical studies, peripheral blood biomarkers will be developed as dynamic indicators for colon cancer immunotherapy.

Conclusions and perspectives

In recent years, ICI-based immunotherapy has brought revolutionary breakthroughs in the treatment of colon cancer. While immunotherapeutic agents continue to be researched and developed, it is also worth focusing on precisely screening beneficial patients through predictive biomarkers. Currently, MSI is the only approved biomarker for screening colon cancer immunotherapy-benefiting patients. However, the results of existing clinical studies indicated the low efficacy of MSI as a predictive biomarker for colon cancer immunotherapy. Several new predictive biomarkers have been proposed in colon cancer immunotherapy. Developments have also been made in the detection method of predictive biomarkers for immunotherapy of colon cancer. In this review, we summarized the currently reported predictive biomarkers in existing studies of colon cancer immunotherapy.

We concluded that immunotherapeutic biomarkers reported in the clinical studies for colon cancer can be divided into four main categories (Figure 1), the first category of biomarkers related to

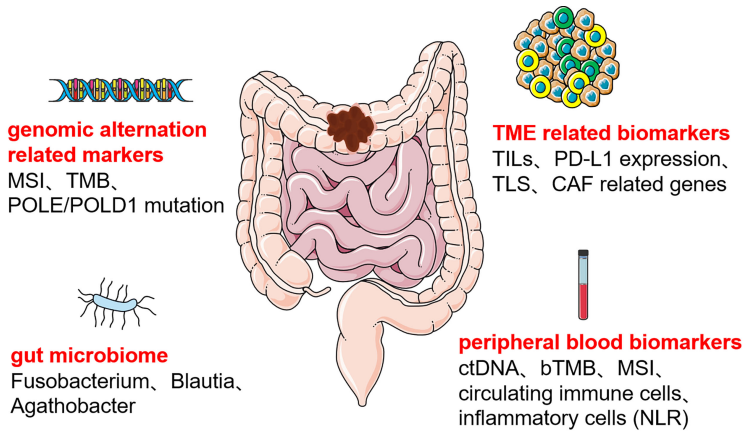

FIGURE 1
Predictive biomarkers of colon cancer immunotherapy MSI, microsatellite instability; TMB, total mutation burden; POLE:/POLD1, DNA polymerase ϵ (POLE) and δ (POLD1); TILs, tumor-infiltrating lymphocytes; PD-L1, programmed cell death-ligand 1; TLS, tertiary lymphoid structure; CAF, cancer-associated fibroblast; ctDNA, circulating tumor DNA; bTMB, blood total mutation burden; NLR, neutrophil-lymphocyte ratio.

genetic alterations, such as MSI, TMB, and POLE/POLD1; the second category of biomolecular markers related to TME, mainly included TILs, PD-L1 expression, TLS, and CAF related genes; the third category is certain specific gut microbiome; the fourth category is peripheral blood biomarkers, such as ctDNA, bTMB, circulating immune cells, and inflammatory cell related indicators. Based on currently reported clinical studies (Table 1), only MSI's predictive role in colon cancer immunotherapy has been demonstrated in a larger cohort. The other immunotherapeutic biomarkers have only been reported in some small cohorts of colon cancer and are pending justification in larger cohorts. Some predictive biomarkers for colon cancer immunotherapy come from database analysis or retrospective studies, also waiting to be demonstrated by large cohort clinical studies. In addition, despite the attention given to the TME and gut microbiota in colon cancer immunotherapy, reliable biomarkers for colon cancer immunotherapy beneficial population selection are still lacking. Peripheral blood markers have been favored by many studies in recent years due to their non-invasive and multi-sampling advantages. However, a unified evaluation criterion is yet to be established. Furthermore, the testing methods of each biomarker are all waiting to be optimized to obtain more accurate testing results, and a unified judgment standard must be developed.

In summary, there is no optimal predictive biomarker for immunotherapy of colon cancer till now, and each biomarker has its limitations. Although the combined application of multi-methods for recognizing multi-indicators could improve the accuracy of biomarkers for colon cancer immunotherapy, large numbers of clinical trials are needed to verify that. With the optimization and improvement of the technology, more accurate biomarkers for predicting immunotherapy of colon cancer will help to stratify patients, which will also greatly improve the prognosis and the overall survival rate of patients

Author contributions

(I) Conception and design: WH, CY, HZ; (II) Administrative support: CY; (III) Provision of study materials or patients: CY, HZ; (IV) Collection and assembly of data: WH, CY, HZ; (V) Manuscript writing: WH, CY, HZ. 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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An *EGFR* L858R mutation identified in 1862 Chinese NSCLC patients can be a promising neoantigen vaccine therapeutic strategy

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Background: This study aimed to develop a vaccine that targets mutation-derived neoantigen in Chinese non-small-cell lung cancer (NSCLC).

Methods: A cohort of 1862 Chinese NSCLC patients who underwent targeted sequencing with a 1021-gene panel was investigated. HLA typing was done using OptiType v1.0 and neoantigens were predicted by netMHCpan v4.0. HLA LOH was inferred using the lohhla algorithm and TMB were quantified by counting the total number of non-synonymous ones based on our panel data. CIBERSORT was utilized to estimate the TME in different *EGFR* mutant subtype by using TCGA data.

Results: HLA-A*11:01(42.59%) was the top one allele and HLA-A*33:03(12.94%) ranked 12th. *EGFR* L858R (22.61%) was the most prevalent gene variant. The binding affinity (IC50 MT = 22.9 nM) and shared frequency (2.93%) of *EGFR* L858R in combination with HLA-A*33:03 were optimal. In a subsequent further analysis on immunological features of *EGFR* mutant subtypes, 63.1% HLA loss of heterozygosity LOH (HLA LOH) and 0.37% (7 of 1862) B2M aberrations were found in our population, both had no significant association with *EGFR* mutant subtypes suggesting that the process of antigen presentation involved HLA LOH and B2M mechanisms in *EGFR* L858R is working. Tumor mutation burden

(TMB) was investigated by utilizing our panel and showed that *EGFR* L858R had the lowest TMB compared with other *EGFR* mutant subtypes. In addition, analysis of 22 immune cell types from The Cancer Genome Atlas (TCGA) data showed *EGFR* L858R was correlated with low level of CD8 T cells, activated CD4 memory T cells and elevated level of macrophage M2 suggesting an inhibited tumor microenvironment (TME).

Conclusion: Our study identified that *EGFR* L858R neoantigen had the potential to generate cancer vaccines in NSCLC patients with HLA A*33:03. The neoantigen-based vaccines may become an effective salvage regimen for *EGFR* L858R subgroup after targeted therapy or immune checkpoint inhibitors (ICIs) failure.

KEYWORDS

EGFR L858R mutation, neoantigen vaccine, HLA A*33:03, immunological features, Chinese NSCLC

Introduction

Lung cancer is still the most common malignancy with morbidity and mortality both ranking first worldwide, and non-small-cell lung cancer (NSCLC) is a subset of lung cancer that has extensive clinical and molecular heterogeneity (1, 2). Epidermal growth factor receptor (*EGFR*) mutations are the most common driver genes in NSCLC, followed by RAS and ALK (3, 4). Only a subset of patients initially responds to targeted therapy, nonetheless, the majority inevitably acquire drug resistance (5–7).

Currently, immune checkpoint inhibitors (ICIs) have achieved positive laboratory results and remarkable clinical responses in the treatment of many kinds of cancer, including NSCLC (8–16). However, in the NSCLC clinical trials, *EGFR* mutant patients benefit less from ICIs than patients with KARS, BRAF, and MET mutations (8, 17–19). Previous studies have reported that antigen expression and presentation deficiency, the low mutation burden, immunosuppressive microenvironment, and upregulation of PD-L1 may be the mechanisms that limited efficacy of ICIs in *EGFR* mutant NSCLC patients (2, 20–23).

Yet, some NSCLC patients whose tumors are harboring *EGFR* mutations do respond to ICIs and studies have continued to focused on the tumor immune phenotype or somatic mutation features to develop novel and more effective treatments for this population. To date, the strategy that utilizes individualized neoantigen vaccines derived from mutated genes against cancers has achieved success in both mouse models and the clinical settings (7). Neoantigens generated from tumor-specific somatic mutations are the optimal targets for T-cells and are capable of mobilizing strong antitumor immune responses (24, 25).

To develop a vaccine that targets individualized neoantigen in NSCLC patients with *EGFR* mutations who do respond to ICIs, we performed a retrospective analysis of 1862 Chinese NSCLC tumor tissues matched with normal tissue samples which were previously profiled using our 1021-gene panel. We then assessed the expression of mutated alleles and predicted possible neoantigens. In this research, we have found that an *EGFR* L858R mutation could be a good target for the development of an individual vaccine for NSCLC patients with HLA A*33:03. We then presented a further investigation on immunological features (HLA LOH, B2M, TMB, and TME) of *EGFR* mutant subtypes to procure the evidence supporting the feasibility of *EGFR* L858R neoantigen. Our results not only provide useful information for predicting response to ICIs, but also introduce a promising treatment for Chinese NSCLC patients with *EGFR* mutations who were failed ICIs therapy and are without alternative therapy.

Abbreviations: NSCLC, non-small-cell lung cancer; ICIs, immune checkpoint inhibitors; EGFR, Epidermal growth factor receptor; 19 del, deletions in exon 19; HLA, human leukocyte antigen; HLA LOH, HLA loss of heterozygosity; TMB, Tumor mutation burden; TCGA, The Cancer Genome Atlas; TME, tumor microenvironment.

Materials and methods

Cohort

Clinical information of patients was collected from our records. Patients who were diagnosed with NSCLC and underwent targeted sequencing with a 1021-gene panel at Geneplus-Beijing (Beijing, China) were deemed eligible for analysis. For each patient tumor and normal (peripheral blood

or normal tissue) samples were available. This study was approved by the Ethics Committee of Fujian Cancer Hospital. Written, informed consent was obtained from all participants before inclusion.

HLA typing

HLA typing was done using the OptiType v1.0 to obtain the four-digit HLA type at each locus of a patient (26). The Allele Frequency Net Database was utilized to retrieve the allele frequency (AF) of alleles in general Chinese Han populations and carrier frequencies were calculated according to this equation: carrier frequency = $1-(1-AF)^2$.

Neoantigen prediction and prioritization

For each patient, manually curated somatic mutations (missense or in-frame indel, AF≥0.05) in coding regions were also retrieved from previous records in our database. Neoantigens were predicted using netMHCpan v4.0 (27). Candidates with IC50 mut <500 nM and IC50 wild >=500 nM were considered for further analysis. A putative neoantigen was considered mutant-specific if the IC50 mut is <500 nM, and especially, it is considered as a "strong binder" if the IC50 mut is <50 nM.

Loss of Heterozygosity (LOH) in HLA genes

The LOH status at all three human leukocyte antigen (HLA) loci was inferred using the lohhla algorithm developed by McGranahan et al. (28). A locus was considered impacted by LOH if the computed *p*-value ('PVal_unique' in the output) was <0.01. A patient with a LOH at an HLA locus was defined as one who had at least one HLA locus impacted by LOH. All other patients (including those who have homozygous alleles at all three HLA loci) were considered not affected by HLA LOH.

Mutation number across four *EGFR* mutation type

Samples were categorized into four sub-groups: with L858R mutation, with deletions in exon 19 (19del), with other *EGFR* mutations, and *EGFR* wild types (WT). Mutations in each sample were quantified by counting the total number of non-synonymous ones. Group-wise Kruskal-Wallis tests were then performed.

NSCLC datasets and preprocessing in TCGA

Somatic mutations and RNA-sequencing (RNA-seq) data were downloaded from TCGA (https://portal.gdc.cancer.gov/). In consideration of no *EGFR* L858R mutation was found in 495 lung squamous cell carcinoma, therefore, we only mined mutation data from lung adenocarcinoma samples. The lung adenocarcinoma cohort was divided into four clusters as *EGFR* L858R (n=21), EGFR 19del (n=21), EGFR other (n=29) and EGFR WT (n=490). TCGA-LUAD (lung adenocarcinoma) FPKM data containing 594 cancer tissue samples were obtained. After exclusion, analysis was performed on a dataset of 513 lung adenocarcinoma patients who have EGFR mutation status data: *EGFR* L858R (n=21), *EGFR* 19del (n=19), *EGFR* other (n=28), *EGFR* WT (n=445).

Inference of infiltrating cells in TME

The CIBERSORT (http://cibersort.stanford.edu/) is an analytical tool developed by Newman et al. (29). To quantify the proportions of immune cells in tissue samples. We used the CIBERSORT algorithm and the LM22 gene signature, which was used to distinguish 22 immune cell phenotypes, including B-cells, T-cells, natural killer cells, macrophages, DCs, and myeloid subsets. We utilized CIBERSORT to estimate the fractions of 22 immune cell types among different *EGFR* mutant subtype.

Statistical analysis

$P < 0.05$ was considered statistically significant. All data were processed using the R software (version 3.6.0), GraphPad 7.0, and AdobeIllustratorCS6.

Results

Diversity and prevalence of HLA class I alleles

Data was collected from 1,862 patients who had been diagnosed with NSCLC and underwent targeted sequencing with a 1021-gene panel.

We recovered the HLA class I alleles (HLA-A, HLA-B, and HLA-C) for each patient from NGS data. We found 172 different alleles, of which 17 were carried by more than 10% of all patients (Figure 1). The most prevalent allele, A*11:01, was found in over 40% of all patients. HLA*11:01 and HLA-A*33:03 allele frequency (AF) are roughly comparable to the numbers

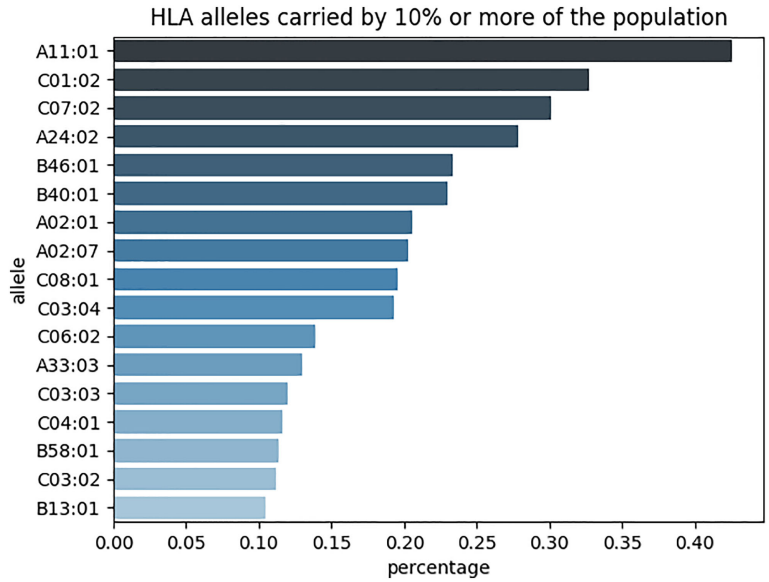

FIGURE 1
17 different HLA class I alleles (HLA-A, HLA-B, and HLA-C) were found in more than 10% of all patients (n=1862).

retrieved from the Allele Frequency Net Database (30) (Supplementary Tables 1, 2). A rarefaction curve shows that the selected patients covered a large portion of HLA alleles, although it did not reach saturation (Supplementary Figure 1). Therefore, the selected population was not biased towards certain allele types and the reported carrier rates were reliable.

Recurrence of genes and mutations

Somatic missense or in-frame indel mutations were selected with an AF greater than or equal to 0.05 for neoantigen prediction and post-prediction analysis. We detected over 10,000 mutations across all patients (about 5 per patient). These mutations affected more than 800 genes. Genes *EGFR* and TP53 were the most frequently mutated genes, they were found mutated in 50% and 40% of all patients, respectively. They were followed by LRP1B and KRAS, which were mutated in 13% and 11% of all patients, respectively (Figure 2A). When inspected at variant level, *EGFR* mutations L858R and E746_A750del were overwhelmingly dominant. The frequencies were 23% and 13% for each, over 7-fold and 4-fold higher than the third mutation on the list. The *EGFR* genotyping results mainly agreed with previous studies in the prevalence of driver mutations in NSCLC patients (3, 4, 31). Interestingly, LRP1B mutations were not among the top at variant level (highest frequency at 0.11%), although the gene was mutated in a moderate proportion of patients (Figure 2B). Despite the aforementioned genes and mutations, a large majority of these genes and mutations were carried by few patients, typically less than 1% of the population.

Overview of putative neoantigens

All 8- to 11-mer peptides were derived from all selected mutations and predicted their binding affinity to the patient's HLA class I complexes to identify patient-specific neoantigens. The prediction was performed for both mutant (MT) and wild type (WT) peptides. We considered a MT peptide a candidate neoantigen if the IC50 MT is smaller than 500 nM and the corresponding IC50 WT is greater than or equal to 500 nM. Furthermore, we categorized candidate neoantigens into "strong binder" and "weak binder" groups by the IC50 MT threshold of 50 nM.

We identified ~1900 candidate neoantigens in approximately 60% of all patients (1122/1862). The number of neoantigen ranged from 1 to 15, with a median of 1. More than half of the patients were predicted to possess only one neoantigen (Figure 3). We further shortened the list to 1438 unique neoantigens. A neoantigen was deemed "unique" if there was no other neoantigens derived from the same mutation and of the same amino acid sequence. About 35% of these unique neoantigens (504/1438) were derived from mutations found in less than 1% of patients. And among these, 19% (98/504) were strong binders (Figure 4A). Of the 934 unique neoantigens derived from frequently mutated genes (genes that were found mutated in more than 1% of all samples), 18% (169/934) were

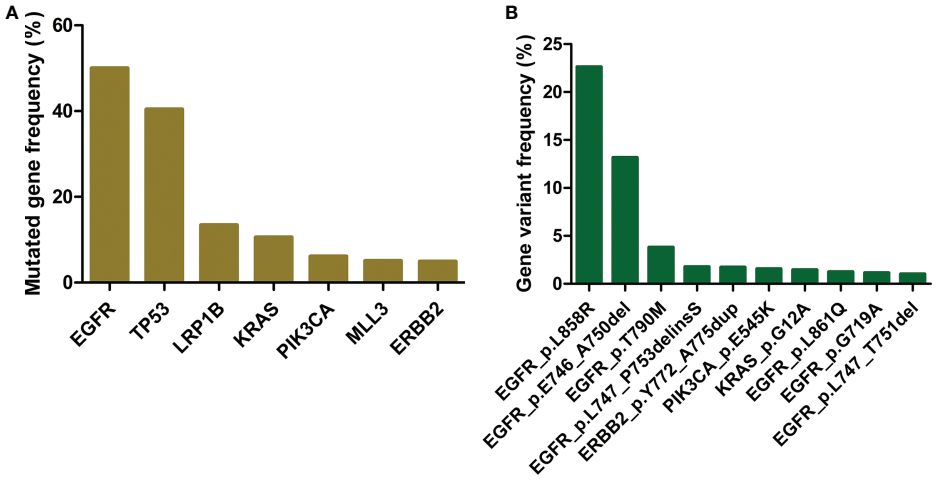

FIGURE 2
Recurrence of genes and mutations. **(A)** Mutated gene frequency among all patients. **(B)** Gene variant frequency among all patients.

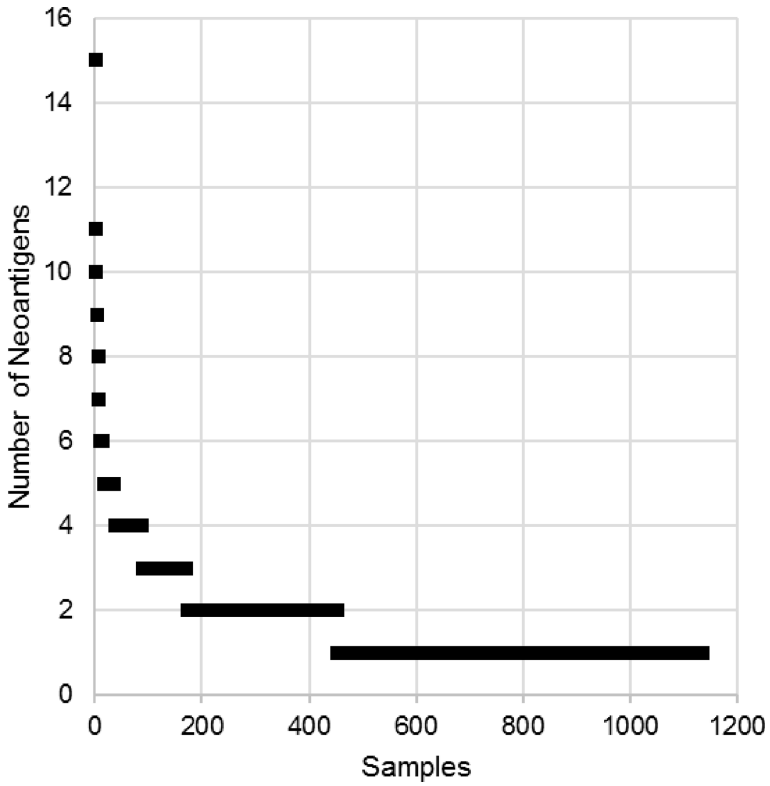

FIGURE 3
The number of neoantigens in each sample.

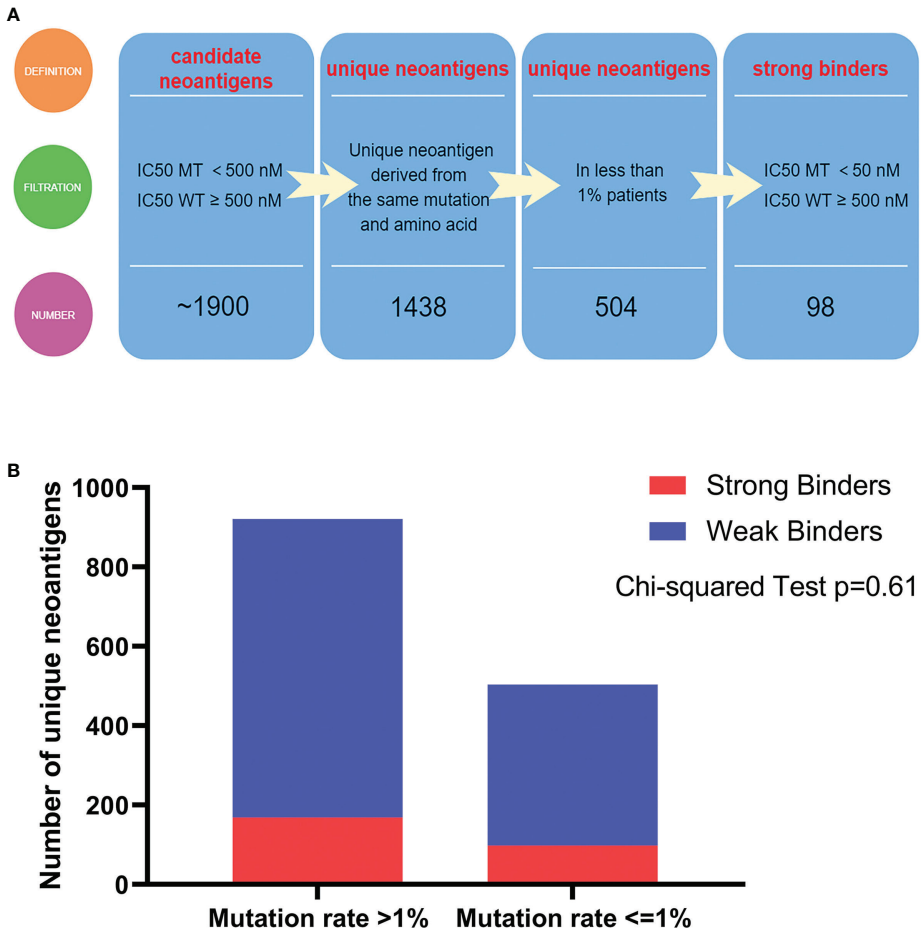

FIGURE 4
Overview of putative neoantigens. **(A)** Analytic pipelines to output putative neoantigens. **(B)** The proportions of strong and weak binders in two groups. The two groups were categorized by the mutation rate >1% samples vs. <=1% samples.

strong binders. Also, there were more than 1% of neoantigens (13/934) with ambiguous binding strength, as they were able to bind different HLA molecules with varying affinities. The neoantigens were further categorized into two groups by the mutation rate of the related genes (>1% samples vs. <=1% samples). No statistically significant difference was found between the proportions of strong and weak binders in the two groups (Chi-square Test, p = 0.61) (Figure 4B). The result indicates that concurrently mutated genes do not relate with increased proportion of strong binding neoantigens.

Quantifying neoantigens

The "neoantigen frequency" was calculated, defined in this study as the number of neoantigens related to a gene divided by the number of all mutations targeting this gene, for each neoantigen-producing gene (32). We did this from two perspectives: (1) the total neoantigen frequency, which reflects the "ability" of a gene to produce neoantigen, and (2) the neoantigen frequency corresponding to a specific HLA allele. Note that the latter might be larger than the former, because when calculating the total neoantigen frequency, neoantigens that were able to bind more than one HLA molecule were only counted once. Genes mutated in less than 1% of all patients were removed. By doing this, we also removed any genes targeted by less than 10 mutations. The *EGFR*, with a neoantigen frequency of 0.646, was the top one neoantigen producing gene, and most of these neoantigens were predicted to bind to A*11:01 (allele-specific neoantigen frequency 0.150). We noticed that some genes that were not so prevalent across patients still exhibited a high neoantigen frequency, like ERBB2 (0.563; mutated in 4.9% of patients), CTNNB1 (0.321; 3.2% of patients), and BRAF (0.357; 3.0% of patients) (Data not shown).

We repeated the above procedures at variant level. Instead of calculating frequency, we counted the number of neoantigens

derived from a mutation directly, in total or HLA-specific. The number of per-mutation neoantigens ranged from 1 to 5. The *EGFR* L858R, the most prevalent mutation, produced four neoantigens. Two mutations were strong binders, while most mutations (except TP53 R110L, which was found in only 0.64% of all patients) produced no more than one strong binder (Figure 5).

Finding shared neoantigens

To find shared neoantigens that could be a good target for generalized neoantigen-based vaccines we further investigated the top two mutations by frequency; *EGFR* L858R and E746_A750del. The two strong binders derived from *EGFR* L858R were HVKITDFGR and RAKLLGAEEK. The latter binds to A*30:01 (IC50 MT = 47.5 nM and IC50 WT = 881.9 nM). Peptide HVKITDFGR binds to three HLA complexes; A*31:01 (IC50 MT = 18.9 nM and IC50 WT = 11653.7 nM), A*33:03 (IC50 MT = 22.9 nM and IC50 WT = 12734.0 nM), and A*68:01 (IC50 MT = 19.6 nM and IC50 WT = 8625.5 nM). The shared frequency of L858R and A*33:03 is 2.93% (22.61% × 12.94%).For the other two the percentages were 1.19% (A*31:01, 22.61% × 5.26%) and 0.28% (A*68:01, 22.61% × 1.24%). The most shared combination is E746_A750del and A*11:01 with a frequency of 5.60%. However, neither of the two neoantigens derived from this mutation was a strong binder (Table 1).

Prevalence of HLA LOH across NSCLC

In order to predict the ability to present neoantigens of different *EGFR* mutant subtypes (*EGFR* L858R, *EGFR* 19del, *EGFR* other rare, *EGFR* WT), we identified HLA LOH in our cohort. We analyzed 1731 tumor exomes and found 639 patients (36.9%) who were heterozygous at all HLA-I loci and 1092 patients (63.1%) who had LOH in at least one HLA-I locus in tumors in total (Supplementary Table 3). The HLA LOH occurrence rate was higher than the 40% reported in a previous study (28). HLA LOH was calculated for the *EGFR* WT (n=828) and *EGFR* mutant tumors harboring *EGFR* L858R (n=380), *EGFR* 19del (n=315), and *EGFR* other (n=139) (Figure 6A). We did not find that HLA LOH had any association with *EGFR* mutation status. Additionally, we examined the HLA LOH of selected HLA (A*33:03, A*31:01, and A*68:01) and also found no difference (Figure 6B).

Some mutations that are vital for antigen presentation and MHC class I expression were detected. In our cohort of 1862 NSCLC patients, only seven tumors were found to harbor β2-microglobulin (B2M) mutations and there was no difference among different *EGFR* mutant subtypes (Supplementary Table 4). No further mutations like TAP1, TAP2, LMP2 and LMP7 were identified in our cohort (Supplementary Table 5).

Association between *EGFR* mutant subtypes and mutation number

To examine whether the *EGFR* mutant status influenced the tumor mutation number, we determined the mutation numbers across *EGFR* mutation subtypes in NSCLC tumors from our cohorts. The median of *EGFR* WT (n=604) was five non-synonymous mutations, *EGFR* L858R (n=421) was three non-synonymous mutations, *EGFR* 19del (n=367) was three non-synonymous mutations, and *EGFR* other (n=145) was four non-synonymous mutations. The mutation number was

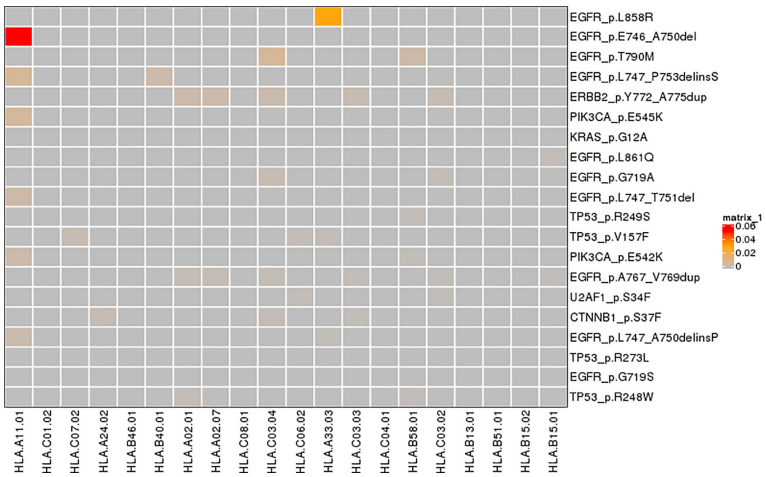

FIGURE 5
Neoantigens derived from a mutation frequency corresponding to a specific HLA allele.

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TABLE 1 Shared neoantigens based on *EGFR* L858R and E746_A750del.

EGFR mutation	EGFR neoepitope	HLA restriction	IC50 MT (nM)	IC50 WT (nM)	shared frequency (%)
L858R	HVKITDFGR	A*31:01	18.9	11653.7	1.19
		A*33:03	22.9	12734.0	2.93
		A*68:01	19.6	8625.5	0.28
	RAKLLGAEEK	A*30:01	47.5	881.9	1.71
E746_A750del	IPVAIKTSPK	A*11:01	158.2	31132.7	5.60
		A*03:01	70.7	30763.0	0.63
		A*03:02	376.4	29825.9	0.05
		A*11:02	158.2	31132.7	0.48
		A*11:20	65.1	26653.4	0.03
		A*68:01	429.6	11669.5	0.16
	AIKTSPKANK	A*30:01	355.0	5721.5	1.00

significantly lower in *EGFR* L858R and *EGFR* 19del tumors compared with *EGFR* other and *EGFR* WT tumors. There was no difference between *EGFR* L858R and *EGFR* 19del, which was different from the previous report that *EGFR* 19del mutant lung cancers had a lower mutation number compared with *EGFR* L858R mutant lung cancers (2, 20) (Figure 7).

Association between *EGFR* mutant subtypes and immune infiltration

When looking at the difference of 22 immune cells in *EGFR* mutant subtypes, *EGFR* L858R mutation were found to be associated with the relatively low level of CD8 T cells (P=0.00032), activated CD4 memory T cells signatures (P=0.0052) and elevated level of macrophage M2 (P=0.02) compared to *EGFR* WT tumors. However, the differences were not significant among *EGFR* L858R, *EGFR* 19del and *EGFR* other sites mutations (Figure 8).

Discussion

NSCLC accounts for about 85% of all lung cancers and is a tumor with a high mutational load (33). Although NSCLC harbors many known driven mutations, the inter-individual genomic heterogeneity is extensive. Distinct molecular subtypes differ in sensitivity to various treatments (2). For instance, for treating *EGFR*-driven lung cancers, *EGFR* TKIs has been the first choice. However, the acquired resistance to TKIs is inevitable (5, 6, 34). As an emerging therapeutic approach with the potential for durable responses, ICIs are not

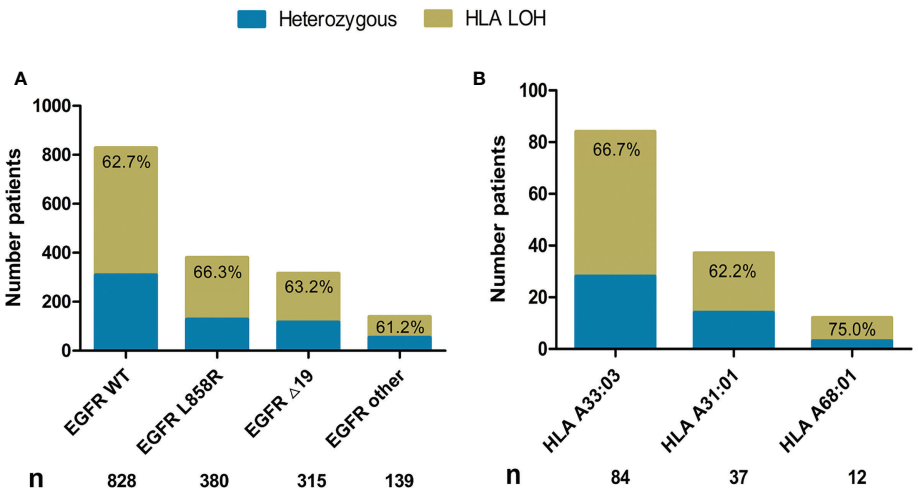

FIGURE 6
Frequency of HLA LOH in NSCLC. **(A)** The HLA LOH rate in the *EGFR* WT (n=828), *EGFR* L858R (n=380), *EGFR* 19del (n=315), and *EGFR* other (n=139). **(B)** The HLA LOH rate in HLA A*33:03 (n=84), HLA A*31:01 (n=37), and HLA A*68:01 (n=12).

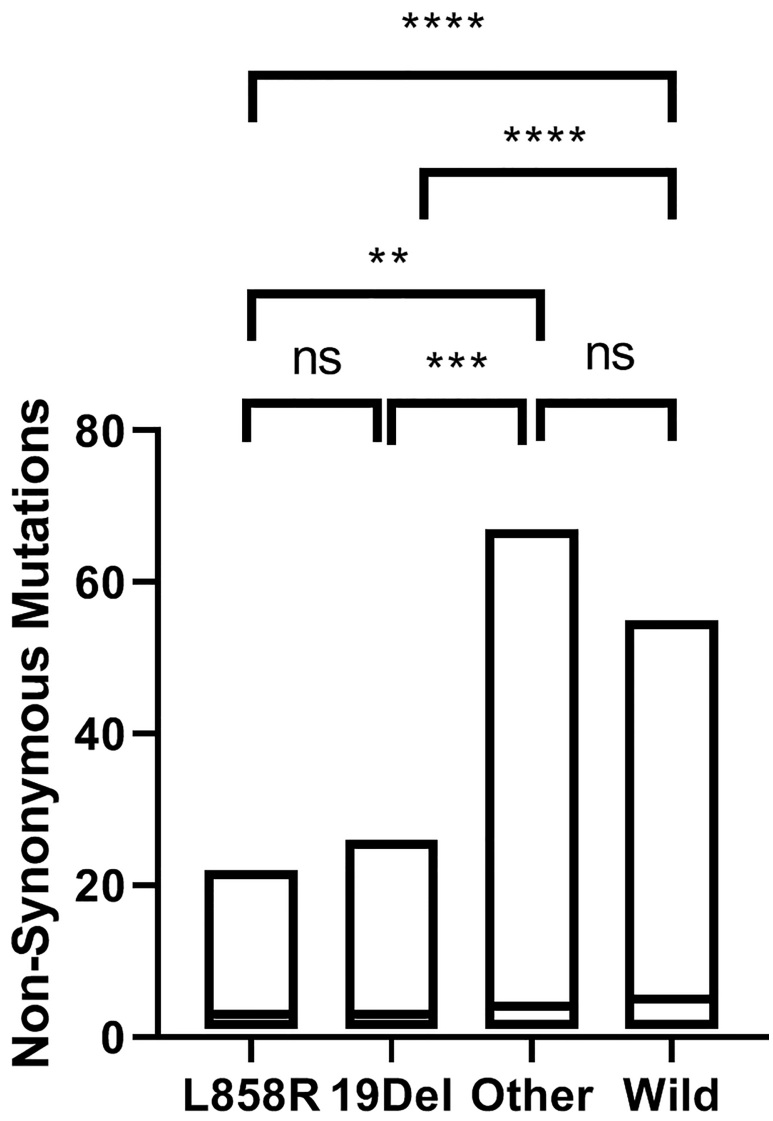

FIGURE 7
Non-synonymous mutations in NSCLC from our cohorts. The median non-synonymous mutations of *EGFR* L858R (n=421) was three, of *EGFR* 19del (n=367) was three, of *EGFR* other (n=145) was four and of *EGFR* WT (n=604) was five. **p < 0.01; ***p < 0.001; ****p < 0.0001 , ns, no significance.

recommended for *EGFR*-driven lung patients due to less benefit derived compared with other molecular subgroups (8, 17, 18, 35). However, an *EGFR* mutation is the most common gene alteration in NSCLC. There is a considerable need to find an effective treatment option to significantly increase immunotherapy efficacy in this subgroup.

In this study, we explored neoantigens in 1862 Chinese NSCLC patients who underwent targeted sequencing with a 1021-gene panel. Even though some of the same mutations were shared among different patients, not every mutation would play a role as a neoantigen as the binding affinity to each patient's own HLA might vary (7, 36, 37). By combining the shared frequency and binding affinity to identify tumor specific somatic mutations, our data revealed that *EGFR* L858R was the top neoantigen producing gene allele, and most of these neoantigens were predicted to bind to A*33:03. We then presented a further analysis on immunological features of *EGFR* mutant subtypes to procure the evidence supporting the feasibility of *EGFR* L858R neoantigen.

A key step in neoantigen presentation and recognition by T-cell receptors is controlled by HLA. Hence, not only the binding affinity of the peptide to the HLA but also loss of HLA expression, which is caused by HLA haplotype loss or mutation of antigen presentation machinery genes such as B2M, needs to be taken into account (28, 38–40). We found that HLA LOH occurred in 63.1% NSCLC in our cohort, higher

FIGURE 8
TME in NSCLC from TCGA database. TME, tumor microenvironment. TCGA, The Cancer Genome Atlas.

than 40% reported in a previous study and had no significant association with *EGFR* mutant subtypes (28). Furthermore, the HLA LOH of selected HLA (A*33:03, A*31:01, and A*68:01) also did not show any difference. We next examined B2M aberrations. Specifically, we found only one form of frameshift mutation in B2M: p.L15Ffs*41 and did not find B2M aberrations to be significantly enriched in any subtypes of *EGFR* mutations. Since B2M is essential to the assembly of all HLA class I complexes (41), and HLA LOH may facilitate immune evasion (38), our negative findings indicated that the *EGFR* L858R may not have a deficiency in neoantigen presentation, at least HLA LOH and B2M mutations did not play a crucial role in the immune resistance of *EGFR* L858R patients.

TMB contributes to enhancing antigenic response through the generation of neoantigens (42, 43). Accordingly, we next sought to evaluate the correlation between the attributes of the TMB and *EGFR* mutant subtypes using our panal. Our panel analyses demonstrated that *EGFR* L858R and *EGFR* 19del had the lowest TMB compared to other *EGFR* rare sites mutants and *EGFR* WT, though no difference was noted in *EGFR* L858R and *EGFR* 19del. This is in line with the lower response rate of *EGFR* mutant NSCLCs treated with ICIs, for which low TMB was deemed to be a major culprit of low efficiency of immunotherapy for *EGFR* L858R NSCLC. However, this was different from other studies that *EGFR* 19del mutant lung cancers had a lower TMB compared with *EGFR* L858R mutant lung cancers (2, 20), might be due to, for instance, different races, histology and stages. Moreover, tumor cells are embedded in the tumor microenvironment (TME), suggesting that intercellular relationships are as important as genomic factors (44, 45). In our study, we estimated the fractions of 22 immune cell types of NSCLC from TCGA and studied the correlation between the TME and *EGFR* mutant subtypes. We found *EGFR* L858R was correlated with lower percentage CD8 cells, lower percentage activated CD4 memory T cells and higher percentage macrophage M2 compared with *EGFR* WT. Taken together, these revealed an inhibited TME in the *EGFR* L858R subgroup.

We assembled the largest cohort of NSCLC cases to explore tumor-specific somatic mutations by targeted sequencing with a 1021-gene panel for developing neoantigen vaccines. In our analysis, the *EGFR* L858R neoantigen was identified in an HLA subtype-specific manner that could be used to generate cancer vaccines in HLA A*33:03 subsets patients. *EGFR* L858R in HLA A*33:03 patients would be relevant to 2.93% of the population. Given that lung cancer is the most common cancer, the percentage of patients who may benefit is considerable. We then proposed that the lower TMB and inhibited TME may be the reason for the week immunogenicity of the *EGFR* L858R subset of NSCLC. There were no deficiencies in the HLA LOH and B2M mechanisms, suggesting that the process of antigen presentation of *EGFR* L858R is working.

Our research has some insufficiency. One limitation was that a 1021-gene panel lacks sufficient sequencing data compared with

WES or WGS, and only covers a proportion of all coding regions. With the exception of B2M, it did not cover gene mutations related to the HLA presentation which have been implicated as resistance mechanisms to ICIs, like TAP1, TAP2, LMP2 and LMP7 (40, 46, 47). However, since the panel covers most concurrently mutated genomic regions, it is capable of capturing necessary information. In addition, as an indispensable component of neoantigen peptide recognition, the T-cell receptor (TCR) repertoire profiling needs to be explored (48). Recent work on NSCLC has investigated whether the TCR repertoire enables assessment of T cell diversity and T cell clonal expansion and indicated that *EGFR* mutant tumors exhibits lower T cell clonal expansion (49, 50). In the future, we plan to perform TCR sequencing to elucidate whether there exist significant differences in the TCR repertoire diversity in *EGFR* mutant subtypes, aiming to investigate the distinct characteristics of TCR repertoire patterns in *EGFR* L858R. Another possible limitation of this study is that we lack available sequencing data to directly compare TME in this cohort. To address this, we utilized the TCGA data source, but this data source does not represent the real tumor immunogenomic landscape in our Chinese cohort. At last, this is a retrospective study and the clinical information like stages and treatment strategies were incomplete. So we could not conduct stratified analysis to explore some underlying mechanisms.

We excluded frameshift mutations from analysis. The rationale behind this is that there is a chance to raise false positives. Such mutations often result in premature termination codons, which cause the degradation of transcripts *via* nonsense-mediated mRNA decay (NMD) before translation (51). An approach to assess NMD efficiency through RNA-Seq has been published (52), but is not applicable here due to the lack of RNA-Seq data. Still, we estimated to what extent our findings are biased. A total of 975 frameshift mutations (915 unique) were detected, spanning 670 samples. We recalculated the mutation frequency for each gene with frameshift taken into account. The top 6 genes by mutation frequency did not change, while the remaining genes were reordered. For some genes, the mutation frequency increased after the recalculation, such as *TP53* and *LRP1B*. This indicates that in some patients only frameshift mutations were detected on these genes. *TP53* mutation frequency increased from 40.44% to 48.34%, indicating that we probably have underestimated its potential role in neoantigen producing. However, the most frequent frameshift mutation *STK11* P281Rfs*6 was shared by only six patients, which translated into a percentage of 0.3% (Supplementary Table 4). This did not serve our purpose of finding shared neoantigens.

In summary, our research identified that *EGFR* L858R neoantigen had the potential to generate cancer vaccines in NSCLC patients with HLA A*33:03 and revealed the possible underlying immunological features between *EGFR* mutant subtypes. Our finding provides the basis for further investigations into which neoantigen-based vaccines may become an effective treatment strategy for patients with *EGFR* L858R mutation.

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

The studies involving human participants were reviewed and approved by the Ethics Committee of Fujian Cancer Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JiL, JuL, S-GH, BL carried out the whole research. X-BZ, J-NX, Y-QZ, XG, Y-TY, XY statistically analyzed all the data and graphed. LC, Y-FH, HL, J-PL help in assays. C-BC, GC and X-WZ developed the concept. X-FW and YC obtained the funds and organized the study. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Authors S-GH, Y-QZ, XG, Y-TY, and XY were employed by company Geneplus.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1022598/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The rarefaction curve shows the selected population was not biased towards certain allele types.

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A prognostic and therapeutic hallmark developed by the integrated profile of basement membrane and immune infiltrative landscape in lung adenocarcinoma

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Basement membranes (BMs) are specialised extracellular matrices that maintain cellular integrity and resist the breaching of carcinoma cells for metastases while regulating tumour immunity. The tumour immune microenvironment (TME) is essential for tumour growth and the response to and benefits from immunotherapy. In this study, the BM score and TME score were constructed based on the expression signatures of BM-related genes and the presence of immune cells in lung adenocarcinoma (LUAD), respectively. Subsequently, the BM-TME classifier was developed with the combination of BM score and TME score for accurate prognostic prediction. Further, Kaplan–Meier survival estimation, univariate Cox regression analysis and receiver operating characteristic curves were used to cross-validate and elucidate the prognostic prediction value of the BM-TME classifier in several cohorts. Findings from functional annotation analysis suggested that the potential molecular regulatory mechanisms of the BM-TME classifier were closely related to the cell cycle, mitosis and DNA replication pathways. Additionally, the guiding value of the treatment strategy of the BM-TME classifier for LUAD was determined. Future clinical disease management may benefit from the findings of our research.

KEYWORDS

basement membrane, immune infiltration, lung adenocarcinoma, prognosis, immunotherapy response, chemotherapy drug screening

Introduction

Basement membranes (BMs) are thin, pliable, dense sheets of extracellular matrices (ECM) that cover the basal surface of epithelial and endothelial cells and are widely distributed around various tissues and cells in all stages of development, from embryo to adult (1, 2). The major components of BMs are collagen IV, laminin, nidogen and perlecan (3). Laminin and type IV collagen form their two-dimensional meshwork structure. Nidogen and perlecan serve as binding bridges between the two networks, assembling BM (4, 5). Diverse BMs regulate the multifaceted cellular biological process, leading to cell polarity, differentiation and migration (6–8). By acting as a physical barrier and regulating molecular exchange within and outside the cell, BM plays an important role in maintaining the integrity of cell structure and tissue separation (9). Further, BM acts as a major barrier that prevents cancer cells from breaching to develop metastases (10). Metastatic cancer is the leading cause of death in patients (11).

Lung cancer has been reported to have the highest cancer incidence and mortality rates worldwide (12, 13). Lung adenocarcinoma (LUAD) is the most prevalent histologic subtype of non-small cell lung cancer (NSCLC), accounting for approximately 40% of lung malignancies (14). According to the lung tumours chapter of the 2021 WHO Classification of Thoracic Tumours, the distinction between minimally invasive adenocarcinoma (MIA) and adenocarcinoma *in situ* (AIS) depends on whether the BM has been breached (15). With treatment or surgical resection, the five-year survival rate for AIS is 100% (16). However, once AIS penetrates the BM and develops into MIA, the prognosis will deteriorate substantially. Therefore, the BM status affects the prediction of LUAD prognosis.

Emerging evidence indicates that the tumour microenvironment (TME) contributes to the initiation and progression of cancer (17). Tumour-infiltrating immune cells in LUAD are highly heterogeneous and govern the intensity and duration of immunotherapy responses (18, 19). During tumour initiation and progression, BMs act as essential modulators in tumour immunity in addition to promoting tumour proliferation and neoangiogenesis and providing protection from chemotherapy-induced apoptosis (20). Besides, BM components also modulate diverse immune cell behaviours. Laminins not only inhibit the activation and function of CD4+ and CD8+ T cells by attenuating T cell receptor signalling but also promote their apoptosis (20). Additionally, the laminin γ2 chain regulates T cell adhesion and migration, causing T cell exclusion (21). In patients with lung tumours, increased collagen expression has been associated with elevated exhausted CD8+ T cells and the reduction of total CD8+ T cells (22). Further, collagen stabilisation correlates with tumour stiffness, thereby influencing T-cell migration (23). In essence, an intense interaction exists between BMs and tumour-infiltrating immune cells.

In this study, BM score and TME score were established based on the characteristics of BM and immune cells, respectively. According to the relationship between BM and metastasis in LUAD, further investigation of the BM score was performed for patients with and without distant metastases. Considering the interaction of the BM and immune infiltration, we developed an integrated BM-TME classifier based on the BM score and TME score for better prognosis prediction and treatment strategy guidance. Patients with LUAD in several cohorts among different subgroups exhibited diverse prognostic outcomes, enrichment pathways, somatic mutation landscape and therapeutic response, suggesting that our research findings may be conducive to the improvement of clinical disease management.

Materials and methods

Data source

Multiple gene expression profile datasets of LUAD samples were collected from The Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus repository. Only patients with LUAD having prognostic data were retained for subsequent research. The detailed information of these cohorts was summarised in Table S1. In this study, the TCGA-LUAD cohort was applied as the training set for constructing the BM score and TME score. Meanwhile, independent validation sets consisted of five datasets [GSE30219 (24), GSE50081 (25), GSE37745 (26), GSE81089 (27) and GSE135222 (28)], including microarray and RNA sequencing data. Additionally, single-cell RNA sequencing data from eight primary and five metastatic LUAD tumours were retrieved from GSE123902 (29) to visualize BM scores in each cell. Gene levels with survival data for 32 cancers were retrieved from UCSC Xena (https://xena.ucsc.edu/) (30). The bulk RNA sequencing data were log2 (TPM + 1) transformed for further analysis.

Screening prognostic BM-related genes and immune cells for establishing BM score and TME score

The set of 160 BM network genes was derived from a recently published paper (31). To identify prognostic-related BM genes in LUAD, we performed differential expressed gene (DEG) analysis, univariate Cox regression analysis and least absolute shrinkage and selection operator (lasso) regression analysis of 160 BM genes in TCGA-LUAD cohort successively. The threshold of DEGs was set as p.adjust < 0.05. CIBERSORT is an algorithm for estimating 22 immune cells composition of various tissue from gene expression signatures and is used in

TCGA-LUAD cohort (32). Subsequently, Kaplan–Meier survival curve estimation was executed to identify the prognostic value of immune cells. Immune cell types with favorable prognosis and p-value of Kaplan-Meier analysis less than 0.01 were selected for constructing the TME model.

Development of BM score, TME score and BM-TME classifier

The coefficients (Coef) for multivariate Cox regression analysis of 20 BM genes and three types of immune cells in TCGA-LUAD cohort were the basis of the establishment of BM score and TME score. To improve the accuracy of both BM and TME models, we conducted 1000 random sampling of all LUAD samples and performed multivariate Cox analyses each time. Furthermore, the standard deviation (SD) values of Coef were acquired for each gene and cell. Their weights in the corresponding models depend on the ratio of the Coef to the SD values. In summary, the BM score was given as the following formula:

$$BM\ score = \sum_{i=1}^{20} \frac{Coef_i}{SD_i} * exp(gene_j)\ .$$

Similarly, the formula for the TME score was as follows:

$$TME\ score = \sum_{j=1}^{3} \frac{-Coef_j}{SD_j} * fra(cell_j)\ .$$

Where $exp(gene_i)$ and $fra(cell_j)$ indicate the expression level of gene i and the fraction of cell j, respectively. Thereafter, the BM-TME classifier was developed based on the median value of the BM and TME score of each data set. The samples of each cohort were classified into "BM_low+TME_high", "Mixed" (BM_low +TME_low, BM_high+TME_high) and "BM_high+TME_low" groups. Depiction of receiver operating characteristic (ROC) curves was utilised to measure the prognostic predictive ability of the BM-TME classifier through the "timeROC" package (33).

Visualization of BM score at the single cell level

To perform the clustering analysis, annotation and visualization for the single cell data, we created Seurat objects for scRNA-seq gene expression matrix (34). Transcriptomes with more than 300 and fewer than 6000 expressed genes were remained. Cells with more than 50000 reads or mitochondrial genes occupying more than 15% reads were filtered out. Then, the top 3000 highly variable genes were selected for reducing the dimensionality using principal component (PC) analysis. Further, t-SNE (t-distributed stochastic neighbor embedding) was used to summarize the top 30 PC and visualize the single cell data. We employed FindNeighbors and FindClusters functions to identify distinct cell clusters. Cell type annotations were determined based on canonical cell type markers collected from Bischoff et al (Table S2) (35). The BM score for each cell was calculated according to the above formula of BM model.

Gene set functional annotation and enrichment analysis

Gene set enrichment analysis (GSEA) was performed to explore the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways potentially associated with BM score and TME score. Weighted gene co-expression network analysis (WGCNA) was applied for the scout of the gene module affecting the BM-TME classifier (36). The genes with the top 5000 median absolute deviation in TCGA-LUAD expression profile were retrieved for WGCNA analysis. Their biological functions were subsequently discovered *via* gene ontology (GO) analysis. The above enrichment analyses were implemented using the "clusterProfiler" package (37). Additionally, proteomaps were generated by importing a list of differentially expressed proteins on an online tool (https://proteomaps.net/) (38).

Somatic mutation and immunotherapy response

The mutation annotation format (MAF) data of TCGA-LUAD cohort was accessible in TCGA database. The "maftools" package was used to create waterfall plots of the top 15 mutated genes to compare the somatic mutation status in different groups of BM-TME classifiers (39). Additionally, the tumour mutational burden (TMB) of each LUAD sample was obtained by calculating the total number of somatic mutations per million bases in the tumour genome following the removal of germ-line mutation. Tumour immune dysfunction and exclusion (TIDE) database infers the function of genes that regulate tumour immunity and predict the response of anti-programmed cell death 1 (anti-PD1) and anti-cytotoxic T-lymphocyte associated protein 4 (anti-CTLA4) for melanoma and NSCLC (40). Genomics of Drug Sensitivity in Cancer (GDSC) database collected two datasets on the sensitivity and response of tumour cells to drugs: GDSC1 and GDSC2 (41). Taking the LUAD cells in the GDSC2 cohort as the training set, the half-maximal inhibitory concentration (IC50) for various chemotherapeutic drugs of patients with LUAD in TCGA-LUAD and GSE30219 cohort was predicted with the "oncoPredict" package (42).

Statistical analysis

All the statistical analysis of this article was completed on R 4.1.0. Correlations between variables were analysed using Pearson and Spearman methods. Comparisons between different subgroup samples were performed using nonparametric tests, including the Wilcoxon and Kruskal–Wallis rank sum tests. A p-value of <0.05 was valuable. "*", "**" and "***" indicated p<=0.05, p<=0.01 and p<=0.001, respectively.

Results

Both BM score and TME score are prognostic valuable but have the opposite effect

For the sake of developing a method to estimate the basement membrane and immune cell status of patients with LUAD, DEG analysis, univariate cox analysis and lasso regression analysis of BM genes and Kaplan-Meier overall survival estimation of immune cells were conducted sequentially in TCGA-LUAD dataset (Figures 1A, B). The details of the workflow for screening 160 BM genes and 22 immune cells were presented in Tables S3, 4. Afterward, BM score and TME score were established with 20 BM genes and three types of immune cells, respectively. Their information was in the Table S5. The heatmaps in Figures 2A, B separately demonstrated the association of BM score and TME score with prognostic-related BM genes and immune cells in five different LUAD cohorts. According to the overall outcome, BM scores were positively correlated with the majority of unfavourable prognostic factors and negatively correlated with favourable prognostic factors. In contrast, the TME score primarily had positive relationships with favourable prognostic immune cells. Besides, the results of Kaplan–Meier overall survival curves revealed that patients with LUAD in the low BM score group had a better survival outcome than those in the high BM score group, however, the situation with TME score was reversed (Figures 2C, D). These results were hardly surprising because the TME score was constructed with negative Coef while the BM score was not. To identify the potential differential KEGG pathways between high and low BM and TME groups, the top three pathways for the results of GSEA were displayed (Figures 2E, F). Both were enriched in the cell cycle pathway. Additionally, pathways associated with BM score included DNA replication and ECM receptor interaction (Table S6). The differentially expressed genes (DEGs) between high and low TME score tumours were enriched in immune-related pathways, such as the T cell receptor and the JAK-STAT signalling pathways (Table S7).

BM score is diminished in immune cells and elevated in tumours with metastatic

To visualize BM score at cell levels and explore potential associations of BM score among multiple types of tissue and cells, t-SNE plots of 23899 cells from eight primary and five metastatic LUAD biospecimens were generated (Figures 3A, B). The main cell types were defined based on the canonical markers for distinct cell types (Figure S1). When compared to stromal or epithelial cells, immune cells had a significantly lower BM score (Figure 3C). Moreover, there was a negative correlation between BM score and the abundance of resting CD4 memory T cells among several cohorts (Figure 3D, S2). These findings suggest that low BM scores in patients with LUAD may be associated with increased immune cell proportions especially resting CD4 memory T cells. Similarly, compared to primary tumours (M0), patients with LUAD who had metastatic tumours (M1) had a significantly increased BM score (Figures 3E, F). Therefore, a high BM score was a red flag of tumour metastasis in patients with LUAD.

BM-TME classifier was an independent prognostic indicator for multiple LUAD subtypes

According to the aforementioned findings, we considered if it could be preferable to combine the BM score and TME score to simultaneously characterise the BM and immune microenvironmental status of the tumour. The BM-TME classifier was then constructed based on the median value of BM score and TME score for each cohort. Kaplan–Meier overall survival curves were used to analyse the prognostic predictive capability of the BM-TME classifier. In TCGA-LUAD cohort, patients in the "BM_low+TME_high" subgroup had the best prognostic outcomes, followed by the "Mixed" subgroup, and the "BM_high+TME_low" subgroup had the worst prognosis (Figure 4A). The same analysis results were observed in GSE30219 and GSE81089 datasets (Figures 4B, C).

WGCNA analysis was performed to scout for the gene module related to the BM-TME classifier (for more details, see Figure S3 and Table S8). As illustrated in Figure 4D, the blue module genes were most strongly correlated with the BM-TME subgroups. Subsequently, GO analysis was conducted for these genes (Figure 4E). Overall, the enrichment analysis results revolve around the biological processes of the cell cycle, such as DNA replication, mitotic spindle, sister chromatid segregation and mitotic nuclear division. It was interesting to discover the combined analysis of BM and TME synergistically correlates to cancer cell proliferation.

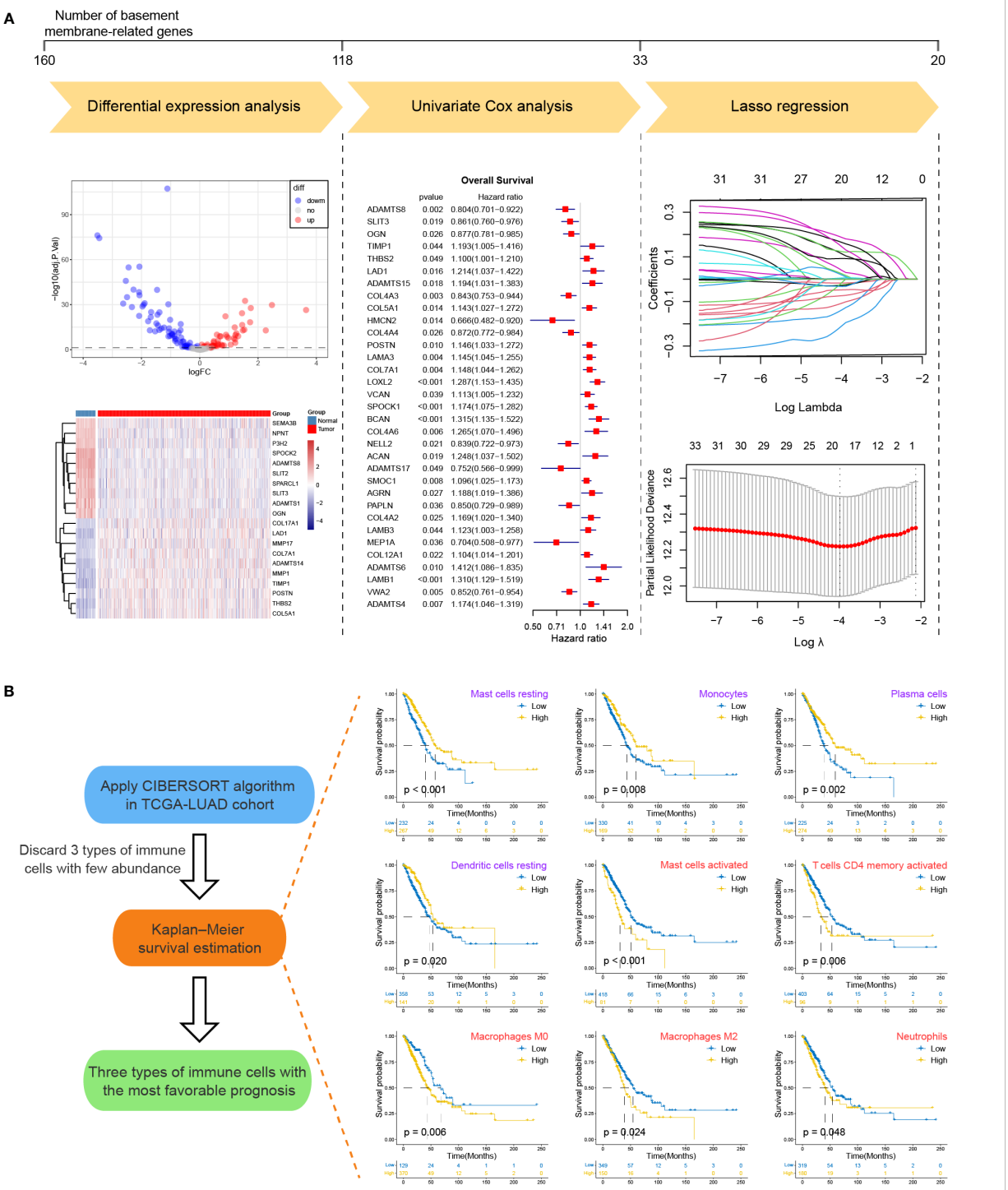

FIGURE 1
The workflow for screening basement membrane-related genes and immune cell types for establishing the BM score and TME score. **(A)** Differential expression analysis, univariate Cox regression analysis and lasso regression analysis were performed for 160 BM-related genes in TCGA-LUAD cohort in succession. The upper line marked the number of BM-related genes before and after each step analysis. Briefly, 20 BM-related genes were selected for the development of BM score. **(B)** The CIBERSORT algorithm was applied to generate the abundance of 22 types of immune cells for TCGA-LUAD samples. After abandoning 3 types of immune cells with few abundances, Kaplan-Meier survival curve estimation was executed for the remaining immune cell types. Kaplan-Meier overall survival curves were shown on the right side only for immune cell types with p-values less than 0.05 (purple indicated favorable prognostic factor while red represented unfavorable prognostic factor). Ultimately, mast cells resting, monocytes and plasma cells were used for the establishment of TME score.

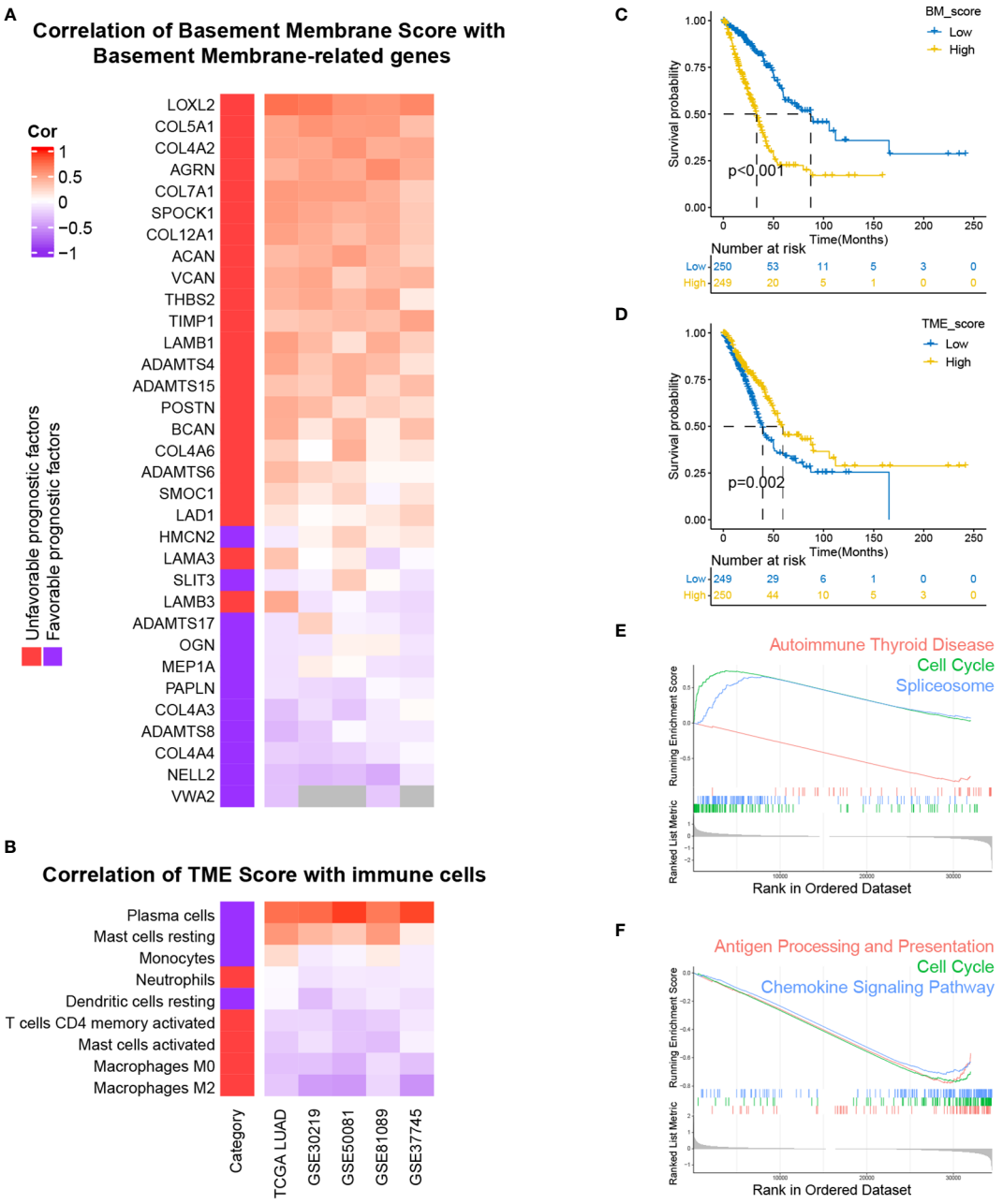

FIGURE 2
Development and performance of BM and TME scores in LUAD, respectively. **(A, B)** The correlation of BM score and TME score with the expression levels of prognostically BM genes and the abundance of prognostically immune cells. **(C, D)** Kaplan–Meier survival curves of high and low BM, TME scores subgroups. **(E, F)** Top three KEGG enrichment pathways based on GSEA analysis of differentially expressed genes (DEGs) between high and low BM, TME scores groups.

Receiver operating characteristic (ROC) analysis indicated that the BM-TME classifier can predict overall survival at 3, 5 and 7 years with the area under the curve of 0.754, 0.686 and 0.698, respectively (Figure 5A). Univariate Cox analysis of five LUAD cohorts revealed that the TNM, stage and BM-TME classifier were all unfavourable prognostic factors (Figure 5B). In addition, the prognosis predictive performance of the BM-TME classifier was comparable to the stage. To investigate and extend the generalised predictive ability of the BM-TME classifier in tumours, a univariate Cox analysis of 32 cancers suggested that the BM-TME classifier was also an unfavourable prognostic indicator for six cancers, including adenoid cystic carcinoma,

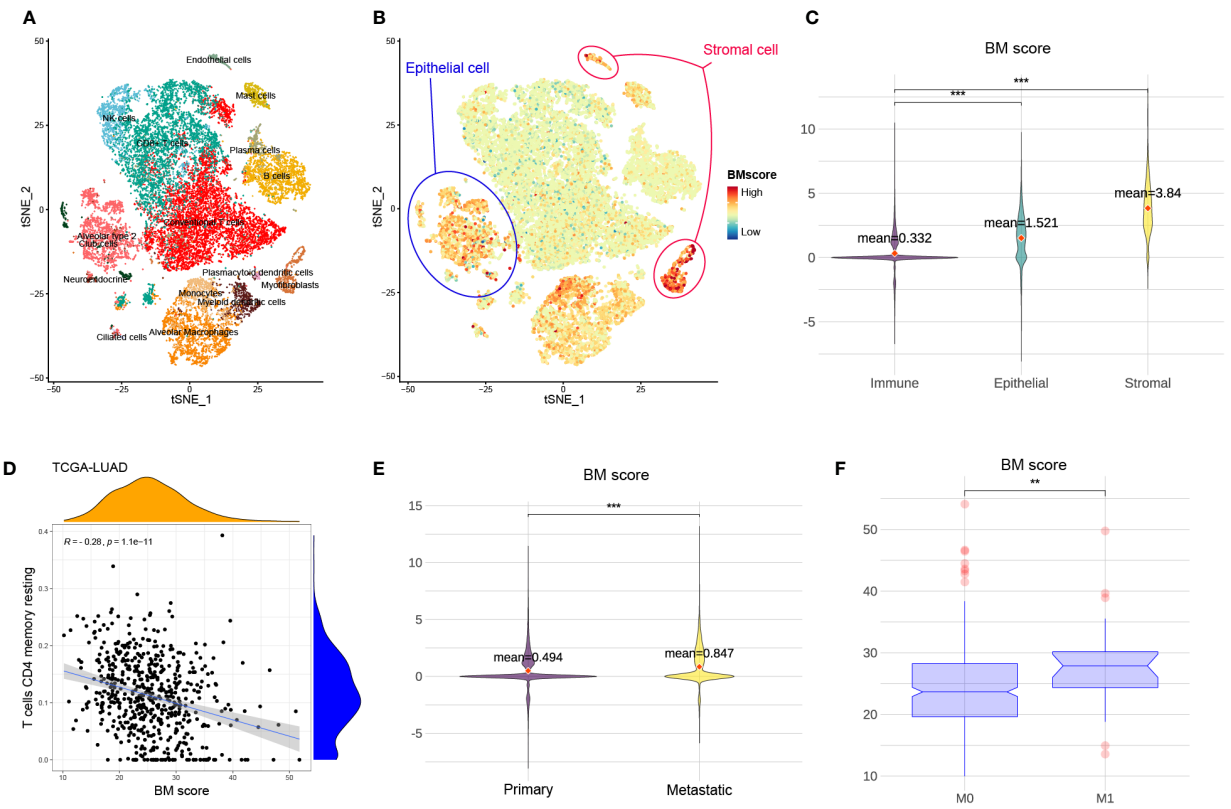

FIGURE 3
The relationship between BM score and different types of cells and tumours. **(A)** t-SNE scatters plot of eight primary and five metastatic LUAD samples with cell annotation. **(B)** The distribution of BM scores at the single cell level. Blue and red circles represent epithelial and stromal cells, respectively. The rest of the scatter plot is filled with immune cells. **(C)** The violin plot demonstrating the difference in BM score among immune, stromal and epithelial cells. And the red dots indicate the average value of BM scores for each group. **(D)** The relationship between BM score and the abundance of T cells CD4 memory resting in TCGA-LUAD cohort. **(E)** The violin plot showing the difference in BM score between primary and metastatic LUAD tumour cells. Further, the red dots indicate the average value of BM scores for each group. **(F)** The distribution of BM score between tumours with non-metastatic (M0) and metastatic (M1). **p<=0.01 and ***p<=0.001, respectively.

mesothelioma, low-grade gliomas, sarcoma, cervical squamous cell carcinoma and head and neck squamous cell carcinoma (Figure S4). Furthermore, Kaplan–Meier overall survival curves of BM-TME classifier in multiple LUAD clinical subtypes were performed (Figures 5C–J). The results revealed that the BM-TME classifier performed effectively in the prognostic prediction of different TNM and stage subtypes of LUAD, which may contribute to validating the general applicability of the BM-TME classifier in predicting prognosis in LUAD.

Different somatic mutation landscapes among BM-TME subgroups

Immune checkpoint therapy provides lasting clinical benefits to oncology patients (43). Thus, the expression patterns of prominent checkpoint genes were further investigated among different BM-TME subgroups. Differential expression was observed among most checkpoint genes in TCGA-LUAD (18/27) and GSE50081 (21/27) cohorts (Figure 6A, S5). Furthermore, the expression of 13 checkpoint genes was downregulated in the "BM_low+TME_high" group compared to those in the "Mixed" and "BM_high+TME_low" groups, such as IDO1, CD274, PDCD1, HAVCR2, and so on. In addition, the "BM_low+TME_high" subgroup exhibited elevated mRNA levels of CD160, BTLA, BTN2A2, BTNL9 and CD47 than the other two subgroups.

Somatic mutations occur and accumulate throughout a person's life. One theory suggests that cancer occurs and develops owing to genetic mutations that accumulate over time (44). We further identified the somatic mutation landscape among different BM-TME subgroups. The top 15 genes in mutation frequency in "BM_low+TME_high" and "BM_high+TME_low" groups are demonstrated with waterfall diagram in Figure 6B. Compared to the "BM_high+TME_low" group, the "BM_low+TME_high" group had a lower frequency

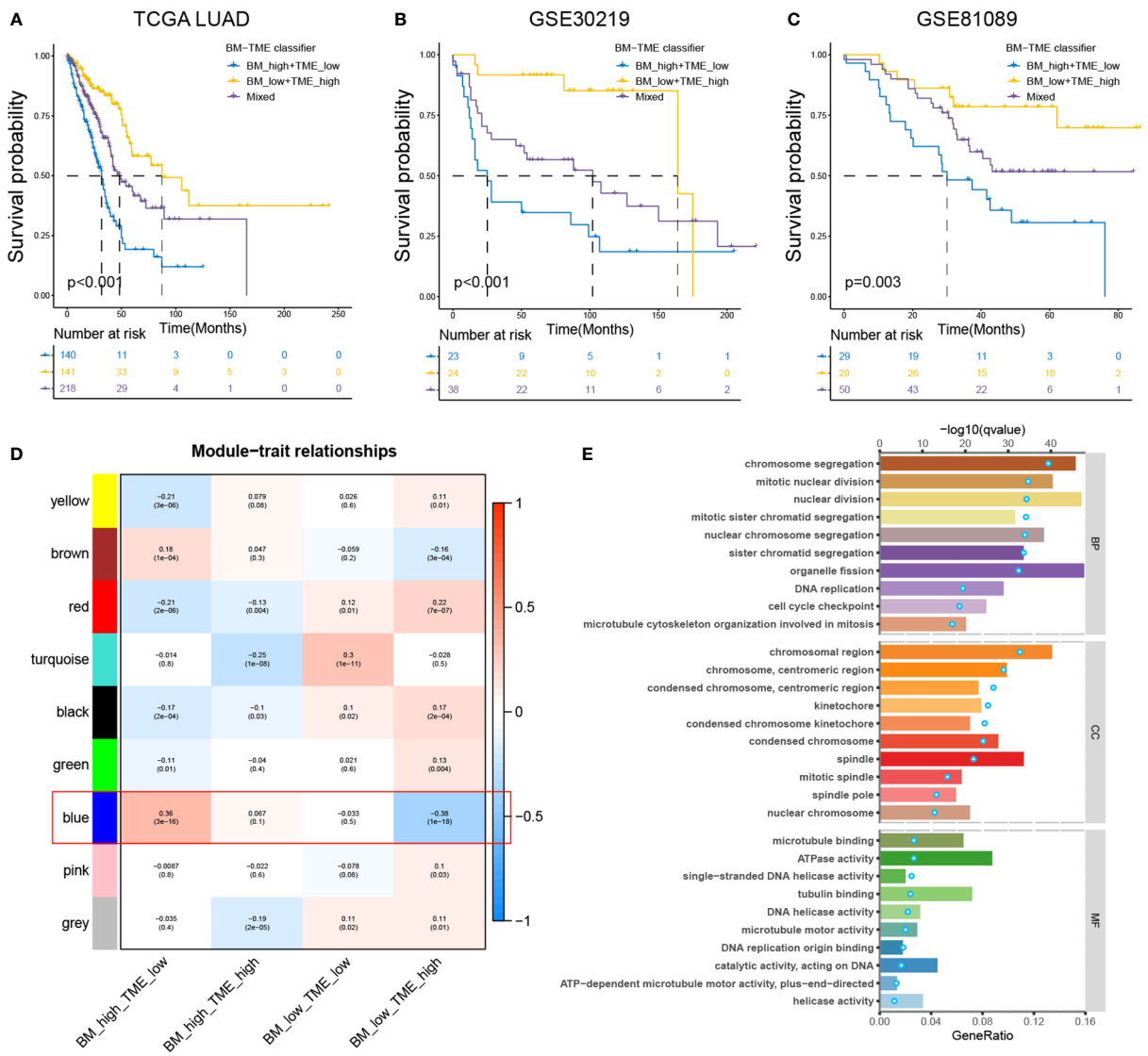

FIGURE 4
Prognostic value and enrichment analysis relevant to BM-TME classifier. **(A–C)** Kaplan–Meier overall survival curves of the training set (TCGA-LUAD cohort) and validation sets (GSE30219 and GSE81089 cohort) based on BM-TME classifier. **(D)** Heat map depicting the correlation between various gene modules of WGCNA analysis and BM-TME subgroups. **(E)** Top ten biological process (BP), cellular component (CC) and molecular function (MF) enrichment pathways based on GO analysis of blue module genes. Bar plots correspond to the lower axis; dot plots correspond to the upper axis.

of both gene mutations and mutations occurring in patients. The mutation frequencies of *tumour protein P53*, *titin*, *cub and sushi multiple domain 3* (*CSMD3*), *low-density lipoprotein receptor-related protein 1B* and SPTA1 were significantly different between these two groups. Besides, "BM_high+TME_low" subgroup patients who had *CSMD3* wildtype may have the worse prognosis (Figure 6C). The "BM_low+TME_high" group had the lowest TMB compared to the other two groups, which corresponded to the results for mutation frequency (Figure 6D).

BM-TME classifier-guided LUAD treatment strategies

Immunotherapy and chemotherapy are currently the most common strategies for cancer treatment. The TIDE module can effectively predict the response to anti-PD1 and anti-CTLA4 therapy in patients with NSCLC (45). We next used TIDE to predict the response of different BM-TME groups to immunotherapy. A diminished BM score and elevated TME score were observed in the responder group compared to those

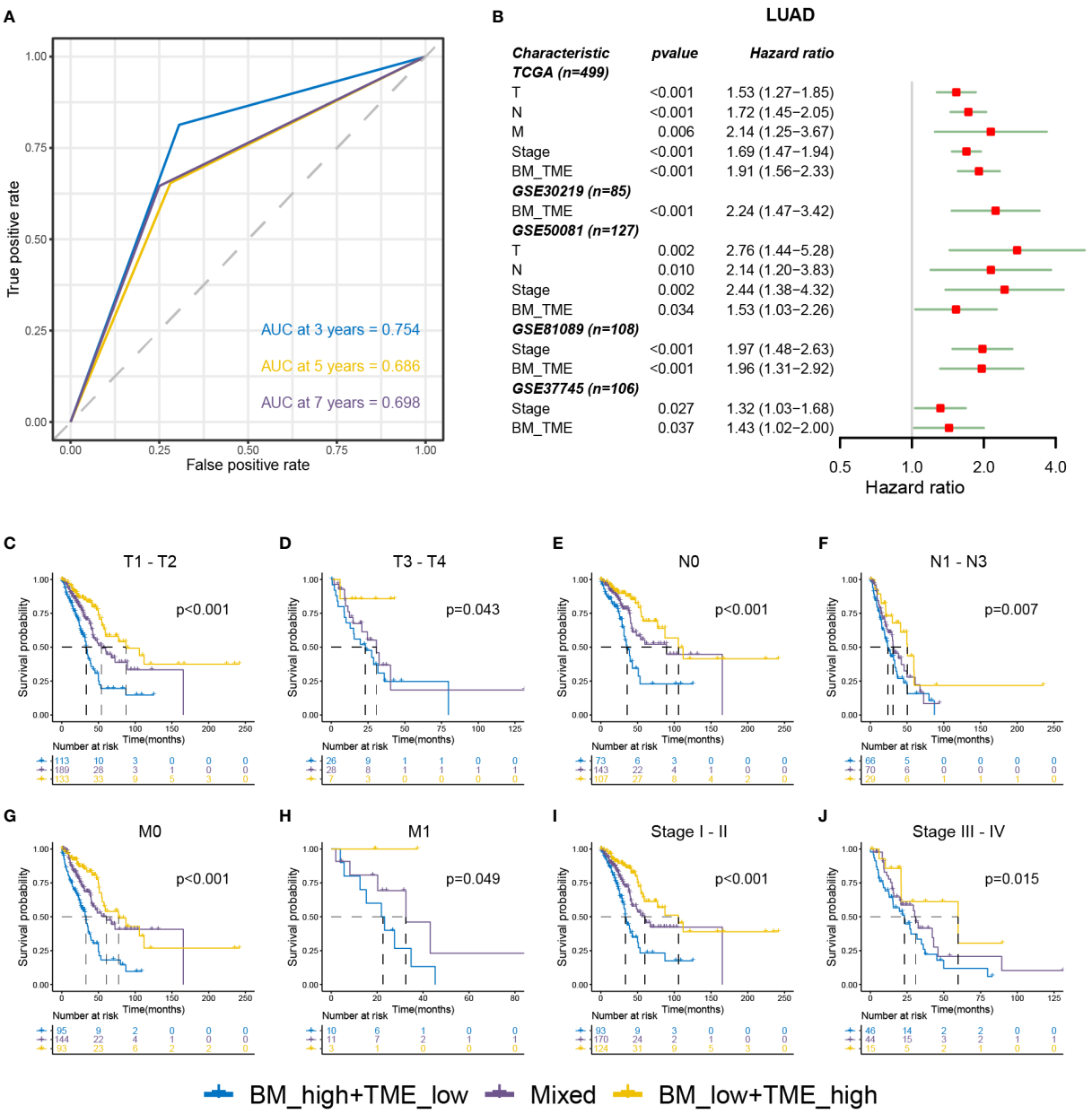

FIGURE 5
Relationships between BM-TME classifier and clinical features in LUAD. **(A)** ROC curves for the 3-, 5- and 7-year overall survival based on the BM-TME classifier in TCGA-LUAD cohort. **(B)** Univariate Cox analysis of clinical characteristics and BM-TME classifier in five LUAD cohorts. **(C–J)** Kaplan–Meier overall survival curves of BM-TME classifier in diverse LUAD clinical subtypes in TCGA-LUAD cohort.

in the non-responder group (Figures 7A, B). Correspondingly, 59%, 34% and 23% of patients in the "BM_low+TME_high", "Mixed" and "BM_high+TME_low" subgroups responded to immunotherapy, respectively (Figure 7C). Additionally, the similar results were discovered in the GSE30219 cohort and another clinical immunotherapy cohort (GSE135222) treated with anti-PD-1/PD-L1 (Figure S6). It was evident that patients in the "BM_low+TME_high" subgroup were more likely to benefit from immunotherapy than those in the other two subgroups. Besides, the proteomaps were used to visually demonstrate and contradistinguish the underlying mechanisms among patients with LUAD in different groups (Figures 7D, E). Interestingly, the proteomaps of "BM_low+TME_high" and responder groups exhibited a considerably high degree of similarity. A similar result was observed between the "BM_high+TME_low" and non-responder groups. This suggests that the BM-TME

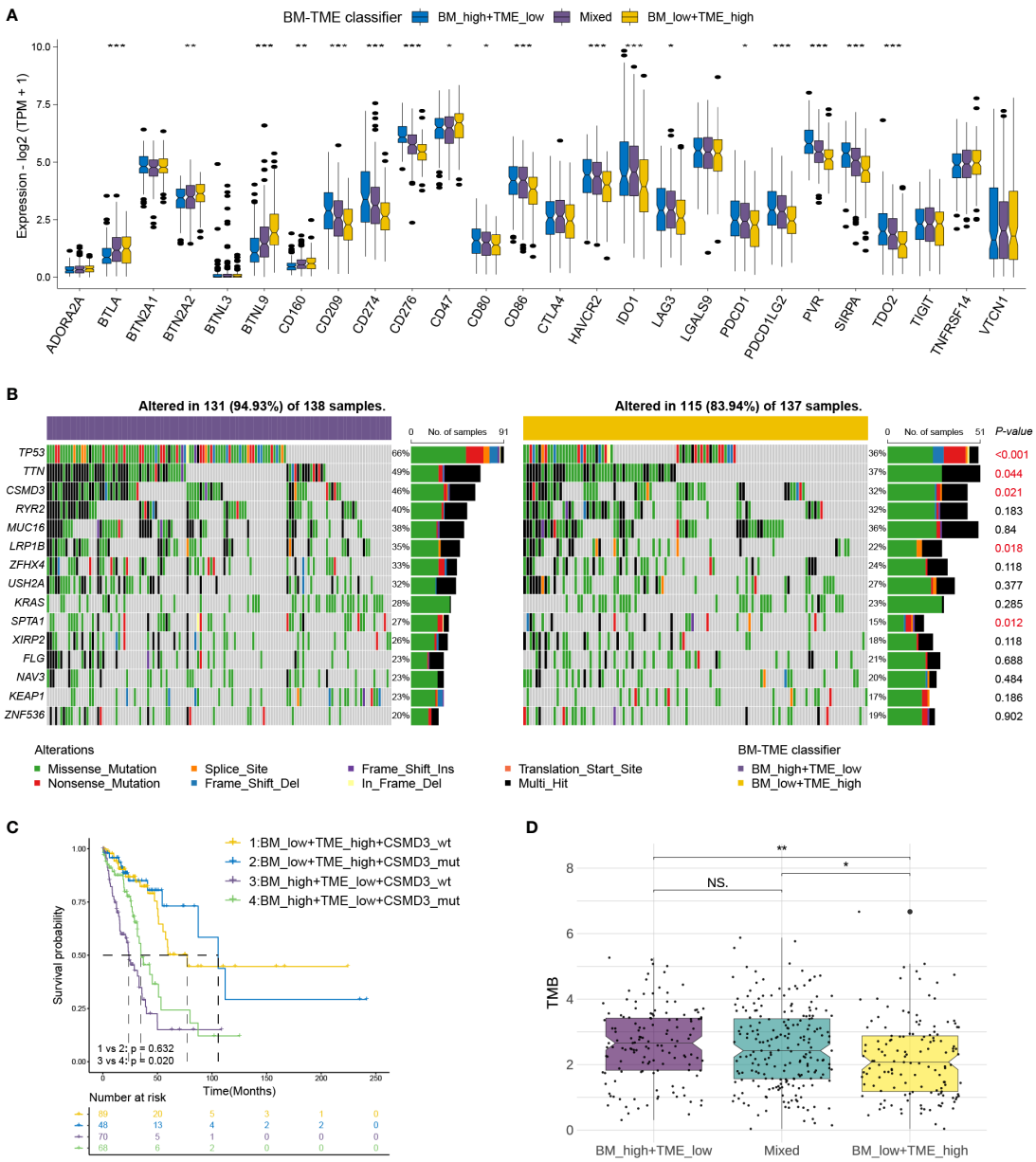

FIGURE 6
Correlations of immune checkpoints and somatic mutation with BM-TME classifier in TCGA-LUAD cohort. **(A)** The differential expression levels of immune checkpoint genes among BM-TME classifier subgroups. **(B)** Waterfall plots depicting the mutation landscape of the top 15 genes with high mutation frequency. *P*-values on the right side displaying the significance of differences in 15 gene mutation frequencies between two BM-TME groups. **(C)** Kaplan–Meier curves of patients with LUAD divided by the *CSMD3* mutation status and BM-TME classifier. **(D)** The distribution of TMB among different BM-TME classifier subgroups. *p<=0.05, **p<=0.01 and ***p<=0.001, respectively. ns, no significance.

classifier can effectively reflect the TME of patients with LUAD and predict the outcome of immunotherapy.

In contrast, sensitivity scores were yielded to predict the IC50 of chemotherapeutic agents using the "oncoPredict" package. As presented in Figures 7F–H, cisplatin, paclitaxel and vinorelbine may be more effective options for "BM_high+TME_low" and "Mixed" subgroups than for the "BM_low+TME_high" subgroup. However, the sensitivity score for cisplatin, which targets the DNA replication pathway, was much higher than those for paclitaxel and vincristine, which target mitosis. In addition, DNA replication and mitotic pathways were enriched in the previous GO function analysis (Figure 4E). These results suggest that chemotherapeutic agents targeting mitosis may be an effective strategy for treating LUAD.

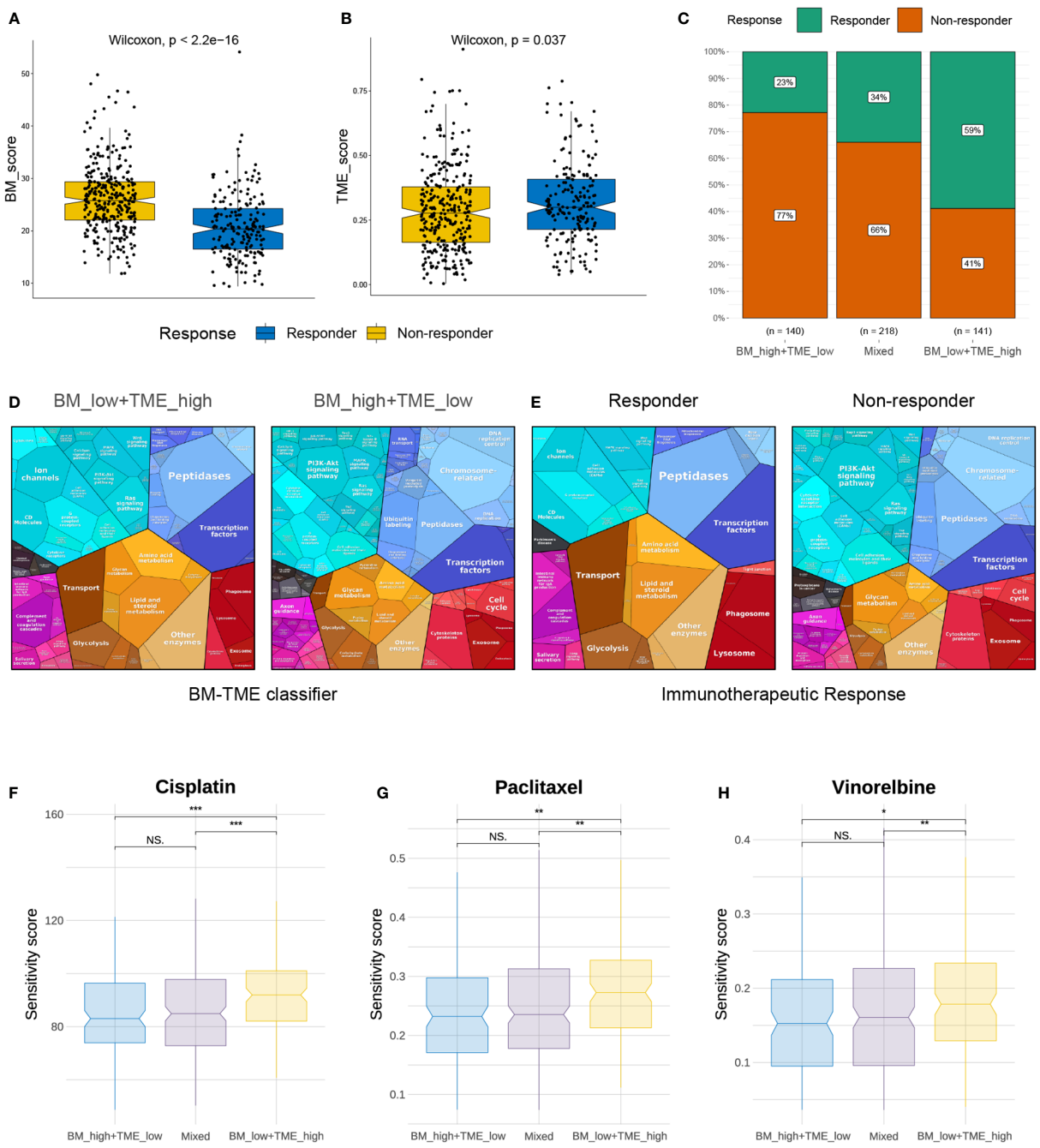

FIGURE 7
Immunotherapy response and chemotherapy drug screening prediction. **(A, B)** Differential distribution of BM score and TME score in immunotherapy response and non-response groups. **(C)** Comparison of immunotherapy responses among different BM-TME classifier groups in TCGA-LUAD cohort. **(D, E)** Proteomaps of the functional analysis results in patients of "BM_low+TME_high", "BM_high+TME_low", immunotherapy responder and non-responder groups. Each KEGG pathway is represented by a polygon, and the size of polygons corresponds to the protein ratio. **(F–H)** Comparison of drug sensitivity to cisplatin, paclitaxel and vinorelbine among different BM-TME subgroups. *p<=0.05, **p<=0.01 and ***p<=0.001, respectively. ns, no significance.

Discussion

BM, a specialised ECM that maintains cell compartmentation and structural integrity, is the predominant barrier that carcinoma cells must consistently breach to form metastases (10, 46). In addition, by weakening T cell activation, BM and its components facilitate tumour progression (20). Based on 20 screened BM genes, the BM score was constructed for prognostic prediction in LUAD. Interestingly, BM score exhibited positive and negative correlations with unfavourable and favourable prognostic factors, respectively, for the 33 prognosis-associated BM genes. Further, elevated BM scores in patients with LUAD implied a poor prognosis, weakened immune cells and an increased risk of developing metastases, revealing that the BM score and its 20 BM genes may be used to describe the status and characteristics of BM in LUAD tumour tissue. Afterwards, the TME score was built based on the presence of the immune cells. GSEA analysis revealed that the cell cycle, the pathway affecting the prognosis of patients with LUAD (47), were associated with both the BM score and TME score, suggesting its important role in the development of LUAD.

Considering the strong interaction between BMs and immune cells, an integrated classifier was established by combining BM score and TME score for comprehensive and accurate prognosis prediction. In six other cancer cohorts and several LUAD cohorts, patients of the "BM_low+TME_high" subgroup exhibited better survival outcomes than those in the other two BM-TME subgroups, demonstrating the universal applicability of the BM-TME classifier in patients with carcinoma. This also implied that patients with various types of cancer shared certain characteristics related to BM and immune infiltration.

GO function enrichment analysis of the gene module that was most relevant to the BM-TME classifier elucidated that its underlying molecular mechanisms predicting prognosis and classification were primarily associated with mitosis and DNA replication processes. LY6K-AS lncRNA and maternal embryonic leucine zipper kinase act as oncogenic molecules by regulating the mitotic process of LUAD cells (48, 49). DEAD-box helicase 59 plays an important role in LUAD development by promoting DNA replication (50). Intriguingly, the commonly used chemotherapeutic agents, vinorelbine, paclitaxel and cisplatin for patients with LUAD target the mitotic and DNA replication pathways, respectively. A study by Gonzalez et al. revealed that a cell cycle-dependent cisplatin-resistant mechanism was associated with mitosis and DNA replication process (51). The aforementioned results revealed that mitosis and DNA replication may serve as promising therapeutic target pathways for LUAD.

The research on somatic mutational signatures with different BM-TME groups remarkably discovered that the expression profile-based BM-TME classifier also reflects DNA heterogeneity. A combination of *CSMD3* mutation status and the BM-TME classifier may cause a better survival prediction. A poor survival outcome was associated with *CSMD3* wildtype, and the same result was discovered in lung squamous cell carcinoma (52). In general, the frequency of mutations in multiple genes was higher in the "BM_high+TME_low" subgroup than in the "BM_low +TME_high" subgroup. Furthermore, higher TMB was observed in patients of the "BM_high+TME_low" subgroup than in those of the "BM_low+TME_high" subgroup. Immune checkpoint disruption can activate the body's natural anti-tumour defence (53, 54), and distinct BM-TME subgroups displayed diverse immune checkpoint expression patterns, indicating that each group may have responded differently to immunotherapy. In addition, 59% of patients in the "BM_low+TME_high" subgroup, 23% of those in the "BM_high+TME_low" subgroup and 34% of those in the "Mixed" subgroup responded to immunotherapy. The similarity between the proteomaps of the "BM_low+TME_high" and immunotherapy response groups reveals certain immune system commonalities between the two patient groups, further demonstrating the efficacy of the BM-TME classifier for directing therapeutic strategies in LUAD.

Overall, we identified the BM score and TME score separately and combined them to establish the BM-TME classifier for LUAD prognostic prediction and treatment strategy guidance. It might be a potential approach for future prognosis estimates and patient stratification for clinical disease management. However, our study had some limitations. First, although the survival prediction value of the BM-TME classifier was validated using several publicly available datasets, there was a dearth of in-house data to fully evaluate its functionality. Second, the classification of patients by the BM-TME classifier was based on the median values of the BM score and TME score for a group of patients. Therefore, we were unable to accurately classify the condition when there was just one patient with LUAD. Similar to the challenges encountered with the definition of the h-TMB cut-offs (55), a significant amount of standardised calibration data is needed for uniform definition in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

Author contributions

XG and CL conceived and designed the study. KC and SL were responsible for the collection and assembly of data, data analysis, interpretation, and the writing of the manuscript. XG and CL provided help in revising the manuscript. Our team is Innovative team of intelligent inspection and active health (ITIH). 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

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Global research landscape and trends of lung cancer immunotherapy: A bibliometric analysis

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Background: Immunotherapy for lung cancer has been a hot research area for years. This bibliometric analysis aims to present the research trends on lung cancer immunotherapy.

Method: On 1 July, 2022, the authors identified 2,941 papers on lung cancer immunotherapy by the Web of Science and extracted their general information and the total number of citations. A bibliometric analysis was carried out to present the research landscape, demonstrate the research trends, and determine the most cited papers (top papers) as well as major journals on lung cancer immunotherapy. After that, recent research hotspots were analyzed based on the latest publications in major journals.

Results: These 2,941 papers were cited a total of 122,467 times. "Nivolumab vs. docetaxel in advanced non–squamous non–small–cell lung cancer" published in 2015 by Borghaei H et al. was the most cited paper (5,854 citations). Among the journals, *New England Journal of Medicine* was most influential. Corresponding authors represented China took part in most articles (904) and papers with corresponding authors from the USA were most cited (139.46 citations per paper). Since 2015, anti–PD–(L)1 has become the hottest research area.

Conclusions: This bibliometric analysis comprehensively and quantitatively presents the research trends and hotspots based on thousands of publications, and further suggests future research directions. Moreover, the results can benefit researchers to select journals and find potential collaborators. This study can help researchers get a comprehensive impression of the research landscape, historical development, and recent hotspots in lung cancer immunotherapy and provide inspiration for further research.

KEYWORDS

bibliometric analysis, lung cancer, PD1/PDL1, clinical trials, pembrolizumab, nivolumab, immunotherapy, tumor mutation burden

Introduction

In recent decades, lung cancer has always become one of the most commonly diagnosed cancers and the leading cause of cancer–related deaths worldwide (1). As a heterogeneous disease, lung cancer is classified as non–small cell lung cancer (NSCLC, ~85%) and small cell lung cancer (SCLC, ~15%) (1). The main treatments for lung cancer involved surgery, radiotherapy, and chemotherapy. In recent years, immune checkpoint inhibitors (ICIs) and targeted therapy have led to a remarkable improvement in the prognosis of patients with lung cancer (2). Programmed cell death 1 (PD–1)/PD1 ligand 1 (PD–L1) interaction is the most frequent target for lung cancer immunotherapy. Blocking this interaction by ICIs leads to increased T–cell activation and enhanced anti–tumor immunity (3). Currently, anti–PD–(L)1 antibodies monotherapy or combined with other therapies have become standard treatments for a large portion of patients with lung cancer, especially patients with advanced lung cancer (1). Lung cancer immunotherapy has been a rapidly growing research area since 2010, with hundreds of articles published every year. It is necessary but challenging for researchers to master research trends and monitor the latest important advances. Therefore, a comprehensive and quantified analysis is required that systemically summarizes important advances, presents the latest research hotspots, and suggests research directions.

Bibliometric analysis is suitable for the comprehensive evaluation of an entire academic discipline including thousands of publications, whereas other major review methods are not (4). Based on the quantitative analysis of structured information from relevant publications, a bibliometric analysis can objectively describe the landscape, research trends, and research hotspots (5). The results are helpful to the researchers in defining the progress of the filed, determining research direction, identifying collaborators, and selecting a target journal for publication (6). Thus, bibliometrics is well suited to quantitatively analyze research trends in lung cancer immunotherapy. In recent years, bibliometric analysis has become increasingly popular in medical research (7, 8). Two bibliometric analyses related to lung cancer immunotherapy have been published (2, 9). However, the first included only papers published before March 2020, which is more than 2 years before the present study was conducted (9). The latter analyzed all articles related to anti–PD–(L)1 for cancer immunotherapy, without limitation of the type of cancer (2). Furthermore, both studies only analyzed the 100 most cited articles, and articles focused on immunotherapy outside of anti–PD–(L)1 were not included. Therefore, a comprehensive, up–to–date, and useful bibliometric analysis of lung cancer immunotherapy is necessary.

The present bibliometric analysis analyzed original articles directly related to clinical immunotherapy for lung cancer published from 2010 to 1 July 2022 and identified the 100 most frequently cited articles (top papers). Furthermore, an additional bibliometric analysis was conducted based on the latest major publications to indicate the latest research hotspots. The objective of this study was to present a comprehensive landscape, research trends, important advances, and current hotspots for researchers. Based on this study, researchers can not only identify key publications, journals, and potential collaborators but may also be stimulated to design more studies.

Methods

Database and paper selection

The Web of Science Core Collection (WoSCC) is one of the most frequently used databases for bibliometric analysis, including more than 10,000 high–quality journals and comprehensive citation records (2). Moreover, the WoSCC document type labels have been shown to be more precise than other databases such as Scopus (10). In this study, we selected the WoSCC Science Citation Index Expanded database for the literature search.

The workflow of this study was shown in (Figure 1). A literature search was conducted on 1 July 2022 for original research articles on lung cancer immunotherapy published since 2010. The authors designed the search strategy as follows to include as many relevant papers as possible while excluding irrelevant papers: 1) keywords were searched only in titles, because some irrelevant papers may contain the search keywords in abstracts; 2) the keywords were “lung cancer” and “immunotherapy”; 3) synonyms of keywords were included as much as possible, and synonyms of “immunotherapy” included specific names of drugs or treatments; 4) papers outside of original studies were excluded; 5) papers containing the term ‘lung metastasis’ were excluded, because these papers were not related to primary lung cancer. The authors performed multiple tests and modifications to ensure the sensitivity and specificity of the search strategy. The detailed search strategy is presented in Supplementary Material S1.

The relevant articles were then identified, and the following information was extracted: title; abstract; keywords; authors; publication time; journal; countries/regions; institutions; the total number of citations; and average number of citations per year (calculated as the number of citations per month × 12). The authors then ranked the papers with the number of citations to identify the 100 top papers.

Statistical analysis

Microsoft Office Excel 2019 software (Microsoft, Redmond, WA, USA) was used for descriptive statistical analysis,

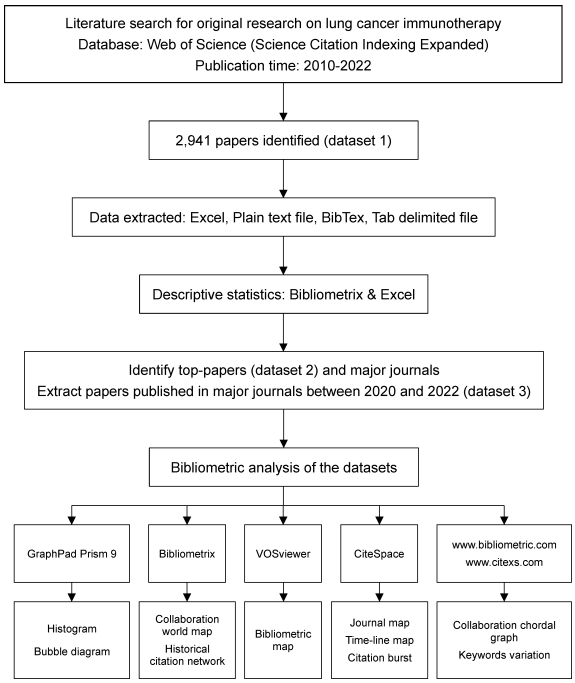

FIGURE 1
The workflow of the present study.

correlation test, and to produce tables. The GraphPad Prism 9 software (Dotmatics, San Diego, CA, USA) was used for plot histograms and bubble diagrams. The "bibliometrix" package was an open–source tool for performing comprehensive science mapping analysis (11). Bibliometrix of R software (v4.1.2) was used for bibliometric analysis and data visualization. VOSviewer (Leiden University, Netherlands) was a software for constructing and viewing bibliometric maps and could display large bibliometric maps in an easy–to–interpret way (12). VOSviewer (v1.6.17) was used to construct bibliographic coupling networks of journals, countries, coauthors, and keywords. Using a customized VOSviewer thesaurus file, the authors merged the synonyms and different derivatives of keywords, countries, and coauthors to better present the networks. The words in VOSviewer networks defaulted to lowercase letters, and the author capitalized some letters to standardize writing. An online platform (https://bibliometric.com) was used to visualize cooperation between countries/regions, and another online platform (https://www.citexs.com) was used to visualize the trends of keyword frequencies. CiteSpace software (v6.1.R1) was used to detect keywords and references with the strongest citation bursts, to construct visualization maps of co–cited references and keywords, and to plot a dual–map overlay of journals. To indicate and visualize the research trends in lung cancer immunotherapy from 2010 to 2022, the authors classified the articles by searching for specific therapies and treatment lines in titles and abstracts. Microsoft Visual Basic for Applications was used to perform a macro for data arrangement and batch retrieval.

To determine whether papers published in highly indexed journals were more cited, the authors conducted a correlation test between average citation per paper per year and (1) 5–year impact factor (IF) and (2) Journal Citation Index (JCI) of journals with a 5–year IF > 5 published at least two papers on lung cancer immunotherapy.

The journals that published the top papers were identified, and their top papers rates (TPR, the percentage of top papers among all relevant papers in a journal) were calculated. Journals with a TPR >5% were considered the top journals on lung cancer immunotherapy. The papers on lung cancer immunotherapy published in top journals since 2020 were identified and analyzed to evaluate recent research hotspots.

Results

The literature search yielded 2,941 original articles on lung cancer immunotherapy published between 2010 and 1 July 2022 (Figure 2A). In recent years, the number of articles published each year has grown rapidly. More than 90% of the articles were published after 2015, while more than 50% of the articles were

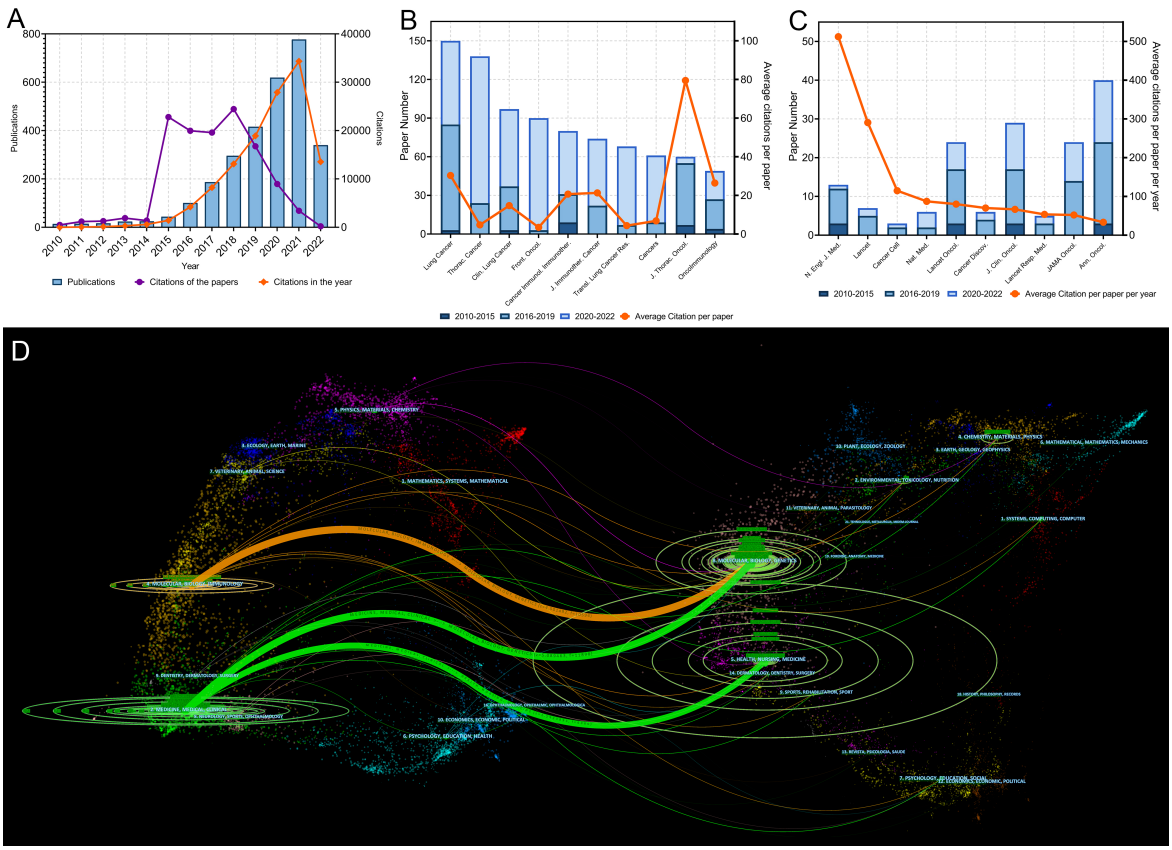

FIGURE 2
(A) Publication and citation number from 2010 to 2022 of the papers on lung cancer immunotherapy. The purple line indicates the total citations of papers published each year. The orange line indicates the total citations of all papers each year. **(B)** Paper numbers and average citations per paper of the top-10 productive journals. **(C)** Top-10 journals with the most citations per paper per year. **(D)** The dual-map overlay of journal categories. The left nodes represent citing journals and the right nodes represent cited journals. The curves represent the citation relationship.

published after 2019. These articles were cited 122,467 times and the median number of citations was 7. Although only 44 papers were published in 2015, they were cited 22,806 times. To present the citation relationship among the key–node papers, a historical direct–citation network was plotted (Supplementary Figure S1). The 25 main references with the strongest citation bursts are listed in Supplementary Figure S2. The bibliographic coupling network of the most co–cited references is shown in Supplementary Figure S3.

The authors ranked the articles with the citation number and identified 100 top papers (Supplementary Table S1). The authors also identified the 100 papers with highest citation per year (Supplementary Table S2). The top papers were cited 76,556 times, which was 62.5% of the number of articles cited on lung cancer immunotherapy. The median number of citations in the top papers was 383.5 (range: 165–5,854). "Nivolumab versus docetaxel in advanced non–squamous non–small–cell lung cancer" published in 2015 by Borghaei et al. had the highest number of citations (5,854) and the second highest average number of citations per year (878.1) (13). "Pembrolizumab versus chemotherapy for PD–L1–positive non–small–cell lung cancer" published in 2016 by Reck et al. had the second highest number of citations (5,287) and the highest average number of citations per year (926.9) (14). Among the 10 most cited articles, 7 were published in the *New England Journal of Medicine* (*N Engl J Med*) (Table 1). Most of the top papers (66 papers) were published between 2016 and 2018. Only 3 top papers were published in 2020 or 2021. "First–line nivolumab plus ipilimumab combined with two cycles of chemotherapy in patients with non–small–cell lung cancer (CheckMate 9LA): an international, randomised, open–label, phase 3 trial" published in 2021 by Paz–Ares et al. was the latest top paper, which had a citation number of 215 and an average citation per year of 161.3 (15).

TABLE 1 The 10 most cited papers in lung cancer immunotherapy from 2010 to 2022[a].

Rank	Title	CorrespondingAuthor	Journal	Year	Total citations	Average citations per year (rank)
1	Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer	Borghaei H	N Engl J Med	2015	5854	878.1 (2)
2	Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer	Brahmer JR	N Engl J Med	2016	5287	946.9 (1)
3	Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer	Chan TA	Science	2015	4908	684.8 (4)
4	Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer	Brahmer J	N Engl J Med	2015	4612	666.8 (5)
5	Pembrolizumab for the Treatment of Non-Small-Cell Lung Cancer	Garon EB	N Engl J Med	2015	3833	541.1 (6)
6	Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial	Herbst RS	Lancet	2016	3217	521.7 (7)
7	Pembrolizumab plus Chemotherapy in Metastatic Non-Small-Cell Lung Cancer	Gandhi L	N Engl J Med	2018	2827	692.3 (3)
8	Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial	Gandara DR	Lancet	2017	2647	488.7 (8)
9	Durvalumab after Chemoradiotherapy in Stage III Non-Small-Cell Lung Cancer	Antonia SJ	N Engl J Med	2017	2002	436.8 (10)
10	Pembrolizumab plus Chemotherapy for Squamous Non-Small-Cell Lung Cancer	Paz-Ares L	N Engl J Med	2018	1568	437.6 (9)

[a]These ten papers were all published on N Engl J Med.

Journals

A total of 496 journals published original articles on lung cancer immunotherapy. Among them, *Lung Cancer* (150 papers), *Thoracic Cancer* (138 papers), and *Clinical Lung Cancer* (97 papers) were the three main journals with the most articles (Figure 2B). Among the top 10 productive journals, the *Journal of Thoracic Oncology* had the highest average number of citations per article (79.47), the average number of citations per article per year (18.80) and IF (20.121), which indicated that it was not only productive but also influential (Table 2).

In particular, the top 10 journals with the highest citations per paper per year differed markedly from the most productive journals (Figure 2C and Table 3). Among them, the *Annals of Oncology* was the most productive (40 papers). Among all journals, the *N Engl J Med* had the highest total number of citations (31,845), average citation per paper (2,449.62), average citations per paper per year (512.22), and local citations (citation

TABLE 2 The top 10 productive journals in lung cancer immunotherapy from 2010 to 2022.

Journals	Paper number	Total citation	Citation per paper	Citation per paper per year[a]	H-index	G-index	IF (2021)
Lung Cancer	150	4543	30.29	9.07	36	60	6.081
Thorac. Cancer	138	652	4.72	2.84	14	4	3.223
Clin. Lung Cancer	97	1427	14.71	5.49	19	34	4.840
Front. Oncol.	90	312	3.47	2.63	9	12	5.738
Cancer Immunol. Immunother.	80	1654	20.68	8.47	22	39	6.630
J. Immunother. Cancer	74	1575	21.28	8.61	22	37	12.469
Transl. Lung Cancer Res.	68	296	4.35	2.79	9	14	4.726
Cancers	61	417	6.84	4.23	13	18	6.575
J. Thorac. Oncol.	60	4768	79.47	18.80	40	60	20.121
OncoImmunology	49	1293	26.39	6.69	19	35	7.723

[a]Papers published in 2022 were not included for calculating citation per paper per year.

TABLE 3 The top 10 journals with highest citations per paper per year in lung cancer immunotherapy from 2010 to 2022[a].

Journals	Paper number	Top paper number	Top paper rate	Total citation	Citation per paper	Citation per paper per year[b]	Local citation[c]	IF (2021)
N Engl J Med	13	12	92.31%	31845	2449.62	512.22	8266	176.079
Lancet	7	5	71.43%	9293	1327.57	290.55	2475	202.731
Cancer Cell	3	2	66.67%	941	313.67	114.86	536	38.585
Nat. Med.	6	2	33.33%	1043	173.83	87.33	913	87.241
Lancet Oncol.	24	15	62.50%	7948	331.17	80.08	2218	54.433
Cancer Discov.	6	3	50.00%	1598	266.33	70.19	709	38.272
J Clin Oncol	29	16	55.17%	6926	238.83	66.69	5272	50.717
Lancet Resp. Med.	5	1	20.00%	706	141.20	53.74	140	102.642
JAMA Oncol.	24	9	37.50%	4118	171.58	52.09	1399	33.006
Ann. Oncol.	40	8	20.00%	4668	116.70	33.38	2744	51.769

[a]Only journals with more than one paper were included.
[b]Papers published in 2022 were not included for calculating citation per paper per year.
[c]Citation number in the current dataset (papers in lung cancer immunotherapy from 2010 to 2022).

number in the current dataset) (8266), which were much higher than others. *N Engl J Med* only published 13 articles on lung cancer immunotherapy, but these articles accounted for 26.00% citations of all articles in this area. Furthermore, *Journal of Clinical Oncology* (*J Clin Oncol*) had high number of local citations (5,272), which indicated that it was highly influential on lung cancer immunotherapy.

A dual map overlay showed the distribution of academic discipline and the citation relationship of journals related to lung cancer immunotherapy (Figure 2D). In the citation relationship indicated by the colored curve, the citing journals are on the left and the cited journals are on the right. This map revealed three primary citation relationship pathways, meaning that papers on molecular/biology/genetics were primarily cited by papers on molecular/biology/immunology and medicine/medical/clinical studies, while papers in health/nursing/medicine were primarily cited by papers in medicine/medical/clinical. The bibliographic coupling network of journals related to lung cancer immunotherapy was conducted (Figure 3A).

The authors identified 111 journals with a 5–year IF > 5 published at least two papers on lung cancer immunotherapy. The correlation test showed that the correlation coefficients between average citation per paper per year and (1) 5–year IF and (2) JCI were 0.893 and 0.887, respectively. Therefore, papers published in highly indexed journals are more likely to be highly cited. However, the authors found that although some journals had high IF, the papers published in these journals were not highly cited. For example, *Sci. Adv.* (5–year IF=16.895) had an average citation per paper per year of only 0.923.

The 100 top papers on lung cancer immunotherapy were published in 27 journals (Supplementary Table S3). The bibliographic coupling network of these journals was conducted (Figure 3B). *J Clin Oncol* (16 papers), *Lancet Oncol.* (15 papers), and *N Engl J Med* (12 papers) were the top three journals with the highest number of papers. The top paper rates (TPR) of the 27 journals were calculated. For journals that published at least two top papers, *N Engl J Med* had the highest TPR (92.31%), followed by *Lancet* (71.43%), *Cancer Cell* (66.67%), *Lancet Oncology* (62.5%) and *J Clin Oncol* (55.17%). The articles on lung cancer immunotherapy published in these journals are highly likely to be top papers. Among the 27 journals, 22 with a TPR >5% were considered the major journals on lung cancer immunotherapy. Since 2020, a total of 110 articles have been published in the major journals (Supplementary Table S4). The three major journals with the most publications between 2020 and 1 July 2022 were *Annals of Oncology* (16 papers), *Clinical Cancer Research* (12 papers), and *J Clin Oncol* (12 papers).

Countries/regions

Researchers from 75 countries/regions contributed to the 2,941 original articles on lung cancer immunotherapy. A network visualization map presented the collaboration relationship and the average publication year of the countries/regions (Figure 3C). However, the corresponding authors only represent 51 of the countries/regions. The corresponding authors from China contributed the most publications (904 papers), followed by the corresponding authors from the United States (536 papers) and Japan (496 papers) (Table 4 and Figure 4A). However, papers by corresponding authors from the United States were cited as high as 74,751 times, with an average citation per paper of 139.46, which was much higher than in other countries/regions. Most studies were conducted by authors from single countries. International collaboration was more common in North American or European countries than in Asian countries. The rate of multiple–country papers in Japan

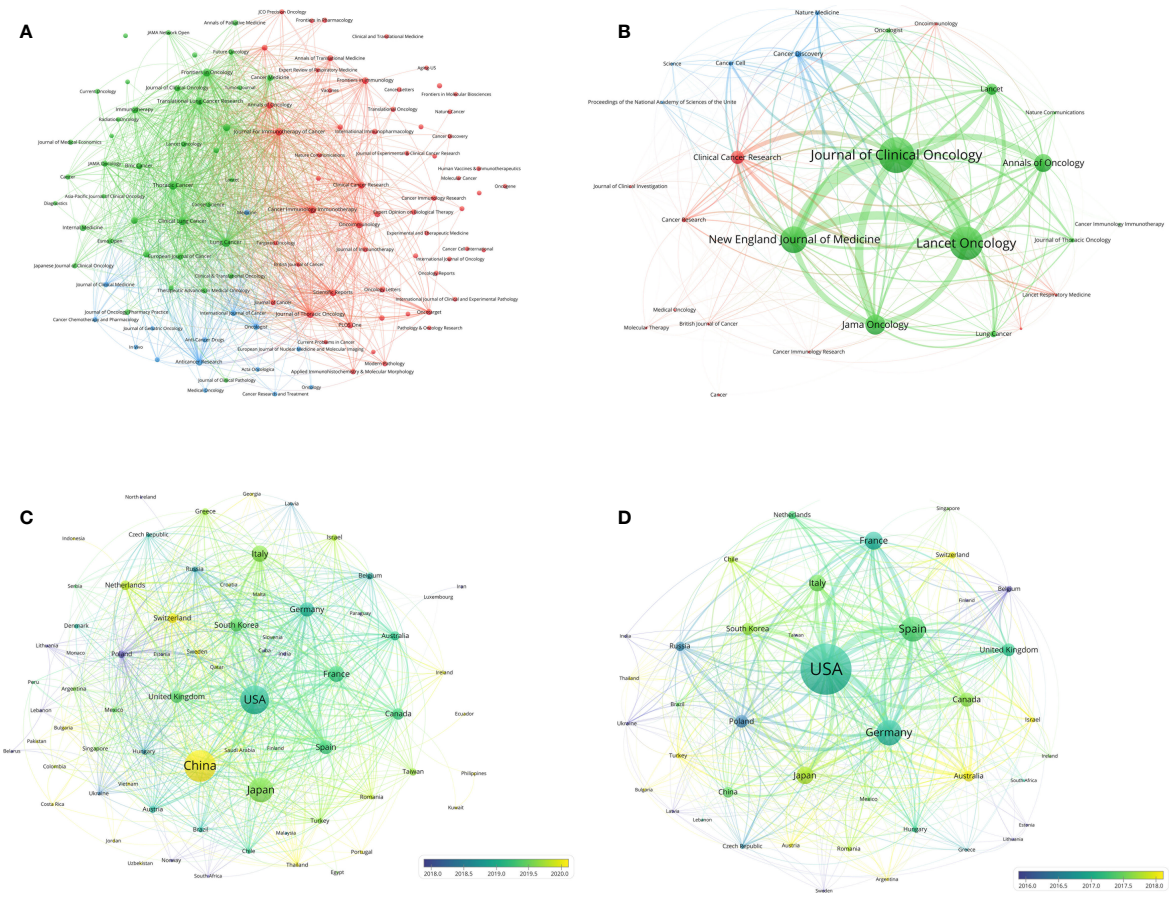

FIGURE 3
(A) Bibliographic coupling of journals with at least five papers related to lung cancer immunotherapy. **(B)** Bibliographic coupling of journals with top papers related to lung cancer immunotherapy. **(C)** Network visualization of countries with papers related to lung cancer immunotherapy. **(D)** Network visualization of countries with top papers related to lung cancer immunotherapy. The circle size represents the number of papers. The breadth of the curves represents the connection strength. The journals in the same color are of similar research areas.

TABLE 4 The top 10 productive countries of corresponding authors of papers in lung cancer immunotherapy from 2010 to 2022.

Countries	Paper number	Percentage (N/2941)	Multiple-country paper rate[b]	Total citation	Citation per paper	Top paper number[a]	Multiple-country top-paper rate
China	904	30.74%	12.39%	11127	12.31	5	20.0%
USA	536	18.23%	33.02%	74751	139.46	61	65.6%
Japan	496	16.87%	3.43%	9383	18.92	7	14.3%
Italy	172	5.85%	21.51%	4240	24.65	5	60.0%
France	149	5.07%	28.86%	4177	28.03	4	100.0%
Korea	114	3.88%	9.65%	2403	21.08	1	100.0%
Germany	79	2.69%	43.04%	2866	36.28	4	100.0%
Spain	63	2.14%	42.86%	5011	79.54	7	100.0%
Canada	52	1.77%	40.38%	1623	31.21	2	100.0%
Netherlands	45	1.53%	37.78%	1155	25.67	2	50.0%

[a]Besides the countries mentioned above, corresponding authors from Belgium and Switzerland contributed one top-paper each.
[b]Percentage of multiple-country top papers among all papers of a country.

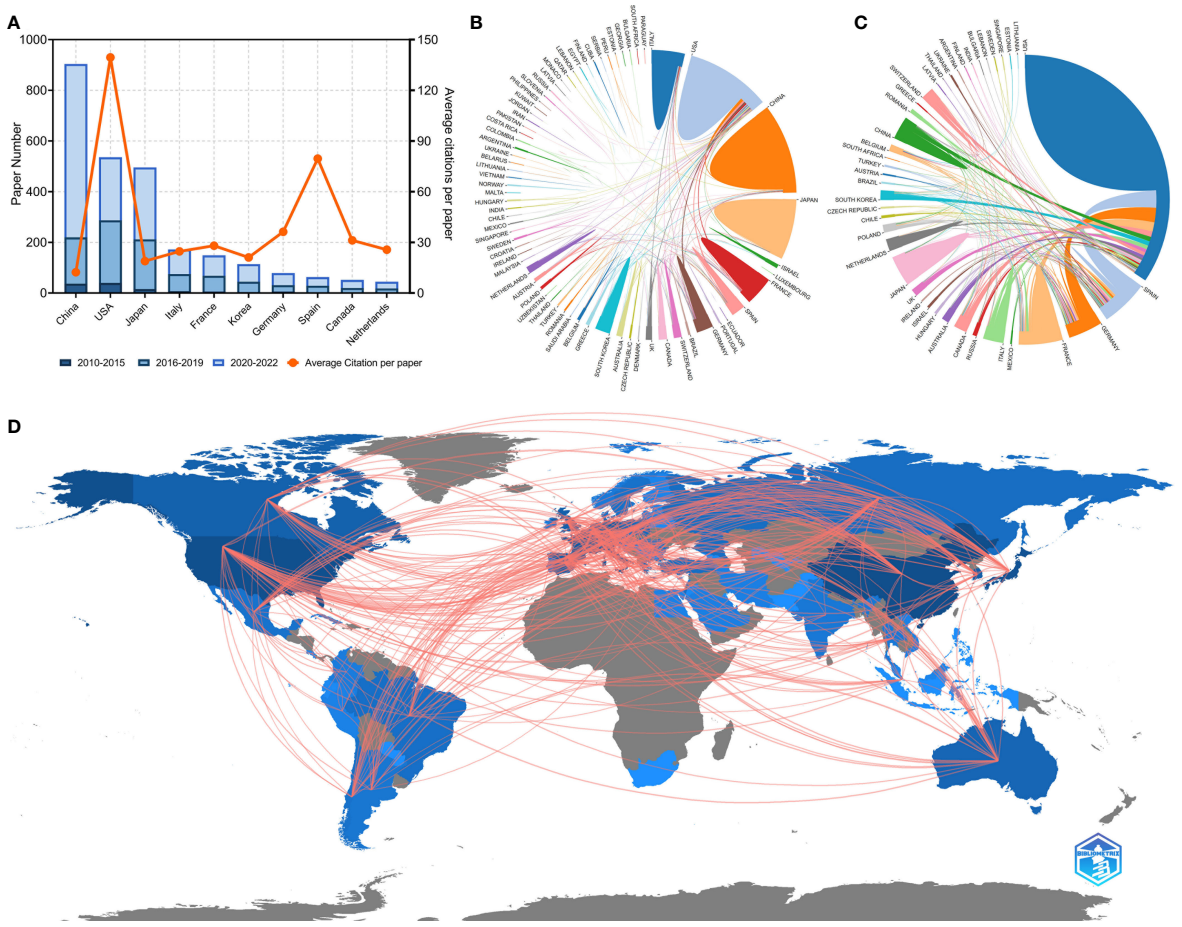

FIGURE 4
(A) Paper number and average citations of corresponding authors' countries. MCP, multiple-country publications; SCP, single-country publications. **(B)** Network mapping of international collaboration base on 2941 papers related to lung cancer immunotherapy. **(C)** Network mapping of international collaboration base on 100 top papers related to lung cancer immunotherapy. **(D)** Visualization world map of publications and collaboration relationship.

(3.42%) was the lowest among the most productive countries. A chordal graph and a collaborative network world map showed the collaboration between countries/regions (Figures 4B, D). The United States collaborated with most countries/regions in this research area. Most studies supported by developing countries/regions were published more recently than those of developed countries.

The 100 top papers were published by authors from 42 countries/regions and corresponding authors from 12 countries/regions. A network visualization map presented the collaboration relationship and average publication year of the countries/regions with the top papers (Figure 3D). The corresponding authors of most of the top papers (61 papers) represented the United States. International collaboration was more common in the top papers than in all the papers on lung cancer immunotherapy (Figure 4C). International collaboration, however, remained rare in China and Japan, which had only one top paper with foreign authors.

Institutions

The authors of the 2,941 papers represented 4,296 institutions. The University of Texas MD Anderson Cancer Center contributed most articles (224 papers) among institutions (Table 5). Seven of the 10 most productive institutions were in China and the other 3 were in the United States. A collaboration network and a cluster analysis of the institutions were conducted (Figure 5A). Most institutions preferred domestic collaboration over international collaboration. International collaboration was common between the institutions with the strongest research strength in their countries.

TABLE 5 The top 10 institutions with the most papers or top papers on lung cancer immunotherapy from 2010 to 2022.

Institutions	Country	Paper number[a]	Percentage (N/2941, %)	Top paper number	Top paper rate	Top paper number rank
Univ Texas Md Anderson Canc Ctr	USA	224	7.62%	14	6.25%	8
Tongji Univ	China	204	6.94%	0	0.00%	N/A
Fudan Univ	China	193	6.56%	0	0.00%	N/A
Sun Yat Sen Univ	China	190	6.46%	1	0.53%	214
Shanghai Jiao Tong Univ	China	182	6.19%	2	1.10%	124
Sichuan Univ	China	153	5.20%	0	0.00%	N/A
Chinese Acad Med Sci and Peking Union Med Coll	China	147	5.00%	0	0.00%	N/A
Mem Sloan Kettering Canc Ctr	USA	138	4.69%	48	34.78%	1
Natl Canc Ctr	China	116	3.94%	6	5.17%	36
Dana Farber Canc Inst	USA	115	3.91%	16	13.91%	5
H Lee Moffitt Canc Ctr and Res Inst	USA	102	3.47%	24	23.53%	3
Johns Hopkins Univ	USA	93	3.16%	19	20.43%	4
Yale Univ	USA	78	2.65%	33	42.31%	2
Univ Calif Los Angeles	USA	68	2.31%	15	22.06%	7
Sarah Cannon Res Inst	UK	47	1.60%	16	34.04%	5
German Ctr Lung Res	Germany	35	1.19%	14	40.00%	8

[a]All papers were included, without limitation of corresponding author's institutions. NA, Not applicable.

A total of 583 institutions contributed to top papers. The three leading productive institutions of the top papers were the Memorial Sloan–Kettering Cancer Center (48 papers), Yale University (33 papers), and the H Lee Moffitt Cancer Center and Research Institution (24 papers). In particular, Yale University had the highest TPR (42.31%). Although some institutions in China contributed to a large number of papers, their number of top papers was low. A collaboration network and cluster analysis of the institutions with top papers was performed (Figure 5B). Compared to the clusters in Figure 5A, the clusters of the institutions with top papers had more obscure boundaries. Collaboration between institutions with top papers was common and less restricted by geographical factors.

Authors

A total of 15,017 researchers contributed to the 2,941 original articles on lung cancer immunotherapy. Reck M was the most cited author in this area (44 papers, 22,380 citations), followed by Hellmann MD (35 papers, 18,839 citations) and Paz–Ares L (25 papers, 18,588 citations) (Table 6). Notably, although only published 6 papers in this area, Lubiniecki GM was the sixth cited author (15,257 citations). Reck M and Brahmer JR published their first papers in 2012 and 2013, respectively, which indicated that they joined this research area early. Other eight most cited authors published their first papers in 2015. A collaboration network and clustering analysis of the coauthors was conducted (Figure 5C). The authors of China and Japan preferred to establish stable collaborations with researchers in their own countries rather than with foreign researchers.

The analysis of corresponding authors might highlight the main contributors to the articles. A total of 2,005 corresponding authors were identified. As corresponding author, Zhang L contributed to most papers (16 papers), but these papers were only cited 450 times (Table 7). Hellmann MD was corresponding author for 14 papers (5,394 citations) and Reck M was corresponding author for 12 papers (2,093 citations). Brahmer JR was the most cited corresponding author with 4 papers (9,966 citations). Notably, Hellmann MD was the only one who was both one of the ten most productive and cited corresponding authors.

A total of 1,498 authors contributed to the 100 top papers. Reck M and Hellmann MD was the most productive authors of the top papers (17 papers each), followed by Horn L (13 papers). A collaboration network and clustering analysis of the coauthors of the top papers was conducted (Figure 5D). International collaboration between authors was common and some close partnerships between Asian researchers and American or European researchers were revealed. Fourteen corresponding authors contributed to at least two top papers (Supplementary Table S5). Hellmann MD was the most productive corresponding author (7 papers), followed by Rizvi NA and Reck M (4 papers each).

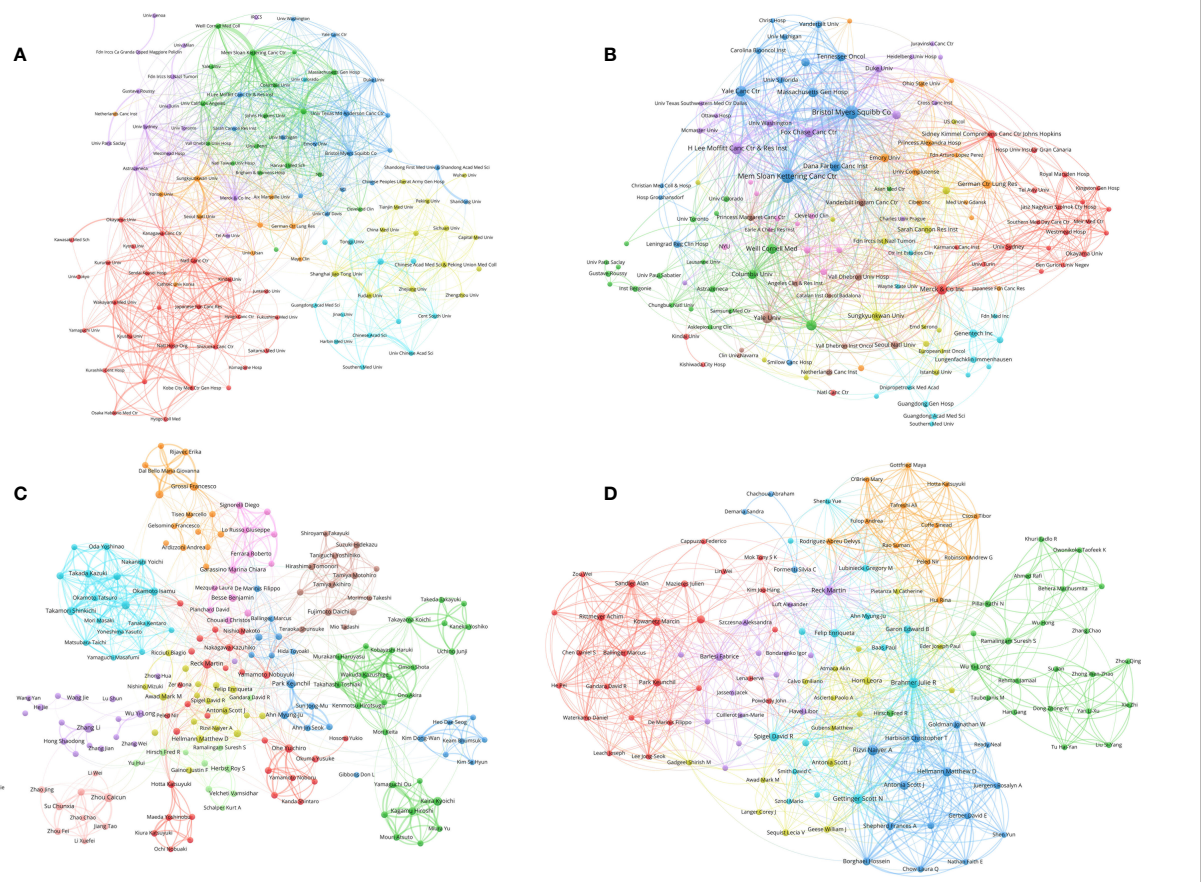

FIGURE 5
(A) Network visualization of institutions with at least 15 papers related to lung cancer immunotherapy. **(B)** Network visualization of institutions with at least 2 top papers related to lung cancer immunotherapy. **(C)** Network visualization of authors with at least 10 papers related to lung cancer immunotherapy. **(D)** Network visualization of authors with at least 2 top papers related to lung cancer immunotherapy. The circle size represents the number of papers. The breadth of the curves represents the connection strength. The institutions in the same color have stronger collaboration with each other.

TABLE 6 Top 10 most cited authors related to lung cancer immunotherapy from 2010 to 2022.

Name	Paper number	Total citation	H-index	Average citations per paper	Articles fractionalized[a]	Top paper number	Top paper rate	First publication year
Reck M	44	22380	29	508.64	4.52	17	38.64%	2012
Hellmann MD	35	18839	32	538.26	2.38	17	48.57%	2015
Paz-Ares L	25	18588	19	743.52	1.88	11	44.00%	2015
Horn L	25	17583	24	703.32	1.66	13	52.00%	2015
Garon EB	22	17190	19	781.36	1.8	8	36.36%	2015
Lubiniecki GM	6	15257	6	2542.83	0.34	5	83.33%	2015
Brahmer JR	21	15116	19	719.81	1.91	6	28.57%	2013
Felip E	24	13763	21	573.46	1.27	10	41.67%	2015
Rizvi NA	17	13704	16	806.12	1.42	12	70.59%	2015
Spigel DR	17	12660	15	744.71	1.31	7	41.18%	2015

[a]Articles Fractionalized = paper number/total number of authors of the papers.

TABLE 7 The top 10 productive and cited corresponding authors in lung cancer immunotherapy from 2010 to 2022.

Most productive corresponding author	Paper number	Total citation	Average citations per paper	Top paper number	Most cited corresponding author	Paper number	Total citation	Average citations per paper	Top paper number
Zhang L	16	450	28.13	0	Brahmer JR	4	9966	2491.50	2
Hellmann MD	14	5394	385.29	7	Borghaei H	5	6057	1211.40	1
Reck M	12	2093	174.42	4	Hellmann MD	14	5394	385.29	7
Fujimoto D	12	551	45.92	1	Chan TA	1	4908	4908.00	1
Wu YL	12	793	66.08	2	Garon EB	8	4280	535.00	2
Takada K	11	171	15.55	0	Herbst RS	3	3441	1147.00	1
Yamada T	11	125	11.36	0	Gandhi L	1	2827	2827.00	1
Awad MM	10	440	44.00	0	Gandara DR	1	2647	2647.00	1
Zhou CC	12	363	30.25	0	Paz-Ares L	6	2483	413.83	3
Kaira K	9	128	14.22	0	Rizvi NA	5	2103	420.60	4

Keywords

Based on author–chose keywords and keyword–plus identified by WoSCC, the hot keywords were analyzed in multiple dimensions. The trends and variation of keyword occurrence frequencies in lung cancer immunotherapy from 2010 to 2022 were analyzed and visualized (Supplementary Figure S4). "Tumor microenvironment", "radiotherapy", "biomarker", and "immune–related adverse events (irAEs)" are recently rising keywords. The top 25 keywords with the strongest citation bursts were identified (Supplementary Figure S5). Vaccine and adoptive cell immunotherapy (ACT) used to be research hotspots, and ICIs became new hotspots since 2015. The co–occurrence and citation network of the keywords of the 2,941 papers was conducted (Figure 6A). The top–keywords included "nivolumab", "pembrolizumab", "docetaxel", "PD–L1", "epidermal growth factor receptor (EGFR)", "survival", and "safety". Recently occurred keywords included "SCLC", "irAEs", "anaplastic lymphoma kinase (ALK)", "biomarker", "atezolizumab", "duvalumab", "radiomics", "tumor mutation burden (TMB)", "tumor burden", "chemoradiotherapy", and "microbiome".

The co–occurrence and citation network of the keywords of the 100 top papers was conducted (Figure 6B). The newly utilized keywords included "bevacizumab", "acquired–resistance", "BRAF", "monotherapy", "vitiligo", "mutational–landscape", "mismatch–repair deficiency", "treatment discontinuation", "tumor–infiltrating lymphocytes (TILs)", and "antibiotics". The keyword co–occurrence and citation network of the 110 recently published papers in major journals was conducted (Figure 6C). The keywords which were different from previous analysis included "heterogeneity", "neoantigens", "adjuvant therapy", "neoadjuvant therapy", "targeted therapy", "overcome resistance", "elderly–patients", "niraparib", and some new molecular targets.

Research trends

The number of publications and the total number of citations per paper per year of article publication of the eight immunotherapies for lung cancer are presented in Figure 7A. Between 2010 and 2022, publications related to vaccines or ACT varied slightly. The number of publications related to ipilimumab (an anticytotoxic T lymphocyte–associated protein 4 [CTLA–4] antibody) has grown slowly since 2012. The number of publications for the two most well–known anti–PD–1 antibodies, nivolumab and pembrolizumab, has increased markedly since 2015. The research on anti–PD–L1 antibodies was reported a little later. In 2016, the first articles on atezolizumab and durvalumab for lung cancer were published.

The variation in the treatment pattern was analyzed and presented in Figure 7B. In recent years, reports related to radiotherapy, single–agent therapy, and first–line therapy have gradually increased. The relationship between the treatment pattern and the immunotherapy modalities was analyzed and is presented in Figure 7C. Among immunotherapies, nivolumab and pembrolizumab had the most related publications. In general, articles on pembrolizumab were slightly more recent than the articles on nivolumab, and pembrolizumab had more related articles on first–line treatment than nivolumab. Compared to first– and second–line treatment, there were fewer articles on adjuvant or neoadjuvant treatment.

A timeline view for the variation of co–cited keywords related to lung cancer immunotherapy is presented in Figure 7D. Keywords were classified into six clusters. Recent research hotspots included "gut microbiota", "tertiary lymphoid structure", "prediction", "gene signature", "sequencing", "extracellular vesicle". A timeline view of the co–cited reference variation related to lung cancer immunotherapy is presented in Figure 7E. The references were classified into 14 clusters. The topics with large yellow nodes, which represented

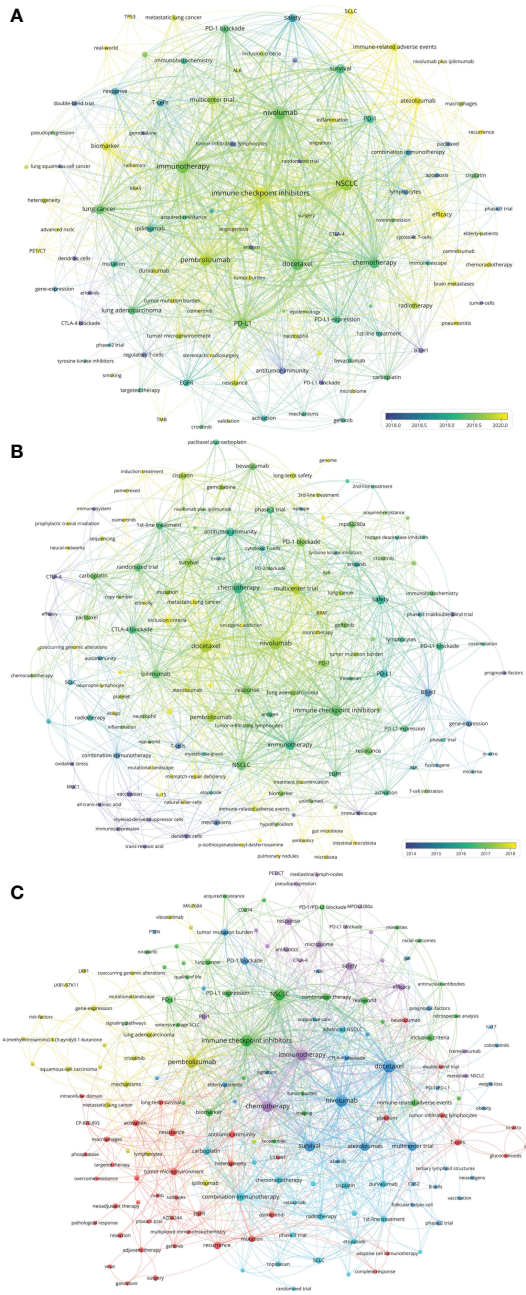

FIGURE 6
(A) Network visualization of keywords that occurred at least 15 times in the papers. **(B)** Network visualization of keywords the top papers. **(C)** Network visualization of keywords in papers published in major journals between 2020 and 2022. The circle size represents the number of papers. The breadth of the curves represents the connection strength.

many recent articles, were research hotspots. Recent hot topics included "clinical outcome", "predictive biomarker", "real–world study", "SCLC", "irAE", and "concurrent chemoradiotherapy (CCRT)".

Discussion

Given its high global disease burden, lung cancer has always been a highly regarded research area. The clinical application of tyrosine kinase inhibitors (TKIs) greatly improved the prognosis of patients with EGFR or ALK gene altered NSCLC (1). In recent years, even for Kirsten rat sarcoma (KRAS) gene alterations, used to considered "un–targetable", new TKIs have emerged (16). However, until the advent of ICIs, the outcomes of patients with SCLC or wild–type driver gene NSCLC remained unsatisfactory. Ipilimumab, an anti–CTLA–4 antibody, improved the prognosis of these patients combined with chemotherapy (17, 18). Subsequently, anti–PD–(L)1 therapy showed even greater efficacy and safety (19, 20). In recent years, anti–PD–(L)1 therapy has become a standard treatment for a large portion of patients with lung cancer. Furthermore, other immunotherapies such as dual–ICIs, cancer vaccine, ACT and T–cell immunoreceptor with Ig and ITIM domain (TIGIT) blockade also showed clinical value in selected lung cancer patients (21–24). Currently, Real–world studies have demonstrated the improvement of OS with the evolution of anticancer pharmacological treatments over the past decade (25). However, primary and acquired resistance, as well as irAEs, limit further improvement in prognosis.

Anti–PD–(L)1 for NSCLC

The historical approach to metastatic NSCLC involved chemotherapy, with an overall survival time (OS) of as short as 8–14 months (26–28). In recent years, the OS of patients with metastatic NSCLC with aberrations of the EGFR/ALK gene has been extended to over 3 years with targeted therapy (29, 30). For patients with NSCLC without targetable gene alteration, immunotherapy was a significant choice. Patients with high expression of PD–L1 or high TMB were more likely to benefit from anti–PD–(L)1 therapy (31, 32). Figure 8 summarizes the outcomes reported by influential studies on advanced NSCLC. Furthermore, adjuvant/neoadjuvant anti–PD–(L)1 therapy has been a research hotspot, which could further reduce the recurrent risk of patients with resectable NSCLC.

Anti–PD–(L)1 for previously treated advanced NSCLC

PD–(L)1 ICIs were initially evaluated as 2nd–line or subsequent treatment for advanced NSCLC. The pioneer phase 3 randomized trials were CheckMate 017 and CheckMate 057, which evaluated nivolumab (an anti–PD–1 antibody) for previously treated squamous and non–squamous NSCLC, respectively, and published their results in 2015 (13, 33). The

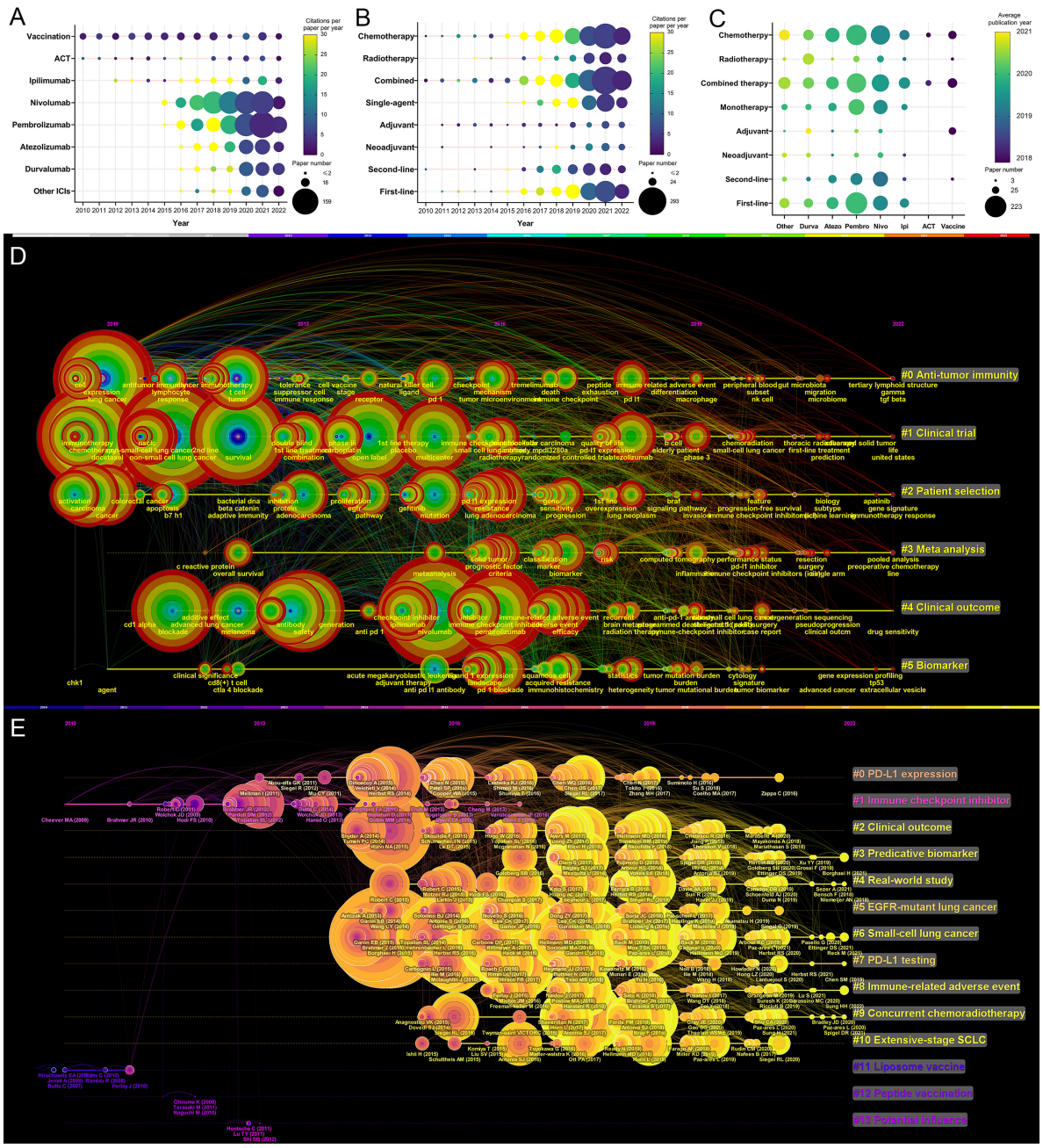

FIGURE 7
(A) Publication number and citations per paper per year of different therapies/drugs. The node size represents the paper number and the color represents the average citations per paper. **(B)** Publication number and citations per paper per year of different treatments. The node size represents the paper number and the color represents the average citations per paper. **(C)** The publication number and average publication year of therapies/drugs for different treatments. The node size represents the paper number and the color represents the average publication year. **(D)** The timeline view for co-cited keywords related to lung cancer immunotherapy. The node size represents the citation number of the reference. The curves between the nodes indicated co-citation relationships. **(E)** The timeline view for co-cited references related to lung cancer immunotherapy. The node size represents the citation number of the reference. The curves between the nodes indicated co-citation relationships. Yellow nodes represent new papers and red nodes represent old ones.

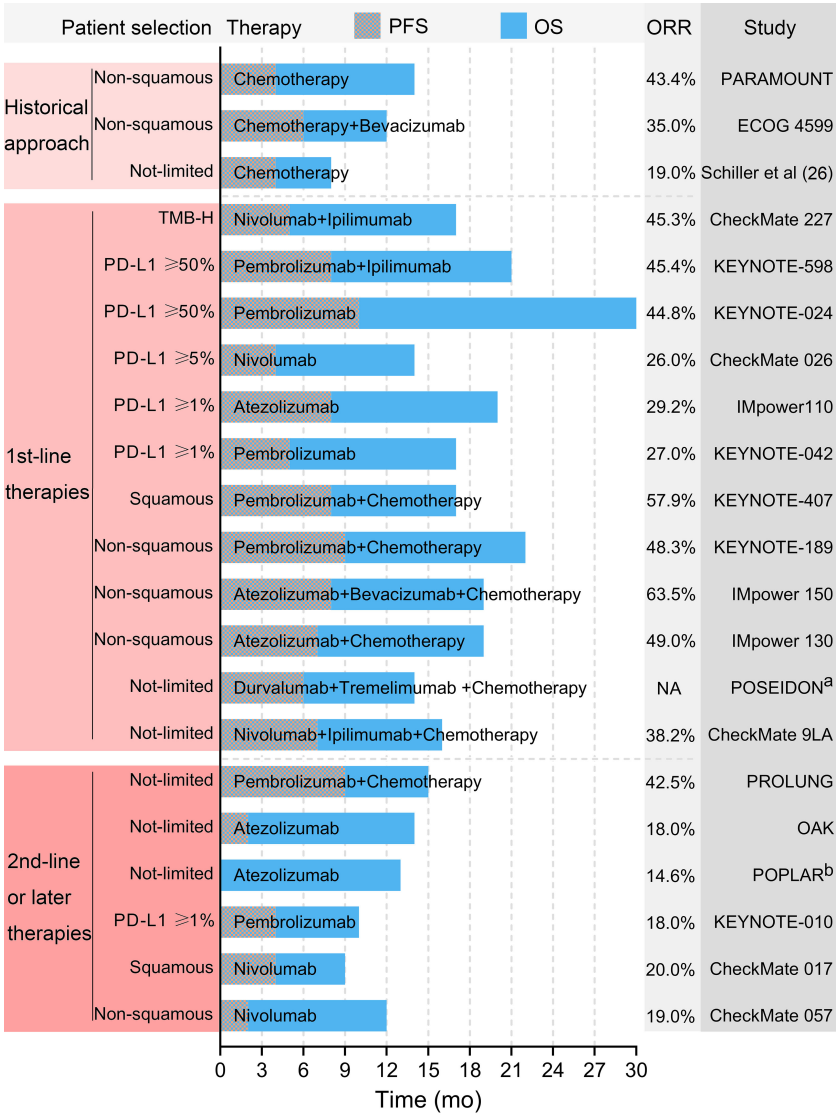

FIGURE 8
Selected outcomes reported by influential studies. [a] The POSEIDON trial did not report the ORR. [b] The POPLAR trial did not report the PFS. PFS, progression-free survival. OS, overall survival. ORR, objectively response rate. TMB-H, high tumor mutational burden.

results showed that nivolumab was more effective and safer than docetaxel regardless of PD–L1 expression in tumor cells, and the expression of PD–L1 was not a prognostic factor in patients treated with nivolumab (13, 33). Subsequently, pembrolizumab (an anti–PD–1 antibody) and atezolizumab (an anti–PD–L1 antibody) were shown, respectively, to be appropriate 2nd–line treatments for advanced NSCLC by the KEYNOTE–010 and POPLAR trials (34, 35). In contrast to previous results, these trials revealed some prognostic or predictive factors in patients treated with ICIs. The KEYNOTE–010 trial only enrolled patients with PD–L1 expressed NSCLC, and found that patients with a tumor proportion score (TPS) for PD–L1 of at least 50% achieved a superior outcome (34). The POPLAR phase 2 trial suggested that although PD–L1 negative patients also benefit from atezolizumab, patients with PD–L1 expressed tumor cells or TIL achieved longer OS (35). The phase 3 OAK trial published in 2017 reported results comparable to those of the POPLAR trial (36).

Although ICIs prolonged the OS of patients, the ORR of anti–PD–(L)1 therapy for previously treated NSCLC was only 14–20% (13, 33–36). Therefore, the combination of anti–PD–(L)1 and anti–CTLA–4 ICIs was evaluated. A phase 1 trial supported that dual–ICIs therapy with specific dose schedules resulted in manageable toxicity and responses regardless of PD–

L1 status (37). However, phase 3 trials (S1400I and ARCTIC) did not support the superiority of dual–ICIs over single agent anti–PD–(L)1 therapy for previously treated patients with advanced NSCLC (38, 39). A recently published trial evaluated dual–ICIs plus palliative radiotherapy for patients with anti–PD–(L)1–resistant metastatic NSCLC. However, radiotherapy did not improve efficacy and this trial was terminated after interim analysis (40). Some trials evaluated ICI plus chemotherapy for previously treated patients with advanced NSCLC to further improve response rate. The phase 2 PROLUNG trial demonstrated that pembrolizumab plus docetaxel resulted in a higher ORR (42.5% vs. 15.8%) and a longer progression–free survival (PFS) (9.5 months vs. 3.9 months) than docetaxel alone for patients with advanced previously treated NSCLC (41). A phase 2 trial (PEMBRO–RT) reported additional stereotactic ablative radiotherapy (SABR) before pembrolizumab improved ORR (36% vs. 18%, P=0.07) for previously treated patients with metastatic NSCLC (42). In particular, patients lacking PD–L1 expression achieved a greater improvement in PFS and OS than patients with PD–L1 expression (42).

Patients with driver genes–altered NSCLC had limited treatment choices after resistance of tyrosine kinase inhibitors (TKIs). ICI plus chemotherapy or antiangiogenic therapy was a considerable advancement for these patients. In 2019, a subgroup analysis of patients with EGFR mutations in the IMpower150 trial demonstrated that atezolizumab plus bevacizumab and chemotherapy resulted in better OS compared to bevacizumab plus chemotherapy (43). A retrospective analysis compared pembrolizumab plus chemotherapy or anlotinib with pembrolizumab alone for previously treated EGFR–mutated NSCLC. The combined therapy resulted in a significantly better prognosis than monotherapy (44).

As a portion of the patients achieved a durable response, the optimal duration of ICI therapy needed to be clarified. However, the results of the phase 3b/4 CheckMate 153 trial demonstrated that patients treated with continuous nivolumab therapy achieved significantly longer OS and PFS than patients treated with fixed–duration nivolumab therapy for 1 year (45). A retrospective analysis suggested that discontinuous treatment due to irAE was correlated with shorter OS compared to continuous anti–PD–1 therapy (46). Although discontinuation of treatment in selected patients with melanoma did not alter the duration of the response, it should be cautious to suspend ICI therapy in previously treated patients with NSCLC (47).

Because an increasing number of patients have received anti–PD–(L)1 therapy as 1st–line treatment in recent years, it is important to establish 2nd–line immunotherapy strategies that could overcome immune–resistance. Some TKIs targeting the tumor microenvironment might synergize with anti–PD–(L)1 therapy. Recently, the LUNG–MAP S1800A phase 2 trial reported that ramucirumab (a CTLA–4/vascular endothelial growth factor [VEGF] inhibitor) plus pembrolizumab achieved improved efficacy (objective response rate [ORR], 28%; median OS, 14.5 months) in patients with NSCLC progressed after anti–PD–(L)1 therapy (48). Similarly, cabozantinb plus atezolizumab and sitravatinib plus nivolumab, respectively, showed antitumor immune activity for immune–failed patients with NSCLC in the COSMIC–021 and MRTX–500 trials (49, 50). Phase 3 trials such as SAPPHIRE are ongoing. In particular, a recently published pooled analysis of five phase 3 trials evaluated the second course of pembrolizumab for patients with progression of the disease after at least 2 years since the end of the first course of pembrolizumab. The results showed that the second course was beneficial to the patients (51). Another study reported that 2nd–line anti–PD–1 therapy after 1st–line anti–PD–L1 therapy or 2nd–line anti–PD–L1 therapy after 1st–line anti–PD–1 therapy could be superior to chemotherapy (52).

Anti–PD–(L)1 has become the standard treatment for patients with advanced NSCLC who progress after chemotherapy. However, the ORR remains unsatisfactory. ICI combined with other therapies may further improve prognosis, but more clinical evidence is required, and tolerability should be evaluated. Combined therapy including anti–PD–(L)1 is considerable for patients resistant to TKI with EGFR–mutated NSCLC. Furthermore, studies evaluating novel, optimal, and individual treatment strategies for patients resistant to anti–PD–(L)1 therapy are needed.

First–line anti–PD–(L)1 therapy for advanced NSCLC

Since 2016, a growing number of influential trials have focused on 1st–line anti–PD–(L)1 therapy for advanced NSCLC. The KEYNOTE–024 trial enrolled untreated patients with advanced NSCLC with PD–L1 TPS of at least 50%. Pembrolizumab monotherapy achieved significantly higher ORR (44.8% vs. 27.8%), longer PFS, and less toxicity than chemotherapy (14). In that trial, patients in the chemotherapy group could switch to pembrolizumab after progression, but patients in the pembrolizumab group still achieved longer OS (30.0 vs 14.2 months) (53). The CheckMate 026 trial enrolled patients with NSCLC with PD–L1 TPS of at least 5%. However, 1st–line nivolumab therapy did not achieve better efficacy than chemotherapy (54). The IMpower110 trial reported similar results that atezolizumab improved the outcome only in patients with high expression of PD–L1, but not in all patients with expression of PD–L1 (55). In contrast, the KEYNOTE–042 trial reported that 1st–line pembrolizumab therapy achieved a better prognosis than chemotherapy for patients with NSCLC in all subgroups (PD–L1 TPS between 1% and 20%, between 20% and 50% and at least 50%) (56).

First–line anti–PD–(L)1 monotherapy showed minor toxicity and improved response in selected patients. Anti–PD–(L)1 combined with other therapies was evaluated to further

improve efficacy and expand indications. In 2016, the phase 2 KEYNOTE–021 trial first demonstrated that 1st–line pembrolizumab plus chemotherapy achieved superior efficacy over chemotherapy (ORR, 55% vs. 29%) for patients with non–squamous NSCLC with/without PD–L1 expression (57). This result was confirmed by the KEYNOTE–189 phase 3 trial (58). Similar results were found in the IMpower 130 trial, which compared atezolizumab plus chemotherapy with chemotherapy (ORR, 49% vs. 32%) as 1st–line treatment for non–squamous NSCLC (59). Furthermore, the IMpower 150 trial further showed that the addition of atezolizumab to bevacizumab plus chemotherapy as the 1st–line treatment further improved ORR (63.5%) in patients with non–squamous NSCLC; however, the reported OS and PFS between IMpower 130 and IMpower 150 were similar (60). Recently, the phase 2 CAPAP lung trial reported that 1st–line camrelizumab (an anti–PD–1 antibody) combined with apatinib and albumin paclitaxel produced encouraging efficacy (ORR, 73.1%) and acceptable safety for advanced non–squamous NSCLC (61). Anti–PD–(L)1 plus chemotherapy was evaluated for previously untreated squamous NSCLC, and the KEYNOTE–407 trial demonstrated the superiority of combined therapy (ORR, 57.9% vs. 38.4%) including pembrolizumab (62).

Nivolumab plus ipilimumab (N+I) was the most common combination of dual–ICIs as the 1st–line treatment for NSCLC. The CheckMate 227 trial only enrolled patients with high TMB, and patients treated with N+I also achieved better efficacy (ORR, 45.3% vs. 26.9%) (63). The CheckMate 277 trial suggested that N +I resulted in better efficacy (ORR, 35.9% vs. 30.0%) and comparable safety to chemotherapy (21). Furthermore, CheckMate 9LA demonstrated that N+I plus two cycles of chemotherapy achieved a higher ORR (38.2%) (15). Similarly, the POSEIDON trial demonstrated that durvalumab plus tremelimumab combined with chemotherapy was superior to chemotherapy alone as 1st–line treatment for metastatic NSCLC (64). However, the KEYNOTE–598 trial did not support the improvement in efficacy of adding ipilimumab to pembrolizumab, but increased toxicity, as the 1st–line treatment for patients with NSCLC with PD–L1 TPS of at least 50% (65). Recently, a randomized trial (SQUINT) demonstrated that 1st–line N+I achieved similar efficacy and superior safety compared to nivolumab plus chemotherapy for advanced squamous NSCLC (66).

Anti–PD–(L)1 plus chemotherapy is suitable for previously untreated advanced NSCLC without PD–L1 expression and driver gene alteration. However, the optimal therapeutic modality for PD–L1 positive NSCLC remains uncertain. In 2021, the US Food and Drug Administration reported a pooled analysis of anti–PD–(L)1 plus chemotherapy vs. anti–PD–(L)1 alone as 1st–line treatment for advanced NSCLC with PD–L1 TPS 1–49% (67). Combined therapy showed better OS (21.4 months vs. 14.5 months) and PFS (7.7 months vs. 4.2 months) than anti–PD–(L)1 monotherapy in these patients. Furthermore, patients ≥75 years old experienced a similar prognosis in both groups (67). A recently published pooled analysis of NSCLC with PD–L1 TPS ≥50% demonstrated the superiority of combined therapy (OS, 25.0 months vs. 20.9 months; PFS, (9.6 months vs. 7.1 months) (68). Similarly, patients ≥75 years old may not benefit from combined therapy (68). In the 2022 World Conference on Lung Cancer (WCLC 2022), Dong et al. reported that adding radiotherapy to first–line anti–PD–1 plus chemotherapy for advanced NSCLC significantly improved OS and PFS (69).

Anti–PD–(L)1 plus chemotherapy has been established as the standard 1st–line treatment for the most advanced patients with NSCLC with no targetable driver gene mutation. Some patients may benefit from combination therapy that includes antiangiogenic or anti–CTLA–4 therapy, but more clinical evidence is warranted. The optimal treatment for older patients or patients with poor tolerance may be anti–PD–(L)1 monotherapy. However, the therapeutic strategy for other special patients, such as patients with KRAS gene alterations or brain metastases, remains unspecified. Future studies are warranted to develop next–generation therapies, establish robust predictive models, and determine individual treatment patterns for specific subgroups of patients.

Novel immunotherapy for advanced NSCLC

The efficacy of current immunotherapy–based treatment remains unsatisfactory and the treatment option for patients with anti–PD–(L)1–resistant NSCLC is limited. Currently, multiple novel immunotherapies targeting different signal pathways are being developed, including lymphocyte activation gene–3 (LAG–3), Janus kinase 1 (JAK1), and T cell immunoreceptors with Ig and ITIM domains (TIGIT) antibodies. Furthermore, bispecific antibodies that could block both PD–(L)1 and VEGF such as KN046, MEDI5752, and AK112 are being evaluated for advanced NSCLC.

LAG–3 blockade (relatlimab) has been shown to be beneficial when combined with nivolumab for advanced melanoma (70). Recently, the phase 2b TACTI–003 trial reported that eftilagimod alpha (a soluble LAG–3 protein) plus pembrolizumab achieved favor efficacy (ORR, 38%; PFS, 6.9 months) as 1st–line treatment for advanced NSCLC (71). An anti–TIGIT antibody tiragolumab plus atezolizumab was considered superior to atezolizumab monotherapy for previously untreated patients with NSCLC in the CITYSCAPE phase 2 trial (24). However, the recently announced interim results of the SKYSCRAPER–01 phase 3 trial that evaluated tiragolumab plus atezolizumab as 1st–line treatment for patients with PD–L1–high metastatic PD–L1 NSCLC did not meet its primary endpoint. Other trials (e.g., KEYVIBE–007, AdvanTIG–302) are ongoing to further evaluate 1st–line anti–TIGIT (e.g.,

vibostolimab, ociperlimab) plus anti–PD–1 therapy for NSCLC. Clinical evidence supporting immunotherapy for other targets, such as interleukin–1β and hematopoietic progenitor kinase 1, is still lacking. In WCLC 2022, a phase 2 trial reported that pembrolizumab plus itacitinib (a JAK1 inhibitor) was effective (ORR, 62%) and safe for metastatic NSCLC with PD–L1 TPS of at least 50% (72). Another phase 2 trial (HUDSON) suggested durvalumab plus ceralasertib (an ATR inhibitor) might be an effective treatment option for patients with NSCLC who failed from chemotherapy and anti–PD–L1 therapy (73).

AK112, a PD–1/VEGF bispecific antibody, plus chemotherapy, showed promising antitumor efficacy for patients who had not previously been treated, failed prior EGFR–TKI, or progressed after anti–PD–(L)1 plus chemotherapy in a recent phase 2 trial (74). Among the patients in the three cohorts, the ORR was reported to be 76.9%, 68.4%, and 40.0%, respectively (74). The preliminary results of trials support the antitumor activity of other bispecific antibodies, but clinical evidence on NSCLC is lacking.

Because combinations of existing drugs can hardly further improve the prognosis and overcome resistance of advanced NSCLC, novel immunotherapies are urgently needed. The main research directions include new targets and bispecific antibodies, but clinical evidence is lacking, and many new approaches have not been successful during clinical trials. In addition, basic research on tumor immune microenvironment may promote the understanding of immune–resistance mechanisms, thus guiding the development of novel approaches.

Adjuvant anti–PD–(L)1 therapy

Because anti–PD–(L)1 therapy showed encouraging efficacy and manageable toxicity for advanced NSCLC, it is reasonable to evaluate anti–PD–(L)1 therapy as adjuvant treatment after radical surgery or chemoradiotherapy. The most influential phase 3 trial that evaluated adjuvant anti–PD–(L)1 for NSCLC was PACIFIC, which first reported that durvalumab after concurrent chemoradiotherapy improved PFS and OS (75, 76). Recent published 5–year results confirmed the superiority of the results achieved with durvalumab over placebo (median OS, 47.5 vs. 29.1 months; median PFS, 16.9 vs. 5.6 months) (77). In 2020, the LUN 14–179 phase 2 trial suggested that consolidation of pembrolizumab after chemoradiotherapy also improved PFS and OS in patients with stage III NSCLC (78). Another nonrandomized phase 2 trial (KEYNOTE–799) reported that pembrolizumab plus concurrent chemoradiation therapy had good efficacy and safety for stage III NSCLC (79). The IMpower010 trial evaluated atezolizumab after chemotherapy in patients with resected stage IB–IIIA NSCLC. The results demonstrated that patients with stage II–IIIA NSCLC and PD–L1 TPS of at least 1% benefited from atezolizumab (80). Recently, the interim results of a phase 3 trial (GEMSTONE–301) reported that an anti–PD–L1 antibody (sugemalimab) after chemoradiotherapy prolonged PFS in patients with stage III NSCLC (81). For patients with advanced NSCLC who could not tolerate concurrent chemoradiotherapy, sequential chemoradiotherapy was a standard treatment. In August 2022, the phase 2 PACFIC–6 trial reported durvalumab after sequential chemoradiotherapy achieved acceptable safety and encouraging efficacy (82). The phase 3 PACFIC–5 trial is ongoing to further evaluate the efficacy and safety of durvalumab after sequential or concurrent chemoradiotherapy for unresectable stage III NSCLC (82).

Clinical trials have provided preliminary evidence supporting the efficacy and safety of anti–PD–(L)1 therapy plus/after radical treatments. Additional studies are needed to determine the optimal dosing strategy and duration of immunotherapy and to clarify the subgroup of patients who would benefit from adjuvant immunotherapy.

Neoadjuvant anti–PD–(L)1 therapy

In recent years, several trials have evaluated anti–PD–(L)1 monotherapy or combined with chemotherapy or SABR as neoadjuvant treatment for resectable NSCLC. In 2018, a pilot study reported that neoadjuvant nivolumab therapy resulted in a major pathological response (MPR) of 45% and the response was correlated with TMB (83). Recently, neoadjuvant pembrolizumab therapy for resectable NSCLC has also been reported to be effective (84, 85). Furthermore, combined neoadjuvant therapy including ICI further improved efficacy. In 2020, a phase 2 trial reported that atezolizumab plus carboplatin and nab–paclitaxel as neoadjuvant therapy achieved a MPR rate of 57% for patients with stage IB to IIIA NSCLC (86). The phase 2 NADIM trial evaluated neoadjuvant nivolumab plus chemotherapy for stage IIIA NSCLC and reported a 2–year PFS of 77.1% (87). The SAKK 16/14 phase 2 trial reported that the addition of perioperative durvalumab to neoadjuvant chemotherapy yielded a MPR of 62% and a 1–year event–free survival (EFS) rate of 73% (88). A phase 3 trial (AEGEAN) is ongoing to further evaluate neoadjuvant durvalumab plus chemotherapy followed by adjuvant durvalumab (89). Another phase 2 trial reported that neoadjuvant toripalimab (an anti–PD–1 antibody) combined with chemotherapy achieved similar results (90). The first phase 3 trial evaluating neoadjuvant anti–PD–(L)1 therapy was CheckMate 816, which published the results in May 2022. This trial demonstrated that neoadjuvant nivolumab plus chemotherapy produced a median EFS of 31.6 months and a complete pathological response rate of 24% for patients with stage IB to IIIA resectable NSCLC (91). Recently, neoadjuvant therapy including both ICI and radiotherapy has been a research hotspot. A phase 2 trial reported that the addition of SABR (24 Gy in three fractions) to neoadjuvant durvalumab therapy

resulted in much higher MPR (53.3% vs. 6.7%) in patients with stage I–IIIA NSCLC (92). Furthermore, the ongoing SQUAT phase trial is evaluating durvalumab plus CCRT (50 Gy in 25 fractions) as neoadjuvant therapy for patients with stage IIIA to IIIB resectable NSCLC (93).

In recent years, neoadjuvant immunotherapy has been investigated more than adjuvant immunotherapy. This may be due to 1) the existing tumor causing an immune response; 2) immunotherapy synergies with chemotherapy and/or radiotherapy; 3) the preoperative immunity may be stronger than postoperative immunity. Therefore, neoadjuvant immunotherapy is theoretically superior to adjuvant immunotherapy. Combined neoadjuvant therapy achieves high MPR, suggesting that combined therapy might be an optimal option for most patients. However, more clinical evidence is still needed to determine the indications and combination strategy of neoadjuvant immunotherapy for patients with resectable NSCLC.

Anti–PD–(L)1 for SCLC

SCLC is highly aggressive and easily develops resistance to antitumor therapies. The standard systemic treatment for SCLC was etoposide plus platinum chemotherapy for several years. The high TMB of SCLC leads to a high neoantigen load, thus promoting potential antitumor immunity (94). In 2016, a phase 3 trial evaluated 1st–line ipilimumab plus chemotherapy for extensive–stage SCLC (ES–SCLC), but the results were negative (95). With evidence of the promising antitumor activity of anti–PD–(L)1 therapy, novel treatment strategies have been evaluated to improve the prognosis of patients with SCLC. Furthermore, because SCLC is heterogeneous and can be classified into four molecular subtypes, future studies are acquired to establish optimal management of SCLC in different subtypes (96).

Anti–PD–(L)1 for previously treated extensive–stage SCLC

SCLC patients who advance after 1st–line chemotherapy have limited treatment options. In 2017, the KEYNOTE–028 phase 1 trial reported pembrolizumab had promising efficacy in patients with previously treated, PD–L1–expressing SCLC (97). Furthermore, a pooled analysis of the data from KEYNOTE–028 and KEYNOTE–158 suggested that the antitumor activity of pembrolizumab was independent of PD–L1 expression (98). The efficacy and safety of nivolumab was also demonstrated in the CheckMate 032 trial (99). Anti–PD–(L)1 therapy plus antiangiogenic therapy or chemotherapy alone could further improve prognosis. The phase 2 PASSION trial demonstrated that camrelizumab (an anti–PD–1 antibody) plus apatinib was effective and safe for previously treated patients with SCLC (100). A phase 2 trial reported that pembrolizumab plus paclitaxel showed moderate activity in previously treated patients with SCLC (101). A phase 1/2 trial evaluated rovalpituzumab tesirine (an antibody–drug conjugate) plus nivolumab with/without ipilimumab for previously treated patients with ES–SCLC. However, the toxicity was not tolerable (102).

Some trials were conducted to evaluate dual–ICIs for previously treated patients with SCLC. In 2016, the CheckMate 032 phase 1/2 trial reported that nivolumab monotherapy and nivolumab plus ipilimumab had antitumor activity and manageable toxicity for SCLC patients previously treated (103). However, the following CheckMate 451 phase 3 trial demonstrated that dual–ICIs were not superior to nivolumab monotherapy as maintenance therapy after first–line chemotherapy for ES–SCLC (104). The results and basic research suggested that patients with high TMB could benefit from dual–ICIs (104, 105). A phase 2 trial reported that the addition of SABR to durvalumab and tremelimumab did not improve the prognosis of patients with recurrent SCLC (106). Furthermore, a phase 1 trial suggested that quavonlimab plus pembrolizumab showed antitumor activity in previously treated patients with ES–SCLC (107).

The 2nd–line chemotherapy frequently used for SCLC included topotecan and amrubicin. The IFCT–1603 and CheckMate 331 respectively compared atezolizumab and nivolumab with chemotherapy as 2nd–line therapy for patients with SCLC. However, the results showed that anti–PD–(L)1 therapy was not superior to chemotherapy (108, 109).

Anti–PD–(L)1 monotherapy showed antitumor activity for previously treated SCLC. However, the efficacy of 2nd–line anti–PD–(L)1 therapy was not superior to chemotherapy. Dual–ICIs did not achieve better efficacy than monotherapy. Antiangiogenic therapy, chemotherapy, or radiation therapy may improve the prognosis, but more clinical evidence is warranted. Furthermore, some trials are ongoing to evaluate anti–PD–(L)1 combined with novel drugs (e.g., LAG–3 blockades and T cell immunoglobulin and mucin domain 3 blockades) for ES–SCLC (94).

First–line anti–PD–(L)1 for extensive–stage SCLC

Potential antitumor immunity of previously untreated patients with SCLC may be more potent than that of heavily treated patients. Therefore, some trials evaluated 1st–line anti–PD–(L)1 therapy plus standard chemotherapy for patients with ES–SCLC. In 2018, the phase 3 IMpower133 trial demonstrated that the addition of atezolizumab improved OS and PFS (20). The subgroup analysis suggested that the efficacy was regardless of the expression of PD–L1 or TMB (110). The CASPIAN trial demonstrated that 1st–line durvalumab plus chemotherapy

yielded better OS than chemotherapy alone (111). However, dual–ICIs (durvalumab and tremelimumab) plus chemotherapy did not improve prognosis than durvalumab plus chemotherapy (112). The KEYNOTE–604 trial demonstrated that the addition of pembrolizumab to chemotherapy also improved PFS (113). Similarly, the phase 3 CAPSTONE–1 trial reported that the addition of adebrelimab (a new anti–PD–L1 antibody) to chemotherapy improved OS (114). A phase 1 trial reported that pembrolizumab plus thoracic radiotherapy after chemotherapy was tolerated for SCLC, but its efficacy was unclear (115).

Anti–PD–(L)1 plus chemotherapy has been shown to be an effective and safe 1st–line treatment for ES–SCLC. Currently, further studies are needed to determine the optimal dosing strategy, duration of treatment, and patient selection. Because thoracic radiotherapy after chemotherapy improves the prognosis of ES–SCLC, the combination of anti–PD–(L)1 plus radiotherapy after chemotherapy should be further evaluated (116). Combination therapy including other treatments (e.g., antiangiogenic therapy and anti–TIGIT–antibodies) may further improve the prognosis, but clinical evidence is lacking.

Anti–PD–(L)1 for limited–stage SCLC

The standard treatment for limited–stage SCLC (LS–SCLC) used to be CCRT. In recent years, several trials have evaluated ICIs plus or after CCRT for LS–SCLC. In 2020, a phase 1/2 trial reported that pembrolizumab plus CCRT resulted in favorable efficacy and safety (117). The STIMULI trial evaluated dual–ICIs after CCRT for LS–SCLC but reported negative results (118). Currently, results from phase 3 trials are lacking, trials including ADRIATIC and AdvanTIG 204 are currently evaluating anti–PD–(L)1, dual–ICIs, and ICI combined with anti–TIGIT antibody plus CCRT for LS–SCLC (119).

Other immunotherapies

In addition to ICIs, lung cancer immunotherapies included vaccination and ACT. Vaccination was generally used for maintenance treatment to enhance the effect of chemotherapy or salvage therapy. In recent years, the combination of vaccine and ICI has been evaluated for lung cancer. The clinical value of some therapeutic vaccines (e.g., TG 4010, BLP25, NEO–PV–01) has been demonstrated (120–124). Vaccines can promote antitumor activity through multiple pathways, so they may synergize with other therapies. More basic and clinical studies are needed to explore the mechanisms of tumor immunology and develop predictive models to develop individual treatments for different subgroups of patients.

ACT has shown anti–tumor activity as salvage therapy for some patients with lung cancer. In recent years, TIL–based ACT has been evaluated for lung cancer. A phase 1 trial reported that TIL therapy was safe and showed a deep and durable response in some patients (125). Furthermore, chimeric antigen receptor (CAR)–modified ACT also showed antitumor immunity in some studies (126, 127). ACT based on novel technique was an effective salvage therapy for some patients with lung cancer. However, the response rate is not satisfactory and clinical evidence is lacking. Additional studies are needed to determine the patients suitable for ACT and to provide more evidence.

Journals, countries, institutions, and authors

Lung Cancer was the most productive journal on lung cancer immunotherapy. Among the top 10 productive journals, *Lung Cancer* had the second highest citation indexes, and the *Journal of Thoracic Oncology* had the highest citation indexes. These two journals were both productive and impactful. The *N Engl J Med* was the most impactful journal in this area. The results of the correlation test suggested papers published in most of the journals with high 5–year IF or JCI are more likely to be highly cited. Among the 27 journals with the top papers, 22 were considered major journals. The articles published in major journals were likely to be impactful. Notably, most of the top papers were published in comprehensive journals, which may be due to the high IFs of these journals.

Researchers from China contributed most of the studies. However, papers by corresponding authors from the USA were much more influential. International collaboration was rare in China and Japan. In contrast, most of the top papers were contributed by authors from multiple countries/regions. Although some studies from developing countries have been published in recent years, studies from Africa or the Middle East are lacking. The most productive institution was the University of Texas MD Anderson Cancer Center. Although some universities in China contributed to many articles, their TPRs were low. Some institutions in developed countries, although their total number of papers was not high, contributed to many top papers. The most productive authors of the top papers were Reck M, Hellmann MD, and Horn L.

This study described the most influential journals, countries, institutions, and authors on lung cancer immunotherapy and presented collaboration networks. The results can help researchers select target journals for publication and find potential cooperative partners.

Research trends and hotspots

Based on thousands of publications, this bibliometric analysis quantitatively and comprehensively presented research trends, status, and hotspots in lung cancer immunotherapy.

Other major review methods, such as systematic literature review and meta–analysis, are unapplicable for this purpose (4).

This study analyzed the research trends. Before 2015, most publications on lung cancer immunotherapy focused on vaccination. The number of publications on vaccination or ACT varied little from year to year. The number of articles on ipilimumab has gradually increased since 2013. From 2015, anti–PD–1 antibodies became the main focus of research, followed by anti–PD–L1 antibodies. In recent years, additional studies have evaluated 1st–line immunotherapy or combined therapy compared to 2nd–line immunotherapy or monotherapy. Adjuvant and neoadjuvant therapy have recently become hotspots. Radiotherapy combined with ICI has recently been a research hotspot, and durvalumab plus radiotherapy was evaluated most compared with other ICIs.

The current research status on lung cancer immunotherapy is: 1) Anti–PD–(L)1 with/without chemotherapy is a standard 1st–line treatment for advanced NSCLC; 2) 1st– or 2nd–line anti–PD–(L)1 plus new immunotherapies may overcome resistance, but high–quality clinical evidence is lacking; 3) neoadjuvant and adjuvant anti–PD–(L)1 therapy has proven to be beneficial, and combined neoadjuvant anti–PD–(L)1 achieves encouraging efficacy; 4) anti–PD–(L)1 plus standard chemotherapy as first– or second–line treatment has favorable efficacy for ES–SCLC; 5) ICI plus CCRT may improve the efficacy for LS–SCLC, but more clinical evidence is needed; and 6) other immunotherapies are effective supplements to anti–PD–(L)1 therapy, and selected patients may benefit from them.

Current research hotspots include: 1) treatment for special patients; 2) treatment for patients who failed after anti–PD–(L)1 therapy; 3) immunotherapy combined with antiangiogenic therapy or radiotherapy; 4) combined neoadjuvant therapy; 5) anti–PD–(L)1 plus CCRT; 6) tumor immune microenvironment and immune–resistance mechanisms; and 7) new immunotherapies. The authors suggest that important future research directions include: 1) the optimal and individual managements for advanced NSCLC and ES–SCLC; 2) basic research and novel treatments to overcome resistance; 3) phase 3 clinical trials evaluating immunotherapy for LS–SCLC or resectable NSCLC; 4) robust predictive models; and 5) mechanisms and efficacy of immunotherapy combined with other therapies.

Limitations

This study has some limitations. 1) This study aims to present the landscape of clinical immunotherapy for lung cancer, and only includes papers directly related to this topic published between 2010 and 2022. Therefore, earlier papers were excluded. Although some basic or clinical publications may have contained the keywords in their abstracts, they were not directly related to the topic. The search strategy excluded them to ensure that the identified papers were directly related to the topic, thereby avoiding interference, and better presenting the real research landscape. 2) Some papers evaluating multiple cancers were excluded, which introduces a bias in this study. However, with a reasonable search strategy, it was impossible to include them while excluding some other irrelevant papers. 3) The citation number was influenced by various confounding factors (e.g., publication time, research area, journal, and author). Therefore, citation number could not accurately represent the influence of a paper. Most of the top papers in this study were published prior to 2020; hence, some recent important papers were omitted, and the top papers could not represent the latest research hotspots. To minimize the impact of publication time, the authors also analyzed the average citation per year of the papers. 4) Due to the large number of papers, it was impossible to read every publication and thoroughly analyze the subareas. Furthermore, the recently published important papers represented the latest research hotspots but were difficult to quantificationally identify. To better present the trends and status of the sub–areas, the authors evaluated the development of subareas and presented the most recent advances including reports from the American Society of Clinical Oncology Annual Meeting and the WCLC. 5) This study focused on clinical studies. Therefore, important basic research studies may have been omitted, and basic immunology was not discussed. 6) Finally, the literature search was conducted only based on the Web of Science database, and papers not included in this database were omitted. This may have led to selection bias and analytical errors.

Conclusions

To our knowledge, this study is the first comprehensive and quantitative bibliometric analysis of original articles on lung cancer immunotherapy. This study demonstrates the research trends and hotspots based on the analysis of 2,941 publications and 100 top papers. In addition, researchers can benefit from the results for selecting target journals for publication of findings and establishing cooperative relationships. The authors suggest that important research directions include: 1) optimal and individual treatment for advanced NSCLC and ES–SCLC; 2) overcoming immune–resistance; 3) clinical trials for resectable NSCLC or LS–SCLC; 4) robust predictive models; and 5) immunotherapy combined with other therapies. This study can help researchers gain a comprehensive picture of the research landscape, historical development, and recent hotspots in lung cancer immunotherapy and can provide inspiration for future research.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

XZ and YanL contributed to the study conception. YanL analyzed the data. YanL, XuC, XH, XiC, SJ, YarL, ZZ, LL, BQ, and YC contributed to the literature review. YanL 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1032747/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Historical direct citation network among the publications.

SUPPLEMENTARY FIGURE 2
Top 25 references with the strongest citation bursts on lung cancer immunotherapy.

SUPPLEMENTARY FIGURE 3
Citation relationship network of the publications with the strongest link strength.

SUPPLEMENTARY FIGURE 4
The trends of the keywords rank of lung cancer immunotherapy.

SUPPLEMENTARY FIGURE 5
Top 25 keywords with the strongest citation bursts on lung cancer immunotherapy.

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Lung cancer patients with chronic obstructive pulmonary disease benefit from anti-PD-1/PD-L1 therapy

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Lung cancer (LC) and chronic obstructive pulmonary disease (COPD) are two of the most fatal respiratory diseases, seriously threatening human health and imposing a heavy burden on families and society. Although COPD is a significant independent risk factor for LC, it is still unclear how COPD affects the prognosis of LC patients, especially when LC patients with COPD receive immunotherapy. With the development of immune checkpoint inhibition (ICI) therapy, an increasing number of inhibitors of programmed cell death-1 (PD-1) and PD-1 ligand (PD-L1) have been applied to the treatment of LC. Recent studies suggest that LC patients with COPD may benefit more from immunotherapy. In this review, we systematically summarized the outcomes of LC patients with COPD after anti-PD-1/PD-L1 treatment and discussed the tumor immune microenvironment (TIME) regulated by COPD in LC immunotherapy, which provides novel insights for the clinical treatment of LC patients with COPD.

KEYWORDS

chronic obstructive pulmonary disease (COPD), immune checkpoint inhibitors (ICIs), PD-1/PD-L1, lung cancer (LC), tumor immune microenvironment (TIME)

1 Introduction

Chronic obstructive pulmonary disease (COPD) is a common and fatal respiratory disease. It is a heterogeneous syndrome consisting of emphysema, chronic bronchitis, and small airway disease, and it affects approximately 251 million people worldwide (1). COPD is a devastating lung disease that is characterized by progressive airflow restriction and is associated with the abnormal inflammatory response of the lung to noxious particles or gases (1, 2). The World Health Organization (WHO) predicts that by 2030, if effective measures are not taken, such as reducing smoking and air pollutants, COPD will become the third leading cause of death globally (3). In addition, during the same period, the number of lung cancer (LC) deaths will rise to 10 million annually, accounting for nearly one-fifth of all cancer deaths worldwide (4, 5).

LC and COPD share common risk factors (such as smoking and other environmental factors), and the pathogenesis of them is the same. As early as the 1980s and 1990s, researchers observed that chronic lung disease might participate in LC progression and explored the relationship between COPD and LC (6, 7). In 1986, Skillrud et al. first proposed that COPD could serve as an important independent risk factor for LC (7). Previous studies reported that respiratory symptoms such as coughing, expectoration, shortness of breath, and chest tightness, as diagnostic criteria for COPD, had a negative impact on the prognosis of LC patients (8–10). However, the application of immune checkpoint inhibitors (ICIs), especially PD-1/PD-L1 inhibitors, in advanced LC, along with evidence of the imbalance of immune checkpoint protein (PD-1 and PD-L1) expression and changes in the immune microenvironment in COPD patients (8, 11, 12), revealed that COPD-related LC may respond better to immunotherapy (8, 13). Therefore, we systematically summarized the outcomes of LC patients with COPD after PD-1/PD-L1 inhibitor therapy and explored the impact of the tumor immune microenvironment (TIME) regulated by COPD on LC immunotherapy.

Abbreviations: LC, Lung cancer; COPD, Chronic obstructive pulmonary disease; AECOPD, Acute exacerbation of chronic obstructive pulmonary disease; ICIs, Immune checkpoint inhibitors; PD-(L)1, Programmed death-1/programmed death-ligand 1 inhibitors; NSCLC, Non-small cell lung cancer; TIME, Tumor immune microenvironment; CD, Cluster of differentiation; DCs, Dendritic cells; TILs, Tumor-infiltrating lymphocytes; Th1/2, T-helper cell type 1/2; Treg, Regulatory T cells; MDSC-s, Myeloid-derived suppressor cells; IL-2, Interleukin 2; TNF-γ/α, Tumor necrosis factor γ/α; TIM3, T cell immunoglobulin domain and mucin domain-3; MDSCs, Myeloid-derived suppressor cells; VEGF, Vascular endothelial growth factor; EWAS, Epigenome wide association study; OS, Overall survival; PFS, Progression-free survival; ORR, Objective response rate; FEV1, Forced expiratory volume in one second; FVC, Forced vital capacity; IRP, ICI-related pneumonia; APCs, Antigen presenting cells; ADR, Adverse drug reactions; MHC, Major histocompatibility complex; Mif, Multiplex fluorescence.

2 Current advances in anti-PD-1/PD-L1 therapy in LC patients with COPD

To understand the current status of immunotherapy in LC patients with COPD, we conducted a rapid systematic review. Inclusion criteria: 1) population: LC patients with COPD at any disease stage; 2) interventions: ICI immunotherapy, with no restrictions on drug regimen and treatment lines; 3) outcomes: the overall survival (OS), progression-free survival (PFS), objective response rate (ORR) and the impact on pulmonary function; 4) research type: randomized controlled trials (RCTs) and cohort studies. Exclusion criteria: 1) duplicate publications; 2) languages other than Chinese or English; 3) conference abstracts or studies without data; 4) literature with inconsistent research purposes; 5) unavailable studies. Then, all English databases (PubMed, Embase, Cochrane Library) and Chinese databases (CNKI, VIP, WanFang databases) from the inception of the database to 8th July 2022 were searched. The search terms were (chronic obstructive pulmonary disease OR COPD OR ventilatory defect OR emphysema) AND (immune checkpoint inhibitor OR pembrolizumab OR atezolizumab OR nivolumab OR durvalumab OR carrelizumab OR toripalimab OR sintilimab OR tislelizumab OR immunity therapy OR PD-1 OR PD-L1) AND (Lung cancer). The search formula of PubMed is shown in Table 1. Finally, a total of 236 articles (135 in English and 101 in Chinese) were retrieved, but only 9 studies met the inclusion criteria and were included. The screening process is shown in Figure 1.

In total, 9 relevant studies were included, 2 from China (8, 14), 4 from Japan (15–18), 1 from the United States (19), 1 from South Korea (20) and 1 from France (21). All studies were cohort studies (prospective 2 and retrospective 7). There were 1044 patients with stage III-IV NSCLC (COPD 432 and non-COPD 612). The ICIs used in the studies included nivolumab, pembrolizumab, atezolizumab, avelumab, camrelizumab, tislelizumab and PD-1/PD-L1 inhibitors.

Seven studies reported OS or median OS (COPD vs. non-COPD, respectively: nonreached vs. 510 days, P<0.05; COPD better, P = 0.0126; 19.5 vs. 11.6 months, P=0.03; 20.6 vs. 10.8 months, P = 0.092; 359 vs. 145 days, P = 0.0350; COPD better, P=0.003; COPD better, P=0.2), and these studies also revealed longer PFS or median PFS in the COPD-LC group (COPD vs. non-COPD: 316 vs. 186 days, P=0.018; COPD better, P = 0.0407; 6.6 vs. 2.7 months, P<0.001; 6.5 vs. 2.3 months, P<0.01; 154 vs. 44 days, P = 0.0491; COPD better, P=0.003; COPD better,

TABLE 1 The search formula of PubMed.

Search number	Query
#1	Search: chronic obstructive pulmonary disease[MeSH Terms]
#2	Search: (((chronic obstructive pulmonary disease[Title/Abstract]) OR (COPD[Title/Abstract])) OR (ventilatory defect[Title/Abstract])) OR (emphysema [Title/Abstract])
#3	#1 OR #2
#4	Search: immune checkpoint inhibitor[MeSH Terms]
#5	Search: ((((((((((immune checkpoint inhibitor[Title/Abstract]) OR (pembrolizumab[Title/Abstract])) OR (atezolizumab[Title/Abstract])) OR (nivolumab [Title/Abstract])) OR (durvalumab[Title/Abstract])) OR (carrelizumab[Title/Abstract])) OR (toripalimab[Title/Abstract])) OR (sintilimab[Title/Abstract])) OR (tislelizumab[Title/Abstract])) OR (immunity therapy[Title/Abstract])) OR (PD-1[Title/Abstract])) OR (PD-L1[Title/Abstract])
#6	#4 OR #5
#7	Search: (Lung cancer[MeSH Terms]) OR (Lung cancer[Title/Abstract])
#8	#3 AND #6 AND #7

P=0.04). In addition, 5 studies reported ORR (COPD vs. non-COPD: 52.63% vs. 90.91%, P<0.05; 75.0% vs. 53.7%, P = 0.0586; 36.4% vs. 20.8%, P = 0.0167; 32.4% vs. 15.9%, P=0.022; 38.2% vs. 20.5%, P=0.028). The 2 other studies showed that FeNO levels, FVC and FEV1 were significantly increased in the COPD-LC group after immunotherapy (P<0.05), while there was no significant change in the LC group (P>0.05).

Overall, these studies suggested that COPD is not a risk factor for LC patients receiving immunotherapy. Conversly, LC patients with COPD may benefit more than non-COPD LC patients, with better PFS, OS and ORR, as well as increased FeNO levels and improved lung function based on FVC and FEV1; the details are specified in Table 2.

3 Potential mechanisms for LC patients with COPD benefiting from PD-1/PD-L1 inhibitor treatment

3.1 Interactions between PD-1/PD-L1 inhibitors and the tumor immune microenvironment (TIME)

3.1.1 Mechanisms of action of PD-1/PD-L1 inhibitors

Effective antitumor immunotherapy mainly relies on the modulation of the tumor microenvironment and restoration of

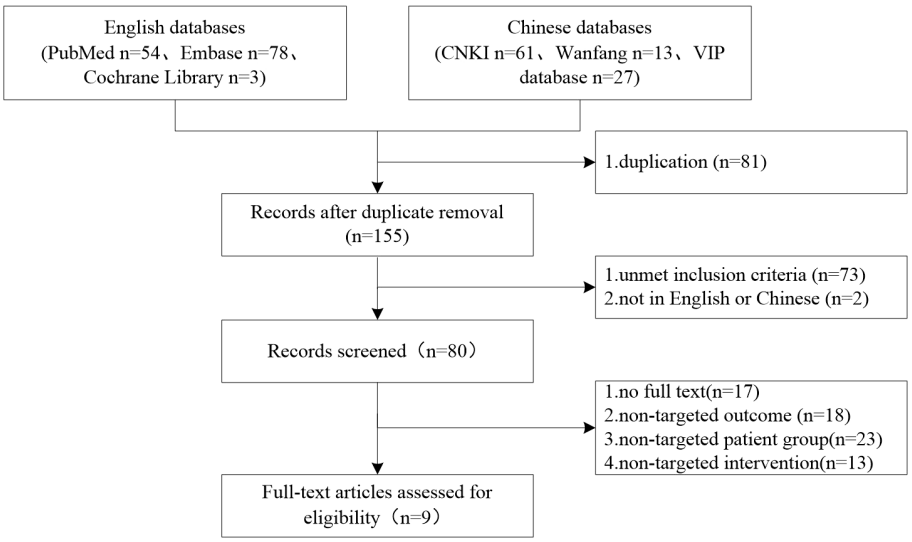

FIGURE 1
Flowchart of the literature search for ICI therapy in LC patients with COPD.

the T-cell response. The activation of T cells requires two signals: the first signal is antigen recognition, which comes from the binding of T-cell antigen receptor (TCR) to cognate antigen presented in the context of major histocompatibility complex (MHC) on the surface of antigen-presenting cells (APCs), and the second signal is provided by the interactions between costimulatory molecules, which are also called 'immune checkpoint' molecules (22). The second signals are divided into two types: costimulatory signals (classical pathways include B7.1/B7.2/B7H2-CD28, CD137 L-CD137, CD70-CD27, CD40-CD40 L) and coinhibitory signals (classical pathways include B7H1/B7DC-PD1, B7.1/B7.2/B7H2-CTLA4, HVEM-BTLA), which perform positive and negative regulatory functions, respectively (23–26).

The PD-1 receptor is expressed on the surface of T cells and primary B cells and plays an important role in the regulation of

TABLE 2 Main characteristics of studies in systematic reviews.

Study ID	Country	Type of Study	Disease Stage	Drug	COPD vs non-COPD		
					Population	Age (years)	Outcome
Jiebai Zhou, 2021 (8)	China	retrospective cohort study	Advanced-stage NSCLC (n=156)	PD-1/PD-L1 inhibitors	65 vs. 91	/	median OS: non-reached vs. 510 days, P<0.05; median PFS: 316 vs. 186 days, HR = 0.56, 95% CI (0.33,0.96), P=0.018
Huo Shufen, 2022 (14)	China	prospective cohort study	Recurrence、IIIA-IV NSCLC (n=30)	Pembrolizumab/Nivolumab/ Carrelizumab/Tislelizumab	19 vs. 11	All: 67.2 ± 9.8	ORR: 52.63% vs. 90.91%, P<0.05; FeNO levels: 26.08 (18.32, 32.91) vs. 21.10 (15.58, 24.84), P<0.05; FVC: 2.98 ± 0.35 vs. 2.62 ± 0.38, P<0.05; FEV1: 1.99 (1.65, 2.18) vs. 1.98 (1.60, 2.20), P<0.05
Yuzo Suzuki, 2019 (15)	Japan	prospective cohort study	IIIB, IV, or unresectable stage IIIA NSCLC (n=95)	Pembrolizumab	41 vs. 54	69 (67–73) vs. 69 (63–75)	ORR: 75.0% vs. 53.7%, P = 0.0586; FeNO levels: COPD increased (P =0.0242), non-COPD didn't (P>0.05) FVC/FEV1: COPD increased (P<0.05), non-COPD didn't (P>0.05)
Shinkichi Takamori, 2020 (16)	Japan	retrospective cohort study	Advanced or recurrent NSCLC (n=257)	Nivolumab/Pembrolizumab/ Atezolizumab	57 vs. 200	61.8 vs.65.0	OS: COPD better, P = 0.0126; PFS: COPD better, P = 0.0407; ORR: 36.4% vs. 20.8%, P = 0.0167
Yusuke Takayama, 2021 (17)	Japan	retrospective cohort study	Advanced-stage NSCLC (n=153)	Nivolumab/Pembrolizumab/ Atezolizumab	71 vs. 82	68.0 ± 9.5 vs. 68.0 ± 10.3	median OS: 19.5 vs. 11.6 months, HR=0.58, 95% CI (0.36, 0.94), P=0.03; median PFS: 6.6 vs. 2.7 months, HR=0.47,95% CI (0.32,0.69), P<0.001; ORR: 32.4% vs. 15.9%, P=0.022
Yoshimi Noda, 2022 (18)	Japan	retrospective cohort study	Advanced-stage NSCLC (n=56)	PD-1/PD-L1 inhibitors	41 vs. 15	70 (66–74) vs. 72 (64–75)	OS: 20.6 vs. 10.8 months, P = 0.092; PFS: 6.5 vs. 2.3 months, P < 0.01
Nicholas M. Mark, 2018 (19)	USA	retrospective cohort study	III-IV NSCLC (n=125)	Pembrolizumab/Nivolumab/ Atezolizumab/Avelumab	60 vs. 65	68.8 ± 7.0 vs. 64.3 ± 10.1	OS: 359 vs. 145 days, P = 0.0350; PFS: 154 vs. 44 days, P = 0.0491
Sun Hye Shin, 2019 (20)	Korea	retrospective cohort study	Advanced-stage NSCLC (n=133)	Pembrolizumab	59 vs. 74	65.3 ± 8.0 vs. 61.0 ± 10.2	OS: COPD better, HR=0.45, 95%CI (0.26,0.78), P=0.003; PFS: COPD better, HR=0.50; 95%CI (0.31,0.79), P=0.003; ORR: 38.2% vs. 20.5%, P=0.028
Jérôme Biton, 2018 (21)	France	retrospective cohort study	Advanced-stage NSCLC (n=39)	Nivolumab	19 vs. 20	64 ± 9 vs. 61 ± 12	OS: COPD better, P=0.2 PFS: COPD better, P=0.04

OS, Overall survival; PFS, progression-free survival; ORR, objective response rate; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

cell differentiation and apoptosis. PD-1 has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) (27). PD-L1 protein is widely expressed in activated T cells, B cells and macrophages and interacts with the receptor PD-1 on T cells to inhibit the activation of T cells and cause apoptosis in these cells, thus exerting suppressive effects on the immune response in various cancer types. Furthermore, the tumor microenvironment can also induce the expression of PD-L1, which induces the apoptosis of antitumor T cells and contributes to the occurrence and growth of tumors (23, 28, 29).

Under normal circumstances, APCs such as macrophages and dendritic cells can capture foreign pathogens or antigens, then process them to bind with MHC molecules, and present them outside the cells for T cells to recognize through the TCR (23, 24). In addition to MHC-TCR contact, costimulatory signals such as CD80/CD86-CD28 activate effective and sufficient T cells and initiate T-cell immune responses (25, 29). To avoid excessive T-cell activation caused by continuous antigen stimulation, coinhibitory molecules such as PD-1 and PD-L1 are transmitted in T cells, thus reducing the proliferation or apoptosis of T cells and avoiding excessive immune activation (22), which could prevent T-cell killing of tumor cells. However, it is necessary to stimulate the immune response of T cells in cancer therapy. Consequently, ICIs (PD-1/L1 inhibitors) can block the combination of PD-1/PD-L1 and negative regulatory signaling, recover the functional activity of T cells, and thus enhance the immune response against tumor cells.

3.1.2 TIME characteristics may be a vital determinant of the efficacy of ICI treatment

The tumor immune microenvironment (TIME) is a complex and dynamic ecosystem that consists of many different cell types, including tumor cells, immune cells and other supporting cells (e.g., fibroblasts, stromal cells and endothelial cells) (30). Circulating immune cells can be recruited by chemokines produced by tumor cells, fibroblasts or inflammatory cells and then migrate to the tumor site through the transendothelial process. As a result of the abnormal components and functions of immune cells in the TIME, tumor cells escape immunity and become drug resistant and metastasize. Among the multiple cell populations in the TIME, people can often find cells associated with acute inflammation (including neutrophils, basophils, and eosinophils), cells associated with innate immunity (including macrophages, NK cells, and DCs), and cells derived from adaptive immune responses (including CD8+ T cells, Th1-/Th2 cells, and B cells) (30, 31). Collectively, the TIME formed by the interaction between immune cells affects the invasion and metastasis of tumor cells, which can further modulate the progression of cancer (32).

As mentioned above, PD-1/PD-L1 blockades in the TIME exerts antitumor effects. On the one hand, as a part of the TIME, PD-1/PD-L1 blockades will change the composition or proportion of some immune cells in the TIME, but importantly, the composition of the TIME itself will induce the expression of PD-L1, affect the binding of PD-1/PD-L1 inhibitors and the subsequent recognition and phagocytosis of the immune response, and thus affect the antitumor effect (33). Existing studies have shown that the expression levels of target proteins, infiltrating T cells, and other types of immune cells in the TIME are closely related to the response to ICIs, which implies that the immune status of the TIME can determine the efficacy of ICIs (31). Studies have also shown that tumor-infiltrating lymphocytes (TILs) have potent and specific antitumor effects and are closely related to cancer prognosis (34–36). In particular, the effectiveness of immune checkpoint therapy can be predicted by CD8+ cells (34, 37). In addition, myeloid cells are heterogeneous immune cells of the innate immune system, represented by macrophages and dendritic cells (DCs), which have a powerful ability to modulate T-cell responses and play an important role in cancer progression. Therefore, any alterations in the TIME may affect the efficacy of ICIs; details are indicated in Figure 2.

3.2 Effects of COPD on the TIME in Patients with LC

Based on previous evidence, COPD has a significant impact on the TIME. We speculate that, on the one hand, the development of COPD is closely linked to the PD-1/PD-L1 pathway and the TIME and might be affected by various factors, such as T-cell apoptosis, altered expression of immune checkpoints on immune cells, and the effect of cytokines on immune cells or tumor cells. Some of the same pathogenesis might lead to a greater benefit of PD-1/L1 inhibitors (38, 39). On the other hand, the trend of COPD affecting some immune cells of the TIME might be consistent with PD-1/PD-L1 inhibitors or beneficial to PD-1/PD-L1 inhibitors to play a synergistic role or enhance the antitumor effects. Therefore, we discussed in detail how COPD affects the ICI efficacy of LC by modulating the TIME.

3.2.1 Cluster of differentiation 4+ (CD4+) cells and T-helper cell type 1/2 (Th1/2)

In the airways and alveolar lumen of COPD patients, the number of CD4+ T cells increases significantly with airflow limitation and emphysema staging (40, 41). Upon encountering specific antigens, the initial CD4+ T cells will activate and differentiate into two effector T-cell subtypes to function. Th1 cells are the main effectors of phagocyte-mediated host immunity, which secrete interleukin 2 (IL-2), interferon γ (INF-γ) and TNF-α and are mainly involved in defense against intracellular pathogens. In contrast, Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and other factors, which are involved in metabolic reactions and defense against parasitic infections (40).

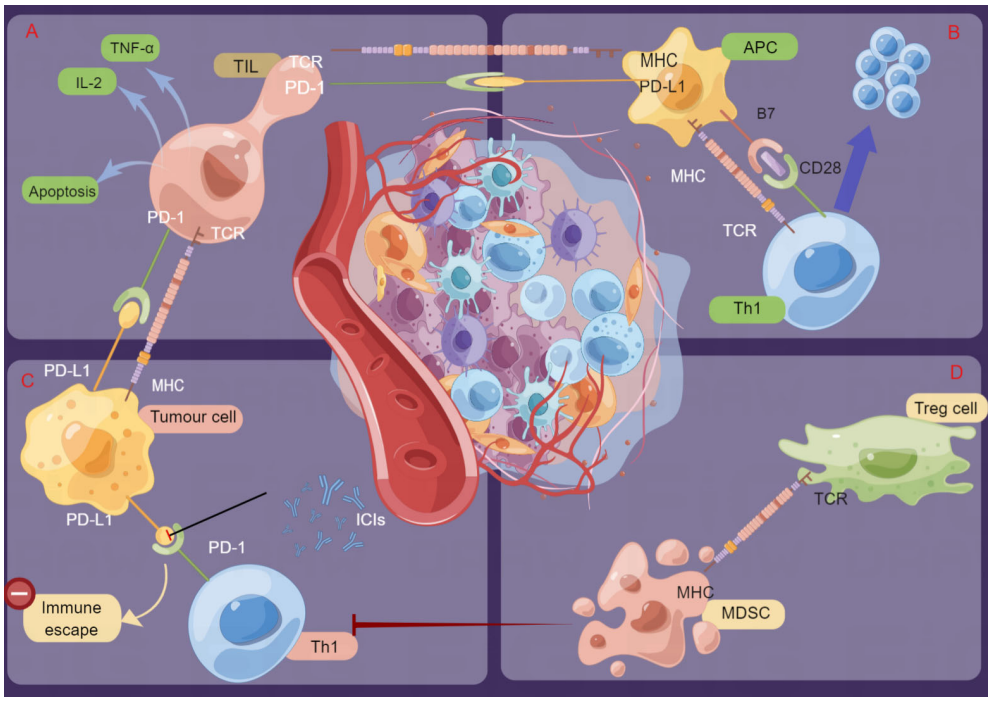

FIGURE 2
Schematic presentation of the mechanisms of immune checkpoint inhibitors (ICIs) and regulation of the tumor immune microenvironment (TIME). Tumor cells bind to PD-1 on the surface of T cells by overexpressing PD-L1 or PD-L2 molecules, thus inactivating T cells for immune escape. ICIs can inhibit these interactions by binding to PD-1 or PD-L1 and then activate cytotoxic T cells and other immune cells to kill tumor cells. **(A)**. TIL: Specific immune response to tumor cells. **(B)** APCs present antigens, stimulate T cells and transmit immune signals. **(C)** Th1: Mediates the cellular immune response and promotes cytotoxic T-cell (CTL) killing. **(D)** MDSCs: Inhibit the body immune cells to play normal innate and adaptive immune functions; Treg: Suppress the immune response of other cells and control self-tolerance) (by Figdraw).

Previous studies have indicated that Th1 cells secrete promyelocytic factors, which have antitumor effects, and Th2 cells secrete anti-inflammatory cytokines, which have tumor-promoting effects, and that the Th1/Th2 ratio is correlated with tumor stage (42). On the other hand, both Th1 and Th2 cells are increased in COPD and acute exacerbation of chronic obstructive pulmonary disease (AECOPD) compared to healthy individuals, and changes in the Th1/Th2 ratio correlate with the severity and prognosis of AECOPD (43). Thereby, we speculate that COPD affects tumor sensitivity to ICIs by affecting the ratio of Th1/Th2, and this hypothesis can be further confirmed by Mark et al. They confirmed that the number of CD8+ and CD4+ lymphocytes increased in COPD patients. Additionally, Th1 differentiation and PD-1 expression were increasingly affected by COPD lung tissue, which implied that the presence of COPD was associated with prolonged progression on-free survival in ICI-treated patients (19). An increased proportion of Th1 cells enhanced the antitumor immune response, and high levels of Th1 cells predicted better clinical outcomes after chemotherapy, while an increased proportion of Th2 cells downregulated the antitumor immune response and predicted worse chemotherapy outcomes (44). Therefore, CD4+ and Th1/2 cells play critical roles in the enhancement of ICI efficacy in LC with COPD.

3.2.2 CD8+ cells and tumor-infiltrating lymphocytes (TILs)

CD8+ cells have become incredibly important in antitumor immunity research due to their direct antitumor cytotoxicity. In tumor immunity, CD8+ cells are activated upon recognizing tumor antigens presented on MHC-I, release IFN-γ to bind to its receptor, and induce the expression of PD-L1 on tumor cells, which could bind to elevated levels of PD-1 on the surface of TILs, thus triggering the inhibition of the PD-1/PD-L1 axis (23). ICIs enhance antitumor activity by blocking the interaction of PD-1 with PD-L1 and eliminating the suppressive effects of CD8+ cells. At the same time, TILs often become dysfunctional ('exhausted') and fail to destroy tumor cells due to prolonged exposure to persistent antigens or chronic inflammation. Surprisingly, clear evidence from earlier animal studies suggests that this phenomenon is reversible and that ICIs can restore the antitumor activity of TILs through PD-1/PD-L1 inhibitors, which in turn leads to a durable response in different subgroups of patients with solid tumors (24, 45, 46). Therefore, T-cell exhaustion is often considered

a marker of tumor specificity and response to ICIs (45). Based on these two points, we hypothesized that the more PD-1/L1 expression or the stronger TIL exhaustion, the stronger the response to ICI may be.

By tissue analysis of NSCLC patients with and without COPD, Biton et al. revealed that COPD severity was positively correlated with CD8+ TIL depletion, as the expression of PD-1 and T-cell immunoglobulin domain and mucin domain-3 (TIM3) was enhanced in CD8+ cells from NSCLC patients with COPD (21). PD-1 and TIM3, as inhibitory receptors that induce T-cell depletion, are also considered markers of T-cell loss of function and tumor progression in NSCLC. Thus, it is evident that T-cell depletion mediated by its inhibitory receptors, such as PD-1, is present in patients with severe COPD. Furthermore, the results demonstrated that NSCLC patients with COPD had a higher survival rate after anti-PD1 therapy than those without COPD. Therefore, this study appears to highlight the effectiveness of PD-1 blockers in unleashing the antitumor CD8+ T-cell response in a subpopulation of patients characterized by strong CD8+ TIL depletion (21). This occurrence explains why COPD patients with fatigue but elevated TIL levels often respond better to ICI therapy than NSCLC patients without COPD.

3.2.3 Regulatory T cells (Treg) and T-helper cell type 17 (Th17)

Treg and Th17 belong to the same T-cell subpopulation and are involved in the pathogenesis of CODP and LC. Th17 cells promote inflammatory responses, while Treg cells suppress them (47). There is an important balance between Th17 and Treg cells, which plays an important role in maintaining the immune environment, and an imbalance can lead to abnormal immune responses locally or systemically (40, 47).

Treg cells, a poor factor in cancer prognosis, can infiltrate tumors and suppress antitumor immunity within the TMIE, thus promoting tumor progression and growth (48). Th17 cells have complex biological functions, and evidence suggests that these cells may paradoxically also contribute to antitumor immunity (40). Under *in vitro* conditions, Th17 cells themselves cannot directly kill tumor cells but achieve antitumor immunity by stimulating tumor cells, promoting T-cell recruitment to tumor sites, and initiating CD8+ T-cell killing (40). Taken together, the development of COPD may alter the Treg/Th17 balance, which in turn affects the effect of immunotherapy on LC.

3.2.4 Myeloid-derived suppressor cells (MDSCs)

MDSCs are a heterogeneous group of cells with significant immunosuppressive activity that promote tumor growth by suppressing effector T-cell function and thereby mediating tumor immune escape (47). Earlier studies indicated that in NSCLC, the synthesis of vascular endothelial growth factor (VEGF) was increased, and VEGF fostered the formation of MDSCs (49).

Meanwhile, Szentkereszty et al. determined that the effect of VEGF and MDSCs on systemic immunity was attenuated by the presence of COPD in patients with advanced NSCLC (50). They measured VEGF and MDSCs in patients with NSCLC or NSCLC combined with COPD. In NSCLC, a significant increase in VEGF and M-MDSCs and G-MDSCs was observed, whereas in NSCLC combined with COPD, M-MDSC scores were raised, yet G-MDSC scores remained constant. The study further analyzed the relationship between serum VEGF concentration and the size of various cell populations, again demonstrating a direct association between higher VEGF and M-MDSCs in NSCLC but the opposite relationship in NSCLC + COPD patients. Consequently, accompanying COPD decreased G-MDSCs and reversed the modulation of M-MDSCs by VEGF. The results suggested that PFS is positively influenced by COPD in advanced NSCLC because COPD supports some effector lymphatic function and alleviates tumor inflammation (50).

Overall, LC patients with COPD benefit more from ICIs due to their ability to alter the TIME. On the one hand, COPD-like chronic inflammation creates a favorable immunosuppressive TIME for tumorigenesis and development. On the other hand, COPD-related alterations in the TIME may lead to associated lung tumors overexpressing PD-L1 and PD-1, which respond better to ICIs.

4 New therapeutic strategies for LC with COPD

In current treatment guidelines, COPD is not an absolute contraindication of immunotherapy for LC patients but is considered a high-risk factor for the use of ICIs. The incidence of checkpoint inhibitor pneumonitis (CIP) in COPD and asthma patients was reported to be 2.3% higher than that in non-COPD or asthma patients (51) because LC patients with COPD require additional supervision after immunotherapy due to their weak lung function and immunity after long-term use of hormonal bronchodilators.

To achieve better therapeutic efficacy, some scholars have proposed identifying novel biomarkers or predictors to assess the risks or benefits of receiving ICIs. For example, Zhou et al. proposed that IL-2R may be used as a potential biomarker for ICI in patients with advanced LC and COPD, as high baseline levels of IL-2R and posttreatment elevations may predict poor prognosis (8). In addition, due to the existence of the same signaling pathways and pathogenic factors between COPD and LC, researchers have developed new drugs based on this idea, such as the development of new potential therapeutic targets using tobacco-related pathogenic mechanisms as an example

(52). Studying the genome-wide association (GWA) of LC and COPD revealed identical single nucleotide polymorphisms (SNPs) in the CHRNA3-CHRNB4-CHRNA5 gene cluster (52), which provides a sound basis for the development of new drugs and therapeutic strategies (52). However, most studies have been limited to animal models or small sample clinical trials. In addition, antagonists of some cytokines have been proposed for use in combination with immunotherapy, such as IL-17 antagonists, which have been proposed for COPD treatment and LC chemoprevention because IL-17 cytokines are associated with cigarette smoke-induced emphysema, and inhibition of IL-17 limits disease progression (19, 53).

5 Discussion and summary

5.1 Summary

We performed a systematic evaluation and first reviewed the efficacy of PD-1/PD-L1 inhibitors during LC combined with COPD after immunotherapy and then analyzed the potential reasons for the benefit to the corresponding population, providing new evidence and viewpoints for clinical treatment. Our findings differ from previous findings that COPD, as an independent risk factor for LC, is generally considered detrimental to patient treatment and prognosis. In contrast, we concluded that LC patients with COPD benefited better from anti-PD-1/PD-L1 therapy, with improved lung function, such as FeNO levels, FEV1, and FVC, and prolonged OS, PFS and ORR. The reason for these benefits may be that COPD alters the TIME of the LC, mainly manifested by increased Th1 expression, increased PD-1 expression on CD8+ cells, enhanced TIL exhaustion, an altered Treg/Th17 ratio, reduced G-MDSCs and reversed effects of VEGF on M-MDSCs.

5.2 Other potential mechanisms by which LC patients with COPD benefit from ICIs

In addition to changes in the TIME, COPD and LC have also been found to have altered epigenetic modifications, including DNA methylation and microRNA regulation (47–49). On the one hand, DNA methylation regulators have long been considered potential biomarkers for assessing the efficacy of ICIs (54), and studies have demonstrated that DNA methylation profiles can effectively infer the proportion of different types of immune cells in the TIME (55), and promoter methylation levels of CTLA4, LAG3, and PD-L1 are associated with efficacy-related immunotherapy (56). On the other hand, several studies have revealed that DNA methylation contributes to COPD development and serves as a potential biomarker for COPD disease prevention, diagnosis and prognostic assessment (57, 58). Therefore, we speculate that the modulation of DNA methylation in COPD and LC affects the efficacy of ICIs. For example, Wauters et al. analysed COPD-driven immune-related signatures by DNA methylation profiling of NSCLC and revealed some differences between LC patients with and without COPD, namely, the different levels of expression and methylation of genes that are primarily involved in the immune response (59). Recently, an epigenome-wide association study (EWAS) carried out the link between the gene methylation of COPD and LC. According to the methylation level and the degree of gene repression, COPD+LC was the highest, COPD was the second, and LC was the lowest (60).

MicroRNAs are short, single-stranded RNAs that play important roles in the pathophysiological processes of many diseases. Previously, some commonly dysregulated microRNAs have been identified in both COPD and LC and can be utilized as novel therapeutic targets, as well as for early diagnosis and prognosis (61). Fathinavid A et al. revealed that miRNA targets such as hsa-miR-15b and hsa-miR-106a are associated with COPD and LC, which are downregulated in COPD but upregulated in NSCLC (61). Yang et al. (62) also stated that the expression level of miR-103 was downregulated in NSCLC and COPD tissues, while it was inversely correlated with tumor stage and tumor size. Meanwhile, miR-106a is an oncogenic miRNA that targets the transcription factor FOXO3, thereby regulating apoptosis, cell cycle arrest, and autophagy-related genes (61, 62). We speculate that unlike LC or COPD alone, microRNAs are dysregulated in LC with COPD, thereby affecting the efficacy of ICIs.

Whether it is the alteration of the TIME or epigenetic (DNA methylation/MicroRNAs), more detailed studies on the molecular mechanism of lung cancer combined with COPD, such as the study of SNPs, exploration of biomarkers, and epigenetic modulation, are needed in the future to provide more basis for the clinical application of ICIs for lung cancer combined with COPD.

5.3 Safety for LC patients with COPD receiving ICIs

Although LC patients with COPD benefit from ICIs, some side effects occur during treatment, such as ICI-related pneumonia (IRP), thyroid toxicity, and dermal toxicity. In particular, IRP is very typical and closely related to the use of ICI in inflammatory situations. IRP is an inflammatory and invasive lung disease associated with ICI, which has a high termination rate and mortality, leading to discontinuation of treatment in LC patients (63, 64). Previous pairwise meta-analyses reported that the incidence of IRP in NSCLC was 3.6-4.1%, and the incidence of IRP in PD-1 inhibitors was higher

than that in PD-L1 inhibitors (64, 65). Moreover, COPD, as an inflammatory disease, is one of the potential risk factors for IRP. Therefore, both the efficacy and safety of ICIs should be carefully considered during the treatment of LC patients with COPD. For IRP that has occurred, international organizations or guidelines recommend that CIP be divided into different types and levels according to imaging and clinical symptoms. The treatment principles are as follows: appropriately delay ICI treatment + symptomatic treatment such as hormone + follow-up (mild IRP, level 1), suspend ICI treatment + symptomatic treatment such as hormone + hospitalization (moderate IRP, level 2), permanently stop ICI + symptomatic treatment such as hormone + hospitalization (severe IRP, level ≥3) (66). Then, for patients without IRP temporarily, we suggest establishing some predictive biomarkers or prediction models to avoid ADR. Some studies have built a prediction model for IRP and used COPD as one of the predictors to judge the probability of IRP occurrence by scoring patients' COPD and other factors (physical fitness score, ages) to provide treatment decisions for the clinical selection of ICI (63).

5.4 Advances in detection of PD-L1 expression

PD-L1 expression is a commonly used biomarker to predict ICI efficacy, and we attempted to find the optimal cut-off value of immunotherapy for LC patients with COPD, but there were no data available on the relationship between the expression level of PD-L1 and the efficacy of immunotherapy. We systematically evaluated the LC group and COPD+LC group, both including PD-L1<1%, 1-49% and ≥ 50%. However, there was no significant difference between the two groups. Therefore, research on the relationship between PD-L1 expression and immunotherapy efficacy may be a novel direction of future research.

Currently, several methods are available to detect the expression of PD-L1. 1) Immunohistochemistry (IHC): IHC staining is a classic method to detect protein expression, and the NCCN guidelines recommend IHC detection of PD-L1 expression in NSCLC. The FDA has also approved a variety of PD-L1 IHC tests as a concomitant diagnosis of whether tumor patients should receive ICIs (67). 2) Multiplex fluorescence (mIF): mIF is a promising tool in the scenario of immunotherapy because it can simultaneously detect and quantify PD-L1 markers with multiple antibody clones and conduct in-depth analysis of the number, density and spatial location of tumor and immune cells. MIF has been widely used at present, and it is expected to be a powerful clinical tool for accurate prognosis and prediction of efficacy, to help accurate prediction of efficacy, and to facilitate accurate screening of patients benefiting from immunotherapy (68, 69). 3) Imaging Mass Cytometry (IMC): This method can simultaneously analyze more than 40 markers on a single tissue slice. More importantly, it can realize the *in situ* detection of protein expression and ensure the integrity and accuracy of data. Especially for some precious microsamples, invalid loss during sample preparation is avoided (70). Previously, Alnajar et al. studied the immune cell repertoire and PD-L1 expression in patients with sarcomatoid urothelial carcinoma (SUC) using IMC, which promoted the understanding of the rare subtype of urothelial carcinoma (UC) (71). 4) Others: In addition to PD-L1, a growing body of data suggests the importance of immune cells and other important biomarkers in the TIME in guiding patients' drug selection and prognostic assessment (31). For example, circulating tumor cells (CTCs) can be detected and PD-L1 expression can be dynamically evaluated in a timely manner to identify drug resistance to the current treatment scheme and guide follow-up treatment. Immunoosmosis detection methods are also emerging, such as high-precision single-cell RNA sequencing and bulk RNA-seq.

5.5 Future perspective

The current review has some limitations: (1) There are few studies related to immunotherapy in LC patients with COPD, and more high-quality prospective studies are needed to verify these conclusions. (2) Most LC patients with COPD are over 60 years old, and the risk of adverse reactions is high, while we did not report the safety outcome. (3) Evidence related to the effects of SCLC and long-acting bronchodilator data on the efficacy of ICIs is lacking. (4) This study did not investigate the effects of CTLA-4 inhibitors on the efficacy in LC patients with COPD.

In general, the following areas should be considered in future research. First, from the perspective of the mechanisms for better efficacy of ICIs in LC patients with COPD, further studies on both the alteration of the TIME and epigenetic regulation (such as DNA methylation and noncoding RNA modulation) are of great importance. Moreover, research on microenvironment-related and immune-related gene SNPs and exploration of biomarkers for predicting the efficacy of ICIs in LC patients may provide a basis for the clinical application of ICIs in the treatment of lung cancer combined with COPD. Second, we should pay attention to the safety of ICI treatment in the COPD +LC population, especially the screening criteria for the expression level of PD-L1 or other biomarkers for ICI-related ADRs, and establish prediction models for IRPs. Last, at present, the monitoring of immunotherapeutic markers is mainly aimed at the tumor itself, but there is no objective monitoring and evaluation of the entire TIME. Therefore, we believe that the TIME should be monitored in the future, including TILs and Tregs, and new biomarkers related to the TIME should be explored.

6 Conclusion

In short, LC patients with COPD could benefit from anti-PD-1/PD-L1 therapy, which may be due to changes in the TIME (an altered Th1/2 ratio and Treg/Th17 ratio, increased consumption of CD8+ cells and TIL and MDSC reversal), ultimately improving lung function and prolonging the OS or PFS of patients. In the future, research on the relationship between the expression level of PD-L1 and the efficacy of immunotherapy, as well as the detailed molecular mechanisms by which COPD affects the TIME and ICI efficacy, would provide a novel basis for ICI treatment in LC patients with COPD.

Author contributions

ML and ZH conceived and designed the review. ML wrote the manuscript. YC prepared the figures. HX, ZH, and TW reviewed and edited 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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Anti-PD-1 antibodies, a novel treatment option for advanced chemoresistant pulmonary lymphoepithelioma carcinoma

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Background: Pulmonary lymphoepithelioma-like carcinoma (LELC) exhibits a unique immune microenvironment, including high PD-L1 expression and abundant infiltrating-immune cells. However, the availability of PD-1/PD-L1 inhibitors in patients with LELC is still not determined.

Methods: A total of 36 cases of pulmonary LELC treated with PD-1/PD-L1 inhibitors were reviewed, including 10 cases from our institute and 26 cases included from the literature. The Kaplan-Meier method and log-rank test were utilized to analyze the survival outcomes of LELC patients receiving immunotherapy, and the factors related to immunotherapy response were further examined.

Results: Of the 10 patients from our institute, the median age was 53.5 years, adrenal glands and distant lymph nodes were the most common metastatic sites, and 4 of 8 (50%) patients had a PD-L1 TPS ≥50%. The median progression-free survival and overall survival in patients from our institute and from the literature were 11.6 and 27.3 months, 17.2 months and not reached, respectively. In all 36 patients, the objective response rate was as high as 57.6%. Patients with higher PD-L1 expression were more likely to have a tumor response, but the association of PD-L1 expression with survival time remains to be determined.

Conclusions: PD-1/PD-L1 inhibitors in patients with pulmonary LELC demonstrated a promising efficacy in retrospective cohorts, and deserve further validation in prospective studies administrating in front-line setting.

KEYWORDS

lung cancer, Lymphoepithelioma-like carcinoma, rare subtype, efficacy, immunotherpay

Introduction

Primary pulmonary lymphoepithelioma-like carcinoma (LELC) is a rare subtype of non-small cell lung cancer (NSCLC) predominantly affecting younger non-smokers in Southeast Asia where nasopharyngeal cancer (NPC) prevails (1, 2). First reported in 1987, it is a non-keratinizing, poorly differentiated, pulmonary-originated squamous cell carcinoma associated with Epstein-Barr Virus (EBV) infection (3). Recently, a genetic study using whole-exome sequencing has revealed that pulmonary LELC possesses a distinct genomic profile from other lung cancers but shares similar alterations with NPC, including constitutive activation of inflammatory nuclear factor kappa B (NF-κB) signaling driven by EBV-encoded oncoprotein latent infection membrane protein 1 (LMP1) and crippled innate antiviral immunity due to losses of type I interferon (IFN) genes (4). In addition, programmed cell death-ligand 1 (PD-L1) upregulation in pulmonary LELC has been recognized as a major culprit to blame for undermining adaptive immune response (4, 5). Indeed, pulmonary LELC is uniquely featured by its inflamed environment but effective immune evasion.

Optimal treatment for advanced pulmonary LELC has not been well established. Our previous study had identified that platinum-based combination chemotherapy with or without radiotherapy could achieve good initial responses (6). However, the majority of advanced tumors eventually progressed from upfront treatments and there are limited therapeutic options to choose from after their resistance to chemotherapy. Actionable oncogenic driver mutations in NSCLC, such as epidermal growth factor receptor (EGFR) mutation and anaplastic lymphoma kinase (ALK) rearrangement, rarely exist in pulmonary LELC (7). PD-1 blockade or its combination with chemotherapy has been proven effective in treating patients with advanced NPC (8–10). Based on the histologic and genetic resemblance of pulmonary LELC to NPC (4, 5, 11), one might naturally surmise that PD-1/PD-L1 inhibitors would emerge as another promising weapon in the therapeutic arsenal against this rare tumor.

Yet, only a handful of case reports have explored the efficacy of anti-PD-1/PD-L1 antibodies in pulmonary LELC (12–21). The rarity of this lethal disease makes it impossible to conduct any convincing clinical trial for this matter. Here, we reported a cohort of ten patients who received anti-PD-1 therapy after routine management failed. To our knowledge, this represents the first and the largest cohort to test PD-1 inhibitors as late-line treatment in patients with advanced pulmonary LELC. We also performed a focused search of the literature with Pubmed to identify studies of blocking PD-1/PD-L1 in pulmonary LELC and attempted to determine predictors of efficacy from collected clinicopathological traits in this enriched population.

Methods

Patients

From July 2017 to September 2020, 10 patients received anti-PD-1 antibodies after progression from previous chemotherapy in the Centro Hospitalar Conde de Sao Januario (CHCSJ), Macau for advanced pulmonary LELCs. Data were collected from the hospital information system. All these cases were confirmed by Epstein-Barr encoding region (EBER) positivity. Otolaryngologists' consultations with nasopharyngoscopy check-ups and imaging tests were applied to rule out NPC or other origins of LELCs. Flat dosing of nivolumab (240mg every 2 weeks) or pembrolizumab (200mg every 3 weeks) were given until progressive disease or intolerable toxicity. Tumor assessments were performed by computed tomography before the anti-PD-1 treatment and every 6 or 9 weeks thereafter. Treatment response was determined according to Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST) (22). Progression-free survival (PFS) was calculated from the date of starting anti-PD-1 treatment to disease progression or death due to any cause. Overall survival (OS) was calculated from the date of starting anti-PD-1 treatment to death due to any cause.

PD-L1 immunohistochemistry and scoring

PD-L1 expressions of 8 patients were assessed, based on sample deriving from primary tumor, by the PD-L1 immunohistochemical (IHC) staining 22C3 pharmDx assay (Dako North America, CarpinteriaCA) that has been approved as a companion diagnostic for use in non–small-cell lung cancer (23). The staining protocol used in this study was as described in the instructions for the commercial assay. Expression was scored using a tumor proportion score (TPS) which is defined as the number of positive tumor cells divided by the total number of viable tumor cells multiplied by 100%.

Literature search

We conducted a literature search for reports of pulmonary LELCs in the Pubmed database and collected 191 studies. We then screened out 11 studies that focused on using PD-1/PD-L1 inhibitors monotherapy or their combination to treat advanced diseases. 10 studies were finally recruited after removing 1 case that was duplicated in the CHCSJ cohort. Studies that contained original information on clinical results of PD-1/PD-L1 inhibitor treatment were screened, including original researches and case reports. Clinicopathological

factors, PD-L1 expression status, tumor response, and survival data were collected.

Statistical analysis

The Mann-Whitney U test was used to analyze the relationship between PD-L1 expression and tumor responses to anti-PD-1/PD-L1 therapy. PFS and OS were assessed using Kaplan-Meier method. Patients were divided into two groups (low/high) according to their PD-L1 expression level and based on the optimal cut-off value of PFS calculated by the "survminer" package of R software. Univariate and multivariate analyses were applied to identify prognostic factors of PFS. R (version 3.6.1, http://www.r-project.org) was used for statistical analyses. Two-tailed value of $p < 0.05$ was considered to be statistically significant.

Results

Clinicopathological features of the CHCSJ cohort

The clinicopathological features of the CHCSJ cohort are described in Table 1. There were 4 males and 6 females. The median age of this cohort was 53.5 years (range, 46-67 years). Adrenal glands and distant lymph nodes were the most common sites of metastases (n=3, respectively). 8 patients had their PD-L1 expression tested: 4 (50%) were with TPS ≥50%, 3 with TPS 1~49%, and 1 with TPS <1%. 6 patients received pembrolizumab while 4 others nivolumab. Anti-PD-1 antibodies were applied in the 2nd line setting in 3 patients, the 3rd line setting in 3 patients, and the 4th line setting in 4 patients. The median duration of anti-PD-1 treatment was 10.5 cycles (range, 1 to 30 cycles) and detailed descriptions of the duration of each treatment were shown in Figure 1.

Efficacy of PD-1 inhibitors in the CHCSJ cohort

8 patients were available for response rate analysis and 2 others died before an imaging-based assessment could be carried out. The objective response rate (ORR) was 62.5% (5/8), while disease control rate (DCR) was 100%. One male patient (12.5%) receiving nivolumab monotherapy as 3rd line treatment achieved a complete response (CR). Partial response (PR) was reached in 4 (50.0%) patients who took pembrolizumab or its combination with chemotherapy in the 2nd to 4th line. 3 others achieved stable disease (SD).

During a median follow-up time of 18.5 months, 7 patients had progressed from PD-1 inhibitors or died from pulmonary LELC. The median PFS was 11.6 months (95% CI 8.8-NR [not reached]) and the median OS was 27.3 months (95% CI 17.4-NR) (Supplementary Figure S1).

Efficacy of PD-1/PD-L1 inhibitors in the literature review

We finally recruited ten reports from 2017 to 2021 and gathered a total of 26 pulmonary LELC patients who were treated with PD-1/PD-L1 inhibitors for their unresectable or metastatic diseases (Table 2). 42.3% of patients received PD-1/PD-L1 inhibitors monotherapy, 34.6% of patients anti-PD-1 antibodies with chemotherapy, and 23.1% of patients anti-PD-1 antibodies with vascular endothelial growth factor receptor (VEGFR)-targeted tyrosine kinase inhibitors (TKIs). 19.2% of patients took immunotherapy in the 1st line, 57.7% of patients in the 2nd line, 23.1% of patients in the 3rd or later lines. Of 25 patients whose responses had been clearly reported, 56% achieved PR and 40% SD. Only 1 patient experienced quick progression after nivolumab. The median PFS was 17.2 months

TABLE 1 Clinicopathological factors of the CHCSJ cohort.

No.	Sex	Age	SmokingStatus	Staging	TPS	Metastaticsites	Anti-PD-1 antibodies	No. of line	BestResponses	PFS (m)	OS (m)	Outcomes
1	F	63	Non-smoker	IVB	95%	Adrenals	Pembrolizumab	4th	PR	16.2	17.4	Deceased
2	F	50	Non-smoker	IVA	90%	Pleura	Pembrolizumab	4th	PR	25.4	27.3	Deceased
3	F	59	Non-smoker	IVB	35%	Lung & liver	Pembrolizumab	2nd	PR	17.5	17.5	Ongoing
4	F	67	Non-smoker	IVB	55%	Lung & NRLNs	Pembrolizuamb+GC	3rd	PR	8.5	8.5	Ongoing
5	M	59	Ex-smoker	IVB	NA	Bone & NRLNs	Nivolumab	3rd	SD	3.5	12.5	Progressed
6	M	60	Non-smoker	IVB	NA	NRLNs	Nivolumab	4th	CR	11.6	12	Ongoing
7	F	46	Non-smoker	IIIA	<1%	None	Pembrolizumab	2nd	SD	8.8	37	Surgery
8	F	48	Current-smoker	IIIB	10%	None	Pembrolizumab	2nd	SD	2	2	Ongoing
9	M	58	Current-smoker	IVB	20%	Pleura & liver	Nivolumab	4th	NA	3.5	3.5	Deceased
10	M	54	Current-smoker	IVB	95%	Adrenals & liver	Nivolumab	2nd	NA	0.3	0.3	Deceased

(NRLNs, Non-regional lymph nodes; GC, gemcitabine plus carboplatin; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NA, not applicable).

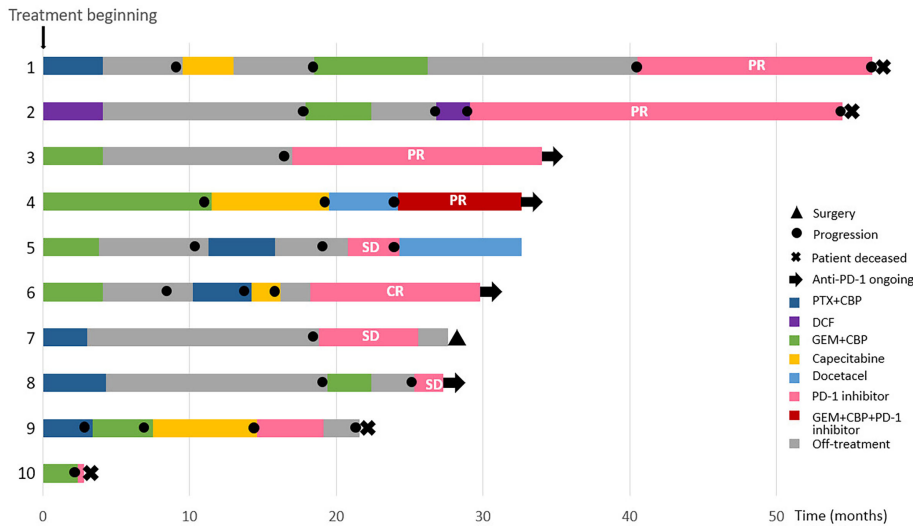

FIGURE 1
Summary of treatment reactions to chemotherapy and anti-PD-1 antibodies in the CHCSJ cohort.

(95% CI 7.7-NR). The median OS was not reached (Supplementary Figure S2).

Relation of PD-L1 expression with efficacy

Altogether, the CHCSJ cohort and literature review contributed 36 pulmonary LELC patients. 30 patients had PD-L1 expression analyzed using TPS. 33 patients had clear tumor response data of anti-PD-1/PD-L1-based therapy. Of 27 patients with both available PD-L1 expression and immunotherapy response data, those who achieved PR had a significantly higher level of PD-L1 expression compared to those who achieved SD (median PD-L1 expression: 80% vs 22.5%, p=0.002, Figure 2A). Utilizing TPS 30% as the cut-off value determined by the "survminer" package of R software, patients with high PD-L1 expression (>30%) had higher ORR (14/17, 82.4% vs 1/10, 10.0%, p<0.001, Figure 2B) than those with low PD-L1 expression (≤30%). Likewise, the PFS of patients with high PD-L1 expression in the CHCSJ cohort was 25.4 months (95% CI 16.2-NR), significantly longer than that of those with low PD-L1 expression (6.2 months, 95% CI 3.5-NR, p=0.027, Figure 3A). However, such survival advantage in patients with high-level PD-L1 expression was not seen in OS analysis (27.3 vs 3.5 months, p=0.628, Figure 3B). Furthermore, there were no significant differences in PFS (17.2 vs 8.8 months, p=0.177, Figure 3C) and OS (22.0 months vs NR, p=0.541, Figure 3D) between those with high and low PD-L1 expression when the two cohorts combined.

Prognostic relevance of clinicoprognostic factors for PFS and OS

During a median follow-up time of 10.5 months (range, 0.3-37 months), 18 patients experienced disease progression and 8 patients died of pulmonary LELCs among 36 patients. The Cox regression analysis was conducted to assess the impact of other clinical factors on PFS and OS. As shown in Table 3, univariate analysis showed that, compared to non-smoking patients, former or current smokers tended to have poor PFS (HR=12.08, p=0.053). However, neither smoking history nor treatment modality became independent predictors in multivariate analysis. In contrast, the difference in OS between never-smokers and ever-smokers was statistically significant (HR 30.74, 95% CI 2.11-447.25, p=0.012). However, other factors including sex, age (≥60y vs <60y), tumor stage (locally advanced vs metastatic), treatment modality (monotherapy or combination), liver metastasis (yes vs no), treatment scenario (<2nd line vs ≥3rd line) and PD-L1 expression (TPS >30% vs ≤30%) did not seem to exert any significant influence on the OS of patients with pulmonary LELCs (Table 4). Furthermore, compared with patients receiving immunotherapy alone, those who received immunotherapy combined with chemotherapy or targeted therapy had statistically non-significant improved PFS (8.8 vs 17.2 months, p=0.128) and OS (15.3 months vs NR, p=0.541, Supplementary Figure S3).

Discussions

Pulmonary LELC is a rare subtype of NSCLC characterized by EBV infection and abundant lymphocyte infiltration. EBV

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TABLE 2 Summaries of the literature review.

No.	References	Sex	Age	SmokingStatus	Staging	TPS	Anti-PD-1 antibodies	No. of line	BestResponses	PFS (m)	OS (m)	Outcomes
1	Xie, et al.	F	56	Non-smoker	IV	30%	Nivolumab+GEM	1st	SD	1	1	Lost f/u
2	Xie, et al.	F	49	Non-smoker	IIIB	60%	Nivolumab+GEM+ anlotinib	2nd	SD	6	6	Ongoing
3	Xie, et al.	M	48	Non-smoker	IVA	15%	Camrelizumab+ apatinib	3rd	SD	7	7	Ongoing
4	Qiu, et al.	F	56	Non-smoker	IVA	80%	Nivolumab	2nd	PR	4.4	4.4	Ongoing
5	Darrason, et al.	F	51	Ex-smoker	IVB	0%	Nivolumab	2nd	NA (PseudoPD)	7	14	Deceased
6	Kim, et al.	F	37	Non-smoker	IIIA	NA	Nivolumab	2nd	PD	0.8	1	Deceased
7	Kumar, et al.	M	56	Ex-smoker	IVA	NA	Nivolumab	4th	PR	21	25	Ongoing
8	Kumar, et al.	F	37	Non-smoker	IIIB	5%	Nivolumab	3rd	SD (PseudoPD)	24	27	Ongoing
9	Narayanan	F	76	Non-smoker	IVA	≥ 50%	Atezolizumab	2nd	PR	4	22	Deceased
10	Wu, et al.	F	58	Non-smoker	IIIA	40%	Sintilimab+anlotinib	2nd	PR	8.3	8.3	Ongoing
11	Wu, et al.	F	53	Non-smoker	IA2	30%	Pembrolizuamb+ nab-PTX	2nd	SD	10.9	10.9	Ongoing
12	Wu, et al.	F	48	Non-smoker	IV	90%	Pembrolizumab	2nd	SD	4.2	4.2	Lost f/u
13	Wu, et al.	F	56	Non-smoker	IVA	80%	Nivolumab	2nd	PR	7.5	15.3	Progressed
14	Wu, et al.	F	63	Non-smoker	IV	5%	Nivolumab+anlotinib	4th	SD	24.5	26	Ongoing
15	Tang, et al.	F	50	Non-smoker	IVB	10%	Nivolumab/Nivolumab +nab-PTX+NDP	2nd	SD (PseudoPD)	5	10	Progressed
16	Fu, et al.	M	68	Current-smoker	IVB	80%	Sintilimab	2nd	SD	3.4	3.4	Ongoing
17	Fu, et al.	F	56	Non-smoker	IVA	30%	Pembrolizumab	2nd	SD	7.7	7.7	Progressed
18	Fu, et al.	M	55	Non-smoker	IVB	90%	Pembrolizumab+ nab-PTX+CBP	1st	PR	9.5	11.8	Progressed
19	Fu, et al.	F	63	Non-smoker	IVB	70%	Pemrolizumab+ pemetrexel	1st	PR	14.4	14.4	Ongoing
20	Fu, et al.	F	70	Non-smoker	IVA	90%	Nivolumab+Anlotinib	4th	PR	15	15	Ongoing
21	Fu, et al.	M	46	Non-smoker	IVB	60%	Nivolumab+Apatinib	5th	PR	17	26.5	Progressed
22	Fu, et al.	F	56	Non-smoker	IVA	80%	Nivolumab+DXT	2nd	PR	17.2	17.2	Progressed
23	Fu, et al.	F	61	Non-smoker	IVB	1-50%	Sintilimab+Anlotinib	2nd	PR	6.4	6.4	Ongoing
24	Fu, et al.	F	54	Non-smoker	IVB	NA	Sintilimab+nab-PTX+CBP	1st	PR	7.5	7.5	Ongoing
25	Fu, et al.	M	43	Non-smoker	IIIC	80%	Sintilimab	1st	PR	3.2	3.2	Ongoing
26	Chen, et al	M	41	Non-smoker	IIIB	NA	Pembrolizumab+ nab-PTX+S1	2nd	PR	4.5	4.5	Progressed

(DXT, docetaxel; nab-PTX, Nanoparticle albumin-bound paclitaxel; GEM, gemcitabine; CBP, carboplatin; S-1 Triflurdine/tipiracil; NA, not applicable; PseudoPD, Pseudoprogression).

infection has been linked to upregulation of PD-L1 expression in malignancies (24). On the other hand, a high level of lymphocyte infiltration and PD-L1 expression are believed to be associated with immunotherapy response (25). However, the efficacy of PD-1/PD-L1 inhibitors in pulmonary LELC needs to be further verified.

In the present study, we described 10 previously treated LELC patients receiving PD-1 inhibitors, with an ORR as high as 62.5%. Of note, in a retrospective study previously reported by our institution, 41 LELC patients treated by 2nd or above chemotherapy had an ORR of 20-25% (6). Likewise, previously treated LELC patients receiving immunotherapy also tended to have longer PFS and OS (6). Furthermore, we performed a literature review, and found that pulmonary LELC tends to have a higher PD-L1 expression and desirable immunotherapy response compared with other subtypes of NSCLC (26). Consistently, in the CHCSJ cohort, our results suggested that the PFS of patients with PD-L1 TPS >30% was significantly longer than those with TPS ≤30%. In contrast, the association of prolonged PFS with higher PD-L1 expression was not observed in patients retrieved from literature or when two cohorts were combined. Furthermore, the prognostic significance (both PFS and OS) of PD-L1 expression levels in LELC patients treated with chemotherapy is also controversial (27–29). Intriguingly, when we focused on the patients of Chinese descent, those with high PD-L1 expression (TPS >30%) tended to have a prolonged

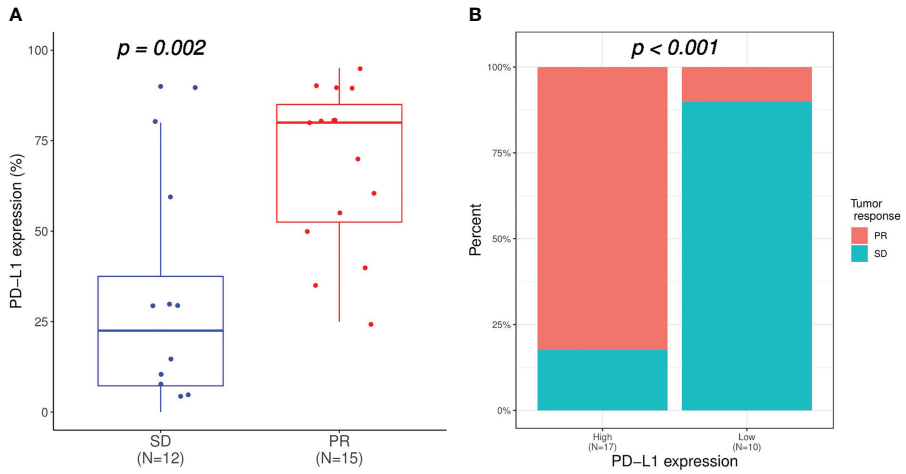

FIGURE 2
Relation of PD-L1 expression with immunotherapy response. **(A)** Comparison of PD-L1 expression between patients achieved SD and PR. **(B)** Comparison of immunotherapy response between patients with high (TPS ≥30%) and low (TPS <30%) PD-L1 expression. SD, stable disease; PR, partial response; TPS, tumor proportion score.

PFS (n=29, p=0.09, data not shown). Even when the study was limited to the patients who received PD-1/PD-L1 inhibitors in China, high PD-L1 expression was significantly associated with prolonged PFS (n=28, p=0.049, data not shown). These results suggest the possibility that patient pedigree and environmental factors may influence the efficacy of immunotherapy in pulmonary LELC. On all accounts, given the desirable efficacy of PD-1/PL-L1 inhibitors in lung and nasopharyngeal cancer, our results support the administration of immunotherapy in patients with pulmonary LELC.

Notably, our results suggested non-smoking patients with pulmonary LELC may have better survival outcomes when compared to ever-smokers. However, the effect of smoking on the tumor immune microenvironment is complicated. On the one hand, smoking has been associated with elevated tumor mutation burden and PD-L1 expression, suggesting better immunotherapy results (30). On the other hand, smoking may impair PD-1/PD-L1 response by inhibiting immune cell infiltration into tumors (31). Studies support that smoking can improve the efficacy of checkpoint inhibitor monotherapy, but does not significantly affect the response of NSCLC to immunochemotherapy combination (32). Specifically, a previous study reported that smoking was an independent predictor of unfavorable survival in patients with pulmonary LELC (33). Overall, the effect of smoking status on immunotherapy outcomes in patients with LELC needs to be further confirmed in larger cohorts.

To the best of our knowledge, the present study reports the largest cohort to date of pulmonary LELC patients treated with PD-1 inhibitors, and our results support the administration of immunotherapy in LELC patients. Nevertheless, several limitations need to be highlighted. Firstly, the relatively small scale of the CHCSJ and literature review cohorts might lead to selection bias. Although we did a thorough screening in Pubmed, only 1 case whose disease progressed after the application of nivolumab was found. This may be the case, meaning the vast majority of advanced pulmonary LELCs responded well or at least with stable disease to anti-PD-1/PD-L1 therapy, or cases that didn't benefit from immunotherapy hadn't been reported and included in this study. Unavailability of the responses in several cases was another reason why the efficacy of anti-PD-1/PD-L1 antibodies should not be overestimated in daily practice. Secondly, some of the public data were lacking. For example, patient fitness and treatment-related adverse events were untouched in this study due to incomplete data and it may have an impact on clinical outcomes of pulmonary LELCs. PD-L1 expressions in some cases were inaccessible and different antibodies such as SP142 or 22C3 were used for TPS evaluation in public data. All may interfere with our endeavor to identify the relationship between PD-L1 expression and the efficacy of anti-PD-1/PD-L1 antibodies, for blueprint study showed interchangeability of 22C3 and other antibodies but lower sensitivity of SP142 in NSCLC TPS IHC assay (34). Thirdly, the study was puzzled by various anti-PD-1/PD-L1 regimens and their combinations with chemotherapy or targeted therapy. However, all these PD-1/PD-L1 inhibitors have been proven to be comparably effective in treating driver mutation-negative NSCLC either on their own or joined by other active agents (35). Nevertheless, combining public data and Macau cases, our analysis made a good summary of how PD-1/PD-L1 antagonists performed in LELCs and proved a positive correlation between PD-L1 expression on the efficacy of immunotherapy. It also raised the evidence of using PD-1/PD-L1

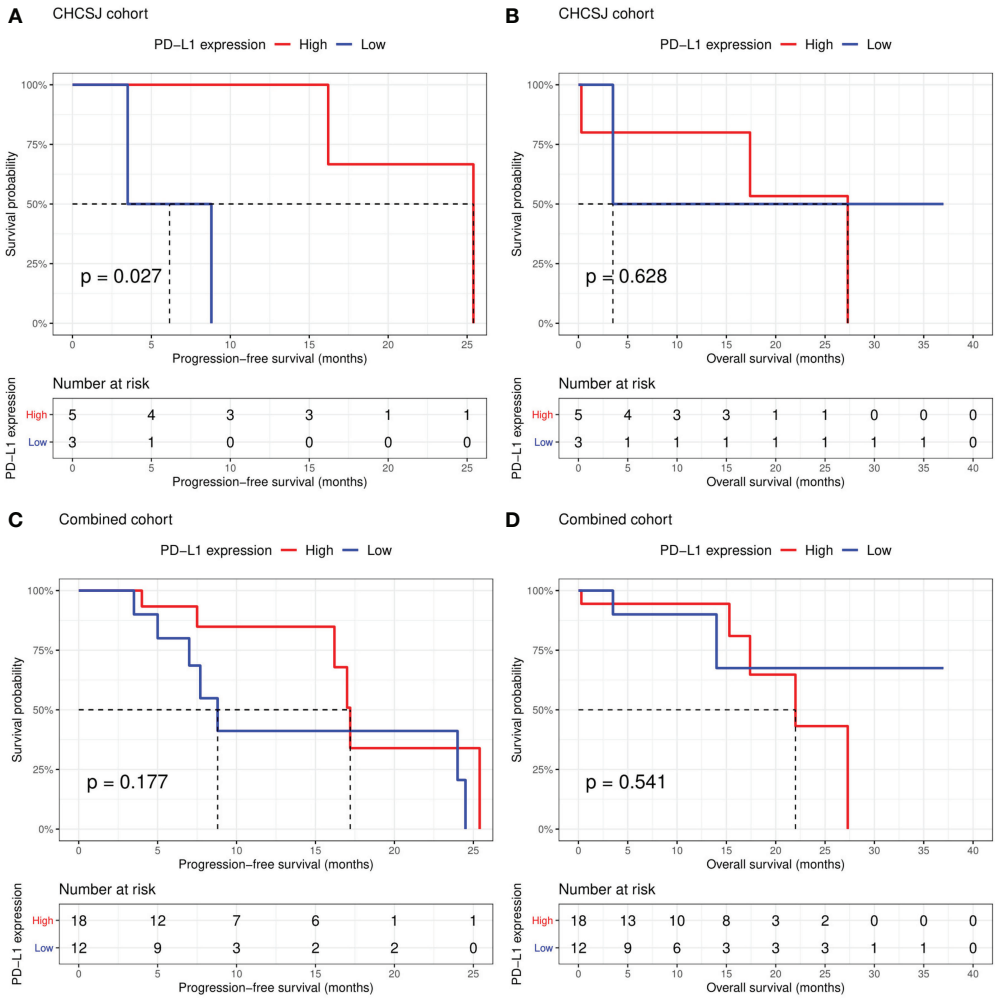

FIGURE 3
Comparison of progression-free survival **(A, C)** and overall survival **(B, D)** between patients with high (TPS ≥30%) and low (TPS <30%) PD-L1 expression in the CHCSJ and combined cohorts. TPS, tumor proportion score.

TABLE 3 Univariate and multivariate analyses of factors for progression-free survival in the combined two cohorts.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Sex (Female vs. Male)	0.48 (0.17,1.35)	0.166	0.54 (0.18,1.59)	0.266
Age (≥60 vs. <60)	0.72 (0.23,2.24)	0.567	-	-
Smoking history (Yes vs. No)	12.08 (0.96,151.4)	0.053	6.26 (0.47,83.78)	0.166
TNM stage (IV vs. III)	0.67 (0.21,2.09)	0.487	-	-
PD-L1 expression (Low vs. High)	2.07 (0.64,6.68)	0.223	-	-
Multiple metastases (Yes vs. No)	0.9 (0.29,2.8)	0.852	-	-
Liver metastasis (Yes vs. No)	1.31 (0.41,4.14)	0.647	-	-
Treatment line (>2 vs. 1-2)	0.49 (0.16,1.55)	0.226	-	-
Combination therapy (Yes vs. No)	0.46 (0.16,1.33)	0.154	0.5 (0.17,1.47)	0.209

TABLE 4 Univariate and multivariate analyses of factors for overall survival in the combined two cohorts.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p*	HR (95% CI)	*p*
Sex (Female vs. Male)	0.79 (0.15,4.15)	0.783	-	-
Age (≥60 vs. <60)	0.98 (0.19,5.1)	0.985	-	-
Smoking history (Yes vs. No)	28.31 (2.35,340.82)	0.008	30.74 (2.11,447.25)	0.012
TNM stage (IV vs. III)	2.41 (0.28,20.51)	0.42	-	-
PD-L1 expression (Low vs. High)	0.59 (0.11,3.11)	0.538	-	-
Multiple metastases (Yes vs. No)	3.26 (0.79,13.51)	0.103	1.83 (0.38,8.69)	0.449
Liver metastasis (Yes vs. No)	3.07 (0.68,13.77)	0.144	3.6 (0.68,19.21)	0.133
Treatment line (>2 vs. 1-2)	0.58 (0.13,2.54)	0.467	-	-
Combination therapy (Yes vs. No)	0.22 (0.01,14.6)	0.249	-	-

inhibitors in advanced pulmonary LELC to a higher level rather than individual experiences.

In conclusion, our results preliminarily examine the efficacy of anti-PD-1 antibodies in patients with pulmonary LELC. Further validation was also warranted for solid conclusions supplementary to the scarce specified data about immunotherapy in pulmonary LELC.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of CHCSJ. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

NZ, YYW and YZW conceived the study. NZ, HT, YL and YZW reviewed medical records and analyzed the data. SY performed pathological review. NZ, HT and YZW drafted the manuscript. YYW revised the draft. 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/fimmu.2022.1001414/full#supplementary-material

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PD-1/PD-L1 inhibitors plus anti-angiogenic agents with or without chemotherapy versus PD-1/PD-L1 inhibitors plus chemotherapy as second or later-line treatment for patients with advanced non-small cell lung cancer: A real-world retrospective cohort study

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Background: The aim of this study was to assessment the efficacy and safety of Programmed cell death protein 1 (PD-1)/Programmed cell death-Ligand protein 1 (PD-L1) inhibitors plus anti-angiogenic agents with or without chemotherapy versus PD-1/PD-L1 inhibitors plus chemotherapy as second or later-line treatment for patients with advanced non-small cell lung cancer.

Methods: In this study, pre-treatment clinical and laboratory indicators from 73 patients with advanced non-small cell lung cancer were retrieved for retrospective analysis. According to the therapy regimes they received, the patients were separated into groups, PD-1/PD-L1 inhibitors plus chemotherapy group (PC group), PD-1/PD-L1 inhibitors plus anti-angiogenic agents' group (PA group), PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus chemotherapy group (PAC group). Cox's proportional hazards regression model and Kaplan-Meier (KM) curves were used to assess the connection between treatment regimens and progression free survival (PFS) and overall survival (OS). In addition, the association of treatment regimens with the risk of disease progression and death was evaluated by subgroup analysis.

Results: The average age of the enrolled patients was 58.2 ± 10.2 years and 75.3% were male. Multivariate analyses showed that patients in PA group (Disease progression: HR 0.4, P=0.005. Death: HR 0.4, P=0.024) and PAC group (Disease progression: HR 0.3, P=0.012. Death: HR 0.3, P=0.045) had a statistically significant lower hazard ratio (HR) for disease progression and death compared to patients in PC group. Kaplan-Meier analysis showed that patients in PA group (mPFS:7.5 vs.3.5, P=0.00052. mOS:33.1 vs.21.8, P=0.093) and PAC group (mPFS:5.1 vs.3.5, P=0.075. mOS:37.3 vs.21.8, P=0.14) had a longer PFS and OS compared to patients in PC group. In all the pre-defined subgroups, patients in PA and PAC groups showed a decreasing trend in the risk of disease progression and death in most subgroups. The patients in PA group (DCR:96.3% vs.58.3%, P=0.001) and PAC group (DCR:100% vs.58.3%, P=0.019) had a better disease control rate (DCR) than patients in PC group.

Conclusion: PD-1/PD-L1 inhibitors plus anti-angiogenic agents with or without chemotherapy were superior to PD-1/PD-L1 inhibitors plus chemotherapy as second or later-line treatment in patients with advanced non-small cell lung cancer.

KEYWORDS

advanced non-small cell lung cancer, second or later-line therapy, PD-1/PD-L1 inhibitors, anti-angiogenic agents, real-world study

Introduction

Lung cancer is one of the cancers that poses the greatest menace to people's health and lives. According to the World Health Organization's International Agency for Research on Cancer's latest "Global Cancer Statistics 2020" data, lung cancer incidence and mortality rates in China were significantly higher, with men accounting for the highest incidence and mortality rates of all malignant tumors, and women accounting for the second highest incidence and first highest mortality rates (1).

85% of lung cancer are non-small cell lung cancer (NSCLC), which is the main type of lung cancer (2). Patients with stage I-III non-small cell lung cancer could be cured surgically, with a 5-year survival rate of approximately 70% (3). The 5-year survival rate for advanced non-small cell lung cancer is only 5% (4). Due to the lack of typical symptoms in lung cancer patients, about 62 percent of non-small cell lung cancer patients receive a stage IV diagnosis at their initial diagnosis (5). The most popular treatments for people with advanced non-small cell lung cancer include chemotherapy, targeted therapy, and immunotherapy (6). Although tyrosine kinase inhibitors (TKIs) have improved survival in patients with advanced non-small cell lung cancer who have a driver-gene, the 5-year survival rate for patients with driver-negative advanced non-small cell lung cancer remains poor due to TKIs treatment unsuitability. Exploring the effectiveness of various treatment methods is therefore urgently needed to assist physicians in perfecting their treatment plans.

The popularity of immune checkpoint inhibitors (ICIs) in the management of advanced non-small cell lung cancer has grown exponentially. Only those with high levels of PD-L1 expression could get PD-1/PD-L1 inhibitor monotherapy. Notwithstanding, combination regimens of PD-1/PD-L1 inhibitors with a variety of other medications (chemotherapy, anti-angiogenic drugs, and other immunotherapeutic agents) are increasingly being explored in immunotherapy clinical research because the benefit of PD-1/PD-L1 inhibitors monotherapy is constrained in this group of people with low or negative PD-L1 expression. KEYNOTE-189 and KEYNOTE-407 are two clinical trials that have produced promising results (7) (8), have discovered that combining PD-1/PD-L1 inhibitors with chemotherapy significantly improved progression-free survival and overall survival in patients with advanced non-small cell lung cancer who were driver-negative, lowering the risk of disease progression and death.

Small molecule inhibitors like anlotinib, apatinib, and lenvatinib, as well as monoclonal antibodies like bevacizumab are anti-angiogenic agents that reduce tumor angiogenesis by disrupting the VEGF signaling pathway, resulting in anti-tumor

effects. Clinical trials, such as BEYOND (9) and ALTER0303 (10), have shown that this class of medicines is effective in the treatment of advanced non-small cell lung cancer. Several clinical trials have published results on whether immunotherapy combined with anti-angiogenic agents could be an effective treatment option. IMpower150 (11) showed that atezolizumab in combination with chemotherapy and bevacizumab was effective in extending progression-free survival and overall survival in patients with advanced non-small cell lung cancer. KEYNOTE-524 (12) showed an objective response rate (ORR) of 33.3% for pembrolizumab in combination with lenvatinib as the first-line treatment for advanced non-small cell lung cancer. These results provide an evidence-based basis for the treatment regimen of PD-1/PD-L1 inhibitors in combination with anti-angiogenic agents.

There are presently just a few second or later-line therapeutic options available for patients with advanced NSCLC. Some clinicians prefer PD-1/PD-L1 inhibitors plus chemotherapy or PD-1/PD-L1 inhibitors plus anti-angiogenic agents with or without chemotherapy, however real-world data on the efficacy of PD-1/PD-L1 inhibitors plus anti-angiogenic with or without chemotherapy therapies is currently lacking. The efficacy and safety of PD-1/PD-L1 inhibitors plus anti-angiogenic with or without chemotherapy versus PD-1/PD-L1 inhibitors plus chemotherapy as second or later-line therapy for advanced non-small cell lung cancer patients were further investigated in this study using real-world clinical data analysis.

Materials and methods

Study subjects and design

Lung cancer patients receiving PD-1/PD-L1 inhibitors at the Guangxi Cancer Hospital from January 1, 2018 to December 1, 2021 were included for further screening, and the screening criteria for the cohort study were as follows: (1) pathologically confirmed primary NSCLC (the 5th edition of the WHO Thoracic Tumor Classification); (2) clinical stage III(unresectable Stage IIIB and IIIC) or IV (the 8th edition of the TNM staging system); (3) exclude cases with primary malignancies in other systems; (4) at least one measurable lesion; (5) receiving PD-1/PD-L1 inhibitors combination therapy (chemotherapy, anti-angiogenic or both) in second or later lines; (6) receiving at least 2 cycles of PD-1/PD-L1 inhibitor combination therapy (21 days for 1 cycle); (7) exclude cases where follow-up information was not available and cases with missing data. A total of 73 patients met these criteria and were ultimately included in this cohort study, and the screening process and results are shown in Figure 1.

PD-1/PD-L1 inhibitors are frequently used in the first-line therapy of advanced NSCLC. Patients who were unable to utilize PD-1/PD-L1 inhibitors in the first-line for different reasons or patients whose EGFR-TKI treatment failed and selected PD-1/PD-L1 inhibitors as follow-up therapy made up a portion of the study's patient population. Another part of the patients failed the first-line PD-1/PD-L1 inhibitors plus chemotherapy, and then used PD-1/PD-L1 inhibitors combination therapy again in the later line. All included patients were treated with PD-1/PD-L1 inhibitors in second or later-line therapy, PD-1 inhibitors included pembrolizumab, nivolumab, camrelizumab, tislelizumab, sintilimab, toripalimab. PD-L1 inhibitors included atezolizumab and durvalumab. Combination regimens with chemotherapeutic agents including pemetrexed, gemcitabine, paclitaxel analogues (docetaxel, paclitaxel, albumin paclitaxel, paclitaxel liposomes), platinum analogues (carboplatin, cisplatin). Anti-angiogenic agents included bevacizumab, anlotinib, apatinib. The patients all got PD-1/PD-L1 inhibitor combination treatment for more than 2 cycles. Based on the therapy plans they underwent, the patients were separated into groups: PD-1/PD-L1 inhibitors plus chemotherapy (PC group), PD-1/PD-L1 inhibitors plus anti-angiogenic medicines (PA group), and PD-1/PD-L1 inhibitors plus chemotherapy plus anti-angiogenic agents (PAC group).

Data collection and assessment

The hospital database's collection of medical records was searched for relevant information. Patients who had not visited the hospital in more than three months were contacted by phone to follow up and got the necessary information, such as the patient's tumor recurrence and prognosis. Tumor lesions were evaluated both before and after treatment, and a CT scan was utilized to gauge how well the therapy responded. All clinical and laboratory indicators were extracted from the patient's medical records. Clinical indicators included pathological type, sex, age, ECOG-PS, clinical stage, smoking history, metastases, line of treatment and treatment plan. Laboratory indicators included the EGFR mutation and PD-L1 tumor cell proportion score (TPS).

Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 was applied to classify effectiveness into four categories: complete response (CR), partial response (PR), stable disease (SD) and progressive disease (PD). Objective response rate (ORR): CR+PR; Disease control rate (DCR)=CR+PR+SD. Adverse events (AEs) were evaluated by the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) 5.0. Progression-free survival (PFS) was calculated from the date of initiation of treatment with the study protocol to the date of disease progression, or to the time of the last follow-up. Overall survival (OS) was the time from the patient's first anti-tumor drug treatment to death or the last follow-up.

The Guangxi Medical University Affiliated Cancer Hospital 's ethical committee gave its approval for this study. All processes and information collection for this study followed the ethical standards of the Research Committee of the Guangxi Medical University Affiliated Cancer Hospital.

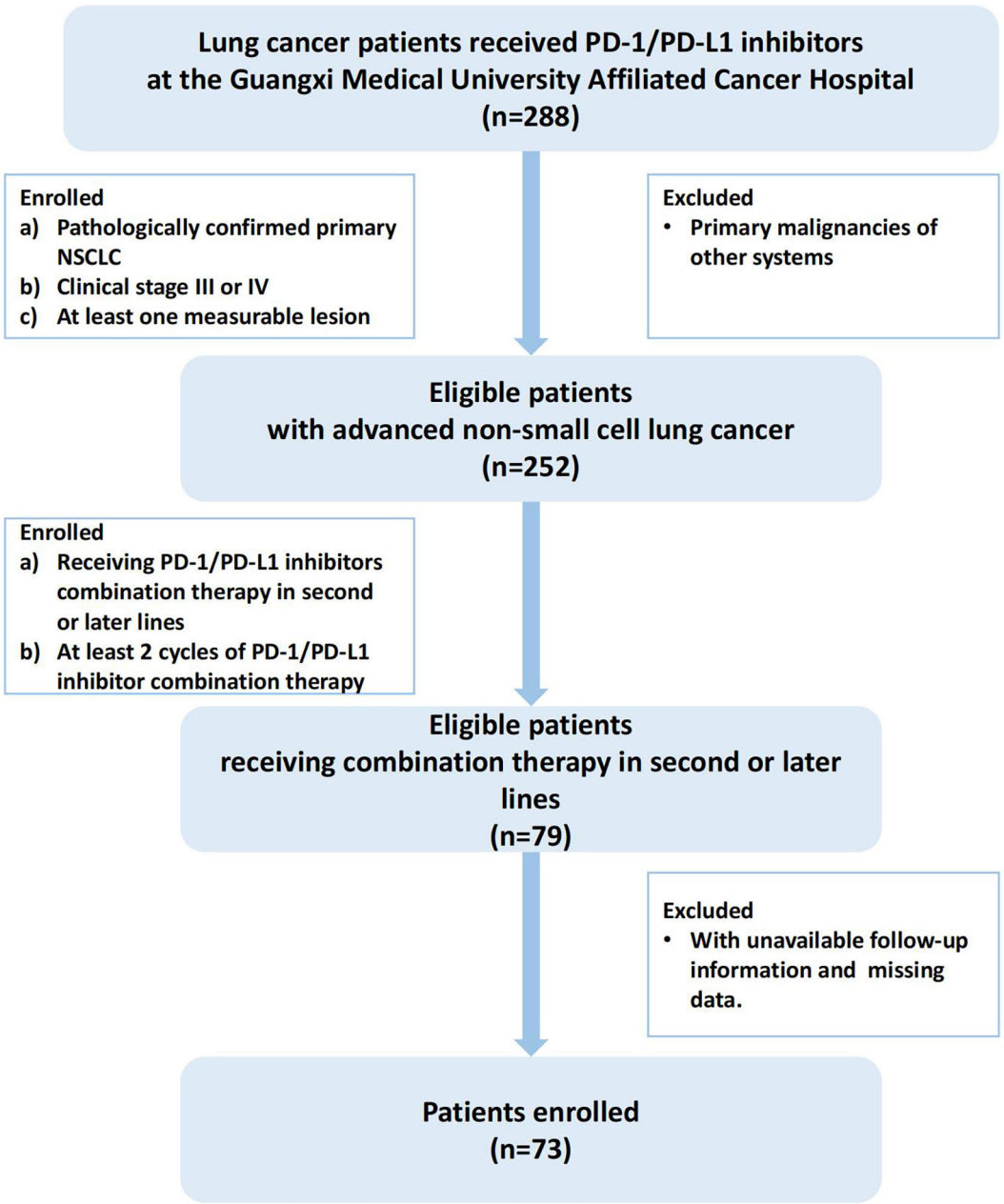

FIGURE 1
Flowchart of the study.

Statistical analyses

All statistical analyses were performed using the statistical package R and EmpowerStats software. We used frequencies, percentages or ratios for categorical variables and means ± standard deviations (SD) for continuous variables. $\chi 2$ or Fisher's exact test (for categorical variables) was used to test for differences between study protocol groups. Survival curves were plotted using the Kaplan-Meier method, and differences were compared using the log-rank test. Cox regression was used for both univariate and multivariate analyses. Hazard ratios (HR) and 95% confidence intervals (CI) were calculated using Cox's proportional hazards regression model. In the multivariate analysis, we adjusted the potentially confounding covariates, the covariates included in the adjustment were screened using EmpowerStats statistics, the screening criteria was: introduction

of covariates in the basic model or removal of covariates from the full model had >10% impact on the regression coefficient of the study protocol groups. Subgroup analyses were used to assess the association between treatment plans and the risk of disease progression and death in different subgroups. In all analyses, P<0.05 was statistically significant.

Results

Baseline characteristics

The clinical baseline data for the patients were shown in Table 1 by the study protocol. The study had 73 patients in all, with a mean age of 58.2 ± 10.2 years and a gender ratio of 75.3% men. The majority of the pathological types were adenocarcinomas (65.8%). 65 patients had an ECOG-PS < 2, and 42 patients had a history of smoking. Ten individuals (13.7%) had a stage III diagnosis, while 63 (86.3%) had a stage IV diagnosis. 39 (53.4%) patients received second-line treatment and 34 (46.4%) received later-line treatment. Sixteen patients had EGFR mutation and 10 patients had PD-L1 TPS ≥ 1%. Age, sex, smoking history, ECOG-PS, pathological type, brain metastasis, liver metastasis, bone metastasis, lung metastasis, line of treatment, clinical stage, EGFR mutation and PD-L1 TPS in the study protocol groups were not statistically significantly different. However, more patients did not have adrenal metastasis (P=0.022).

Univariate analyses of the relationship between study protocol groups and the risk of disease progression and death

Tables 2, 3 demonstrated the results of the univariate analysis. In terms of disease progression, there was no statistical significance in age, sex, smoking history, ECOG-PS, pathological type, brain metastasis, liver metastasis, bone metastasis, lung metastasis, adrenal metastasis, clinical stage, line of treatment, EGFR mutation and PD-L1 TPS. In terms of study protocol groups, the risk of disease progression was reduced by 70% (95% CI=0.2-0.6, P<0.001) in PA group patients and 50% (95% CI=0.2-1.1, P=0.071) in PAC group patients compared to PC group patients.

Patients receiving later-line treatment had a statistically significant 60% decreased risk of death than those receiving second-line treatment (P=0.002). There was no statistical significance for the remaining clinical markers. For the study protocol, patients in the PA group had a 40% (95% CI=0.3-1.1, P=0.100) lower likelihood of dying than those in the PC group, while those in the PAC group had a 60% (95% CI=0.2-1.3, P=0.126) lower risk.

Multivariate Cox regression analyses after adjusting the potentially confounding covariates

To avoid interaction of clinical characteristics parameters, multivariate Cox regression analysis was used to determine the independent predictability of PA group and PAC group in terms of disease progression and death (Tables 4, 5).

Based on the results of the multivariate Cox's proportional hazards regression model, we could see that PA group (Disease progression: HR 0.4, P=0.005. Death: HR 0.4, P=0.024) and PAC group (Disease progression: HR 0.3, P=0.012. Death: HR 0.3, P=0.045) had a statistically significant lower hazard ratio (HR) for disease progression and death compared to patients in PC group.

Kaplan–Meier analyses

The Kaplan-Meier curves of PFS for patients in PC group compared to patients in PA group and PAC group were shown in Figure 2, and the Kaplan-Meier curves of OS for patients in PC group compared to patients in PA group and PAC group were shown in Figure 3. Kaplan-Meier analysis showed a median PFS of 3.5 months (95% CI=2.0-5.6) for patients in PC group and 7.5 months (95% CI=4.9-NA) for patients in PA group. 5.1 months (95% CI=3.3-NA) for patients in PAC group. The difference in PFS between patients in PC group and patients in PA group was significant (P=0.00052). In terms of OS, the median OS was 21.8 months (95% CI=16.2-34.4) for patients in PC group, 33.1 months (95% CI=21.1-NA) for patients in PA group and 37.3 months (95% CI=35.0-NA) for patients in PAC group.

Stratified analyses

Stratified analyses were conducted to observe subgroup effect size trends for the study. The results of the stratified analyses of the risk of disease progression (Figures 4, 5) and death (Figures 6, 7) for patients in PA group and PAC group compared to those in PC group were represented by forest plots. Based on the results of the stratified analyses, it can be seen that patients in PA group and PAC group had a significantly lower risk of disease progression in most subgroups compared to patients in PC group, the interaction between the groups was not statistically significant. In terms of risk of death, patients in PA group and PAC group also had a significantly lower risk of death in most subgroups compared to patients in PC group. Moreover, it is worth noting that the risk of death was significantly higher for patients diagnosed with stage III in PAC group compared to those in PC group. However, the risk of death was reduced for patients diagnosed

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TABLE 1 Baseline clinicopathological features.

	Total n = 73	PC group n =36	PA group n =27	PAC group n=10	P-value
Age	58.2 ± 10.2				0.177
<65	51 (69.9%)	26 (72.2%)	16 (69.3%)	9 (90.0%)	
≥65	22 (30.1%)	10 (27.8%)	11 (40.7%)	1 (10.0%)	
Sex					0.931
female	18 (24.7%)	9 (25.0%)	7 (25.9%)	2 (20.0%)	
male	55 (75.3%)	27 (75.0%)	20 (74.1%)	8 (80.0%)	
Smoking History					0.944
never	31 (42.5%)	16 (44.4%)	11 (40.7%)	4 (40.0%)	
ever	42 (57.5%)	20 (55.6%)	16 (59.3%)	6 (60.0%)	
ECOG-PS					0.995
<2	65 (89.0%)	32 (88.9%)	24 (88.9%)	9 (90.0%)	
≥2	8 (11.0%)	4 (11.1%)	3 (11.1%)	1 (10.0%)	
Pathological Type					0.282
adenocarcinoma	48 (65.8%)	21 (58.3%)	18 (66.7%)	9 (90.0%)	
squamous cell carcinoma	20 (27.4%)	13 (36.1%)	6 (22.2%)	1 (10.0%)	
others	5 (6.8%)	2 (5.60%)	3 (11.1%)	0 (0.00%)	
Brain Metastasis					0.843
no	54 (74.0%)	26 (72.2%)	21 (77.8%)	7 (70.0%)	
yes	19 (26.0%)	10 (27.8%)	6 (22.2%)	3 (30.0%)	
Liver Metastasis					0.502
no	57 (78.1%)	27 (75.0%)	23 (85.2%)	7 (70.0%)	
yes	16 (21.9%)	9 (25.0%)	4 (14.8%)	3 (30.0%)	
Bone Metastasis					0.262
no	51 (69.9%)	25 (69.4%)	21 (77.8%)	5 (50.0%)	
yes	22 (30.1%)	11 (30.6%)	6 (22.2%)	5 (50.0%)	
Lung Metastasis					0.712
no	48 (65.8%)	22 (61.1%)	19 (70.4%)	7 (70.0%)	
yes	25 (34.2%)	14 (38.9%)	8 (29.6%)	3 (30.0%)	
Adrenal Metastasis					0.022
no	59 (80.8%)	32 (88.9%)	22 (81.5%)	5 (50.0%)	
yes	14 (19.2%)	4 (11.1%)	5 (18.5%)	5 (50.0%)	
Line of treatment					0.746
2	39 (53.4%)	18 (50.0%)	16 (59.3%)	5 (50.0%)	
>2	34 (46.6%)	18 (50.0%)	11 (40.7%)	5 (50.0%)	
Clinical Stage					0.765
III	10 (13.7%)	6 (16.7%)	3 (11.1%)	1 (10.0%)	
IV	63 (86.3%)	30 (83.3%)	24 (88.9%)	9 (90.0%)	
EGFR mutation					0.461
negative	37 (50.7%)	17 (47.2%)	15 (55.6%)	5 (50.0%)	
positive	16 (21.9%)	8 (22.2%)	4 (14.8%)	4 (40.0%)	
unknown	20 (27.4%)	11 (30.6%)	8 (29.6%)	1 (10.0%)	
PD-L1 TPS					0.167
<1%	7 (9.60%)	3 (8.30%)	1 (3.70%)	3 (30.0%)	
≥1%	10 (13.7%)	6 (16.7%)	3 (11.1%)	1 (10.0%)	
unknown	56 (76.7%)	27 (75.0%)	23 (85.2%)	6 (60.0%)	

ECOG-PS, Eastern Cooperative Oncology Group Performance Status; EGFR, Epidermal Growth Factor Receptor.
PD-L1 TPS, Programmed Cell Death-Ligand 1 Tumor cell Proportion Score.
PC group: PD-1/PD-L1 inhibitors plus Chemotherapy.
PA group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents.
PAC group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus Chemotherapy.

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TABLE 2 Univariate cox regression analysis of clinical indicators to predict risk of disease progression.

	N	HR	(95% CI)	p value
Age				
<65	51 (69.9%)	1		
≥65	22 (30.1%)	0.7	(0.4, 1.3)	0.247
Sex				
female	18 (24.7%)	1		
male	55 (75.3%)	1.1	(0.6, 2.1)	0.69
Smoking History				
never	31 (42.5%)	1		
ever	42 (57.5%)	0.7	(0.4, 1.2)	0.148
ECOG-PS				
<2	65 (89.0%)	1		
≥2	8 (11.0%)	0.9	(0.3, 2.2)	0.767
Pathological Type				
adenocarcinoma	48 (65.8%)	1		
squamous cell carcinoma	20 (27.4%)	0.8	(0.4, 1.4)	0.412
others	2 (5.60%)	1	(0.3, 2.8)	0.961
Brain Metastasis				
no	54 (74.0%)	1		
yes	19 (26.0%)	1.8	(1.0, 3.3)	0.045
Liver Metastasis				
no	57 (78.1%)	1		
yes	16 (21.9%)	1.5	(0.8, 2.8)	0.169
Bone Metastasis				
no	51 (69.9%)	1		
yes	22 (30.1%)	1.3	(0.7, 2.3)	0.364
Lung Metastasis				
no	48 (65.8%)	1		
yes	25 (34.2%)	1.3	(0.8, 2.3)	0.314
Adrenal Metastasis				
no	59 (80.8%)	1		
yes	14 (19.2%)	1.1	(0.6, 2.2)	0.774
Line of treatment				
2	39 (53.4%)	1		
>2	34 (46.6%)	1.2	(0.7, 2.1)	0.453
Stage				
III	10 (13.7%)	1		
IV	63 (86.3%)	1.5	(0.7, 3.6)	0.32
EGFR mutation				
negative	37 (50.7%)	1		
positive	16 (21.9%)	1.2	(0.7, 3.6)	0.523
unknown	20 (27.4%)	0.5	(0.3, 1.0)	0.061
PD-L1 TPS				
<1%	7 (9.60%)	1		
≥1%	10 (13.7%)	1.8	(0.7, 4.8)	0.229
unknown	56 (76.7%)	0.7	(0.3, 1.5)	0.362
Treatment				
PC group	36 (49.3%)	1		
PA group	27 (37.0%)	0.3	(0.2, 0.6)	<0.001
PAC group	10 (13.7%)	0.5	(0.2, 1.1)	0.071

HR, Hazard Ratio; CI, confidence interval; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; EGFR, Epidermal Growth Factor Receptor; PD-L1 TPS, Programmed Cell Death-Ligand 1 Tumor cell Proportion Score.
PC group: PD-1/PD-L1 inhibitors plus Chemotherapy.
PA group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents.
PAC group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus Chemotherapy.

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TABLE 3 Univariate cox regression analysis of clinical indicators to predict risk of death.

	N	HR	(95% CI)	p value
Age				
<65	51 (69.9%)	1		
≥65	22 (30.1%)	0.6	(0.3, 1.3)	0.221
Sex				
female	18 (24.7%)	1		
male	55 (75.3%)	1.2	(0.6, 2.3)	0.647
Smoking History				
never	31 (42.5%)	1		
ever	42 (57.5%)	0.8	(0.5, 1.5)	0.516
ECOG-PS				
<2	65 (89.0%)	1		
≥2	8 (11.0%)	0.8	(0.3, 2.0)	0.666
Pathological Type				
adenocarcinoma	48 (65.8%)	1		
squamous cell carcinoma	20 (27.4%)	1.2	(0.6, 2.3)	0.688
others	2 (5.60%)	0.8	(0.3, 2.4)	0.732
Brain Metastasis				
no	54 (74.0%)	1		
yes	19 (26.0%)	0.5	(0.2, 1.0)	0.051
Liver Metastasis				
no	57 (78.1%)	1		
yes	16 (21.9%)	1.1	(0.5, 2.1)	0.817
Bone Metastasis				
no	51 (69.9%)	1		
yes	22 (30.1%)	0.9	(0.5, 1.7)	0.855
Lung Metastasis				
no	48 (65.8%)	1		
yes	25 (34.2%)	1.2	(0.6, 2.2)	0.583
Adrenal Metastasis				
no	59 (80.8%)	1		
yes	14 (19.2%)	1	(0.4, 2.1)	0.932
Line of treatment				
2	39 (53.4%)	1		
>2	34 (46.6%)	0.4	(0.2, 0.7)	0.002
Stage				
III	10 (13.7%)	1		
IV	63 (86.3%)	0.6	(0.2, 1.7)	0.349
EGFR mutation				
negative	37 (50.7%)	1		
positive	16 (21.9%)	0.5	(0.3, 1.1)	0.523
unknown	20 (27.4%)	0.7	(0.3, 1.4)	0.286
PD-L1 TPS				
<1%	7 (9.60%)	1		
≥1%	10 (13.7%)	1	(0.3, 3.0)	0.969
unknown	56 (76.7%)	0.4	(0.2, 1.1)	0.067
Treatment				
PC group	36 (49.3%)	1		
PA group	27 (37.0%)	0.6	(0.3, 1.1)	0.1
PAC group	10 (13.7%)	0.4	(0.2, 1.3)	0.126

HR, Hazard Ratio; CI, confidence interval; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; EGFR, Epidermal Growth Factor Receptor; PD-L1 TPS, Programmed Cell Death-Ligand 1 Tumor cell Proportion Score.
PC group: PD-1/PD-L1 inhibitors plus Chemotherapy.
PA group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents.
PAC group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus Chemotherapy.

TABLE 4 Unadjusted and adjusted cox proportional hazards model for disease progression.

	N	Unadjusted HR (95% CI)	P-value	Fully Adjusted HR (95% CI)	P-value
Treatment					
PC group	36 (49.3%)	1.0		1.0	
PA group	27 (37.0%)	0.3 (0.2, 0.6)	<0.001	0.4 (0.2, 0.7)	0.005
PAC group	10 (13.7%)	0.5 (0.2, 1.1)	0.071	0.3 (0.1, 0.8)	0.012

Fully adjusted model adjusts for Smoking history; Pathological Type; Brain Metastasis; Bone Metastasis; Adrenal Metastasis; EGFR mutation; PD-L1 TPS.
PC group: PD-1/PD-L1 inhibitors plus Chemotherapy.
PA group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents.
PAC group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus Chemotherapy.
HR, Hazard Ratio; CI, confidence interval; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; EGFR, Epidermal Growth Factor Receptor.
PD-L1 TPS, Programmed Cell Death-Ligand 1 Tumor cell Proportion Score.

TABLE 5 Unadjusted and adjusted cox proportional hazards model for death.

	N	Unadjusted HR (95% CI)	P-value	Fully Adjusted HR (95% CI)	P-value
Treatment					
PC group	36 (49.3%)	1.0		1.0	
PA group	27 (37.0%)	0.6 (0.3, 1.1)	0.1	0.4 (0.2, 0.9)	0.024
PAC group	10 (13.7%)	0.4 (0.2, 1.3)	0.126	0.3 (0.1, 1.0)	0.045

Fully adjusted model adjusts for Age; ECOG-PS; Brain Metastasis; Adrenal Metastasis; Line of therapy; EGFR mutation; PD-L1 TPS.
PC group: PD-1/PD-L1 inhibitors plus Chemotherapy.
PA group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents.
PAC group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus Chemotherapy.
HR, Hazard Ratio; CI, confidence interval; ECOG-PS, Eastern Cooperative Oncology Group Performance Status; EGFR, Epidermal Growth Factor Receptor.
PD-L1 TPS, Programmed Cell Death-Ligand 1 Tumor cell Proportion Score.

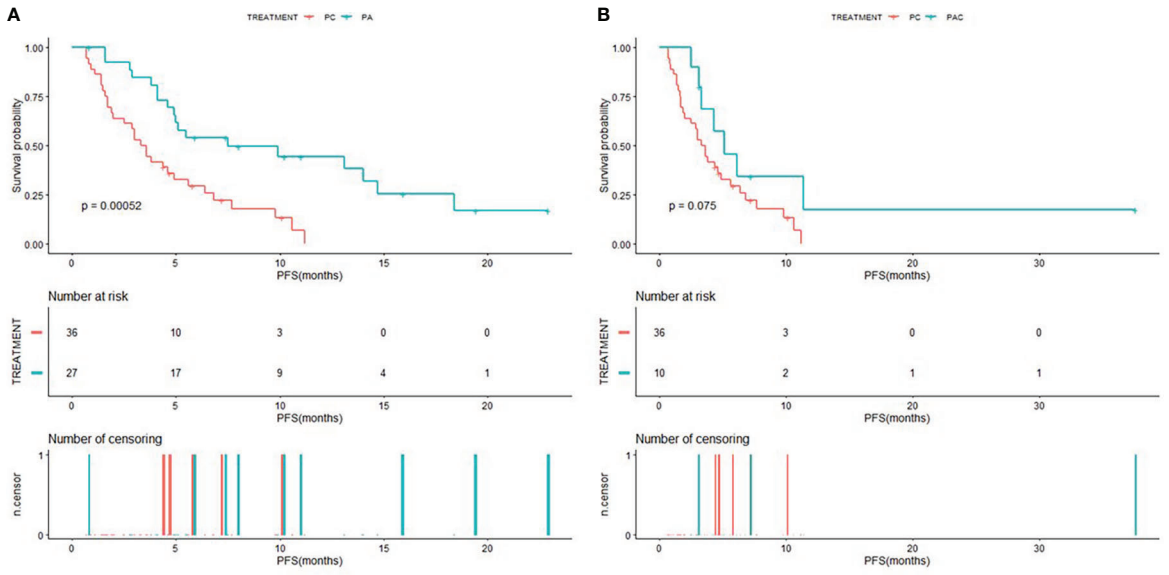

FIGURE 2
Kaplan-Meier (KM) curves of PFS **(A)** of patients in PC group versus PA group and PFS **(B)** of patients in PC group versus PAC group.

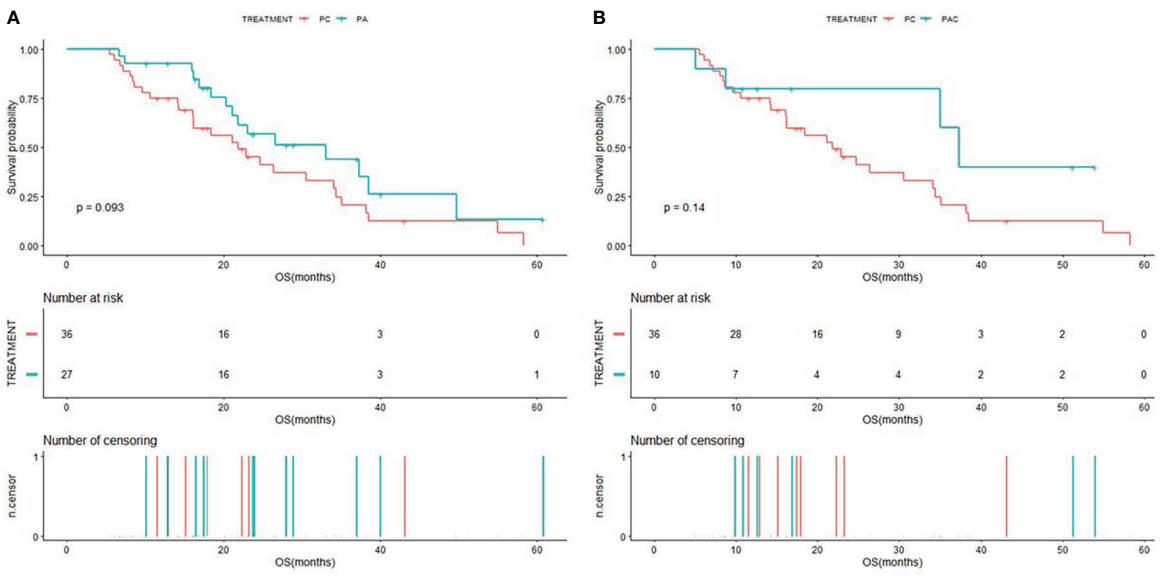

FIGURE 3
Kaplan-Meier (KM) curves of OS **(A)** of patients in PC group versus PA group and OS **(B)** of patients in PC group versus PAC group.

with stage IV in PAC group compared to those in PC group, with a P value of <0.0001 for the interaction analysis, which means that the prognosis may be different for patients in PAC group with different stage

Confirmed objective response

The result of the confirmed objective response rates in the three groups were displayed in Table 6. None of the patients in the three groups were able to achieve complete response. The objective response rate (ORR) for the three groups was 13.9% in PC group, 11.1% in PA group and 20% in PAC group. The patients in PA group (DCR:96.3% vs.58.3%, P=0.001) and PAC group (DCR:100% vs.58.3%, P=0.019) had a better disease control rate (DCR) than patients in PC group.

Toxicities analyses

Table 7 showed the incidence of treatment-related adverse events in the study protocol, with blood toxicity (27.4%) being the most common adverse event and abnormal renal function (1.4%) and pneumonia (1.4%) occurring relatively infrequently. Patients in PA group (18.5% vs.0%, P=0.011) and PAC group (30% vs.0%, P=0.008) had a greater proportion of hypertension than patients in PC group.

Discussion

The management of lung cancer has advanced quickly in this era of diverse medicines. However, there is still a bottleneck in the availability of second or later-line therapy for advanced non-small cell lung cancer. The TAX317 study (13) results showed that docetaxel used in second-line treatment of driver-negative advanced NSCLC significantly increased overall survival when compared to best supportive care (7 months vs 4.6 months), and the TAX317/TAX320 (14) studies established docetaxel as the standard chemotherapy regimen for second-line treatment of NSCLC. The paradigm of second or later-line therapy for patients with advanced NSCLC has been further altered by the development of immunotherapeutic drugs. The KEYNOTE-010 research (15) demonstrated that pembrolizumab resulted in longer OS than docetaxel in patients with PD-L1 TPS≥1%. The outcomes of CheckMate 078 (16) demonstrated that the effectiveness and safety performance of nivolumab in Chinese patients was similar to the worldwide trials CheckMate 017 and 057 (17) (18), with a median overall survival of 11.9 months in the nivolumab group compared to 9 months in the docetaxel group. When compared to docetaxel, nivolumab dramatically increased patient survival, lowering the chance of mortality by 36%. In the meanwhile, patients with PD-L1 TPS≥1% in OAK research (19), a phase 3 clinical research with atezolizumab, had a median OS of 15.7 months compared to 10.3 months when compared to docetaxel. In the ORIENT-3 study (20), the median

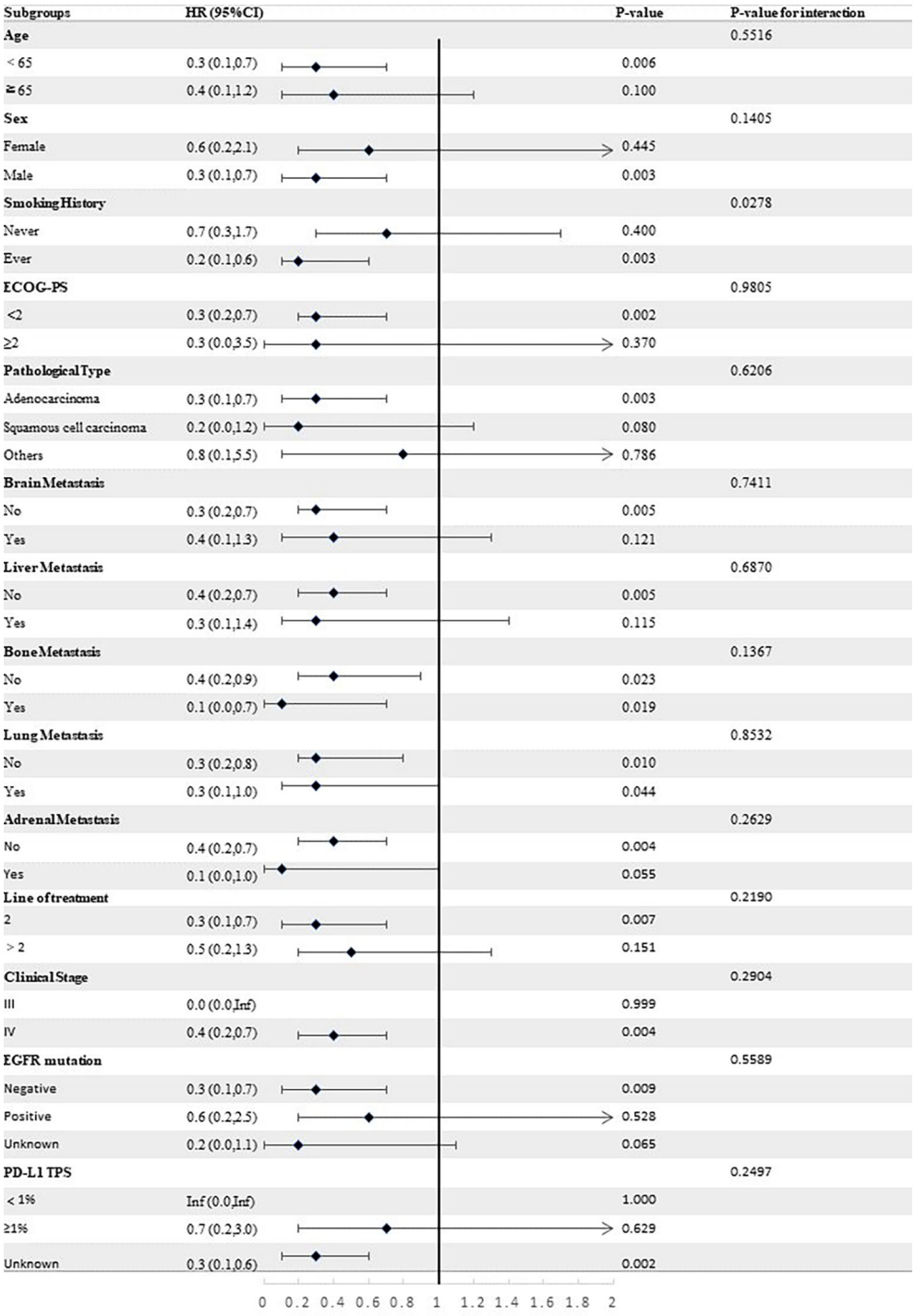

FIGURE 4
Forest plot of risk of disease progression for patients in PA group compared to PC group in different subgroups.

PFS was also much longer in the sintilimab group, coming in at 4.30 months compared to 2.79 months in the docetaxel group. The RATIONALE303 study (21) was designed to evaluate the efficacy and safety of tislelizumab versus docetaxel in second or later-line treatment of patients with advanced NSCLC, showing a median PFS of 4.1 months vs. 2.6 months and a PFS rate of 23.3% vs. 5.7% at 12 months. The ALTER0303 study (10) demonstrated a median OS extension of 3.3 months for

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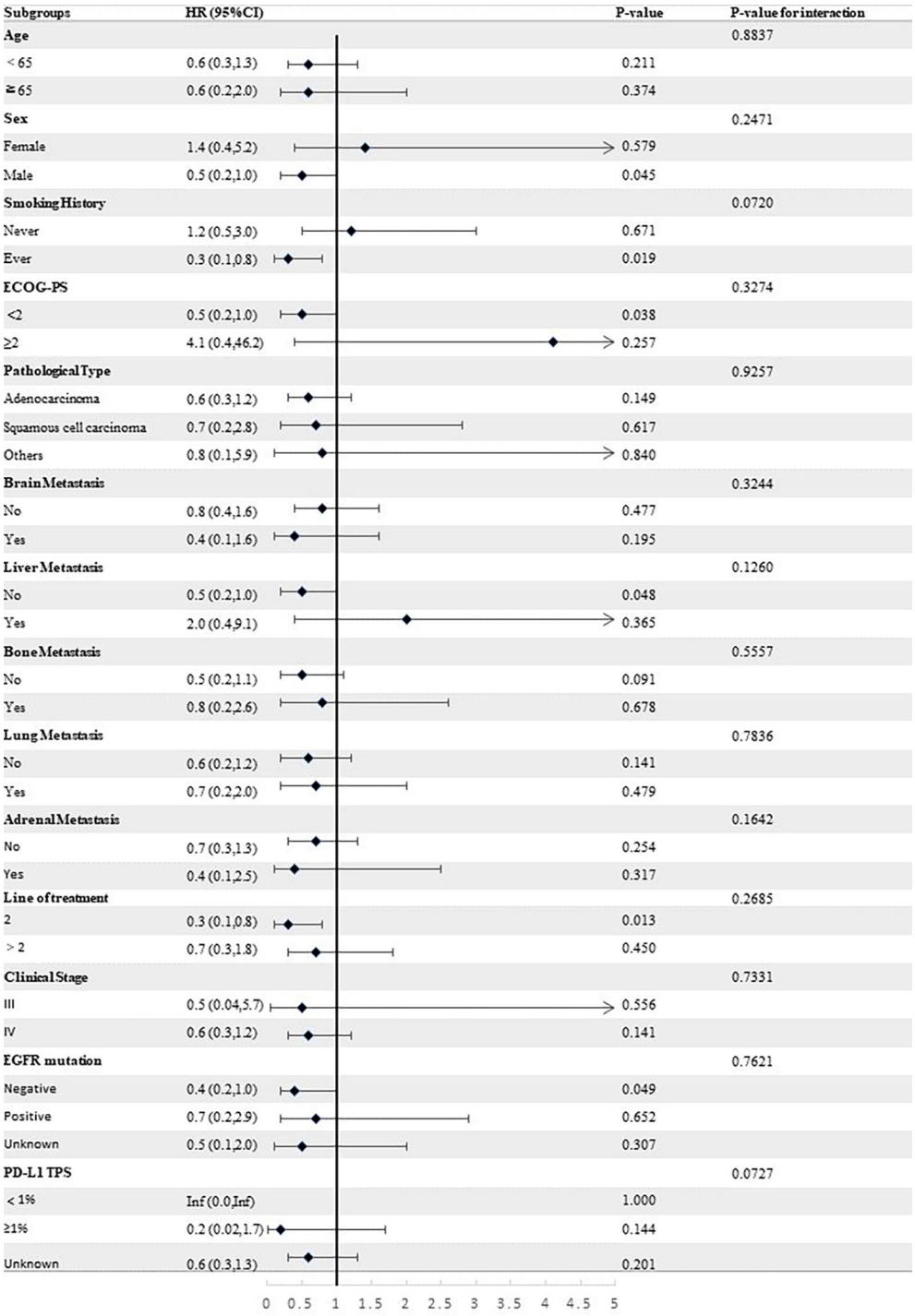

FIGURE 5
Forest plot of risk of disease progression for patients in PAC group compared to PC group in different subgroups.

patients in the anlotinib arm compared to the placebo arm (9.6 months vs 6.3 months); and a median PFS extension of 4.0 months (5.4 months vs 1.4 months). Based on the results of the above, chemotherapy, PD-1/PD-L1 inhibitors or anti-angiogenic agents monotherapy have been approved by the Food and Drug Administration (FDA) and the National Medical Products Administration (NMPA) of China for the second or later-line treatment of patients with driver-negative advanced non-small

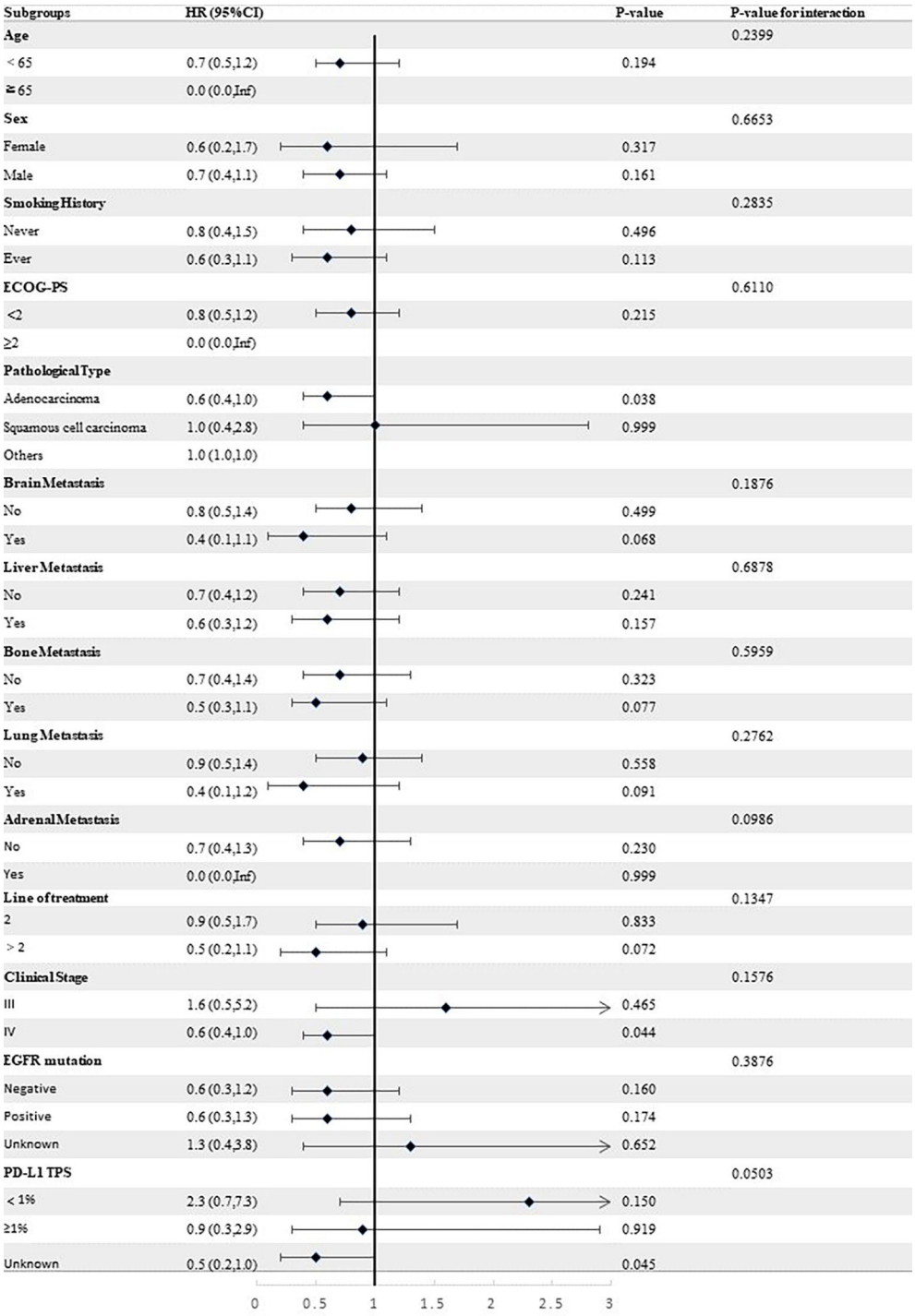

FIGURE 6
Forest plot of risk of death for patients in PA group compared to PC group in different subgroups.

cell lung cancer. Due to the paucity of scientific evidence supporting the use of combination regimens in the second or later-line treatment of patients with advanced non-small cell lung cancer, a number of clinical studies are being conducted worldwide to further examine the viability of combination regimens.

In this retrospective study, we fully evaluated the efficacy and safety of PD-1/PD-L1 inhibitors plus anti-angiogenic agents

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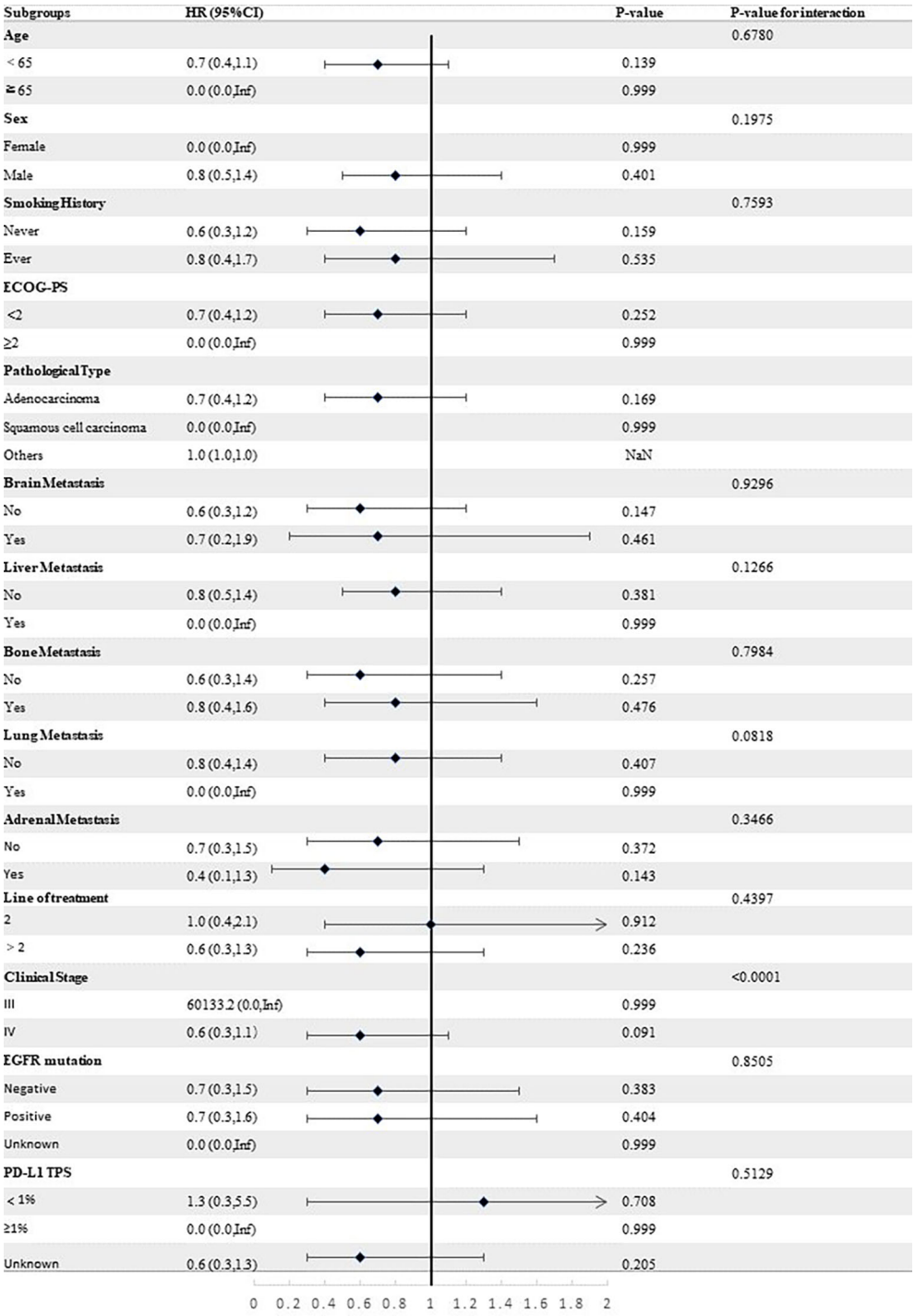

FIGURE 7
Forest plot of risk of death for patients in PAC group compared to PC group in different subgroups.

with or without chemotherapy in the second or later-line treatment of patients with advanced non-small cell lung cancer. The results showed that PD-1/PD-L1 inhibitors plus anti-angiogenic agents with or without chemotherapy were superior to PD-1/PD-L1 inhibitors plus chemotherapy in terms of progression-free survival in second or later-line treatment, and that two different combination regimens (PD-1/PD-L1 inhibitors plus anti-angiogenic agents and PD-1/PD-L1

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TABLE 6 Summary of confirmed response assessed by RECIST version 1.1.

Confirmed Response	PC group	PA group	PAC group
Best response			
Complete response (CR)	0 (0%)	0 (0%)	0 (0%)
Partial response(PR)	5(13.9%)	3(11.1%)	2(20%)
Stable disease(SD)	16(44.4%)	23(85.2%)	8(80%)
Progressive disease (PD)	14(38.9%)	1(3.7%)	0(0%)
Not evaluable	1(2.8%)	0(%)	0(0%)
Objective response rate(ORR)	13.90%	11.10%	20%
P-value	0.802		
Disease control rate (DCR)	58.30%	96.30%	100%
P-value	<0.001		
PC vs PA	P=0.001		
PC vs PAC	P=0.019		

RECIST: Response Evaluation Criteria In Solid Tumors.
Objective response rate(ORR)= Complete response (CR)+ Partial response(PR).
Disease control rate (DCR)= Complete response (CR)+ Partial response(PR)+ Stable disease(SD).
Not evaluable= Patients who did not have 1 postbaseline imaging assessment.
PC group: PD-1/PD-L1 inhibitors plus Chemotherapy.
PA group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents.
PAC group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus Chemotherapy.

inhibitors plus anti-angiogenic agents plus chemotherapy) reduced the risk of disease progression by 60% and 70%, respectively, compared to the PD-1/PD-L1 inhibitors plus chemotherapy, with consistent trends in results in subgroup analyses, and a better median PFS than the PD-1/PD-L1 inhibitors plus chemotherapy. In terms of overall survival, the multivariate Cox regression analysis showed a significantly lower risk of death with the two different combination regimens compared to the PD-1/PD-L1 inhibitors plus chemotherapy regimen, with statistically significant. In terms of Kaplan-Meier survival curves, patients in the two different combination regimens survived significantly better than those in the PD-1/PD-L1 inhibitors plus chemotherapy regimen. The overall survival data from this single center, small sample retrospective study should be further investigated in a multi-center study with a larger sample size because they may be statistically biased. Additionally, data on the overall survival of PD-1/PD-L1 inhibitors combined with anti-angiogenic agents, with or without chemotherapy regimens, warrant further investigation. Patients receiving one of the two alternative combination regimens had a considerably higher DCR than those receiving chemotherapy plus PD-1/PD-L1 inhibitors in terms of effectiveness. Although there was a greater prevalence of hypertension in the two distinct combination regimens, the majority of adverse events were grade 1-2, indicating the safety and tolerability of this treatment method. According to the findings of our retrospective study, patients who received PD-1/PD-L1 inhibitors plus anti-angiogenic agents with or without chemotherapy had numerically better OS and PFS than participants in the previous KEYNOTE-010, OAK, CheckMate 078, ORIENT-3, and RATIONALE303 studies. This proves the reliability of the data from our retrospective study.

Additionally, several academics have offered convincing explanations for the processes at play when PD-1/PD-L1 inhibitors are combined with other agents. Chemotherapy slows the development of tumors mostly by halting the cell cycle, preventing DNA replication, upsetting cellular metabolism, or blocking microtubule assembly (22). Through increased production of 2-microglobulin and changes to the peptide antigen repertoire expressed on HLA class I, gemcitabine can considerably upregulate the expression of human leukocyte antigens (HLA)-A, B, and C. Topotecan, which increases HLA class I expression by activating the NF-B/Interferon/MHC-I signaling axis, exhibits a similar behavior (23). Oxaliplatin and anthracycline are two examples of cytotoxic chemotherapy agents that can cause immunogenic cell death and activate the body's natural defenses against tumors (24). Additionally, through enhancing mitochondrial biogenesis, pemetrexed increase the activation of tumor-infiltrating lymphocytes (TILs) (25). In animal models, it has been shown that chemotherapy and PD-1/PD-L1 inhibitors work in concert (26).

The tumor microenvironment (TME), a dynamic ecosystem, is made up of a variety of soluble chemicals, fibroblasts, stromal cells, blood vessels, tumor cells, and immune cells (27). Regulatory T cells (Tregs), myeloid-derived suppressor cells

TABLE 7 Incidence of adverse events (AEs).

Event	PC group	PA group	PAC group	Total	P-value
Blood toxicity	12(33.3%)	6(22.2%)	2 (20%)	20(27.4%)	0.586
Abnormal liver function	2(5.6%)	2(7.4%)	0(0%)	4(5.5%)	1.000
Abnormal renal function	0(0%)	1(3.7%)	0(0%)	1(1.4%)	0.500
Rash	2(5.6%)	1(3.7%)	1(10%)	4(5.5%)	0.628
Hypertension	0(0%)	5(18.5%)	3(30%)	8(11%)	0.003*
Abnormal thyroid function	2(5.6%)	4(14.8%)	3(30%)	9(12.3%)	0.085
Cardiotoxicity	8(22.2%)	2(7.4%)	0(0%)	10(13.7%)	0.146
Pneumonia	1(2.8%)	0(0.0%)	0(0%)	1(1.4%)	1.000

PC group: PD-1/PD-L1 inhibitors plus Chemotherapy.
PA group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents.
PAC group: PD-1/PD-L1 inhibitors plus anti-angiogenic agents plus Chemotherapy.
*PC vs PA P-value=0.011; PC vs PAC P-value=0.008.

(MDSCs), tumor-associated macrophages (TAMs), and immature dendritic cells (ImDCs) are among the numerous immunological suppressor cells encountered in the TME (28). The abnormal morphology of tumor vascular endothelial cells and the loose connections between endothelial cells and different basement membranes ultimately lead to a heterogeneous blood perfusion of tumor cells and a hypoxia and acidosis microenvironment (29). Hypoxia further promotes infiltration of these suppressive immune cells by inducing the expression of chemokines that recruit suppressive immune cells. For example, in the presence of C-C Motif Chemokine Ligand 22 (CCL22) and C-C Motif Chemokine Ligand 28 (CCL28), Tregs are further promoted into the tumor cells (30). Vascular Endothelial Growth Factors (VEGFs), which include VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and Placental Growth Factor (PIGF), are a group of secreted glycoproteins that are crucial for the angiogenesis of TME (31). VEGFR-1, 2 and 3 are the three VEGF receptors, and the pro-angiogenic effect of VEGF is primarily mediated by the binding of VEGF-A and VEGFR-2 receptors. The VEGF/VEGFR signaling pathway inhibits anti-tumor immune responses not only by inducing a hypoxic microenvironment, but also through other complex mechanisms to produce immunosuppressive effects.

Dendritic Cells (DCs) are specialized antigen-presenting Cells (APCs) that play a key role in the antitumor immune response. In the presence of tumor antigens, DCs migrate and become mature during the migration process. Mature DCs activate T cells to exert their anti-tumor effects (32). Immature DCs (ImDCs), because of the absence of co-stimulatory molecules, result in the inability of T cells to activate properly. According to a publication, VEGF inhibits DC maturation by binding to VEGFR-2 on their surface and activating the NF-kB signaling pathway (33). The maturation and differentiation of DCs were hampered by high amounts of VEGF in a mouse model, which provided additional confirmation of this conclusion (34).

Myeloid-derived Suppressor Cells (MDSCs) conduct a variety of mechanisms to suppress anti-tumor immune responses, such as depleting lymphocytes of nutrients, reducing the viability of transit lymphocytes, generating oxidative stress and inducing Tregs to differentiate (35). It has been shown that VEGF causes an increase in MDSCs and suppresses anti-tumor immune responses because VEGF expression over-activates Janus Kinases 2/Signal Transducer and Activator of Transcription 3 (Jak2/STAT3) signaling, leading to aberrant myeloid differentiation in tumors (36). In a mouse tumor model, the concentration of intratumoral MDSCs correlated with the concentration of VEGF, and the infusion of VEGF into tumor-free normal mice significantly increased the level of MDSCs in mice (37).

Tumor-associated macrophages (TAMs) come in two varieties: M1 and M2. A number of pro-inflammatory substances, immune activators, and chemokines are released by M1 TAMs, and these substances have anti-tumor effects through cytophagocytosis, acute pro-inflammatory reactions, and immune activation responses. By secreting immunosuppressive factors, cytokines and growth factors, M2 TAMs inhibit the proliferation and activation of T cells, regulate and promote the Th2 immune response, promote tumor cell growth, participate in tumor angiogenesis and promote tumor infiltration and metastasis (38). VEGF signaling promotes a step-change in TAMs from the M1 to the M2 phenotype, in addition to recruiting TAMs into tumors (39).

Regulatory T Cells (Tregs) inhibit the action of T cells on tumors (40). Tregs are activated and their immunosuppressive function is enhanced by Neurofibrillin-1 mediation, and VEGF binds directly to Neurofibrillin-1 and induces Tregs to migrate into the tumor (41). It has also been shown that VEGF expression is positively correlated with the level of Tregs in

tumors and that VEGFR-2 is more abundant in Tregs compared to other T cells (42, 43), suggesting an important role for VEGF signaling in the activation and induction of Tregs. VEGF further exerts immunosuppressive effects by affecting Tregs.

T-cells may directly destroy tumor cells, which causes an immune response that is anti-tumor. The finding that VEGF was found to have a low expression in tumor-derived T cells (44) was validated, pointing to a relationship between VEGF and T cells. Further research indicated that the severe thymic atrophy caused by VEGF-A infusion in a tumor-bearing mouse model was caused by a significant decrease in CD4+/CD8+ thymocytes (45). More research was done on this phenomenon, and it was shown that co-repressor molecule production in CD8+ T cells enhances T cell depletion, which is also boosted by VEGF-A (46). VEGF not only affects the antitumor immune response by influencing the activation of T cells, but also reduces the expression of adhesion molecules on immune cells and endothelial cells that are essential for T cell infiltration, such as Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Cell Adhesion Molecule-1 (ICAM-1) (47), thereby further affecting the immune response.

Activated immune cells can control tumor angiogenesis directly and indirectly at the same time. CD8+ T cells play a key role in inhibiting tumor angiogenesis through the secretion of Interferon-γ (IFN-γ). Endothelial cell proliferation is reduced and migration is diminished in response to IFN-γ, which promotes the secretion of IFN-Inducible Protein-10 (IP-10) and Monokine Induced by Interferon Gamma (MIG), which react with chemokine receptor 3 to inhibit endothelial cell proliferation and tumor angiogenesis (48, 49), thereby normalizing vasculature and promoting effector T cell infiltration. IFN-γ also downregulates VEGF-A and upregulates chemokine CXC ligands 9, 10 and 11, which together stimulate vascular maturation by enhancing the recruitment of pericytes (50). The IFN-γ/STAT1 signaling pathway promotes the reprogramming of M1-like TAMs and contributes to vascular normalization (51). CD4+ Th1 cells can also contribute to tumor vascular normalization through the production of IFN-γ in TME. In multiple mouse models, depletion of CD4+Th1 cells reduced pericyte coverage and increased aberrant tumor vessels, while activation of CD4+Th1 cells improved vascular normalization (52, 53). Immune cells can also directly influence the phenotype and function of tumor vessels through various cytokines, such as cytokines that inhibit tumor angiogenesis (interferon-α, interleukin-12, interleukin-18 or tumor necrosis factor) and chemokines (CXCL9, CXCL10 or CCL21) (54–56). One of the essential preconditions for immunological activation is anti-VEGF/VEGFR medication therapy, and strong evidence shows that immune cell activation furthers vascular normalization, creating a positive feedback loop between immunotherapy and anti-angiogenic therapy. Figure 8 provides an illustration of the pertinent processes.

On data related to the use of PD-1/PD-L1 inhibitors plus anti-angiogenic agents for second or later-line treatment of advanced non-small cell lung cancer, a phase I clinical study evaluated the use of ramucirumab in combination with pembrolizumab in the later-line treatment of different malignancies and included 27 patients with NSCLC, showing an ORR of 30% (57). Zhou Na et al. published the results of a phase IB clinical study of camrelizumab in combination with anlotinib as a second or later-line treatment option for patients with advanced non-small cell lung cancer (58), which showed that camrelizumab in combination with anlotinib showed some effectiveness with an overall median PFS of 8.2 months and a median OS of 12.7 months for patients. Among the different anlotinib dose groups, the cohort group with anlotinib 12 mg demonstrated better efficacy and safety. The European Society for Medical Oncology (ESMO) 2021 reportd the results of the first interim analysis of the ORIENT-31 study (59) of sintilimab in combination with bevacizumab and chemotherapy in EGFR-mutated non-squamous NSCLC that has failed EGFR-TKI therapy, in the intention-to-treat population, patients in the sintilimab in combination with bevacizumab and chemotherapy arm achieved a significant prolongation of PFS compared to patients in the chemotherapy arm based on blinded independent imaging assessment committee assessment (median PFS: 6.9 months vs 4.3 months, HR=0.464, 95% CI: 0.337-0.639, p<0.0001).Wang Peiliang et al. conducted a retrospective analysis of the efficacy and safety of PD-1 inhibitors plus anlotinib in patients with advanced non-small cell lung cancer after failure of previous systemic therapy (60), which also showed anti-tumor activity and tolerable adverse effects of immune checkpoint inhibitors in combination with anti-angiogenic agents. Zhang Fan's team and Hu Ran's team also conducted a retrospective study on the efficacy and safety of immune checkpoint inhibitors in combination with chemotherapy and anti-angiogenic drugs as a second or later-line treatment option for advanced non-small cell lung cancer (61, 62), demonstrating the feasibility of immune checkpoint inhibitors in combination with chemotherapy and anti-angiogenic drugs in the second or later-line treatment. All of these findings provide some evidence-based evidence for the use of PD-1/PD-L1 inhibitors plus anti-angiogenic agents.

In conclusion, our study has a number of advantages: First and foremost, it was a real-world study that accurately reflected how PD-1/PD-L1 inhibitors combined with anti-angiogenic agents with or without chemotherapy would appear in the real world. Second, to show the connection between the research protocol and the risk of disease progression and death, the study applied rigorous statistical adjustments to reduce the impact of confounding factors. The study's theoretical underpinnings were also well-established. A stratified analysis was then performed to confirm the consistency of our results within the subgroup. The study's findings are valuable for physicians in that they may be used to create more effective treatment strategies for various individuals in a clinical environment.

Despite the significance of the study's findings, there are a few restrictions on it. First of all, because the cases were gathered

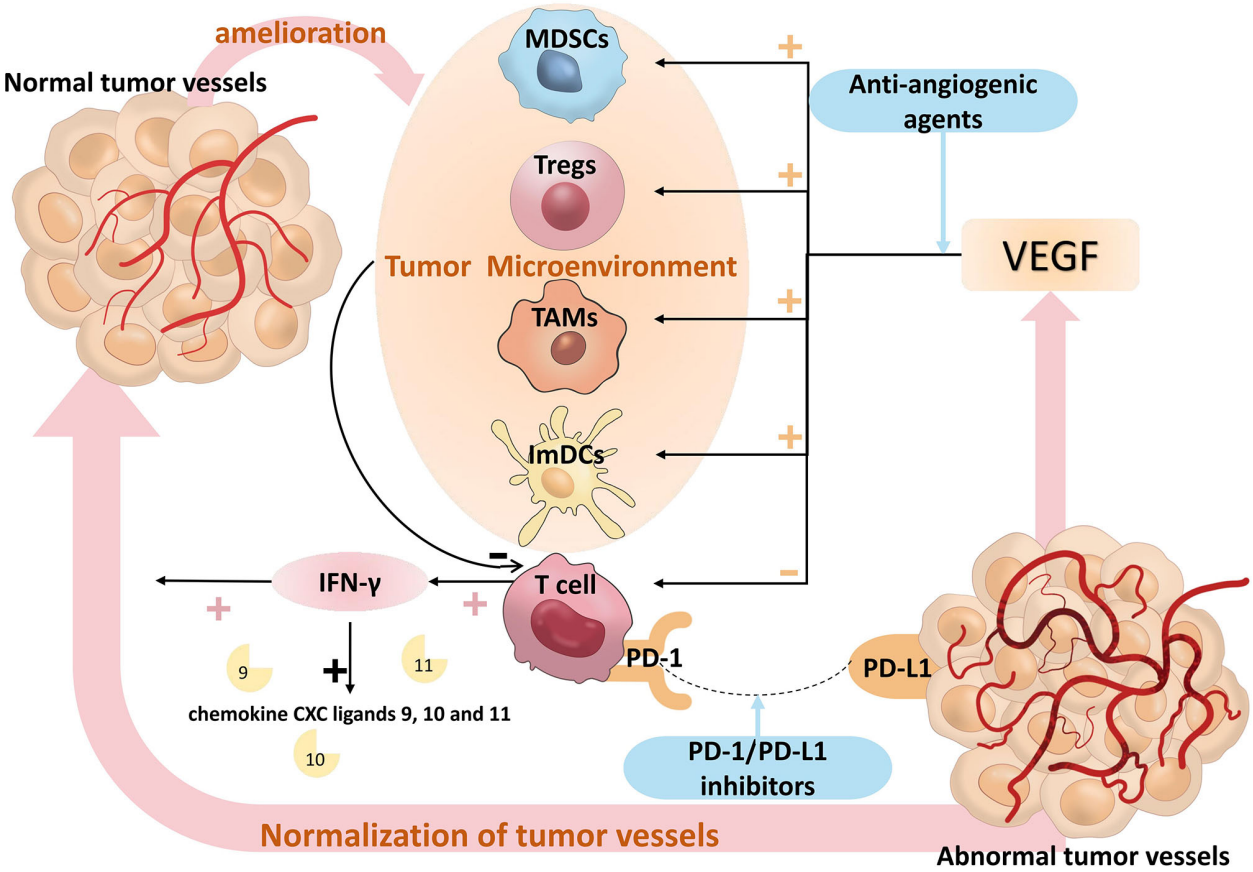

FIGURE 8
The relevant mechanisms of PD-1/PD-L1 inhibitors plus anti-angiogenic agents. T cells can be suppressed by members of the VEGF family, and VEGF can augment the suppressive actions of ImDCs, Tregs, TAMs, and MDSCs, which prevent T cells from having an anti-tumor impact. Anti-angiogenic agents inhibit the VEGF signaling pathway, which in turn activates T cells. T cells improve the tumor microenvironment by releasing IFN-γ, and IFN-γ upregulates chemokine CXC ligands 9, 10, and 11 to promote normalization of tumor vessels, thereby attenuating the effect of VEGF. PD-1/PD-L1 signaling pathway attenuates the anti-tumor activity of T cells, PD-1/PD-L1 inhibitors reactivate the anti-tumor activity of T cells, T cells improve the tumor microenvironment by releasing IFN-γ, IFN-γ upregulates chemokine CXC ligands 9, 10 and 11 to promote normalization of tumor vessels, thereby attenuating the effect of VEGF, which form a positive feedback loop between immunotherapy and anti-angiogenic therapy. VEGF, Vascular Endothelial Growth Factor; ImDCs, Immature Dendritic Cells; Treg, Regulatory T cells; TAMs, Tumor Associated Macrophages; MDSCs, Myeloid Derived Suppressor Cells. IFN-γ, Interferon-γ.

from a single center, the retrospective form of the research made it easy to add selection bias and skew the results of the relationships that were found. Second, the study's limited sample size could have produced some insufficient statistical findings, and a sizable prospective clinical trial might be required to further support the study's findings.

Conclusion

PD-1/PD-L1 inhibitors plus anti-angiogenic agents with or without chemotherapy were superior to PD-1/PD-L1 inhibitors plus chemotherapy as second or later-line therapy in patients with advanced non-small cell lung cancer. This conclusion needs to be further validated in large-scale and prospective clinical trials.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by The Guangxi Medical University Affiliated Cancer

Hospital 's ethical committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SC conducted the statistical analysis of the data and wrote the Chinese manuscript. HWe and WZ helped to perform the analysis and translated the article and created some of the graphics. WJ, RN, SZ, LT, HWa, CS, JH and AZ obtain informed consent from patients and administering treatment of patients and provide the clinical data of patients. YZ given some constructive discussions make revisions to the paper. QY conceptualized the research process and guided its conduct. 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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Acetylcholine receptor binding antibody–associated myasthenia gravis, myocarditis, and rhabdomyolysis induced by tislelizumab in a patient with colon cancer: A case report and literature review

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Despite the intriguing therapeutic prospects offered by immune checkpoint inhibitors (ICIs), immune-related adverse events (irAEs) become an increasingly important safety issue. Herein, we report a patient with locally advanced colorectal cancer (LACRC) who received anti-programmed cell death protein 1 (PD-1) (tislelizumab) therapy, then developed weakness of the limbs and drooping eyelids. He experienced sequential irAEs including severe myasthenia gravis, myocarditis, and rhabdomyolysis. Although many irAEs caused by tislelizumab have been reported, the cooccurrence of severe myasthenia gravis, myocarditis, and rhabdomyolysis caused by tislelizumab has not been described. The patient responded well to methylprednisolone and intravenous immunoglobulin therapy. This case illustrates the severe toxicity caused by ICIs, highlighting the importance of early prevention, early diagnosis, and appropriate management of irAEs. Multidisciplinary discussions should be held to improve the prognosis of patients.

KEYWORDS

tislelizumab, myocarditis, myositis, Myasthenia Gravis, immune-related adverse events, locally advanced colorectal cancer

Introduction

In recent years, immune checkpoint inhibitors (ICIs) have been used to treat multiple types of tumors. Programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) immunocheckpoint inhibitors represent one of the most significant breakthrough in the treatment of advanced malignancies (1). PD-1 inhibitors facilitate restore the endogenous anti-tumor T cell response by blocking the binding of PD-1 to PD-L1 and PD-L2. PD-1 inhibitors, such as nivolumab, have been approved by the US Food and Drug Administration (FDA) for a wide range of cancers, such as melanoma, renal cell carcinoma (RCC), colon cancer, bladder cancer, and Hodgkin's lymphoma since 2014 (2). Despite the impressive efficacy, ICI-related toxicities (i.e., immune-related adverse events and irAEs) should not be neglected, as many irAEs such as myocarditis and rhabdomyolysis are covert and fatal (3, 4). Therefore, the use of ICIs should be accompanied by vigilance against the occurrence of serious irAEs. Herein, we report a patient with locally advanced colorectal cancer (LACRC) who developed tislelizumab-induced multiple organ irAEs including myasthenia gravis, myocarditis, myositis, liver damage, and kidney damage. The patient was admitted to the neurological care unit (NCU) and his symptoms improved significantly after intravenous immunoglobulins (IVIGs) and corticosteroids treatments. We hope to provide reference for the prevention and treatment of clinically related adverse reactions.

Case report

A 65-year-old man was diagnosed with LACRC and received curative surgery in 3 years ago due to repeated, intermittent hematochezia and black stool. He was previously healthy with no autoimmune medical history. The tumor was staged as a T3N0Mx rectal adenocarcinoma as per the TNM (American Joint Committee on Cancer TNM Staging Handbook) staging system. He underwent five cycles of 5-fluorouracil and oxaliplatin (FOLFOX) chemotherapy, and his cancer was stable during the follow-up period from 2018 to 2021. A year ago, his carcinoembryonic antigen (CEA) levels were elevated to 25.43 ng/ml (normal range 0–5 ng/ml) and enhanced CT of the abdomen revealed recurrence of colon cancer and multiple enlarged metastases in the right anterior superior diaphragm and left inguinal region, suggesting the progression of disease. Therefore, molecular genotyping and microsatellite status was performed, and he was diagnosed as high microsatellite instability/defective mismatch repair (MSI-H/dMMR) phenotype colorectal cancer. Because therapeutic failure appears with FOLFOX therapy, then FOLFIRI chemotherapy (folic acid/fluorouracil/irinotecan) was applied for the 6^{th} cycle chemotherapy without adverse effects. Tislelizumab was given to the patient intravenously (200 mg/day for 20 days, BeiGene, China) since December 9, 2021, and FOLFIRI scheme was conducted concomitantly. Twenty days later, the patient experienced weakness of the limbs, followed by bilateral eyelid ptosis, and was admitted to our department. Three days after admission, he had pigmented urine and developed cardiac symptoms such as palpitations. Neurological examination showed that the patient had head drop and complete bilateral gaze paralysis in all directions. Muscle strength of the limbs was 4/5, and the deep tendon reflex was absent. Electromyography (EMG) was normal. His neostigmine test result was positive, which showed clinically significant improvements in quantitative Myasthenia Gravis score (QMG) (> 3 points) (5). Laboratory results revealed significantly elevated levels of myocardial enzymes: creatine kinase (CK) (11920 U/L, normal range 50–310 U/L), CK isoenzyme (CK-MB) (244.2 U/L, normal range 0–25.0 U/L), lactate dehydrogenase (LDH) (1582 U/L, normal range 120 - 250 U/L), α-hydroxybutyric acid (AHB) (1120 U/L, normal range 72–182 U/L), myoglobin (7863 mg/L, normal range 1.5–70 mg/L) and cardiac troponin (cTnI) (0.839 ng/ml, normal range < 0.034 ng/ml). Abnormal liver function was also observed as evidenced by increased levels of alanine aminotransferase (ALT) (313.0 U/L, normal range 9–50 U/L) and AST (587.3 U/L, normal range 15 - 40 U/L). Additionally, his urine protein was positive, urine red blood cell count was 39.1, and his blood urea was 10.17 mmol/L (normal range 2.86–7.14 mmol/L); serum creatinine level was 140 umol/L (normal range 57–111 umol/L), suggesting kidney injury. We demonstrated reduced cortisol levels in the morning (75.89 nmol/L, normal range 244–619 nmol/L) and (77.88 nmol/L, normal range 244–619 nmol/L) at night. His ACTH (adrenocorticotropic hormone) levels were also decreased in the morning (0.22 pmol/L, normal range 1.6–13.9 nmol/L) and (0.22 pmol/L, normal range 1.6–13.9 nmol/L) at night. The patient had hypopituitarism as indicated by low plasma cortisol levels and plasma ACTH levels. His tumor marker CEA was elevated (9.47 ng/ml, normal range 0–5 ng/ml), however, was significantly decreased compared with levels prior to tislelizumab treatment. Antinuclear antibodies (ANA) were 1:100 positive. Serum IgG, IgA, IgM, and complement C3 and C4 were all within normal range. Other autoimmune tests, including antimitochondrial antibody (AMA), perinuclear anti-neutrophil cytoplasmic antibody (p-ANCA), cytoplasmic anti-neutrophil cytoplasmic antibody (c-ANCA), and ceruloplasmin, were all within normal range. Cerebrospinal fluid (CSF) routine test showed no abnormalities in cell counts, protein, and IgG levels. His autoimmune neuromuscular disease antibody tests by radioimmune assay (RIA) revealed elevated levels of anti-acetylcholine receptor antibody (AChR) (0.947 nmol/L, normal range 0–0.5 nmol/L), and anti-titin antibody was determined by enzyme-linked immunosorbent assay (ELISA) and the results showed positive (2.537, normal range < 1). The rest tests for autoimmune neuropathies or paraneoplastic syndrome were all negative. Grade 4 tislelizumab-induced irAEs including MG, myocarditis, myositis, liver damage, and kidney damage was diagnosed according to the Common Terminology Criteria for

Adverse Events (CTCAEs) v5.0. The patient received methylprednisolone at 1g/day intravenously for 3 days, afterward the dose of methylprednisolone was gradually tapered down. IVIG treatment was given at 0.4g/kg per day for 3 days simultaneously. Despite no significant improvement of clinical symptoms, the patient's myocardial enzymes decreased gradually 2 days after the treatment. Five days after admission, the patient complained tightness of the chest and had difficulty in breathing. Arterial blood gas analysis showed type I respiratory failure. The patient was transferred to the NCU for respiratory support, and dyspnea improved 6 days later. Non-invasive positive pressure ventilation was applied for respiratory support. Approximately 20 days after admission, the levels of CK-MB, CK, LDH, AHB, Myo, ALT, AST, troponin, urine microproteins, and kidney function gradually returned to normal (Figure 1). The patient was discharged from hospital 23 days after admission with conventional home ventilator support (intermittent), as he still had difficulty in breathing when he discharged from hospital. After discharge, the patients went to the community rehabilitation center intermittently for rehabilitation training, and the serum indicators (liver enzymes, CK, troponin, etc.) were closely monitored in the community hospital. The patient regained the autonomous walking and weaned off the breathing machine entirely 3 months later. (The symptoms and treatment of the patient were summarized in Figure 2).

Discussion

We present a 65-year-old male patient with tislelizumab-induced multiple organ irAEs, who manifested as weakness of the limbs, drooping eyelids, oculomotor nerve paralysis, dyspnea, rhabdomyolysis, and palpitations. The patient responded well to corticosteroids and IVIG treatment; the symptoms gradually improved. This is the first report of occurrence of polymyositis with MG in colorectal cancer (CRC) patients treated with tislelizumab.

LACRC is defined as CRC stage II (cT3–4, N0)/stage III (any cT, N+) (6). Except for patients with oligometastatic disease, most patients with LACRC have incurable disease (7). The 5- and 10-year survival rates of patients after successful surgical or ablative interventions are approximately 40 and 20%, respectively (8–10). Traditional surgical resection cannot meet the needs of all patients, and MSI-H/dMMR LACRC has low sensitivity to chemotherapy. Most clinical studies demonstrated that patients with MSI-H/dMMR solid tumors received obvious benefits from ICIs (11–13). Thus, ICIs may be a breakthrough for the treatment of colon cancer.

PD-1 inhibitor tislelizumab (BGB-A317) is a humanized IgG4 monoclonal antibody that inhibits the binding of FcγR to macrophages, thus eliminating antibody-dependent phagocytosis and improving T cell clearance (14, 15). Chinese authorities approved tislelizumab for Hodgkin's lymphoma by the National Medical Products Administration (NMPA) in December 2019. PD-L1/PD-L2–associated cell signaling can lead to cytokine production and a reduction in tumor cell death associated immune response. The PD-1/PD-L1 cell signaling can be blocked by tislelizumab (15). Tislelizumab was approved in China due to its antitumor potential in a variety of malignant tumors including relapse or refractory classical Hodgkin lymphoma, urothelial carcinoma, non-small cell lung cancer, and hepatocellular carcinoma. Moreover,

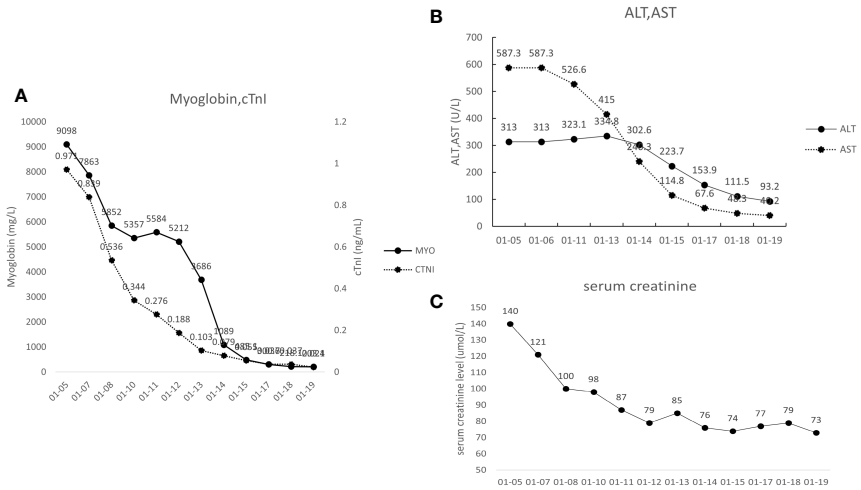

FIGURE 1
(A) Changes to the patient's cardiac markers. Myo, myoglobin; cTNT, cardiac troponin T. **(B)** Changes to the patient's liver function indexes. ALT, alanine aminotransferase; AST, aspartate aminotransferase. **(C)** Changes to the patient's serum creatinine.

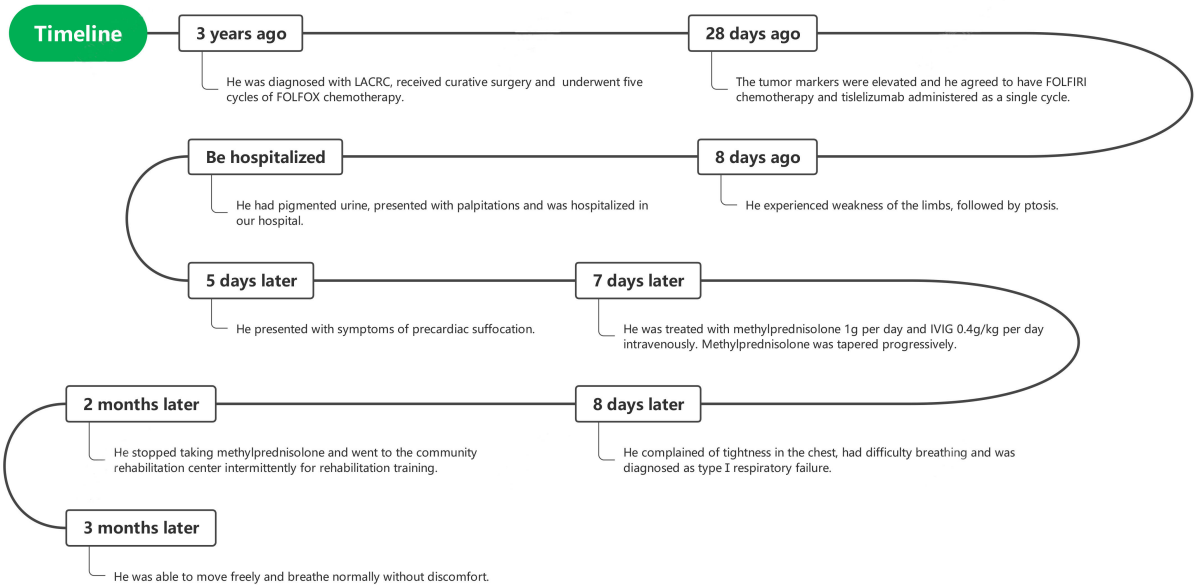

FIGURE 2
Flow chart of patient condition during simulation progression.

results from clinical trials showed tislelizumab had beneficial effects in treating tumors in multiple systems. On March 11, 2022, the NMPA officially approved tislelizumab for adult patients with MSI-H type or dMMR type solid tumor, which brings new options and hope for the treatment of many advanced solid tumors including CRC.

However, due to the increase in clinical use of PD-1 inhibitors, the associated irAEs increased as well (16). According to the European Society for Medical Oncology (ESMO) guidelines (17), Skin toxicity, gastrointestinal toxicity, liver toxicity, pneumonia, and endocrine disruption are common side effects of PD-1 inhibitors. Rare immune-related toxicities include cardiotoxicity, neurotoxicity, rheumatic immunotoxicity, nephrotoxicity, and ophthalmology have also been reported before (18–20). Although the incidence is less than 1%, they often develop in an explosive way, which endangers the life of patients (21) and requires great attention. In a retrospective analysis of a World Health Organization (WHO) pharmacovigilance database (Vigilyze) (4), 52 of 131 fatal immunotherapy-related adverse effects (39.7%) were attributed to myocarditis, and more than a quarter of the fatal myocarditis patients were associated with myositis and MG. PD-1 inhibitor associated myositis/myocarditis with MG often occurs early in treatment and deteriorates rapidly.

Twenty days after tislelizumab therapy, our patient developed weakness of limbs and drooping eyelids, accompanied by chest tightness and pigmented urine. Blood biochemical examination showed that myocardial enzyme, CK and aminotransferase were significantly increased, and CK was more than 50 times the upper limit of normal value. The diagnosis of myasthenia gravis was confirmed because the patient had head drop, facial weakness, ptosis, dysphonia, and shortness of breath, and his anti-AChR antibody was positive. A recent study summarized the onset of ICI-induced MG ranged from 6 to 106 days after the first dose (22). Although patients with Guillain–Barre' syndrome (GBS) may also present with oculomotor paralysis, limb weakness, and loss of tendon reflex, and GBS has been reported in patients with Non-Hodgkin's Lymphoma of the colon during chemotherapy (23), GBS in our patient can be excluded as autoimmune peripheral polyneuropathy antibody were negative and EMG was normal. Myasthenia and myositis caused by paraneoplastic syndrome have also been reported (24, 25); however, paraneoplastic antibodies were all negative; therefore, paraneoplastic syndrome can be excluded. Myositis caused by the combination of atorvastatin and nivolumab has also been reported (26); the patient did not take any statins or other drugs that might cause rhabdomyolysis. After comprehensive analysis, ICI-related adverse effects affecting multiple organ systems were considered. Only two cases of tislelizumab-induced irAEs occurred during treatment of small-cell lung cancer and ureteral epithelium (27) have been previously reported (28). Autoantibodies associated with myositis, peripheral polyneuropathy or paraneoplastic syndrome was not detected in both of them. As ICI is rarely used in the treatment of colon cancer, our report provides a new reference for ICI irAEs.

The mechanism of irAEs has not been fully understood to date. It was reported that tumor tissues and striated muscle (myocardium and skeletal muscle) had cross-reactive antigens (29, 30). Consequently, the distinct T-cell receptors misled the immunological system by targeting dissimilar antigens with clonal T-cell receptors across tumor and muscle samples. According to cases of ICI-induced myositis or myocarditis in previous reports, the skeletal muscle and myocardium biopsy revealed a greater number of mononuclear cells, particularly $CD8^+$ T cells, resulting in the development of irAEs (22, 29, 31). Cytokines or chemokines released from immune cells can also cause immune-mediated tissue damage (32–35). Researchers have demonstrated that genetic factors play a pivotal role in the development of ICI-associated irAEs in patients with arthritis (36), diabetes (37–39), and pruritus (40). The gut microbiome has been suggested to contribute to experimental irAEs (41, 42) and colitis in melanoma patients (43–45).

MG is a rare antibody-mediated neuromuscular disease, whereby predominant anti-AChR antibodies attack the muscle endplate, leading to fatigable weakness in skeletal muscles (46). Furthermore, in retrospective case-control study researchers found neuromuscular decompensation to be more pronounced in patients with anti–PD-1 treatment (47). It has been more previously reported that patients with a history of MG or positive anti-AChR antibody presented a myasthenic crisis after ICI treatment (48–51) compared with patients without a history of MG. For patients with a history of MG, the use of ICIs might activate the T-cell autoimmune response and may induce MG. For acetylcholine receptor antibody-positive MG patients, high doses of corticosteroids alone can exasperate the myasthenic crisis, and ASCO guideline recommend high doses of corticosteroids combined with gamma globulin or plasma replacement as first-line treatment (17).

IrAEs mainly involves skin, endocrine, liver, gastrointestinal and lung, and other rare immune-related toxicities include nervous

TABLE 1 ICI-induced adverse effects, clinical manifestations, and treatment.

Adverse reactions	Clinical manifestations	Treatment
Hepatobiliary diseases (53)	Increased levels of AST, ALT, γ-glutamyl transferase, and bilirubin; autoimmune hepatitis	Hepatic irAEs can require discontinuation of checkpoint inhibitor therapy and treatment with immunosuppressive agents (54).
Endocrine diseases (53, 55)	Hypothyroidism, hyperthyroidism, or other thyroid disorder; adrenocortical insufficiency, diabetes, and secondary hyperglycemia	Some hormone deficiencies can be managed with the corresponding hormone replacement (56).
Blood and lymphatic system disorders (53)	Anemia, leukopenia, neutropenia, thrombocytopenia, hemophilia, and hemolytic anemia	Glucocorticoids are the first line of therapy; IVIG or rituximab can be considered in difficult cases. Neutropenic patients can be treated with G-CSF (57).
Neurological system disorders (19, 58)	Neuromuscular dysfunction (myasthenia gravis), encephalitis, myelitis, cerebral vasculitis, Guillain–Barre Syndrome, and non-infectious meningitis	Corticosteroids were the most frequent treatment, followed by IVIg and plasma exchange (PEX).
Cardiovascular diseases (59)	Pericarditis, myocarditis, and vasculitis	Treatment of ICI-associated myocarditis includes ICI discontinuation, supportive management, and glucocorticoids (60). Prednisone (0.5–2.0 mg/kg), followed by 4–6 weeks taper upon symptoms improvement, is recommended (30, 61).
Respiratory diseases (62, 63)	Pulmonary infections, cough, chest pain, hemoptysis, dyspnea, organizing pneumonia, autoimmune alveolitis, ARDS, and sarcoid-like granulomatosis	Intravenous steroid therapy with intravenous methylprednisolone along with empirical antibiotic therapy should be administered. Bronchoscopy and/or bronchoalveolar lavage are typically performed, and transbronchial biopsy can be considered in a serious condition.
Gastrointestinal diseases (64)	Gastritis, nausea, decreased appetite, esophagitis, gastritis, ileitis, colitis, and pancreatitis	Symptomatic treatment. There is also evidence that infliximab and vedolizumab can be used to treat ICI-induced colitis (35, 65, 66).
Mucosal or cutaneous disease (64)	Rash, pruritus, and vitiligo	The use of topical glucocorticoids is effective for treating low-grade skin reactions, but systemic glucocorticoids are required for high-grade reactions (35).
Musculoskeletal and connective tissue disorders (67, 68)	Arthralgias and rhabdomyolysis	Most patients can be managed with non-steroidal anti-inflammatory drugs or intra-articular glucocorticoid injections (35).
Kidney and urologic diseases (18)	Urinary tract infection and nephritis	Nephrology symptomatic treatment (69).
Ocular diseases (20)	Conjunctivitis, scleritis, episcleritis, uveitis, blepharitis, retinitis, and optic neuritis	Ophthalmic symptomatic treatment (70).
Systemic symptoms (64)	Fever, weight gain, and fatigue	Symptomatic treatment.

system, heart, rheumatoid immunity, and kidney (52). ICI-induced adverse effects are summarized in Table 1. Most irAEs, despite being severe in some cases, can be managed and reverted by ceasing immunotherapy and taking steroids, so early diagnosis and treatment is very important (71). Additionally, if a significant irAE is suspected, high-dose corticosteroids must be administered promptly, and patients with persistent symptoms may require escalation to other immunosuppressive therapies. For example, IVIG or plasmapheresis can be used in severe neurologic toxicity. Myocarditis can be treated with infliximab, mycophenolate mofetil, or anti-thymocyte globulin (72–74). Despite the presence of abnormal cardiac biomarker testing, our patient's surface ECG showed no evidence of atrioventricular conduction delay, and the electrophysiology study showed normal conduction. According to the ASCO guideline (55), these changes were classified as grade III cardiovascular toxicities, suggesting early (i.e., within 24h) initiation of high-dose corticosteroids. Our patient responded well to our treatment. Palpitations and shortness of breath improved, and his troponin gradually returns to normal, so he did not receive additional immunosuppressive therapy. Whenever high-dose corticosteroids fail to produce an immediate response, early institution of cardiac transplant rejection doses of corticosteroids (methylprednisolone 1 g every day) and the addition of either mycophenolate, infliximab, or antithymocyte globulin should be considered (55). Research has shown that the recurrence rate of any grade of irAE is between 25 and 50%. In general, it is recommended that patients suffering from severe irAEs discontinue their ICI treatment permanently (75–77).

Neurological irAEs have unique presentation, including disorders of the central nervous system, peripheral nerves, neuromuscular junctions, and muscles. It is possible for a single patient to have multiple neurological disorders during ICI treatment (78). Myasthenia and myositis are the most common overlap syndromes (79–81). A mixture of MG and myositis can present both clinical manifestations and laboratory findings, such as fatigue, appetite loss, proximal limb weakness, dropped head, dysphagia, respiratory insufficiency, and anti-striational antibodies. As an overlapping condition, myocarditis is also observed (82). In cases where myasthenia and myositis overlap, anti-striational antibodies including titin, ryanodine receptor, muscular voltage-gated potassium channel, Kv1.4 were detected in approximately 75% patients (22, 79, 83). A positive anti-striational antibody rate of 75% is expected to be a biomarker that can be used to diagnose overlapping myasthenia and myositis (84, 85). The anti-titin antibody was positive in our patient, which nicely supported this viewpoint. The T cell–mediated autoimmune mechanism against molecules in the skeletal and heart muscles may be important in the pathogenesis of these overlapping conditions (82). It is possible that peripheral blood may contain T cells that are autoreactive to muscle autoantigens such as titin, Kv1.4, and others (79). ICI treatment activates autoreactive CD8+ T cells, resulting in myositis and myocarditis. Also, autoreactive CD4+ T cells produce anti-AChR and anti-striational antibodies as a result of activation (82).

There is no tendency for neurological irAEs to appear with certain types of underlying cancer, according to epidemiological studies. Neurological irAEs are not associated with the brand of ICI. Nevertheless, atezolizumab can cause autoimmune encephalitis in Asian cancer patients at an unexpectedly high rate (86, 87).

There are several limitations of this case report. First, to further understand the underlying mechanisms of the irAEs, we intended to collect the muscle biopsy specimens from our patient and monitored the cytokine activation and immune cell infiltration. Considering that the muscle biopsy is an invasive test, the patient refused further diagnostic work-up. Second, pulmonary function tests are useful to assess severity of myasthenia gravis at the time of diagnosis and to monitor disease course. It is a pity that our patient cannot cooperate with the pulmonary function test.

Our case report provides experiences in managing multiple irAEs induced by tislelizumab in a patient with colon cancer. Despite its exciting therapeutic prospects, it is becoming an important safety concern. Monitoring immune parameters, making early differential diagnoses, and glucocorticoid therapy as soon as necessary play a crucial role in the treatment of patients who suffer from multiple irAEs, especially when they develop potential lethal complications such as myocarditis.

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

Every author has made substantial contributions to the manuscript. SW drafted the article and contributed to editing and revision. DP, HZ, MX, LP, RW contributed to patient follow-up. MZ has substantively edited 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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Immune-based combination therapy for esophageal cancer

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Esophageal cancer (EC) is an aggressive malignancy raising a healthcare concern worldwide. Standard treatment options include surgical resection, chemotherapy, radiation therapy, and targeted molecular therapy. The five-year survival rate for all stages of EC is approximately 20%, ranging from 5% to 47%, with a high recurrence rate and poor prognosis after treatment. Immunotherapy has shown better efficacy and tolerance than conventional therapies for several malignancies. Immunotherapy of EC, including immune checkpoint inhibitors, cancer vaccines, and adoptive cell therapy, has shown clinical advantages. In particular, monoclonal antibodies against PD-1 have a satisfactory role in combination therapy and are recommended for first- or second-line treatments. Here, we present a systematic summary and analysis of immunotherapy-based combination therapies for EC.

KEYWORDS

esophageal cancer, immunotherapy, chemotherapy, radiotherapy, combination therapy

Introduction

Esophageal cancer (EC) is the seventh most common cancer, with 570,000 cases diagnosed yearly, and the sixth highest cause of cancer-related mortalities, with 509,000 deaths per year worldwide (1). The five-year survival rate for all stages of EC is approximately 20% in China and the USA and only 12% in Europe (2, 3). EC consists of two principal histological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). ESCC occurs mainly in the upper and middle parts of the esophagus and is the primary type of EC in Asia and Eastern Europe. EAC occurs mainly in the lower segment of the esophagus, near the stomach or the junction of the gastroesophageal wall, and primarily affects people in Europe and North America (4). Key risk factors of ESCC include smoking, alcohol consumption, hot drinking, and malnutrition. In contrast, risk factors for EAC include smoking, obesity, gastroesophageal reflux disease, and Barrett's esophagus (5, 6). The incidence of ESCC

has been declining while that of EAC has been increasing rapidly, especially in Western men (7).

Traditional therapies for patients with EC include surgery, chemotherapy, chemoradiotherapy, and targeted therapy (8). Traditionally, surgery has been the most common treatment for EC. However, surgery is not suitable for cancer patients diagnosed with distal metastases or advanced stages. American Society of Clinical Oncology (ASCO) recommends chemoradiotherapy or chemotherapy before surgery for most patients with locally advanced EC (9, 10). However, most patients relapse quickly after the initial therapy, with serious adverse events including systemic toxicity and multidrug resistance (8). Thus, novel and effective drugs are needed and expected to improve overall survival (OS).

Immunotherapy is a promising modality for cancer treatment, having anti-tumor effects and increasing the OS of patients with various cancers. However, several clinical trials of immunotherapy for EC indicate that their clinical results remain challenging as a single agent (11). Therefore, a great effort has been focused on developing novel strategies to extend clinical benefits to non-responder populations. One of the strategies is the combination of immune therapy with other systemic therapeutics.

Immune checkpoint inhibitors therapy in esophageal cancer

The advent of immunotherapy has transformed cancer treatment. As part of its normal function, it can augment or change how the immune system works to curb or slow tumor growth. Several treatment modalities for immunotherapy include immune checkpoint inhibitors (ICIs), cancer vaccines, and adoptive cell therapies (ACTs). However, the major obstacle to immunotherapy is the presence of an immunosuppressive microenvironment, leading to tumor escape from immune surveillance. There are many causes of immunosuppression, including immune checkpoints highly expressed in cancer cells, heterogeneity, and low immunogenicity of tumor antigens (12–15). Immune checkpoint molecules are key co-stimulatory or co-inhibitory signals of the immune response in protecting the host from tissue damage, playing important roles in maintaining self-tolerance and preventing autoimmunity (16–18). However, when tumor cells hyperactivate inhibitory signals, these ligand-receptor pair interactions between tumor cells and T cells negatively regulate T cell activation (19–21). Immune checkpoint molecules include PD1 and CTLA-4 on T-cells and programmed death-ligand 1 (PD-L1) and B7-1/B7-2 on antigen presenting cells (APCs) and tumor cells. Additionally, PD-L1 expression has been studied in several cancers, which can be used to predict the response to ICIs in different cancer types. Immune checkpoint therapy has shown promising clinical responses in several cancers, such as malignant melanoma, head and neck cancer, lung cancer, gastrointestinal adenocarcinoma, and ESCC (22, 23).

The clinical exploration of immunotherapy for patients with advanced EC followed breakthroughs in several areas, including melanoma and lung cancer. The earliest exploration began with the third-line treatment of EC. The ATTRACTION-1 study was the first to explore the efficacy of a PD-1 monoclonal antibody in advanced esophageal squamous cancer (24). In 2018, the Journal of Clinical Oncology published the results of the KEYNOTE-028 study of an EC cohort, followed by the KEYNOTE-180 and KEYNOTE-181 clinical studies of pembrolizumab for third- and second-line treatment of advanced EC, respectively (25–29). Following ATTRACTION-1, the ATTRACTION-3 study of nivolumab versus chemotherapy was conducted as a second-line treatment for advanced ESCC. PD-1 inhibitors, camrelizumab and sintilimab, have advanced the course of domestic PD-1 inhibitors in the second-line treatment of EC. The ESCORT study evaluated the efficacy and safety of camrelizumab compared to the investigator's choice of chemotherapy in treating patients with advanced or metastatic ESCC who failed first-line chemotherapy. This study is the first randomized, controlled, multicenter phase III clinical study with the largest enrollment of patients with advanced esophageal squamous cancer in China who failed first-line standard chemotherapy. Currently, advanced EC has entered the era of immunotherapy, breaking the treatment bottleneck and providing patients with better options (Figure 1).

Pembrolizumab is a humanized IgG4 kappa monoclonal antibody that selectively blocks the interaction of the PD-1 receptor with its receptors PD-L1 or PD-L2. The US Food and Drug Administration (FDA) currently approves this drug for treating breast cancer, hepatocellular carcinoma, melanoma, non-small cell lung cancer, and esophageal or gastroesophageal junction (GEJ) cancer. In KEYNOTE-028, KEYNOTE-180, and KEYNOTE-181 clinical trials, researchers evaluated the safety and efficacy of pembrolizumab monotherapy (27, 30–32). Based on two clinical trials, KEYNOTE-180 and KEYNOTE-181, the FDA approved pembrolizumab as a second-line treatment for advanced or metastatic ESCC. Currently, ASCO recommends pembrolizumab as a first-line drug in combination with chemotherapy for refractory locally advanced or metastatic esophageal and gastroesophageal junction adenocarcinomas and squamous cell carcinomas, regardless of PD-L1 expression.

Nivolumab is a monoclonal anti-PD1 antibody approved by the FDA to treat advanced melanoma, advanced non-small cell lung cancer, advanced renal cell carcinoma, urothelial carcinoma, and squamous cell carcinoma of the head and neck (33–37). The ATTRACTION-1 phase II single-arm trial evaluated the efficacy of nivolumab monotherapy in patients with advanced EC who were refractory to or intolerant of fluoropyrimidine-, platinum-, and taxane-based chemotherapy (24, 38). Based on the ATTRACTION-1 phase II trial, the ATTRACTION-3 phase III trial compared nivolumab monotherapy with taxane monotherapy (paclitaxel or

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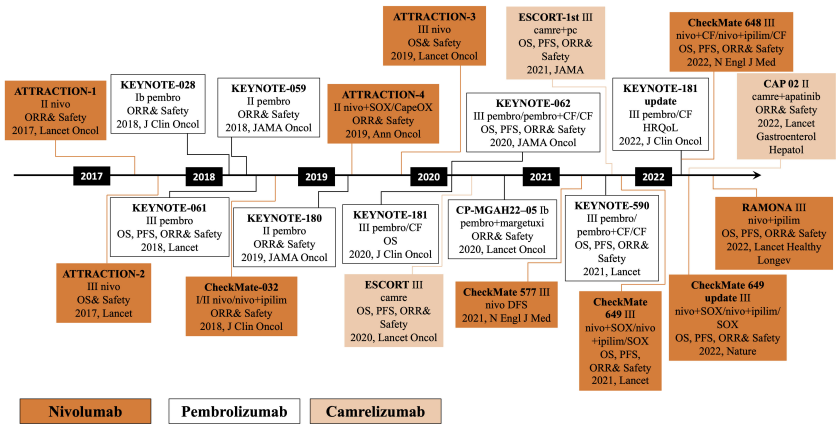

FIGURE 1
Development of chemoradiotherapy combined with immunotherapy in esophageal cancer. pembro, pembrolizumab; nivo, nivolizumab; camre, camrelizumab; ipilim, ipilimumab; OS, overall survival; PFS, progression-free survival; ORR, objective response rate; DFS, disease-free survival; health-related quality-of-life, HRQoL; S-1 plus oxaliplatin, SOX; capecitabine plus oxaliplatin, CapeOX; paclitaxel plus cisplatin, PC.

docetaxel) in patients with advanced ESCC after prior fluoropyrimidine and platinum chemotherapy. It concluded that nivolumab monotherapy was one of the promising therapeutic modalities in EC (39, 40). Subsequently, nivolumab was approved by the Pharmaceuticals and Medical Devices Agency and the FDA for advanced EC refractory to fluoropyrimidine- and platinum-based drugs.

Camrelizumab is a humanized anti-PD-1 monoclonal antibody independently developed by China. Phase I clinical trials showed that camrelizumab was well tolerated by patients with advanced solid tumors and showed anti-tumor activity (41–43). In China, camrelizumab has been approved for the treatment of several malignancies, such as Hodgkin's lymphoma, advanced hepatocellular carcinoma, non-small cell lung cancer (NSCLC), and EC. In addition, camrelizumab exhibited encouraging efficacy in some patients with advanced ESCC in a wider phase I dose-escalation and expansion study (NCT02742935) (44). Currently, camrelizumab is approved as second-line therapy for ESCC in China.

Cancer vaccine in esophageal cancer

Traditional prophylactic vaccines protect humans from diseases caused by viruses or bacteria by exposing people to weakened or killed germs with preserved immunogenicity but lost antigenicity. Prophylactic vaccines, such as the chickenpox vaccine, are administered to healthy people to avoid disease in the future (45). Therapeutic vaccines are administered to treat existing malignancies. Cancer cells originate from the healthy cells of the host. The process of carcinogenesis is believed to involve the accumulation of somatic mutations by stepwise progression, resulting in cancer cells closely resembling normal cells to a certain extent (46). Besides immune cell exhaustion, the side effects of cancer therapies or aging contribute to the severe debilitation of the immune response (47–49). Therefore, treatment vaccines for cancer face severe challenges because of tumor-induced immunosuppression, immune evasion, and the aging immune system (50, 51). Over the past 40 years, only two therapeutic cancer vaccines have been approved in the United States and the European Union: sipuleucel-T and talimogene laherparepvec. Currently, to improve the effectiveness of cancer vaccines, choosing optimal antigens and highly potent vaccine vectors and quelling tumor-mediated immunosuppression have been described as the most important considerations in the design of therapeutic vaccines.

The application of a therapeutic vaccine for EC focuses on New York esophageal squamous cell carcinoma 1(NY-ESO-1) and melanoma-associated antigen (MAGE-A), both well-known cancer-testis antigens (CTAs) with re-expression in numerous cancer types. Owing to their restricted expression patterns and ability to elicit immune responses, CTAs are promising candidates for cancer vaccines (52, 53). Bujas et al. and Forghanifard et al. analyzed the expression profiles of MAGE-A4 and NY-ESO-1 using immunohistochemistry and relative mRNA expression, respectively. Both showed overexpression of MAGE-A4 and NY-ESO-1 in patients with EC (54–56). Kageyama et al. confirmed the safety and immunogenicity of the CHP-NY-ESO-1 vaccine by comparing the effectiveness of repeated inoculation with 100 μg or 200 μg CHP-NY-ESO-1 (57). Several studies have explored the combined application of cancer vaccines and immune adjuvants. Ishikawa conducted a clinical trial on a CHP-NY-ESO-1 vaccine combined with poly-

ICLC and observed that the combination treatment group exhibited better antibody responses than cancer vaccine alone (58). A phase I study of vaccination with NY-ESO-1f peptide combined with Picibanil OK-432 and Montanide ISA-51 in patients with cancers expressing the NY-ESO-1 antigen enrolled six patients with EC and observed an increase in NY-ESO-1 antibody response and CD4 and CD8 T cell response in nine of ten patients (59), indicating the importance of dendritic cell-based cancer vaccines. Since dendritic cells (DCs) are the dominant antigen-presenting cells and strong activators of T cells, numerous studies have investigated the use of peptide-pulsed DCs as cellular vaccines (53, 60, 61). Narita conducted a phase I/II clinical trial in ESCC, demonstrating that anti-tumor immunotherapy with a SART1 peptide-pulsed DC vaccine may not bring clinical and survival benefits (62). However, the vaccine was well tolerated, with acceptable side effects. Several clinical trials have indicated the safety and feasibility of WT1 peptide-pulsed DC vaccinations, with WT1-specific immunity augmented (63, 64). Some trials have also investigated the combination of multiple highly immunogenic human leukocyte Antigen HLA-restricted epitopes of overexpressed CTAs in patients with ESCC, showing promising anti-tumor activity (65–67).

Adoptive cell therapy in esophageal cancer

ACT, including chimeric antigen receptor (CAR)-and T cell receptor (TCR)-engineered T cell therapies and tumor-infiltrating lymphocytes (TILs), exhibits effective and rapid therapeutic effects on tumors. Typically, modification of T cells is a stepwise process *in vitro*, involving extracting the patient's T cells; isolating, modifying, and expanding them ex vivo; and then returning them to the patient. Standard gene transfer methods include transient mRNA transfection, lentiviral transduction, and retroviral vector transduction.

The CAR molecule is major histocompatibility complex (MHC)-independent, and recognition and binding of specific antigens primarily depend on extracellular domains, namely single chain variable fragments (68). Therefore, the tumor immune escape elicited by low human leukocyte antigen (HLA) expression can be avoided. Owing to its potent and long-lasting anti-tumor functions, the clinical response of hematological tumors has shown great success. Genetically engineered autologous CD19 targeted CAR-T cells were the first therapeutic modality approved by the FDA for treating relapsed or refractory hematological malignancies such as lymphocytic leukemia and B-cell lymphoma. In addition, Autologous B-cell maturation antigen-targeted CAR-T cell therapy products for multiple myeloma have shown outstanding anti-tumor activity (69). Nevertheless, despite the remarkable achievements of CAR-T cell therapy in treating liquid tumors, the widespread use of CAR-T cell therapies to treat solid tumors is comparatively modest because of the various roadblocks (69).

Currently, preclinical trials with EC-associated antigens for CAR-T cell therapy targeting HER2, EphA2, MUC1, B7, and H3 are ongoing. Although clinical responses and results are less satisfactory in some solid cancers, Several clinical trials of CAR-T cell therapy against EC are investigating and evaluating (NCT03706326, NCT03740256, NCT03013712, NCT04581473) (70).

An alternative genetically modified T-cell immunotherapy is TCR-T therapy, exhibiting a broader treatment effect. In contrast to CAR-T cells, the TCR-T cell construct is a heterodimer consisting of α and β chains. Antigen recognition by the αβTCR is core to the function of the adaptive immune system (71). Recognition and binding of T cells to antigens depend on the specific matching of TCRs with HLA, resulting in T cells distinguishing rare foreign pMHCs from abundant self pMHC molecules (72–74). Compared with CAR-T cell therapy, TCR-T cell therapy could act on more targeted antigens. In addition to antigens expressed on the surface of cells, intracellular antigens can also be recognized once processed and presented by MHC molecules (75).

Much research on TCR-T cell therapy mainly concentrates on solid tumors (76) rather than liquid tumors, targeting CTAs, including NY-ESO-1, MAGE-A3, and MAGE-A4. Moreover, preclinical and clinical trials of TCR-T-cell therapy for EC are ongoing.

Immunotherapy in combination with chemoradiotherapy

PD1/PD-L1 inhibitors

Previous studies showed chemoradiation combined with immunotherapy to have good anti-tumor effects. This combination treatment includes three action mechanisms: (1) Chemoradiation can kill tumor cells, release tumor antigens, and increase the recognition of T cells (77). (2) Chemoradiation can increase the expression of antigens on the surface of tumor cells (78). In addition, studies have shown that chemotherapy increases the molecular expression of MHC-I, thereby strengthening immune system recognition of tumor cells (79). (3) Radiotherapy and chemotherapy destroy the tumor microenvironment and increase T-cell infiltration (80–82). In addition, some studies have shown that radiotherapy has a range of effects, such as activating dendritic cells, reducing the level of regulatory T cells in tumors, expanding the lineage of T cells, and enhancing T cell metastasis (Figure 2). The combination of immune checkpoint therapy and chemoradiotherapy has

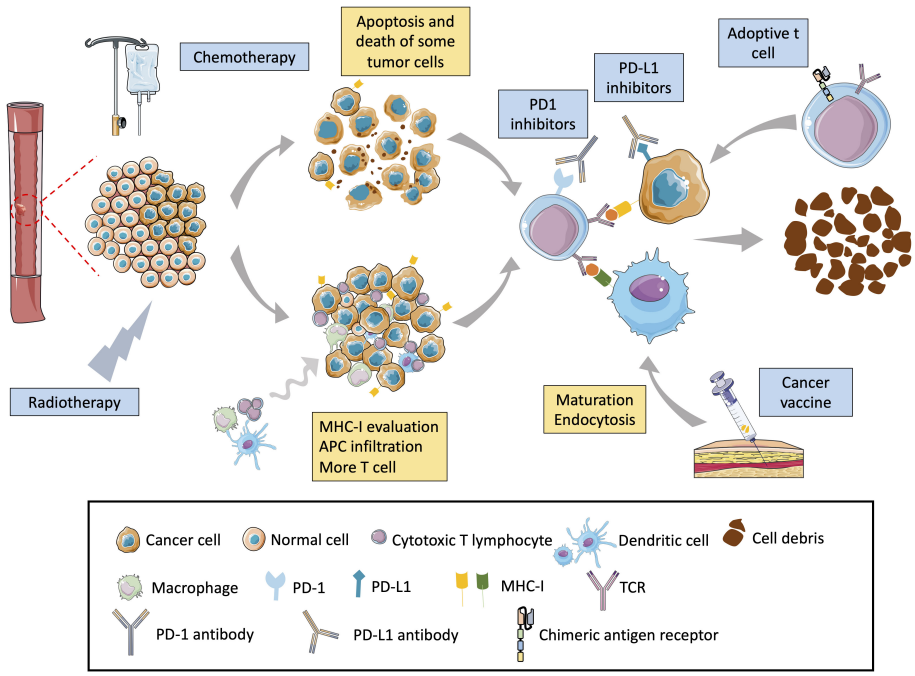

FIGURE 2
Mechanism of chemoradiation–immunotherapy combination in esophageal cancer. Chemoradiation can directly lead to the death of cancer cells by apoptosis, necrosis, and autophagy, promoting the release of tumor-specific antigens by tumor cells and increasing the chances of immune cells finding cancer cells. Chemoradiation can also directly destroy the DNA of cells, causing cancer cells to produce neoantigens and triggering an immune response. In addition, chemoradiation can upregulate the expression of tumor MHC-I that can better present tumor-specific antigens and enhance tumor visibility by cytotoxic T cells. Radiotherapy can modulate the tumor microenvironment, increase the tumor microenvironment, and promote the migration of cytotoxic T cells to the tumor. Radiotherapy also upregulates the PD-L1 expression level on the cancer-cell surface, enhancing the therapeutic effect of PD-L1 antibodies.

shown promising clinical responses in several cancers, such as malignant melanoma, head and neck, lung, gastrointestinal adenocarcinoma, and ESCC.

Pembrolizumab-based monotherapy can prolong the OS and progression-free survival (PFS) of patients with ESCC, with acceptable treatment-related adverse effects. The FDA has approved pembrolizumab to treat ESCC (PD-L1 CPS ≥ 10). Clinical trials of pembrolizumab and chemotherapy combination for the treatment of EC have achieved significant results, and more clinical trials are underway.

The KEYNOTE-059 study, a multi-cohort, phase II, non-randomized clinical trial, compared the effects of pembrolizumab in combination with chemotherapy to those of chemotherapy alone as first-line treatment in patients with advanced gastric or gastroesophageal junction adenocarcinoma (Table 1). The study enrolled 25 and 31 patients, respectively. This trial demonstrated encouraging anti-tumor activity as a first-line treatment and acceptable safety. However, because the sample size was small, it needs to be validated in a larger population. The phase III randomized clinical trial KEYNOTE-062 evaluated the efficacy and safety of first-line treatment in patients with untreated, advanced gastric/GEJ (G/GEJ) cancer with a PD-L1 CPS of ≥ 1 (Table 1). The study enrolled 763 patients randomly divided into pembrolizumab (n = 256), pembrolizumab plus chemotherapy (n = 257), or chemotherapy (n = 250) treatment groups. This clinical trial demonstrated clinically meaningful OS of pembrolizumab in patients with untreated, advanced G/GEJ cancer with PD-L1 CPS of ≥ 1, especially in those with PD-L1 CPS of ≥ 10 and high microsatellite instability (MSI) tumors. However, the benefits of pembrolizumab and chemotherapy combination were not superior to those of chemotherapy alone, regardless of PD-L1 CPS of more than 1 or 10 (83–85). These data are consistent with those of the KEYNOTE-061 study, confirming the utility of PD-L1 high expression and suggesting pembrolizumab for frontline therapy (86). The results provide a reliable basis for further research on pembrolizumab in combination with chemotherapy.

KEYNOTE-590 (NCT03189719) was a randomized, placebo-controlled, double-blind, phase 3 study to evaluate the anti-tumor activity of pembrolizumab plus chemotherapy in comparison with that of chemotherapy alone as the first-line treatment in patients with unresectable, locally advanced, or metastatic EC or Siewert type 1 GEJ cancer (Table 1). A total of 749 patients were enrolled in a randomized 1:1 trial of pembrolizumab plus chemotherapy and chemotherapy alone.

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TABLE 1 Clinical Trial of Immunotherapy in Combination with Chemoradiotherapy.

NCT Number	Phase	Role	Conditions	regime	mOS(m)	mPFS(m)	ORR (%)	mDFS (m)
KEYNOTE-059 (NCT02335411) cohort 2	II	first-line	advanced G/GEJ adenocarcinoma	PENBRO+CHEM	13.8	6.6	60	NA
KEYNOTE-062 (NCT02494583)	III	first-line	untreated, locally advanced/ unresectable, or metastatic G/GEJ cancer (PD-L1 CPS≥1)	PENBRO+CHEMO (CF/CAP) vs PLACEBO+CHEMO	12.5 vs 11.5	6.9 vs 6.4	48.6vs 37.2	NA
KEYNOTE-590 (NCT03189719)	III	first-line	advanced EC and Siewert type 1 GEJ cancer	PENBRO+CHEMO (CF)vs PLACEBO +CHEMO	12.4 vs 9.8	6.3 vs 5.8	45vs 29.3	NA
ATTRACTON-4 (NCT02746796)	III	first-line	HER2-negative, unresectable advanced or recurrent G/GEJ cancer in Asian	NIVO + CHEMO (SOX/CAPOX) vs PLACEBO+CHEMO	17.45 vs 17.15	10.45 vs 8.34	57 vs 48	NA
CheckMate649 (NCT02872116)	III	first-line	advanced or metastatic G/GEJ, and EAC	NIVO + CHEMO vs CHEMO	14.4 vs 11.1 (PD-L1 CPS≥ 5)	7.7vs 6.0(PD-L1 CPS≥ 5)	60 vs 45(PD-L1 CPS≥ 5)	NA
CheckMate 648 (NCT03143153)	III	first-line	advanced ESCC	NIVO + CHEMO (CF) vs CHEMO	15.4 vs. 9.1 (PD-L1 CPS≥ 1) 13.2 vs. 10.7	6.9 vs 4.4(PD-L1 CPS≥ 1) 5.8 vs 5.6	53 vs 20(PD-L1 CPS≥ 1) 47 vs 27	NA
NCT03222440	Ib	first-line	locally advanced ESCC	CAMRE+RADIO	16.7	NA	73	11.7
ESCORT-1st (NCT03691090)	III	first-line	advanced or metastatic ESCC	CAMRE+CHEMO (CP) vs PLACEBO +CHEMO	15.3 vs 12	6.9 vs 5.6	72.1 vs 62.1	NA
NIC-ESCC2019 (NCT04225364)	II	neoadjuvant treatment	resectable ESCC	CAMRE+CHEMO (CP)	NA	NA	66.7	NA
ORIENT-15 (NCT03748134)	III	first-line	unresectable, locally advanced recurrent or metastatic ESCC	SINTILI+CHEMO (CP/CF) + PLACEBO+CHEMO	17.2 vs 13.6 (PD-L1 CPS≥ 10) 16.7 vs 12.5	8.3 vs 6.4(PD-L1 CPS ≥10) 7.2 vs 5.7	78.8 vs 57.5 (PD-L1 CPS ≥10) 75.5 vs 56.9	NA

mOS, median overall survival; mPFS, median progression-free survival; ORR, median objective response rate; mDFS, median disease-free survival; EC, esophageal cancer; GEJ, gastro-esophageal junction; PEMBRO, pembrolizumab; CHEMO, chemotherapy; NIVO, nivolumab; CF, 5-fluorouracil and cisplatin; CAP, capecitabine; SOX, S-1 plus oxaliplatin; CAPOX, capecitabine plus capecitabine; CP, cisplatin plus paclitaxel; CAMRE, camrelizumab; RADIO, radiotherapy; SINTILI, sintilimab; NA, no assessment.

The KEYNOTE-590 study combined immunotherapy and chemotherapy to derive the advantages of immunotherapy enhanced by synergistic effects. The study showed a significant improvement in median PFS (mPFS), documenting the short-term efficacy of mPFS, objective response rate (ORR), and the long-term effect of median OS (mOS). Thus, the combination of pembrolizumab and chemotherapy can provide a comprehensive survival benefit to patients. Compared with the KEYNOTE-062 and ATTRACTION-4 trials that did not indicate a distinct difference in OS between combination therapy and mono-chemotherapy, the OS in KEYNOTE-590 was significantly improved. This may be due to the sample size differences and the use of post-study anti-tumor agents. By comparing ICIs in combination with chemotherapy as first-line treatment and ICI monotherapy as second- or third-line treatment, more significant survival benefits and higher levels of PD-L1 expression in tumors were observed in the former. This phenomenon could be because previous therapy generated tolerance, immunosuppressive tumor microenvironments, and the synergistic effect of ICIs plus chemotherapy. The limitations of this trial include no separation of adenocarcinoma and squamous cell carcinoma in the study, no stratifying analysis based on PD-L1 status, and unknown HER-2 status (87). Since combining pembrolizumab with chemotherapy in KEYNOTE-590 trials as first-line treatment provided meaningful improvement, FDA approved pembrolizumab plus chemotherapy as first-line therapy for advanced or metastatic esophageal or GEJ cancer.

Definitive chemoradiotherapy is the standard treatment modality for unresectable EC or distant metastasis. Based on previous study, A double-blind, phase III randomized placebo-controlled study KEYNOTE-975 (NCT04210115) currently in progress is evaluating the efficacy and safety of pembrolizumab plus definitive chemoradiotherapy compared to placebo plus definitive chemoradiotherapy as first-line treatment for patients with esophageal carcinoma. The data from this trial have not been reported hitherto.

The FDA approved nivolumab in 2021 for patients receiving neoadjuvant radiotherapy for completely resected esophageal or GEJ cancer with the residual pathological disease (88). In

addition, clinical trials of combination chemotherapy for EC have also made significant progress, and the exploration of treatment options for combination chemotherapy is ongoing.

ATTRACTION-4 (NCT02746796) is a randomized, multicenter, double-blind, placebo-controlled, phase II/III clinical trial in patients with previously untreated, unresectable, advanced, or recurrent G/GEJ cancers (Table 1). Based on the safety and efficacy of nivolumab plus chemotherapy observed in the phase II trial of ATTRACTION-4 (89), the latest phase III clinical research results were published in The Lancet ontology. A total of 724 patients were randomly assigned to receive nivolumab plus chemotherapy and chemotherapy in a 1:1 ratio. Compared to CheckMate649, this trial was conducted in Asia, including Japan, South Korea, and Taiwan. It demonstrated that the PFS (hazard ratio (HR): 0.68; 98.51% confidence interval (CI): 0.51–0.90; p = 0.0007), ORR, and more durable responses favored nivolumab plus chemotherapy over chemotherapy alone. However, the OS difference was insignificant (HR: 0.90, 95% CI: 0.75–1.08; p = 0.26) in this trial, showing superior OS with the combination therapy in CheckMate649. The disparity between the ATTRACTION-4 trial and CheckMate-649 study could be attributed to differences in subsequent anti-tumor treatment modalities. *Post-hoc* interaction analyses suggested that most of the baseline characteristics were not determinants of treatment outcomes in either ATTRACTION-4 trial or CheckMate-649 study. Furthermore, no new safety issues were observed. Nivolumab combined with chemotherapy showed a manageable safety profile. A limitation of ATTRACTION-4 was the absence of an assessment between the PD-L1 CPS and each endpoint. In the CheckMate-649 and KEYNOTE-062 trials, the survival advantage of nivolumab plus chemotherapy and pembrolizumab plus chemotherapy was more significant in patients with a higher PD-L1 CPS than in those with a lower PD-L1 CPS. In conclusion, nivolumab in combination with chemotherapy has the potential to become a new first-line treatment for Asian patients with HER2-negative, unresectable advanced or recurrent G/GEJ cancers (90, 91).

Compared with the ATTRACTION-4 clinical trial conducted primarily in Asian populations, the CheckMate 649 trial was conducted in different countries with a broader population.

CheckMate649 (NCT02872116) is a randomized, multicenter, open-label, phase III study that was conducted in patients with untreated, unresectable, non-HER2-positive gastric, GEJ, or EAC to evaluate the efficacy and safety of nivolumab-plus-chemotherapy (Table 1). Among the 1,581 advanced patients enrolled regardless of PD-L1 CPS expression, 789 patients were treated with nivolumab-plus chemotherapy, and 792 were treated with chemotherapy alone. In all randomly assigned patients or patients with a PD-L1 CPS of ≥ 5, the combination therapy group displayed better OS and PFS than the group subjected to chemotherapy alone. Statistical hierarchical testing suggested that OS and PFS benefit magnitudes were relative to the PD-L1 CPS cut-offs. The higher the PD-L1 CPS cut-off, the greater the OS and PFS benefits. For patients with PD-L1 CPS ≥ 5, evidence for using chemo-immune combination therapy regimens is relatively strong. However, the suitability of patients with a PD-L1 CPS of < 5 for chemotherapy in combination with immunotherapy requires further active exploration and search for relevant biomarkers. Examples include tumor mutational burden (TMB), copy number variant load, and neutrophil-to-lymphocyte ratio. The safety profile of nivolumab plus chemotherapy was acceptable and consistent with known individual treatments. Based on this trial, the FDA approved nivolumab plus chemotherapy as a new standard first-line treatment for previously untreated patients with advanced G/GEJ cancer and EAC (89, 92).

Doki et al. compared the first-line treatment with nivolumab-based therapy in patients with previously untreated, unresectable advanced, recurrent, or metastatic ESCC. The experimental design of CheckMate 648 relied heavily on the success of CheckMate 649. CheckMate 648 (NCT03143153) clinical trial is a randomized phase 3 study that enrolled 970 patients. Patients were randomly divided into three groups in a 1:1:1 ratio to receive nivolumab plus chemotherapy, nivolumab plus the monoclonal antibody ipilimumab, or chemotherapy alone (Table 1). This trial demonstrated significant OS and PFS clinical benefits associated with nivolumab plus chemotherapy in patients with the proportion of PD-L1-positive tumor cells to total tumor cells is greater than one percentage. Furthermore, the proportion of patients with numerically higher objective response rates and longer durations of response in the nivolumab plus chemotherapy cohort was larger than those in the chemotherapy alone cohort, among patients with the proportion of PD-L1-positive tumor cells to total tumor cells is greater than one percentage. Additionally, a pre-planned exploratory subgroup analysis showed that tumor cell PD-L1 expression and PD-L1 combined positive score had clinical utility (93).

Camrelizumab has been approved as second-line therapy for ESCC, and its combination with chemotherapy has been approved as first-line therapy for patients with advanced or metastatic ESCC in China. The following is a summary of the main clinical trials of camrelizumab combined with chemotherapy.

A phase Ib study (NCT03222440) investigated the safety and feasibility of definitive radiotherapy plus camrelizumab as a first-line treatment for locally advanced ESCC (Table 1). Twenty patients were recruited for the study. The trial demonstrated promising clinical results and an acceptable safety profile. Additionally, predictive biomarkers and specific status and function of T-cell subsets were assessed by analyzing the tumor microenvironment and systemic immune status (94). Based on these preliminary results, a phase III, randomized, double-blind, placebo-controlled study of camrelizumab versus placebo in combination with concurrent chemoradiationin these patients (NCT04426955) was conducted this year.

The ESCORT-1st (NCT03691090) study was a randomized, double-blind, placebo-controlled, phase 3 trial conducted between December 3, 2018, and May 12, 2020 (Table 1). This trial enrolled 596 eligible patients from 60 hospitals in China. All

patients were randomly assigned (1:1) to the camrelizumab-chemotherapy and placebo-chemotherapy groups. The ESCORT-1st study has three highlights: First, the enrollment criteria are tailored for the Chinese population. As 90% of patients with EC in China are diagnosed with squamous carcinoma, the ESCORT-1st study was designed to include a population of Chinese patients with squamous EC, in line with the actual pathology in China. Second, the paclitaxel plus cisplatin regimen is China's most widely used chemotherapy for advanced EC. Therefore, the ESCORT-1st study is more suitable for the Chinese scenario than the fluorouracil+cisplatin regimen used in overseas clinical studies for advanced EC. Third, camrelizumab plus chemotherapy showed superior efficacy and safety results than placebo plus chemotherapy. The analysis of this clinical trial data suggested that camrelizumab plus chemotherapy showed better OS and PFS than placebo plus chemotherapy, with an acceptable adverse event profile similar to monotherapy. No new adverse events were identified. Furthermore, the results also showed statistically significant improvements in health-related quality-of-life metrics with camrelizumab plus chemotherapy compared to that with placebo plus chemotherapy. There are also several limitations to the trial, including the absence of correlation between PD-L1 expression status and efficacy of camrelizumab plus chemotherapy and the discovery of predictive biomarkers (95). Overall, there are still many directions for this study worth exploring.

Camrelizumab showed promising anti-tumor activity in ESCC as a first-line treatment, and researchers have explored the effect of camrelizumab as neoadjuvant therapy. The phase II trial NIC-ESCC2019 (NCT04225364) assessed camrelizumab plus chemotherapy in resectable ESCC as a neoadjuvant option in China (Table 1). The trial showed the combination therapy's feasibility, safety, and efficacy and indicated that neoadjuvant chemoimmunotherapy might have a better response in lymphatic metastases than in primary lesions (96). However, further research is required due to the small sample size.

Currently, the NICE-2 (NCT05043688) study is designed as a three-arm, multicenter, prospective, randomized, phase II clinical trial to evaluate the efficacy and safety of camrelizumab plus chemotherapy (IO-CT) and camrelizumab plus chemoradiation therapy (IO-CRT) versus CRT as preoperative treatments for locally advanced ESCC. The primary endpoint is the complete pathological response rate, and secondary endpoints include event-free survival, R0 resection rate, and adverse events. Patient enrollment in this trial started in September 2021. It is still in the recruitment stage (97).

Camrelizumab is a PD-L1 monoclonal antibody independently developed by China, and the current clinical trials related to camrelizumab are mainly conducted in China. Although it is approved for second-line treatment of esophageal cancer in China, its application is still very limited worldwide. In the future, it is worth looking forward to carrying out Camrelizumab-related clinical trials for EC in more regions outside China.

Additionally, sintilimab is a domestic anti-PD-1 monoclonal antibody used in China. It was first approved by the National Medical Products Administration (NMPA) for patients with relapsed or refractory classical Hodgkin's lymphoma after two or more lines of systemic chemotherapy. Subsequently, the NMPA approved sintilimab in combination with chemotherapy as first-line treatment for NSCLC and in combination with IBI305 as first-line treatment for hepatocellular carcinoma. Therefore, investigators have evaluated sintilimab in combination with chemotherapy for the treatment of EC. ORIENT-15 (NCT03748134) is a global, randomized, double-blind phase III study that evaluated the efficacy and safety of sintilimab combined with chemotherapy versus chemotherapy alone as the first-line treatment in patients with unresectable locally advanced, recurrent, or metastatic ESCC (Table 1). At the data cut-off, all 659 patients were enrolled and randomized into sintilimab/placebo plus chemotherapy in a 1:1 ratio. Based on the OS, PFS, and ORR analysis, investigators have suggested that the combination of sintilimab and chemotherapy can be considered a new first-line treatment in patients with advanced or metastatic ESCC (98). However, because this study is ongoing, more evidence of its efficacy and safety profile may be forthcoming, along with a longer follow-up.

Cancer vaccine

CRT has been widely used to treat patients with irresectable ESCC. Nonetheless, not all patients are resistant to chemoradiotherapy, and many relapse. To date, cancer vaccines have shown promising results in therapy and a manageable safety profile for EC (Figure 2). Therefore, combining chemoradiotherapy with cancer vaccines may be an effective way to treat EC.

The purpose of phase I clinical study (NCT00632333) of multiple-epitope peptide vaccines combined with CRT was to evaluate its safety and efficacy. As a result, all 11 patients with unresectable chemo-naïve ESCC showed peptide-specific cytotoxic T lymphocyte responses to at least one of the five peptide antigens during vaccination. After the 8th peptide vaccination in combination with CRT, 54.5% (6 of 11) achieved CR, along with 45.5% (5 of 11) showing PD. All patients tolerated the combination therapy well and did not experience serious adverse effects (99). However, the number of patients in this study was small, and there was no control group. Therefore, it is difficult to explain the practical effects of a cancer vaccine in combination with CRT.

Fujiwara et al. conducted a phase I/II open, non-randomized, single-arm clinical trial (UMIN 000000669) between July 1, 2007, and July 1, 2011, to investigate the safety and efficacy of labeled DC combined with systemic

chemotherapy for EC. Five patients were enrolled in this study. This study demonstrated that the accumulation of DC in primary tumors injected with labeled DC did not migrate to the lymph nodes from primary tumors. No DC accumulation was observed elsewhere. Additionally, there were no changes in the antibody titers of the 28 tumor antigens analyzed by the enzyme-linked immunosorbent assay. However, the clinical responses of the five enrolled patients were absent (100).

Wang et al. conducted a clinical trial to observe the efficacy of a combination of radiotherapy and dendritic cells loaded with apoptotic heat-shock EC cell antigens. There was a remarkable increase in the expression of serum IL-2, IL-12, and IFN-γ and the proportion of IFN-γ^{+}CD8^{+}T cells in the treatment group, compared to that in the baseline and control groups (all $P < 0.05$). The 1- and 2-year survival rates improved with vaccination. Only two patients had a mild fever. This clinical trial recruited 40 patients randomly divided into experimental and control groups in a 2:1 ratio. However, because of the study's small sample size, patients being in an early-stage EC, and follow-up time being relatively short, more multicenter trials are necessary for combining radiotherapy and DC-based cancer vaccines (101).

To date, most vaccines in combination with chemoradiotherapy against EC are in the developmental stage—the clinical application of combining immunotherapy with tumor vaccines for EC warrants further exploration.

Adoptive cell therapy

Adoptive cell therapy is also synergistic with chemoradiotherapy (Figure 2). Sato et al. conducted two phase-I trials of adoptive γδT cell therapy combined with chemotherapy. One was for treatment-refractory recurrent or metastatic EC (r/mEC) (γδT-monotherapy-P1, UMIN000001419) and the other for r/mEC with no prior systemic therapy (DCF-γδT-P1, UMIN000008097). The results of the 26 γδT-monotherapy patients enrolled suggest no survival benefits and no severe adverse events. Eight patients received docetaxel, cisplatin, and 5-fluorouracil (DCF) chemotherapy plus adoptive γδT cell therapy, and a better clinical response was obtained, similar to that in the DCF mono-chemotherapy previously reported. All treatment-related adverse events were associated with DCF chemotherapy but not with γδT injection. However, this was a phase I study with a small sample size designed to evaluate the safety. Therefore, large, randomized phase 2 controlled studies are warranted (102).

Combination between immunotherapies

Currently, several clinical trials evaluate the function of ICI monotherapy, such as anti-PD-1/PD-L1 and anti-CTLA-4, in patients with EC. These trials showed favorable results for ICIs as second-line or higher-line therapies, with relatively few side effects. In addition, combination treatment with ICIs has shown promising clinical benefits in some malignant tumors, such as advanced renal cell carcinoma, advanced melanoma, and advanced NSCLC (103–106). However, the role and efficacy of combination therapies with anti-PD-1 and anti-CTLA-4 antibodies in EC is ongoing.

The CheckMate-032 (NCT01928394) study aimed to assess the efficacy and safety of nivolumab and nivolumab plus ipilimumab in patients with metastatic esophagogastric cancer in western countries (Table 2). Patients (n = 160) were randomly divided into three groups: nivolumab 3 mg/kg, nivolumab 1 mg/kg plus ipilimumab 3 mg/kg, and nivolumab 3 mg/kg plus ipilimumab 1 mg/kg. The CheckMate-032 study was the first to demonstrate the potential clinical benefits and manageable safety profile of nivolumab and nivolumab plus ipilimumab in patients with chemotherapy-refractory esophagogastric cancer. Based on the efficacy and safety of different doses, the phase III CheckMate-649 study selected NIVO1 + IPI3 for further evaluation. Additionally, investigators explored potential predictive biomarkers, including tumor PD-L1 and MSI status. However, further studies are warranted because of the small sample size (107, 108).

CheckMate-648 is the largest randomized, global phase III study to date in which ICIs are based on the first-line treatment of advanced ESCC, with a total of 970 patients from multiple countries and regions around the world, including China. In addition, it is the only phase III clinical study evaluating the first-line treatment of advanced ESCC in the first-line dual immune (PD-1 inhibitor + CTLA-4 inhibitor) "de-chemotherapy" regimen; it received widespread attention. The CheckMate648 (NCT03143153) study recruited patients with previously untreated, unresectable advanced, recurrent, or metastatic ESCC who received treatment with nivolumab plus chemotherapy, nivolumab plus the monoclonal antibody ipilimumab, or chemotherapy monotherapy in a 1:1:1 ratio (Table 2). The results of nivolumab plus chemotherapy compared with those of chemotherapy alone are presented above. Of note, OS and objective response were significantly better with nivolumab plus ipilimumab than with chemotherapy among patients with tumor cell PD-L1 expression of ≥ 1% and in the overall population. However, the PFS differences between the two groups did not meet the criteria for statistical significance. The incidence of grade 3 or 4 treatment-related adverse events among those who received nivolumab plus ipilimumab was 32% and that for chemotherapy alone was 36%, lower than that of nivolumab plus chemotherapy (47%). The incidence of treatment-related deaths was similar across groups (93).

RAMONA (NCT03416244) is a multicenter, open-label, phase II trial conducted in 34 centers in Germany to investigate the safety of nivolumab and ipilimumab as second-line therapy in elderly patients with advanced ESCC and

TABLE 2 Clinical Trial of Combination between Immunotherapies.

NCT Number	Phase	Role	Conditions	regime	mOS(m)	mPFS(m)	ORR (%)	mDFS (m)
CheckMate-032 (NCT01928394) cohort2	I/II	adjuvant treatment	chemotherapy-refractory EC in Western	NIVO1+IPI3 vs NIVO3	6.9 vs 6.2	1.4 vs 1.4	24 vs 12	NA
CheckMate-032 (NCT01928394) cohort3	I/II	adjuvant treatment	chemotherapy-refractory EC in Western	NIVO3 + IPI1	4.8	1.6	8	NA
CheckMate648 (NCT03143153)	III	first-line	advanced ESCC	NIVO+IPI vs CHEMO	13.7 vs. 9.1(PD-L1 CPS≥1) 12.7 vs 10.7	4 vs 4.4(PD-L1 CPS≥1)	35 vs 20(PD-L1 CPS≥1) 28 vs 27	NA
RAMONA (NCT03416244)	II	second- line	advanced ESCC (age≥65)	NIVO+IPI	2.7	6.9	NA	NA

mOS, median overall survival; mPFS, median progression-free survival. ORR, median objective response rate. mDFS, median disease-free survival; NIVO, nivolumab; IPI, ipilimumab; IPI1, ipilimumab 1 mg/kg; IPI3, ipilimumab 3 mg/kg; NIVO1, nivolumab 1 mg/kg; NIVO3, nivolumab 3 mg/kg; NA, no assessment.

additional comorbidities (Table 2). It enrolled 66 eligible patients between May 2018 and August 2020. The patients received combined nivolumab and ipilimumab therapy and nivolumab alone in a 2:1 ratio. With the median follow-up of 6.8 months (3.4–15.4), the mOS of 7.2 months was significantly improved compared to that in the previous control cohort receiving standard chemotherapy (p = 0.0063). Treatment-related adverse events were observed in 42 patients. Adverse events of grade 3 or worse occurred in 54 (82%) of the 66 patients, and serious adverse events occurred in 45 (68%) patients. Overall, grade 3–5 treatment-related adverse events occurred in 13 (20%) patients, with no difference between the patients who received nivolumab monotherapy (five [23%] of 22) or combination therapy (eight [18%] of 44) (109).

Many experts and researchers believe that double immunotherapy's adverse reaction rate is lower than that of chemotherapy. Patient tolerance is good, but the serious adverse reactions caused by immunotherapy, such as bone marrow suppression and gastrointestinal reactions, are more difficult to deal with than adverse reactions caused by chemotherapy. For example, some experts believe that the 32% incidence of adverse events of CheckMate 648 grades 3 or 4 is too high. Although the efficacy of double immunotherapy in advanced EC is remarkable, its side effects cannot be denied.

Immunotherapy in combination with targeted drugs

Targeted drugs have significant efficacy in the treatment of tumors due to their small toxicity and high specific advantages (110–112). Apatinib is a small-molecule anti-angiogenic agent that targets the VEGFR-2 tyrosine kinase. Anti-angiogenic agents can increase the infiltration of immune effector cells into tumors and reprogram the tumor microenvironment through the normalization of the tumor vasculature. The addition of anti-angiogenic agents can enhance cancer immunotherapy. Apatinib is the second agent approved by the China Food and Drug Administration for treating advanced metastatic gastric cancer. Several preclinical and clinical trials have demonstrated its vigorous anti-tumor activity and acceptable safety in advanced gastric cancers and ECs.

A single-arm, open-label, investigator-initiated phase II study of apatinib monotherapy evaluated its effectiveness and safety profile in patients with unresectable, metastatic EC (113). The phase II clinical trial ESO-Shanghai 11 evaluated the efficacy and adverse effects of oral apatinib in patients with chemotherapy-refractory ESCC (114). The results of the two clinical trials showed that apatinib monotherapy has the potential to be an efficient and secure second-line or higher treatment for patients with ESCC. Zhao et al. conducted a trial to evaluate the safety and efficacy of apatinib combined with neoadjuvant chemotherapy in patients with locally advanced ESCC compared with chemotherapy alone. They concluded that combination therapy has promising outcomes in the treatment of ESCC (115).

A single-center phase II clinical trial (NCT03603756) investigated the efficacy and safety of camrelizumab plus apatinib in combination with chemotherapy as the first-line treatment (Table 3). This trial enrolled 30 patients between August 7, 2018, and February 23, 2019. The primary endpoint ORR was 80% (19/26), achieving the prespecified primary endpoint. Compared to chemotherapy in a previous clinical trial, combination therapy showed encouraging clinical outcomes. Because the mOS between the PD-L1 CPS ≥ 10 and < 10 subgroups was similar, the results of subgroup analysis showed that the clinical effect was not directly associated with PD-L1 CPS. However, since this was a small sample size, single-arm experiment, a larger clinical trial is needed to assess the relationship between PD-L1 expression and clinical response (114, 116).

The CAP 02 (NCT03736863) trial explored the efficacy of camrelizumab in combination with apatinib as second-line

TABLE 3 Clinical Trial of Immunotherapy in Combination with Targeted Drugs.

NCT Number	Phase	Role	Conditions	regime	mOS (m)	mPFS (m)	ORR (%)	mDFS (m)
NCT03603756	II	first-line	unresectable, locally advanced recurrent or metastatic ESCC	CAMRE+apatinib+ CHEMO(NP)	19.43	6.85	80	NA
CAP 02 (NCT03736863)	II	second-line	advanced ESCC	CAMRE+apatinib	15.8	6.8	34.6	NA
CP-MGAH22–05 (NCT02689284)	Ib-2	first-line	previously treated, HER2-positive GEJ adenocarcinoma	PEMBRO +margetuximab	12.48	2.73	18.48	NA

mOS, median overall survival; mPFS, median progression-free survival. ORR, median objective response rate. mDFS, median disease-free survival; CAMRE, camrelizumab; CHEM, chemotherapy; NP, liposomal paclitaxel plus nedaplatin; PEMBRO, pembrolizumab; NA, no assessment.

therapy for advanced ESCC (Table 3). This single-arm, open-label, phase II study was conducted at eight centers in China and enrolled 52 patients. The objective response rate in this study was close to that observed in CheckMate 648 (47%) and KEYNOTE-590 (45%), indicating anti-tumor activity of camrelizumab plus apatinib in ESCC. However, considering the inconsistencies in the conditions of the three clinical trials, this result should be interpreted with caution. *Post-hoc* analyses of the correlation between PD-L1 expression and clinical responses indicated promising clinical responses, regardless of the amount of PD-L1 expressed. However, the mechanism of action of camrelizumab in combination with anti-angiogenic drugs remains unclear. Larger-scale clinical validation is needed (116). Subsequent camrelizumab plus apatinib protocol was designed as a cohort study of follow-up treatment options for patients who failed first-line immunotherapy. It is believed that this study can bring more therapeutic hope to immune-resistant populations and will further confirm the advantages of anti-angiogenesis combined with immune regimens.

Margetuximab, a novel, investigational, Fc-engineered, anti-HER2 monoclonal antibody, offers more effective antibody-dependent cellular cytotoxicity than trastuzumab-mediated innate immune cells. In a phase 1 study of patients with refractory HER2-positive gastroesophageal adenocarcinoma, margetuximab monotherapy resulted in an objective response rate of 10% (two of 20 patients) and enhanced adaptive immunity (117). Anti-HER2 agents have also been reported to increase PD-L1 expression in tumor cells. In preclinical models, synergistic anti-tumor activity has been observed when anti-HER2 therapeutic approaches are combined with anti-PD-1 antibodies.

Therefore, Catenacci et al. conducted a clinical trial to evaluate the safety, tolerability, and anti-tumor activity of margetuximab plus pembrolizumab in previously treated patients with HER2-positive gastroesophageal adenocarcinomas. CP-MGAH22–05 (NCT02689284), a single-arm, open-label, phase Ib–2 dose-escalation, cohort-expansion study, enrolled 95 patients between February 11, 2016, and October 2 (Table 3). The median follow-up was 19.9 months. Margetuximab plus pembrolizumab cohort showed manageable safety and tolerability. In addition, no dose-limiting toxicities were observed during the dose-escalation phase (118).

Immunotherapy has become the standard for the first- and second-line treatment of advanced EC. However, the issue of no standard treatment for immune-resistant populations has begun to receive clinical attention. The mechanism of immune resistance is very complex. More studies, such as double immune combination, immune combination anti-EGFR monoclonal antibody, immune combined cyclin inhibitors, and immune combined epigenetic drugs, need to be carried out in the future to increase the effect of combination therapy and bring more clinical benefits to patients.

Future direction and conclusion

In recent years, clinical trials have emphasized that ICIs combined with chemotherapy or radiotherapy may achieve greater therapeutic effects on various cancers than ICIs alone. For example, immunotherapy has shown encouraging clinical results in the treatment of EC. Immune checkpoint therapy has been rapidly developed for the treatment of EC. For example, based on Keynote-590, the FDA approved pembrolizumab in combination with platinum and fluoropyrimidine-based chemotherapy for patients with metastatic or locally advanced esophageal or GEJ carcinoma who are not candidates for surgical resection or definitive chemoradiation. Based on CheckMate-648, FDA approved nivolumab in combination with fluoropyrimidine- and platinum-based chemotherapy and nivolumab in combination with ipilimumab as the first-line treatment for patients with advanced or metastatic ESCC.

Gastric cancer and EC have high heterogeneity, and there are great differences in the biological characteristics and clinical characteristics of Chinese and Western patients, bringing difficulties to clinical research. For example, in the CheckMate-649 study, the degree of benefit for patients in the Chinese subgroup was significantly higher, showing that patients in different countries and regions can receive different levels of benefits. Therefore, more influencing factors should be

considered in the future when conducting a hierarchical analysis. In addition, China has a strong and unmet clinical demand for EC drug treatment, therefore, more research on Chinese patients with EC is needed.

While immune checkpoint-based therapy is promising, only a small proportion of patients benefit from immunotherapy. Therefore, accurate screening of target populations and combination therapy will become the main direction of future research on advanced EC. Further, immune-related side effects cannot be ignored. Another area of research is finding strong predictive and prognostic biomarkers or comprehensive biomarkers to optimize treatment strategies. Many clinical trials have explored PD-L1 positivity, TMB, MSI, and T cell inflammatory gene expression profiles as biomarkers. However, because these trials differ in the chemotherapy backbone, anatomical and histological differences, PD-L1 diagnostic antibodies, and positive definitions of PD-L1, any crossover trial comparison should be made with caution.

Tumor vaccines offer a therapeutic approach that helps to direct the immune system to recognize cancer-associated antigens and achieve anti-tumor effects. Tumor vaccines mainly include whole-cell, molecular, and DC vaccines. Although preclinical and clinical trials on cancer vaccine monotherapy have yielded preliminary results, combining tumor vaccines with other regimens requires further exploration. In addition, the discovery and utilization of new antigens have contributed to the development of cancer vaccines.

Several ACTs have shown promising clinical utility in treating EC with acceptable toxicity. Further research is needed to reduce toxicity and improve the efficiency of this strategy. TILs are important candidates for the ACT and have demonstrated anti-tumor activity in preclinical and clinical studies to treat several solid tumors, including melanoma and ovarian cancer. Researchers have also found that TIL is significantly associated with survival in patients with EC, but current TIL-based studies for treating EC have not been reported.

Immunotherapy-based combination therapies have shown positive effects in EC, and more clinical trials are still underway. Nevertheless, more research is needed to identify new targets and expand immunotherapy to the first line. In addition, identifying better biomarkers to provide prognostic information and guide therapy is critical to the breadth of precision oncology. Further studies on the tumor microenvironment, the molecular mechanisms of response, and resistance to checkpoint inhibitors will also be instructive for immunotherapy.

With the increasing application of combination treatment regimens in the first-line treatment of patients with advanced gastric cancer and EC, further developing a second-line treatment plan after a patient's treatment fails is still controversial. Clinicians may choose chemoradiotherapy alone or chemoradiotherapy plus anti-angiogenic drugs or continue to use combination regimens. In the future, the provision of more standardized clinical treatments for such patients needs to be studied further. In addition, whether the combination therapy plan of reduction and exemption can move the frontline forward and apply it to the perioperative treatment of patients with early EC also requires corresponding thinking and exploration by oncology clinicians.

Author contributions

HW contributed to investigation, illustration, writing, and editing. YX, FZ contributed to investigation and writing-review. JL, JY contributed to writing-review and editing. 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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Decreased monocyte-to-lymphocyte ratio was associated with satisfied outcomes of first-line PD-1 inhibitors plus chemotherapy in stage IIIB-IV non-small cell lung cancer

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Objectives: Immune-checkpoint inhibitors (ICIs) combined with chemotherapy are more widely used than monotherapy and have shown better survival in patients with advanced non-small cell lung cancer (NSCLC) without oncogenic driver alterations. The monocyte-to-lymphocyte ratio (MLR) might predict the treatment outcomes of ICI therapy in advanced NSCLC patients but has not yet been investigated. In addition, the cutoff of MLR is controversial. Therefore, the present study aimed to explore the associations between changes in MLR at the initial stage of treatment and clinical outcomes in stage IIIB-IV NSCLC patients receiving first-line PD-1 inhibitor combined with chemotherapy.

Methods: The present study included 139 stage IIIB-IV NSCLC patients treated with first-line PD-1 inhibitor combined with chemotherapy. The blood results were assessed 10 days before initiation of PD-1 inhibitor-based combination therapy (time point 1, baseline) and before the third cycle of combined therapy (time point 2). Compared to altered MLR, neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR) in baseline and in time point 2, patients were divided into decreased MLR/NLR/PLR and increased MLR/NLR/PLR groups. The objective response rate (ORR), progression-free survival (PFS), and the association with the changes in blood indicators were analyzed.

Results: A total of 48 patients were categorized in the decreased MLR group and 91 in the increased MLR group. Patients with decreased MLR had a significantly higher ORR in the univariate (P<0.001) and multivariate (P<0.001) Cox proportional hazards models. On the other hand, decreased MLR was significantly associated with prolonged PFS in the univariate (P=0.007) and multivariate (P=0.016) analyses. Next, 91 patients comprised the decreased NLR group and 48 as the increased NLR group. Patients with decreased NLR exhibited high ORR (P=0.001) and prolonged PFS in univariate analysis (P=0.033). Then, 64 patients comprised the decreased PLR group and 75 the increased PLR group. Decreased PLR was significantly

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associated with high ORR in univariate (P<0.001) and multivariate (P=0.017) analyses. The subgroup analyses showed that decreased MLR was significantly associated with satisfactory outcomes in patients with all PD-L1 expressions.

Conclusion: Decreased MLR was associated with high ORR and long PFS and might have a potential predictive value in patients with stage IIIB-IV NSCLC treated with first-line PD-1 inhibitor combined with chemotherapy. In addition, changes in MLR might have predictive value in all PD-L1-expressing populations. Decreased NLR and PLR also showed improved survival, suggesting that changes in NLR and PLR may be complementary to predicting prognosis.

KEYWORDS

non-small cell lung cancer (NSCLC), prognostic value, immune checkpoint inhibitors (ICI), chemotherapy, monocyte-to-lymphocyte ratio (MLR)

Introduction

The use of immune-checkpoint inhibitors (ICIs), specifically those targeting PD-1 or its programmed cell death ligand-1 (PD-L1), yields a durable response and prolongs the survival of patients with advanced non-small cell lung cancer (NSCLC) with high PD-L1 expression (1). However, the efficacy of monotherapy in patients is limited (2). Many studies have shown that chemotherapy-immunotherapy combinations achieved prolonged progression-free survival (PFS) and overall survival (OS) in advanced NSCLC patients regardless of the PD-L1 expression level and also improved survival than chemotherapy alone (3). ICIs, in combination with chemotherapy, have gradually become the first-line therapy for advanced NSCLC patients without oncogenic driver alterations (4). However, some patients suffer from treatment resistance, exhibiting severe immune-related adverse events (irAEs) (5). Therefore, finding precise and reliable biomarkers for immunotherapy plus chemotherapy is imperative.

To date, predictive biomarkers for ICIs plus chemotherapy have not been identified. PD-L1 expression and tumor mutation burden (TMB) are candidate biomarkers for ICIs plus chemotherapy (6). PD-L1 on tumor cells has been the predictive biomarker of response to ICIs, however, its predictive value is not accurate as the PD-L1 tumor proportion score (TPS) groups might achieve significant survival benefits, including those with TPS <1% (7). In addition, PD-L1 exhibits temporal changes in the expression and intra-tumoral heterogeneity and can only be tested on tissue specimens *via* an invasive operation (8, 9). The assessment of PD-L1 expression during treatment becomes challenging. Blood-based TMB (bTMB) can be detected from the blood and has several advantages over tissue biopsy, such as easily repeated collection over time. Nonetheless, it also has shortcomings as a marker of therapeutic effects. Currently, no distinct thresholds define high or low bTMB levels. In addition, the correlation between bTMB and prognosis presents a non-linear association, and establishing a cutoff becomes challenging (10, 11). Therefore, it lacks effective and convenient markers for immunotherapy plus chemotherapy.

Inflammation can affect disease progression and the survival of many cancers (12). Over the last few years, the prognostic and predictive value of inflammatory-related peripheral blood biomarkers in NSCLC patients receiving immunotherapy has been investigated in-depth. Several studies have shown that high neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) are prognostic markers associated with poor survival in advanced NSCLC patients with immunotherapy (13, 14). However, these studies mainly explored the cutoff, but the inflammatory markers are not linearly related to patient outcomes (15). Therefore, the cutoff of the selected inflammatory markers was uncertain and controversial. Moreover, inflammatory cells, including monocytes and lymphocytes, are commonly associated with tumor prognosis. Monocytes affect the tumor microenvironment by inducing angiogenesis and immune tolerance, and the spread of tumor cells and monocytes has a stronger phagocytosis compared to other blood cells (16). A decrease in the level of lymphocytes induces the release of several suppressive immunological mediators (17). Previous studies have shown that a decrease in the monocyte-to-lymphocyte ratio (MLR) is significantly associated with the good effects of nivolumab monotherapy (18). These findings suggested that MLR is a promising biomarker to predict the survival benefit of ICIs in advanced NSCLC patients. However, the MLR has predictive value for chemotherapy-immunotherapy combinations in advanced NSCLC patients without sensitive driver mutations, but has not yet been investigated.

MLR, NLR, and PLR are readily available inflammatory biomarkers, and the test is clinically convenient and practically noninvasive. We can also monitor blood parameters dynamically for a prolonged duration. However, it is difficult to determine cutoff values for these inflammatory markers. Therefore, the present study aimed to investigate the predictive role of changes in MLR at the initial stage of treatment in stage IIIB-IV NSCLC patients treated with first-line PD-1 inhibitor plus chemotherapy. In addition, whether changes in NLR and PLR can predict the prognosis of patients was investigated.

Materials and methods

Patients

We retrospectively identified and included 139 stage IIIB-IV NSCLC patients treated with a first-line PD-1 inhibitor

(pembrolizumab, sintilimab, or tislelizumab) combined with chemotherapy at Shanghai Chest Hospital, from January 2019 to June 2021. The inclusion criteria for patients were as follows: (1) histopathological confirmation of NSCLC; (2) initial stage IIIB-IV or recurrence after curative surgery; (3) receiving first-line PD-1 inhibitor treatment combined with chemotherapy. The exclusion criteria were as follows: (1) sensitized EGFR/ALK/ROS1 alteration; (2) underwent surgery after the combination treatment; (3) exposed to infection and antibiotics within 7 days before blood draw; (4) data not available (Figure 1).

The study was approved by the ethics review board at Shanghai Chest Hospital (Shanghai, China). All participants provided written informed consent.

Treatment and data collection

Patients were assessed every 2-3 months by computed tomography (CT) scan. The clinical features and laboratory parameters of the patients, including age, gender, smoking history, tumor histology, TNM staging, and PD-L1 expression, were obtained from medical records. Also, the blood results and the incidence of irAEs were recorded. The patients were followed up regularly during the treatment.

Patients received the following therapy: 200 mg pembrolizumab, 200 mg sintilimab, or 200 mg tislelizumab intravenously every 3 weeks. The treatment was continued until tumor progression, development of unacceptable drug toxicity, or death. The combination chemotherapy was platinum-based doublet chemotherapy, while the other drugs, including pemetrexed, docetaxel, paclitaxel/nab-paclitaxel, paclitaxel liposomes, vinorelbine, and gemcitabine were administered according to tumor histology. Peripheral blood samples were collected 10 days before initiation of PD-1 inhibitor-based combination therapy (time point 1, baseline) and before the third cycle of combined therapy (time point 2). If the disease progressed early, before the expected time point 2, a sample of peripheral blood was collected when assessing the disease progression by CT. Complete blood counts, including absolute monocyte count (AMC), absolute neutrophil count (ANC), absolute lymphocyte count (ALC), and platelet count, were recorded at the baseline (time point 1) and at the third cycle of combined therapy (time point 2) (19).

MLR was defined as the ratio of AMC to ALC, NLR as the ratio of ANC to ALC, and PLR as the ratio of platelet counts to ALC. Inflammatory biomarkers (IBs), including MLR, NLR and PLR, were calculated in two time points. The first was before initiation of PD-1 inhibitor-based combination therapy, during which the blood indicators were known as pre-inflammatory biomarkers (pre-IBs).

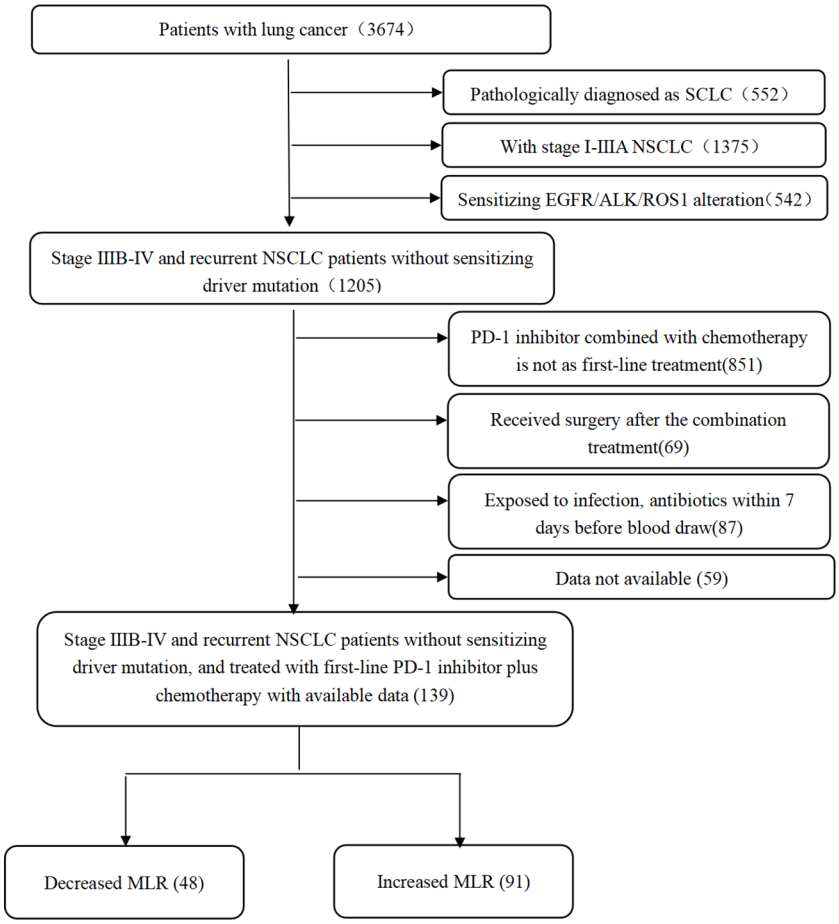

FIGURE 1
Patient disposition. NSCLC, non-small cell lung cancer; MLR, monocyte-to-lymphocyte ratio.

The other stage was prior to the therapy, termed as post-inflammatory biomarkers (post-IBs). The delta-IBs were calculated as follows: delta-IBs=post-IBs/pre-IBs−1. If delta-IBs were >0, it was defined as an increase, while ≤ 0 was deemed as a decrease.

PD-L1 expression was analyzed using PD-L1 immunohistochemistry (IHC) 22C3 pharmDx (Dako), and positive membranous staining of at least 1% of the tumor cells was defined as positive, while <1% was defined as negative. Next, we subdivided the positive status into the high (≥50%) and low (1–49%) expression categories.

Statistical analysis

Objective response rate (ORR) was defined as the percentage of patients who achieved a complete response (CR) or partial response (PR) among all the treated patients. PFS was defined as the duration from the receiving first-line PD-1 inhibitor combined with chemotherapy to the date of first documented disease progression or death.

χ^2 test was used to examine the differences in baseline and patient characteristics between the decreased and increased groups. Kaplan-Meier survival curves were generated and the log-rank test was applied to examine the survival difference between the two groups. Factors associated with ORR were tested with logistic regression in univariate and multivariate analyses. The Cox proportional hazards model was applied to calculate the hazard ratios (HRs) and evaluate the factors independently associated with PFS. Receiver operating characteristic (ROC) curves were used to compare the baseline values and the changes in MLR, NLR, and PLR. SPSS 25.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism software (Prism 8) was used for all the statistical analyses. A two-sided p-value<0.05 was considered statistically significant.

Results

Characteristics of patients

A total of 139 stage IIIB-IV NSCLC patients treated with first-line PD-1 inhibitor combined with chemotherapy participated in this study. The baseline characteristics of these patients are summarized in Table 1. Most patients were >65-years-old (51.8%), and the male proportion was 79.9%, smokers were 74.8%, and 71.9% were in stage IV or had recurrence after surgery (71.9%). Moreover, 79 (56.8%) patients had non-squamous cell carcinoma (including adenocarcinoma and lymphoepithelioma-like carcinoma), 44 (31.7%) had squamous cell carcinoma, and 16 (11.5%) had NSCLC not otherwise specified. For PD-L1 expression, 35 (25.2%) patients had a TPS of ≥50%, 45 (32.4%) patients had a TPS of 1–49%, 42 (30.2%) patients had a TPS of ≤1%, and 17 (12.2%) patients were yet to be diagnosed.

A total of 48 (34.5%) patients had decreased MLR before the third cycle of PD-1 inhibitor-based combination therapy, while 91 (65.5%) had increased MLR. These patients were divided into decreased and increased MLR groups. Similarly, 139 patients were divided into decreased and increased NLR groups and decreased and increased PLR groups. According to Table 2, the demographic and clinical characteristics of the patients between the decreased and increased groups did not show any significant differences (P>0.05).

Analysis for ORR

The ORR for patients in the decreased and increased MLR groups was 68.8% and 25.3%, respectively (P<0.001). The ORR for decreased and increased NLR groups was 50.5% and 20.8%, respectively (P<0.001), and that for decreased and increased PLR group patients was 57.8% and 25.3%, respectively (P<0.001) (Figure 2).

Next, we performed univariate and multivariate analyses for ORR and found that age, sex, smoking history, histological type, Tumor Node Metastasis (TNM) stage, radiotherapy, and irAEs had no significant association with ORR (P>0.05). Patients with 1–49% PD-L1 expression showed significantly higher ORR compared to patients with negative PD-L1 expression in univariate analysis [odds ratio (OR)=2.50, 95% confidence interval (CI): 1.04–5.88; P=0.039]. Patients with at least 50% PD-L1 expression showed significantly higher ORR compared to negative PD-L1 expression in multivariate analysis (OR=3.13, 95% CI: 1.06–9.09; P=0.039). Decreased MLR was significantly associated with high ORR in univariate (OR=6.50, 95% CI: 3.01–14.08; P<0.001) and multivariate (OR=6.75, 95% CI: 2.45–18.62; P<0.001) analyses. Decreased NLR was significantly associated with high ORR in univariate (OR=3.88, 95% CI: 1.73–8.72; P=0.001) analysis, but no association was observed with ORR in multivariate analysis (P>0.05). Decreased PLR was significantly associated with high ORR in univariate (OR=4.04, 95% CI: 1.97–8.29; P<0.001) and multivariate (OR=3.13, 95% CI: 1.23–7.97; P=0.017) analyses. Therefore, high PD-L1 expression and decreased MLR and PLR are independently associated with high ORR (Figure 3 and Supplementary Figure 1).

Analysis for PFS

The Kaplan-Meier plots in Figure 4 show a decrease in MLR and NLR at the third cycle of PD-1 inhibitor-based combination therapy from baseline, which was significantly associated with prolonged PFS (MLR: HR, 0.53; 95% CI: 0.33–0.84; P=0.007; NLR: HR, 0.63; 95% CI: 0.41–0.96; P=0.033). However, a decrease in PLR did not show any significant association (P=0.156).

In the univariate analysis for PFS, no significant differences were detected with respect to patient age, sex, smoking history, histological type, TNM stage, and irAEs. However, the decreased MLR suggested longer PFS (HR=0.53, 95% CI, 0.33-0.84, P=0.007). The decreased NLR also suggested prolonged PFS (HR=0.63, 95% CI, 0.41–0.96, P=0.033). Next, we found that PD-L1 expression (TPS<1%) was associated with shorter PFS compared to both PD-L1 expression (1≤TPS ≤ 49%) (HR=0.50, 95% CI: 0.30–0.84, P=0.009) and PD-L1 expression (TPS≥50%) (HR=0.49, 95% CI: 0.27–0.88, P=0.017). Patients who received radiotherapy had prolonged PFS (HR=0.59, 95% CI: 0.35–0.99; P=0.045). To identify the independent predictors,

10.3389/fimmu.2023.1094378

TABLE 1 Patient characteristics.

Characteristic	Patients
Total number	139
Age (years), n (%)	
<65	67 (48.2)
≥65	72 (51.8)
Gender, n (%)	
Male	111 (79.9)
Female	28 (19.1)
Smoking history	
Never	35 (25.2)
Current/former	104 (74.8)
Histology, n (%)	
Squamous	44 (31.7)
Non-squamous*	79 (56.8)
NOS	16 (11.5)
TNM stage, n (%)	
IIIB-IIIC	39 (28.1)
IV/Recurrence	100 (71.9)
PD-L1 expression, n (%)	
TPS≥50%	35 (25.2)
1%≤TPS ≤ 49%	45 (32.4)
TPS<1%	42 (30.2)
Unknown	17 (12.2)

*Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma.
NOS, not otherwise specified; PD-L1, programmed cell death-Ligand 1; TPS, tumor proportion score.

Cox multivariate analysis was performed. In multivariate analyses, decreased MLR was significantly associated with prolonged PFS (HR=0.52, 95% CI: 0.30–0.88; P=0.016), and patients who had irAEs were significantly associated with prolonged PFS (HR=0.45, 95% CI: 0.24–0.83; P=0.011). In addition, patients who received radiotherapy were significantly associated with long longer PFS (HR=0.52, 95% CI: 0.28–0.98; P=0.043), and PD-L1 expression (TPS<1%) was associated with shorter PFS compared to PD-L1 expression (TPS≥50%) (HR=0.56, 95% CI: 0.31–0.99, P=0.048). NLR was not an independent predictive factor for PFS (P>0.05) (Figure 5 and Supplementary Figure 2).

Subgroup analysis of patients according to changes in MLR

All subgroup analyses showed that the decreased MLR group had significantly prolonged PFS compared to the increased MLR group, especially for subgroups of male, non-smokers, non-squamous, and ≥50% TPS (Supplementary Figure 3A). All subgroup analyses suggested high ORR for the decreased MLR group (Supplementary Figure 3B).

Predictive value of changes in MLR, NLR, and PLR was superior to baseline MLR, NLR, PLR

The ROC curves were constructed using the baseline MLR, NLR, and PLR or changes in MLR, NLR, and PLR as the test variables. Specifically, we used different PFS time points (1, 2, and 3 years) and ORR as the state variable to plot time-dependent ROC curves. The area under the curve (AUC) represents the discriminative power of the test, and the closer the AUC is to 1.0, the higher the authenticity of the detection method. Then, the confidence interval (CI) for the AUC was calculated.

The results are shown in Supplementary Figures 4, 5. The ROC curves showed that the AUC of changes in MLR were significantly larger than baseline MLR (AUC at 3 years: 0.813>0.152, P<0.001) or had a larger trend (AUC at 2 years: 0.661>0.412, P=0.071). The AUC of changes of NLR was larger than the baseline NLR (AUC at 2 years: 0.690>0.316, P<0.001; AUC at 3 years: 0.657>0.107, P<0.001). The AUC of changes in PLR was also larger than the baseline PLR (AUC at 2 years: 0.598>0.286, P<0.001). Therefore, we speculated that the dynamic changes of MLR, NLR, and PLR were superior to baseline MLR, NLR, and PLR for accurately predicting the long-term efficacy of first-line ICIs plus chemotherapy.

Discussion

In this study, we showed that the decreased MLR was significantly associated with high ORR and long PFS in patients with stage IIIB-IV NSCLC treated with first-line PD-1 inhibitor combined with chemotherapy. The decreased NLR and PLR were also related to improved treatment outcomes (PFS or ORR). This finding suggested that altered MLR might be a predictive biomarker for patients with stage IIIB-IV NSCLC treated with first-line PD-1 inhibitor combined with chemotherapy. The predictive role of changes in MLR was effectuated regardless of the PD-L1 expression level. The changes in NLR and PLR might also have a specific predictive value and could be a supplement to predict prognosis.

The current results of this study could be explained by the following reasons. Firstly, monocytes can promote tumor progression and are recruited to primary or metastatic tumors, differentiating to tumor-associated macrophages (TAMs) (20). Secondly, lymphocytes are crucial in the host immune response and have potent anticancer activities that inhibit tumor cell proliferation and metastasis. Furthermore, it is speculated that increased lymphocyte levels are associated with improved clinical outcomes for various cancer types (21, 22). These studies supported our finding that the decreased MLR is associated with satisfactory treatment outcomes.

Furthermore, we observed that changes in MLR are associated with the treatment outcomes in advanced NSCLC patients treated with immunotherapy plus chemotherapy. Although previous studies have discussed the value of inflammatory markers in immunotherapy, only a few have mentioned their role in immunotherapy combined with chemotherapy. According to the guidelines, immunotherapy combined with chemotherapy has gradually become the most common treatment in advanced NSCLC patients without sensitizing driver mutations. Therefore, investigating the role of

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TABLE 2 Correlation between blood parameters and clinicopathological characteristics.

Characteristic	MLR			NLR			PLR		
	Decrease (n=48)	Increase (n=91)	P	Decrease (n=91)	Increase (n=48)	P	Decrease (n=64)	Increase (n=75)	P
Age (years), n (%)									
<65	25 (52.1)	42 (46.2)	0.506	44 (48.4)	23 (47.9)	0.961	34 (53.1)	33 (44.0)	0.283
≥65	23 (47.9)	49 (53.8)		47 (51.6)	25 (52.1)		30 (46.9)	42 (56.0)	
Gender, n (%)									
Male	39 (81.3)	72 (79.1)	0.766	71 (78.0)	40 (83.3)	0.458	54 (84.4)	57 (76.0)	0.220
Female	9 (18.7)	19 (20.9)		20 (22.0)	8 (16.7)		10 (15.6)	18 (24.0)	
Smoking history									
Never	11 (22.9)	24 (26.4)	0.655	26 (28.6)	9 (18.8)	0.205	14 (21.9)	21 (28.0)	0.407
Current/former	37 (77.1)	67 (73.6)		65 (71.4)	39 (81.2)		50 (78.1)	54 (72.0)	
Histology, n (%)									
Squamous	19 (39.6)	25 (27.5)	0.069	26 (28.6)	18 (37.5)	0.298	23 (36.0)	21 (28.0)	0.142
Non-squamous*	21 (43.7)	58 (63.7)		56 (61.5)	23 (47.9)		31 (48.4)	48 (64.0)	
NOS	8 (16.7)	8 (8.8)		9 (9.9)	7 (14.6)		10 (15.6)	6 (8.0)	
TNM stage, n (%)									
IIIB-IIIC	11 (22.9)	28 (30.8)	0.327	21 (23.1)	18 (37.5)	0.072	17 (26.6)	22 (29.3)	0.717
IV/Recurrence	37 (77.1)	63 (69.2)		70 (76.9)	30 (62.5)		47 (73.4)	53 (70.7)	
PD-L1 expression, n (%)									
TPS≥50%	14 (29.2)	21 (23.1)	0.821	27 (29.7)	8 (16.7)	0.264	17 (26.6)	18 (24.0)	0.743
1%≤TPS ≤ 49%	16 (33.3)	29 (31.9)		30 (33.0)	15 (31.2)		23 (35.9)	22 (29.3)	
TPS<1%	13 (27.1)	29 (31.9)		25 (27.5)	17 (35.4)		17 (26.6)	25 (33.3)	
Unknown	5 (10.4)	12 (13.1)		9 (9.8)	8 (16.7)		7 (10.9)	10 (13.4)	
Radiotherapy									
Yes	8 (16.7)	21 (23.1)	0.377	21 (23.1)	8 (16.7)	0.377	13 (20.3)	16 (21.3)	0.883
No	40 (83.3)	70 (76.9)		70 (76.9)	40 (83.3)		51 (79.7)	59 (78.7)	
irAEs									
Yes	13 (27.1)	14 (15.4)	0.097	18 (19.8)	9 (18.8)	0.884	12 (18.7)	15 (20.0)	0.853
No	35 (72.9)	77 (84.6)		73 (80.2)	39 (81.2)		52 (81.3)	60 (80.0)	

*Non-squamous tumor included adenocarcinoma, lymphoepithelioma-like carcinoma.
MLR, monocyte-to-lymphocyte ratio; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; NOS, not otherwise specified; PD-L1, programmed cell death-Ligand 1; TPS, tumor proportion score; irAEs; immune-related adverse events.

MLR in combination therapy was crucial. In addition, the inflammatory markers were usually investigated by identifying a specific cutoff. However, it is difficult to determine the cutoff value, and the correlation between changes in inflammatory markers and treatment outcomes was non-linear (15). The current study showed that changes in inflammatory markers were better than baseline inflammatory markers for longer-term efficacy prediction. Therefore, continuous monitoring of changes in these inflammatory markers during treatment might be a more reasonable method than identifying a cutoff for those patients who can benefit from immunotherapy combined with chemotherapy for a long time.

To the best of our knowledge, the expression of PD-L1 is an imperfect predictive marker, as even patients with positive PD-L1 may not benefit from PD-1 inhibitors. However, for patients with negative PD-L1 expression, PD-1 inhibitors might exhibit a satisfactory therapeutic effect. Therefore, we conducted a subgroup analysis of PFS and ORR according to the PD-L1 expression (TPS<1% *vs.* 1%≤TPS ≤ 49% *vs.* TPS≥50%) and found that decreased MLR was significantly associated with prolonged PFS in patients with PD-L1 expression. In addition, all subgroup analyses with PD-L1 expressions suggested high ORR for the decreased MLR group, especially the 1%≤TPS ≤ 49% and TPS<1% subgroup. These results suggested that

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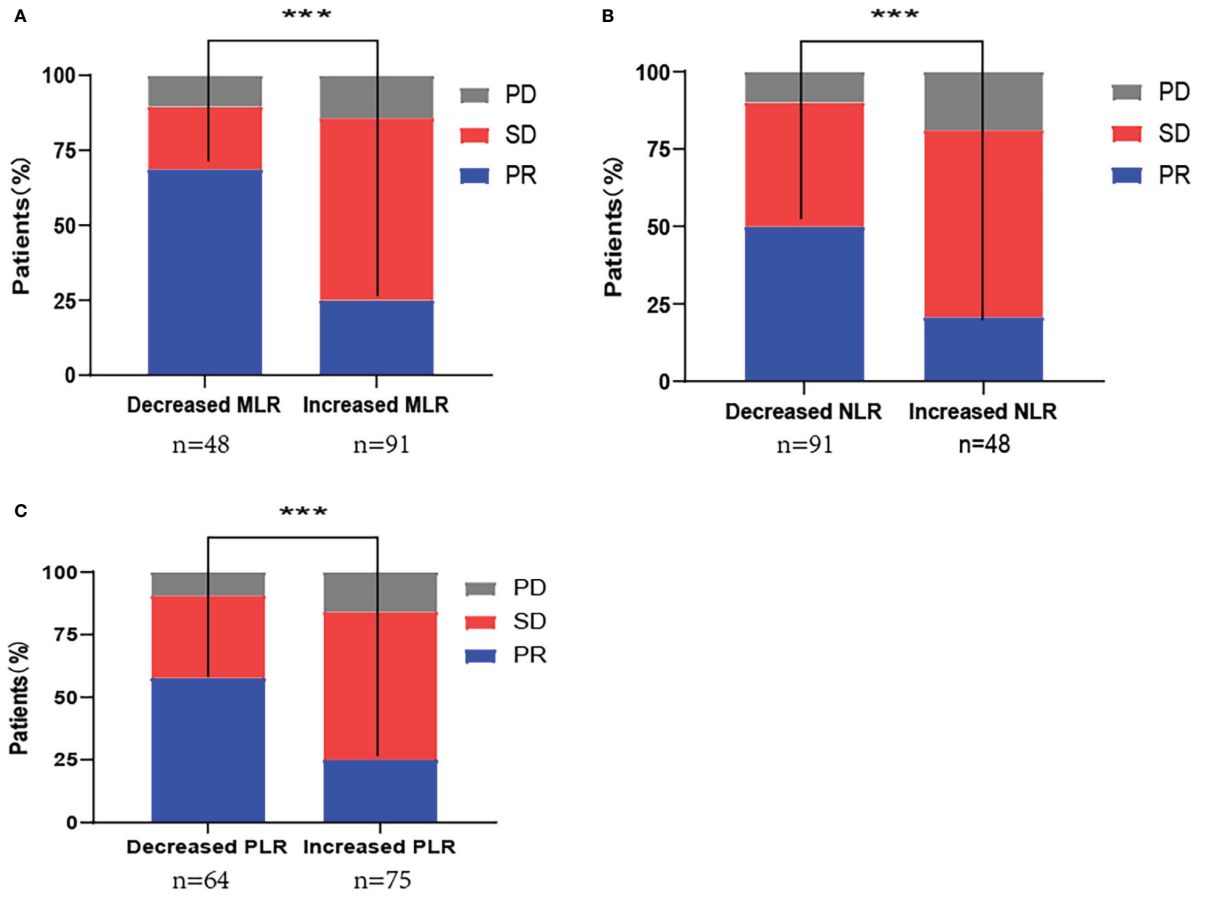

FIGURE 2
First-line PD-1 inhibitor combination with chemotherapy response distribution by changes in **(A)** monocyte-to-lymphocyte ratio (MLR); **(B)** neutrophil-to-lymphocyte ratio (NLR); **(C)** platelet-to-lymphocyte ratio (PLR). PD, progressive disease; SD, stable disease; PR, partial response; ***P<0.001.

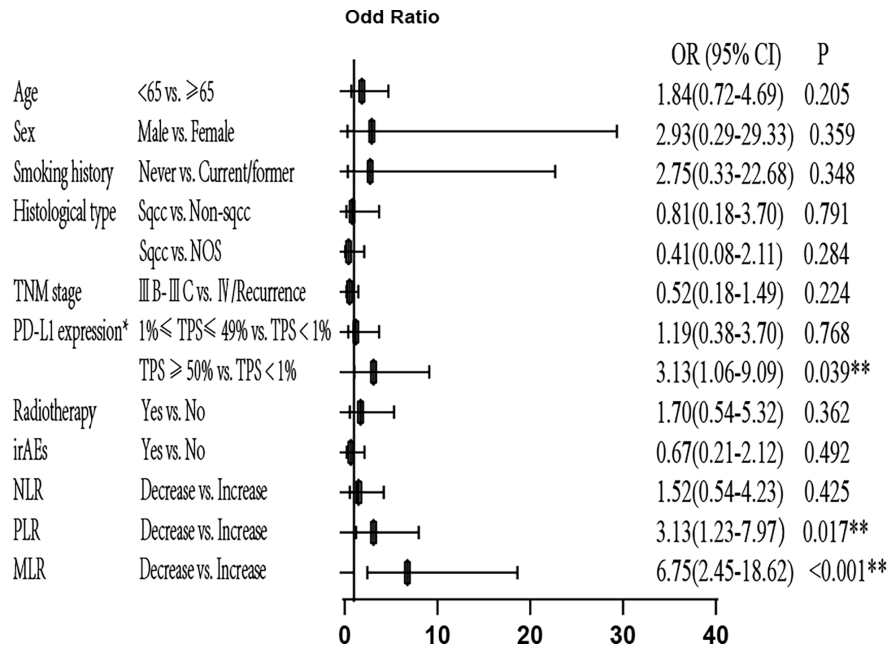

FIGURE 3
Multivariate analysis of ORR. *Only for patients with available PD-L1 expression data (patients with unknown PD-L1 expression were excluded). ORR, objective response rate; OR, odds ratio; PD-L1, programmed cell death-ligand 1; TPS, tumor proportion score; irAEs, immune-related Adverse Events; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; MLR, monocyte/lymphocyte ratio; **P<0.05 indicates statistical significance.

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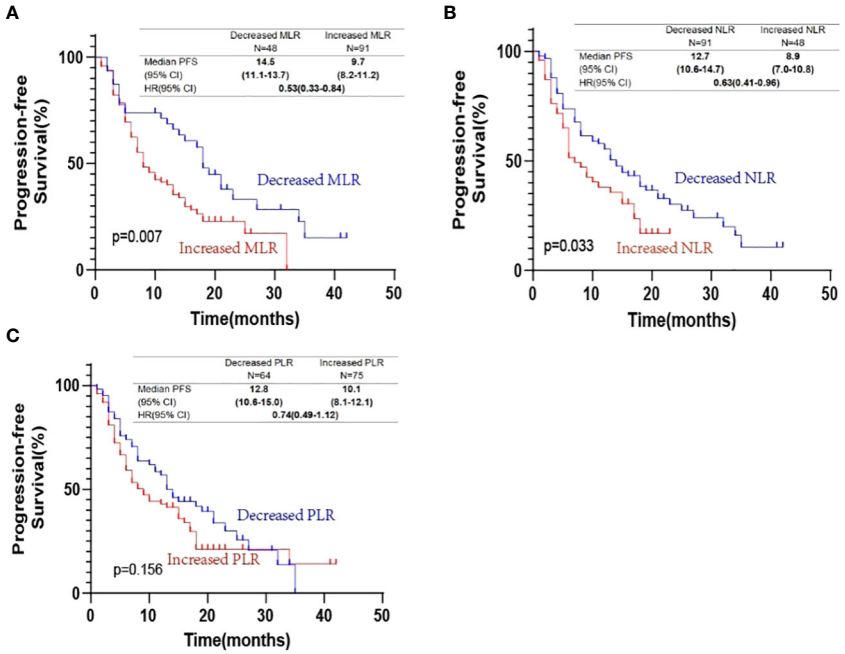

FIGURE 4
Kaplan-Meier progression-free survival curves according to changes in **(A)** monocyte-to-lymphocyte ratio (MLR); **(B)** neutrophil-to-lymphocyte ratio (NLR); **(C)** platelet-to-lymphocyte ratio (PLR).

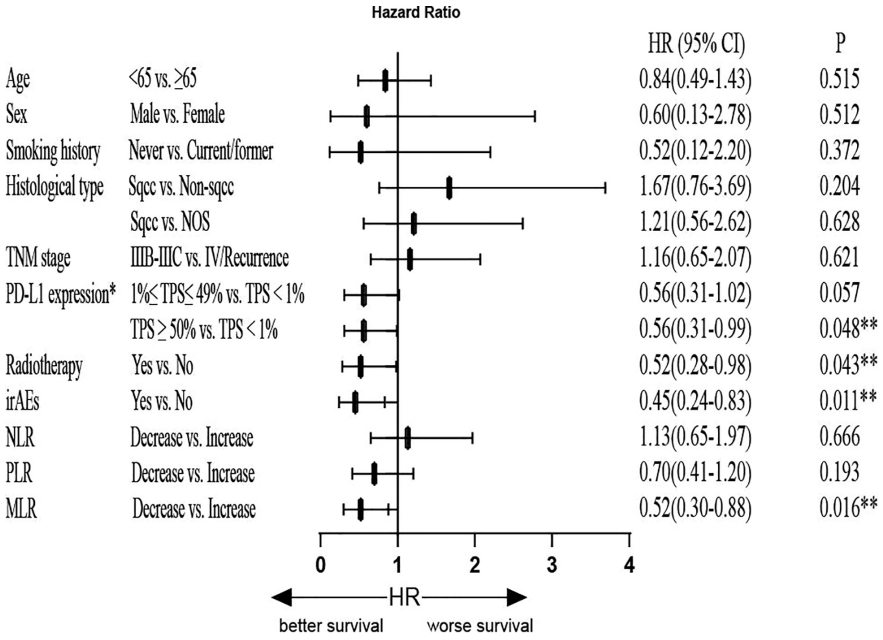

FIGURE 5
Multivariate Cox regression analysis of PFS. *Only for patients with available PD-L1 expression data (patients with unknown PD-L1 expression were excluded). PFS, progression-free survival; HR, hazard ratio; PD-L1: programmed cell death-Ligand 1; TPS, tumor proportion score; irAEs, immune-related adverse events; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; **P<0.05 indicates statistical significance.

changes in MLR might have a predictive value in all PD-L1-expressing populations, while the predictive value of PD-L1 in patients with negative expression is not effective. Therefore, changes in MLR might have a more effective predictive value than PD-L1 expression in patients with TPS<1%. Thus, we could assess the efficacy of PD-1 inhibitors combined with chemotherapy in different populations by observing the changes in MLR and PD-L1 expression, as inflammatory markers are readily available.

Nevertheless, the present study had some limitations. Firstly, this was a retrospective analysis with manual data extraction and entry, which could cause selection bias or introduce confounding factors. Nonetheless, several covariates reflected the disease characteristics and treatment effects that might interfere with the current analysis. Secondly, this study was performed in a single medical center, and only 140 patients were included, thereby limiting the generalizability of the results and necessitating large prospective studies in the future. However, our data maturity was high, and most patients were continuously followed up with progression events. Finally, due to insufficient observation time, we could not collect the mature data of OS, but blood indicators could be monitored dynamically, and subsequent data could be obtained easily.

In conclusion, decreased MLR was associated with improved treatment outcomes in patients with stage IIIB-IV NSCLC treated with first-line PD-1 inhibitor combined with chemotherapy. In addition, changes in MLR might have a predictive value in all the PD-L1-expressing populations. Decreased NLR and PLR also had a trend of enhanced survival, and changes in NLR and PLR might be complementary in predicting prognosis.

Data availability statement

The datasets presented in this article are not readily available because Our data came from unfinished topics. Requests to access the datasets should be directed to zxychest0109@163.com.

Ethics statement

The studies involving human participants were reviewed and approved by Shanghai Chest Hospital (Shanghai, China). The patients/participants provided their written informed consent to participate in this study. 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

LZ and AX: Investigation, Writing - original draft, Writing - review and editing and Formal analysis. SW, JX: Data curation. YS, RZ: Visualization. JL, TC, WZ: Investigation and Resources. YL, XiZ: Project administration. BH, HZ: Supervision. WN: Investigation, Resources, Writing - review and editing and Conceptualization. XuZ: Writing-review and editing, Conceptualization and Formal 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.

The reviewer FZ declared a shared affiliation with the authors to the handling editor at the time of review.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1094378/full#supplementary-material

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Meta-analysis of neoadjuvant immunotherapy for non-metastatic colorectal cancer

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Background: Immunotherapy has been approved for the treatment of metastatic colorectal cancer. The efficacy and safety of neoadjuvant immunotherapy for the treatment of non-metastatic colorectal cancer remains unclear. We tried to explore clinical effect of neoadjuvant immunotherapy in the treatment of non-metastatic colorectal cancer.

Methods: We searched the databases (PubMed, Wanfang Embase, Cochrane Library and China National Knowledge Infrastructure databases) to obtain suitable articles up to September 2022. The primary outcomes of pathological complete response (pCRs), major pathological response (MPR), objective response rate (ORR), R0-resection and anus preserving rate were collected and evaluated. Secordary outcomes (pCRs and MPR) of subgroup analysis between deficient mismatch repair/microsatellite instability-high group (dMMR/MSI-H) and proficient mismatch repair/microsatellite stable group (pMMR/MSS) and outcomes for rectal cancer were analyzed for the final results.

Results: We included ten articles and 410 cases of non-metastatic colorectal cancer with neoadjuvant immunotherapy. There were 113 (27.5%) cases with the dMMR/MSI-H status and 167 (40.7%) cases with the pMMR/MSS status. pCRs was found in 167/373 (44.6%) patients (ES: 0.49, 95% CI: 0.36 to 0.62, P<0.01, chi^2 = 65.3, P<0.01, I^2 = 86.2%) and MPR was found in 194/304 (63.8%) patients (ES: 0.66, 95% CI: 0.54 to 0.78, P<0.01, chi^2 = 42.55, P<0.01, I^2 = 81.2%) with the random-effects model and huge heterogeneity. In the subgroup analysis, pCRs was higher in the dMMR/MSI-H group than the pMMR/MSS group in the fixed-effects model with minimal heterogeneity (OR: 3.55, 95% CI: 1.74 to 7.27, P<0.01, chi^2 = 1.86, P=0.6, I^2 = 0%). pCRs was found in 58/172 (33.9%) rectal cancer patients (ES: 0.33, 95% CI: 0.26 to 0.40, P<0.01, chi^2 = 3.04, P=0.55, I^2 = 0%) with the fixed-effects model and little heterogeneity.

Conclusion: Neoadjuvant immunotherapy could increase pCRs and MPR rate for non-metastatic colorectal cancer. Neoadjuvant immunotherapy could achieve better pCRs rate in dMMR/MSI-H group than in the pMMR/MSS group. Neoadjuvant immunotherapy could be another treatment option for non-metastatic colorectal cancer.

Systematic review registration: https://www.crd.york.ac.uk/prospero/#myprospero, identifier CRD42022350523.

KEYWORDS

neoadjuvant immunotherapy, non-metastatic colorectal cancer, meta-analysis, dMMR/MSI-H group, pMMR/MSS group

Background

The incidence of colorectal cancer is high, and it brings a serious threat to human health (1). Neoadjuvant therapy has been widely used in the clinical treatment, and it is one of the important modes of colorectal cancer (2, 3). Neoadjuvant therapy for rectal cancer is currently based on neoadjuvant chemo(radio)therapy, while neoadjuvant therapy for colon cancer and resectable metastatic colorectal cancer is mostly based on chemotherapy drugs (4, 5). Neoadjuvant chemo(radio)therapy for rectal cancer is the classic mode of neoadjuvant therapy (6, 7). ORR (objective response rate) and pCR (pathological complete response) rate of colorectal cancer after neoadjuvant chemotherapy are 40% and 5% respectively, while the pCR rate of colorectal cancer after neoadjuvant chemo(radio) therapy is about 10%-15% (8, 9).

In 2015, the KEYNOTE-016 study (NCT01876511) indicated that dMMR/MSI-H metastatic colorectal cancer could significantly benefit from programmed death ligand-1 (PD-L1) monoclonal antibody immunotherapy (10). But for pMMR/MSS metastatic colorectal cancer, immunotherapy could not achieve similar clinical efficacy with dMMR/MSI-H metastatic colorectal cancer (11). Guidelines have recommended that immunotherapy is suitable for dMMR/MSI-H metastatic colorectal cancer, but there are no relevant guidelines recommending whether neoadjuvant immunotherapy could be used in non-metastatic colorectal cancer.

The original NICHE study cohort reported the final efficacy data at the 2022 ASCO Annual Meeting, it showed that 30% of pMMR/MSS patients and all the dMMR/MSI-H colorectal cancer patients could respond to neoadjuvant nivolumab plus ipilimumab (12). However, the clinical efficacy and scope of neoadjuvant immunotherapy for colorectal cancer remains unclear (13). Therefore, we collected relevant articles of neoadjuvant immunotherapy for non-metastatic colorectal cancer. We tried to explain the clinical effects of neoadjuvant immunotherapy for non-metastatic colorectal cancer and further compared the difference between dMMR/MSI-H group and pMMR/MSS group in the subgroup analysis.

Abbreviations: MINORS, methodological index for non-randomized studies; pCR, pathological complete response; MPR, major pathological response; ORR, objective response rate; RCT, randomized controlled trial; PNCT, prospective comparative nonrandomized trial; RNCT, retrospective nonrandomized controlled trials; cCR, complete clinical response; SBRT, stereotactic body radiation therapy; IMRT, implementation of intensity-modulated radiotherapy NAT, neoadjuvant therapy; CNKI, China National Knowledge Infrastructure; dMMR, deficient mismatch repair; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; pMMR, proficient mismatch repair; MSS, microsatellite stable; CRM, circumferential resection margin; EMVI, extramural venous invasion; CI, confidence interval; OR, Odds ratios; OS, Overall survival; SMD, Standardized mean difference.

Methods

Literature search

The protocol has been registered on the PROSPERO website (CRD42022350523, https://www.crd.york.ac.uk/prospero/#myprospero). The Supplementary Material 1 showed the details. The meta-analysis was performed according to the PRISMA guidelines (Supplementary Material 2).

According to the design and purpose of the article, we conducted the relevant literature search in the Embase, PubMed, Cochrane Library, CNKI (China National Knowledge Infrastructure) and Wanfang databases (up to September 2022). The search terms were "colorectal cancer" and "neoadjuvant immunotherapy".

The PICO model was followed to guide our literature research in the subgroup analysis: population, intervention, comparator and outcomes. The population included non-metastatic colorectal cancer patients. The intervention was dMMR/MSI-H group. The comparator was pMMR/MSS group. The outcomes included pCRs and MPR.

Inclusion and exclusion criteria

The inclusion criteria were as follows: (1) non-metastatic colorectal cancer, (2) single-arm study, cohort study, prospective study, retrospective study and RCTs, (3) the included patients performed neoadjuvant immunotherapy.

The exclusion criteria were as follows: (1) metastatic colorectal cancer; (2) case reports, meeting, letter and other unsuitable types; (3) no neoadjuvant immunotherapy.

Data extraction and quality assessment

Two reviewers (GYZ and FJW) searched the relevant literatures and sorted the useful clinical data independently with the help of the revised version of MINORS (methodological index for non-randomized studies) (14). The revised version of MINORS was used for the quality assessment of observational or non-randomized studies (15). The third reviewer (LZ) resolved the inconsistencies between the above two authors.

The relevant clinical data was shown by the tables. Tables 1–4 showed the baseline data (such as sex, country, age, MMR status, tumor location and so on), the primary outcomes (MPR, pCRs, and so on), secondary outcomes (MPR and pCRs) and outcomes for rectal cancer (MPR, pCRs, and so on). The details of clinical stage and pathlologic stage were shown in the Supplementary Materials 3, 4 respectively. The details of postoperative complications, adverse events of neoadjuvant therapy and neoadjuvant immunotherapy

TABLE 1 Characteristics of the included articles.

Study	Country	Year	Case	Age	Sex (male/female)	ECOG (0-1)	Tumor diameter(cm)	Median distance from anal verge (cm)	CEA≥5 ng/mL
Bando H 2022	Japan	2022	44	60.6	29/15	44	NR	NR	NR
Chalabi M 2022	Netherlands	2020	40	61.9	18/22	40	NR	NR	NR
Hu H 2022	China	2022	34	49	23/11	34	NR	NR	NR
Kothari A 2022	America	2022	9	55.9	5/4	NR	NR	NR	NR
Li YJ 2021	China	2021	24	65	15/9	NR	5.1 (2.1-7.5)	4 (3-7)	NR
Lin Z 2021	China	2021	30	57	17/13	30	5.4 (2.1-10.0)	4.7 (1.9-9.0)	13
Liu ZX 2022	China	2022	94	58	48/46	94	NR	NR	NR
Rahma OE 2021	America	2021	90	NR	60/30	NR	NR	NR	NR
Shamseddine A 2020	Lebanon	2020	13	62.2	9/4	13	NR	10 (3-14)	NR
Zhang X 2022	China	2022	32	44	17/15	32	NR	NR	NR

Study	CRM	EMVI	MMR status			Tumor location			Lynch syndrome
			dMMR/MSI-H	pMMR/MSS	unknown	right colon	transverse colon	left colon	
Bando H 2022	NR	NR	5	39	0	0	0	44	NR
Chalabi M 2022	NR	NR	21	19	0	22	3	15	7
Hu H 2022	NR	NR	19	0	17	15	6	13	5
Kothari A 2022	NR	NR	9	0	0	6	0	3	3
Li YJ 2021	10	10	NR	NR	NR	0	0	24	NR
Lin Z 2021	NR	NR	1	28	1	0	0	30	NR
Liu ZX 2022	NR	NR	26	68	0	9	1	83	NR
Rahma OE 2021	NR	NR	NR	NR	NR	0	0	90	NR
Shamseddine A 2020	NR	NR	0	13	0	0	0	13	NR
Zhang X 2022	NR	NR	32	0	0	11	4	17	NR

dMMR, deficient mismatch repair; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; pMMR, proficient mismatch repair; MSS, microsatellite stable; CRM, circumferential resection margin; EMVI, extramural venous invasion; pCR, pathological complete response; MPR, major pathological response; ORR, objective response rate; cCR, complete clinical response; NR, no record.
The orders of additional information were range, standard deviation, percentage or NR (if not reported).

TABLE 2 primary outcomes.

Study	MPR (%)	pCRs (%)	R0 resection (%)	anus preserving rate (%)	ORR (%)	cCR (%)
Bando H 2022	17 (38.8)	14 (31.8)	NR	NR	NR	NR
Chalabi M 2022	23 (65.7)	14 (40)	NR	NR	NR	NR
Hu H 2022	29 (85.5)	26 (76.4)	34 (100)	NR	NR	NR
Kothari A 2022	9 (100)	8 (88.8)	NR	NR	5 (55.5)	NR
Li YJ 2021	10 (50)	6 (30)	19 (95)	16 (80)	18 (75)	3 (12.5)
Lin Z 2021	18 (66.6)	13 (48.1)	27 (100)	24 (99.0)	NR	NR
Liu ZX 2022	57 (60.6)	39 (41.5)	94 (100)	NR	83 (88.3)	NR
Rahma OE 2021	NR	22 (31.9)	65 (94)	41 (59.4)	NR	11 (13.9)
Shamseddine A 2020	6 (50)	3 (25)	NR	NR	NR	NR
Zhang X 2022	25 (86.2)	22 (75.9)	29 (100)	NR	29 (100)	3 (9.4)
Total	194 (63.8)	167 (44.6)	268 (98.5)	81 (69.8)	135 (88.9)	17 (14.1)

dMMR, deficient mismatch repair; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; pMMR, proficient mismatch repair; MSS, microsatellite stable; CRM, circumferential resection margin;
EMVI, extramural venous invasion; pCR, pathological complete response; MPR, major pathological response;
ORR, objective response rate; cCR, complete clinical response; NR, no record.
The orders of additional information were range, standard deviation, percentage or NR (if not reported).

plan were shown in Supplementary Materials 5–7 respectively. We obtained no further information after we contacted the relevant authors of the included studies.

Statistical analysis

Stata 11.0 and RevMan 5.0 software was used to analyze the dichotomous data, and it was evaluated by relative risks (ORs or RRs) with 95% confidence intervals. Random effects models and fixed effects model were used to analyse the data with huge heterogeneity (I^2≧50%) and for little heterogeneity (I^2<50%) respectively. Publication bias was assessed by the funnel plots.

Results

Study selection

364 relevant studies were obtained after medical database searching. After we remove the duplicate literatures (N=58), not non-metastatic colorectal cancer (N=105), not neoadjuvant

TABLE 3 secondary outcomes.

Study	MPR (%)		pCRs (%)	
	dMMR/MSI-H	pMMR/MSS	dMMR/MSI-H	pMMR/MSS
Bando H 2022	3 (60)	14 (37.8)	3 (60)	11 (29.7)
Chalabi M 2022	19 (95)	4 (27)	12 (60)	2 (13.5)
Hu H 2022	NR	NR	NR	NR
Kothari A 2022	9 (100)	0	8 (88.9)	0
Li YJ 2021	NR	NR	NR	NR
Lin Z 2021	1 (100)	17 (65.4)	1 (100)	12 (46.2)
Liu ZX 2022	17 (65.4)	40 (58.8)	15 (57.7)	24 (35.3)
Rahma OE 2021	NR	NR	NR	NR
Shamseddine A 2020	NR	6 (50)	NR	3 (25)
Zhang X 2022	25 (86.2)	0	22 (75.9)	0

dMMR, deficient mismatch repair; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; pMMR, proficient mismatch repair; MSS, microsatellite stable; CRM, circumferential resection margin; EMVI, extramural venous invasion; pCR, pathological complete response; MPR, major pathological response;
ORR, objective response rate; cCR, complete clinical response; NR, no record.
The orders of additional information were range, standard deviation, percentage or NR (if not reported).

10.3389/fimmu.2023.1044353

TABLE 4 outcomes for rectal cancer.

Study	MPR (%)	pCRs (%)	R0 resection (%)	anus preserving rate (%)	ORR (%)	cCR (%)
Bando H 2022	17 (38.8)	14 (31.8)	NR	NR	NR	NR
Li YJ 2021	10 (50)	6 (30)	19 (95)	16 (80)	18 (75)	3 (12.5)
Lin Z 2021	18 (66.6)	13 (48.1)	27 (100)	24 (99.0)	NR	NR
Rahma OE 2021	NR	22 (31.9)	65 (94)	41 (59.4)	NR	11 (13.9)
Shamseddine A 2020	6 (50)	3 (25)	NR	NR	NR	NR
Total	51 (49.5)	58 (33.9)	111 (96.5)	81 (69.8)	18 (75)	14 (13.6)

dMMR, deficient mismatch repair; MSI-H, microsatellite instability-high; MSI-L, microsatellite instability-low; pMMR, proficient mismatch repair; MSS, microsatellite stable; CRM, circumferential resection margin; EMVI, extramural venous invasion; pCR, pathological complete response; MPR, major pathological response;
ORR, objective response rate; cCR, complete clinical response; NR, no record.
The orders of additional information were range, standard deviation, percentage or NR (if not reported).

immunotherapy (N=54) and other literatures (Figure 1). We finally included ten articles with 410 non-metastatic colorectal cancers. There were 113 (27.5%) cases with the dMMR status and 167 (40.7%) cases with the pMMR status, while 130 (31.7%) cases remain unknown MMR status (16–25). In the subgroup analysis, the patients of Rahma OE 2021 and Li 2021 do not know the MMR status, Kothari A 2022, Shamseddine A 2020 and Zhang X 2022 are all belong to the dMMR status, so the above studies cannot be included in the subgroup analysis. Table 1 and Supplementary Materials 3, 4 showed the baseline data of the included studies. Eight English study and two Chinese studies were included, all the included studies achieved 12 points with high-moderate quality according to MINORS standard, the literature quality scores and the specific informations were in the Supplementary Material 8.

The vast majority of neoadjuvant therapy regimens are neoadjuvant chemoradiotherapy (long-course radiotherapy: 4500 cGy in 25 fractions or short-course radiotherapy: 2500 cGy in 25 fractions with or without CAPOX and FOLFOX) plus pembrolizumab, ipilimumab or nivolumab (200 mg) for local advanced rectal cancer. Avelumab (10 mg/kg), toripalimab (3 mg/kg) and camrelizumab (200 mg) were immunotherapy drugs in Shamseddine A 2020, Hu H 2022 and Lin Z 2021 respectively. The specific informations were in the Supplementary Material 7. Pembrolizumab, ipilimumab or nivolumab (200 mg) are mainly immunotherapy drugs for local advanced rectal cancer. Pembrolizumab and ipilimumab are mainly immunotherapy drugs for colon cancer. The proportion of clinical stage III patients before neoadjuvant immunotherapy was 77.2%, and the proportion of clinical stage III patients after neoadjuvant immunotherapy decreased to 20.7%. The proportion of T3-T4 patients and N1-N2 patients before neoadjuvant immunotherapy was 98.2% and 81.8% respectively, while the proportion of T3-T4 patients and N1-N2 patients after neoadjuvant immunotherapy was 18.3% and 12.6% respectively (Supplementary Materials 3, 4). According to the RECIST criteria, ORR was 88.9% after neoadjuvant immunotherapy. pCRs and cCR was observed in 167(44.6%) and 17(14.1%) patients after neoadjuvant immunotherapy(Table 2). Table 2 showed the information of primary outcomes (pCRs, MPR, ORR, R0 resection

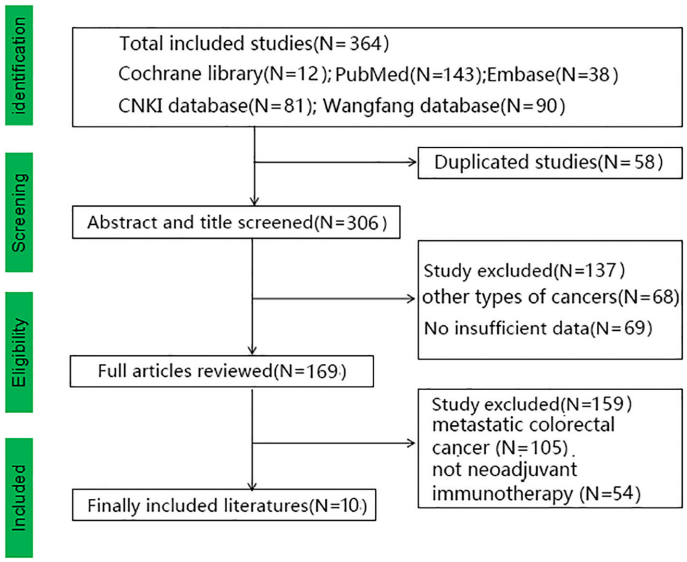

FIGURE 1
Study selection followed by PRISMA diagram.

10.3389/fimmu.2023.1044353

and anus preserving rate). Table 3 showed the information of secondary outcomes (pCRs and MPR) between dMMR/MSI-H group and pMMR/MSS group. Table 4 showed the information of outcomes for rectal cancer.

Primary outcomes: pCRs, MPR, ORR, R0-resection and anus preserving rate

10 studies reported the clinical data of pCRs, pCRs was found in 167/373 (44.6%) patients (ES: 0.49, 95% CI: 0.36 to 0.62, P<0.01, chi^2 = 65.3, P<0.01, I^2 = 86.2%, Figure 2A) with the random-effects model and huge heterogeneity. MPR was reported by 9 studies, MPR was found in 194/304 (63.8%) patients (ES: 0.66, 95% CI: 0.54 to 0.78, P<0.01, chi^2 = 42.55, P<0.01, I^2 = 81.2%, Figure 2B) with the random-effects model and little heterogeneity.

ORR was reported by 4 studies, MPR was found in 135/152 (88.9%) patients (ES: 0.88, 95% CI: 0.82 to 0.93, P<0.01, chi^2 = 3.92, P=0.14, I^2 = 48.9%, Figure 2C) with the fixed-effects model and little heterogeneity. R0-resection rate was found in 6 studies, R0-resection was found in 268/272 (98.5%) patients (ES: 0.98, 95% CI: 0.96 to 0.99, P<0.01, chi^2 = 3.48, P=0.36, I^2 = 0%, Figure 2D) with the fixed-effects model and little heterogeneity. Anus preserving rate was reported by 3 studies, anus preserving rate was found in 81/116 (69.8%) patients (ES: 0.76, 95% CI: 0.57 to 0.95, P<0.01, chi^2 = 12.53, P<0.01, I^2 = 84%, Figure 2E) with the random-effects model and huge heterogeneity.

Secondary outcomes (subgroup analysis): pCRs and MPR (dMMR/MSI-H vs pMMR/MSS group)

4 studies reported the clinical data of pCRs for dMMR/MSI-H and pMMR/MSS group, pCRs was higher in the dMMR/MSI-H group than the pMMR/MSS group in the fixed-effects model with minimal heterogeneity (OR: 3.55, 95% CI: 1.74 to 7.27, P<0.01, chi^2 = 1.86, P=0.6, I^2 = 0%, Figure 3A). 4 studies reported the clinical data of MPR for dMMR/MSI-H and pMMR/MSS group, MPR was similar in the dMMR/MSI-H group and the pMMR/MSS group in the random-effects model with huge heterogeneity (OR: 3.75, 95% CI: 0.73 to 19.26, P=0.11, chi^2 = 8.61, P=0.03, I^2 = 65%, Figure 3B).

Outcome for rectal cancer (pCRs, MPR, R0-resection and Anus preserving rate)

5 studies reported pCRs of rectal cancer, pCRs was found in 58/172 (33.9%) patients (ES: 0.33, 95% CI: 0.26 to 0.40, P<0.01, chi^2 =

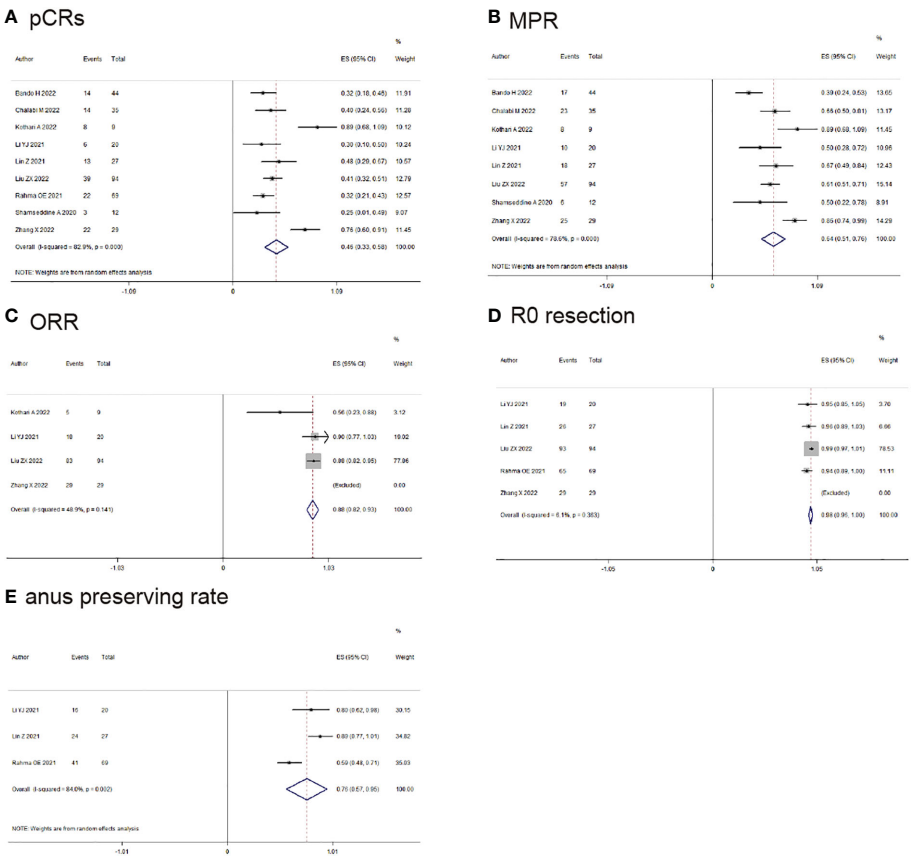

FIGURE 2
Primary outcomes of neoadjuvant immunotherapy for non-metastatic colorectal cancer. **(A)** pathological complete response (pCRs); **(B)** major pathological response(MPR); **(C)** objective response rate (ORR); **(D)** RO-resection; **(E)** anus preserving rate.

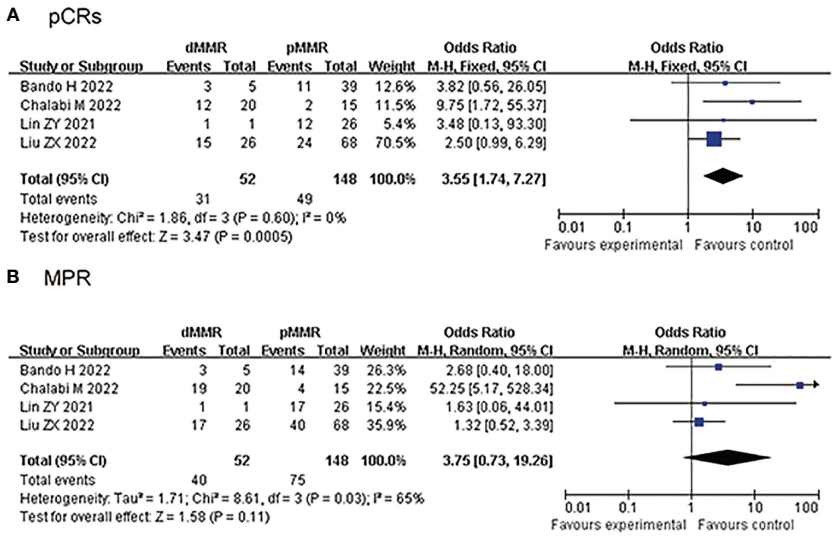

FIGURE 3
Secondary outcomes of neoadjuvant immunotherapy for non-metastatic colorectal cancer. (dMMR/MSI-H vs pMMR/MSS group). **(A)** pathological complete response (pCRs); **(B)** major pathological response (MPR).

3.04, P=0.55, I^2 = 0%, Figure 4A) with the fixed-effects model and little heterogeneity. 4 studies reported MPR of rectal cancer, MPR was found in 51/103 (49.5%) patients (ES: 0.50, 95% CI: 0.40 to 0.59, P<0.01, chi^2 = 5.77, P=0.12, I^2 = 48%, Figure 4B) with the fixed-effects model and little heterogeneity. R0-resection was found in 111/116 (96.5%) patients (ES: 0.95, 95% CI: 0.91 to 0.99, P<0.01, chi^2 = 0.21, P=0.90, I^2 = 0%, Figure 4C) with the random-effects model and huge heterogeneity. Anus preserving rate was found in 81/116 (69.8%) patients (ES: 0.76, 95% CI: 0.57 to 0.95, P<0.01, chi2 = 12.53, P<0.01, I^2 = 84%, Figure 4D) with the random-effects model and huge heterogeneity.

Publication bias

The publication bias was visualized by RevMan 5.0 software with the clinical date of pCRs rate. We found that the points were evenly distributed in the forest plot.

Discussion

Tumor immunity refers to a series of normal physiological processes in which the immune system in the body recognizes and

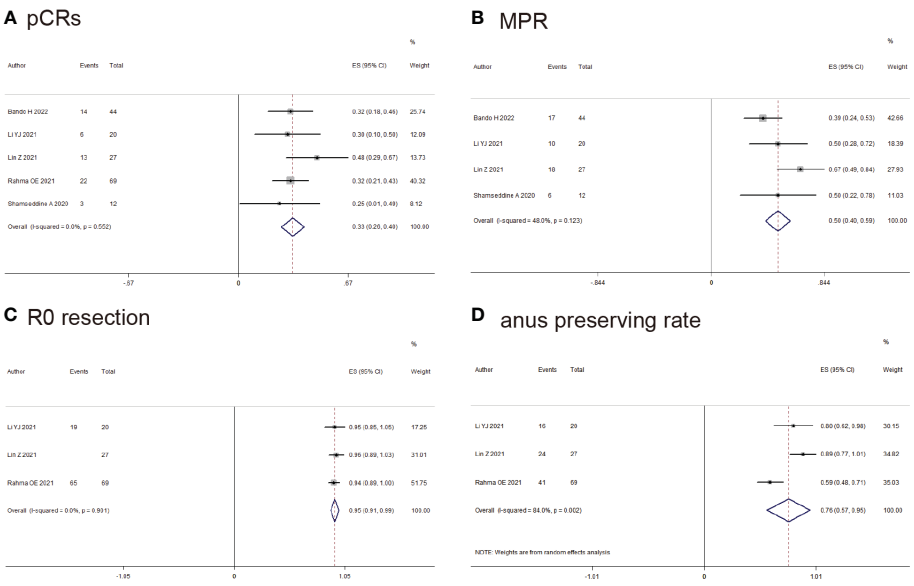

FIGURE 4
Outcomes of neoadjuvant immunotherapy for rectal cancer. **(A)** pathological complete response (pCRs); **(B)** major pathological response (MPR); **(C)** RO-resection; **(D)** Anus preserving rate.

kills tumor cells (26). Tumor cells could express checkpoint inhibitors (PD-L1 molecules) by themselves. When PD-L1 binds to PD-1, it decreased the activation of T cells around the tumor and immune attack of cells in the body, and finally induced the tumor immune escape (27). However, PD-L1 or PD-1 monoclonal antibody could block tumor immune escape and restore the anticancer function of the autoimmune system (28). Tumor immunotherapy has been widely used in clinical treatment, and has achieved good clinical effects in many kinds of cancers (29). KEYNOTE-177 study also confirmed that immunotherapy could improve ORR and survival time in dMMR/MSI-H metastatic colorectal cancer. Some guidelines have pointed out that immunotherapy could be used for metastatic colorectal cancer, especially for dMMR/MSI-H metastatic colorectal cancer (30).

But there are few studies focusing on neoadjuvant immunotherapy for non-metastatic colorectal cancer, and the value of neoadjuvant immunotherapy for non-metastatic colorectal cancer is not fully elucidated (13, 31). We tried to use the available clinical data and explain the clinical effects of neoadjuvant immunotherapy for non-metastatic colorectal cancer.

Novelty of the study

Firstly, the study attempted to evaluate the effect of neoadjuvant immunotherapy for non-metastatic colorectal cancer. There was no similar meta-analysis, only several reviews presented the overview of neoadjuvant immunotherapy for non-metastatic colorectal cancer. Second, we compared the effects of neoadjuvant immunotherapy in the dMMR/MSI-H group and pMMR/MSS group in the subgroup analysis. Third, the meta-analysis explored many other outcomes (pCRs, MPR, ORR, R0-resection and Anus preserving rate) to clarify the advantages and disadvantages of neoadjuvant immunotherapy for non-metastatic colorectal cancer.

Outcome results

pCRs, MPR, ORR, R0-resection and Anus preserving rate

The pCR rate of neoadjuvant chemotherapy for colorectal cancer was about 5%, and the pCR rate of neoadjuvant chemoradiotherapy for rectal cancer was about 10-15% (5, 32). In our study, pCR rate of neoadjuvant immunotherapy was 38.3% for non-metastatic colorectal cancer patients. The ORR rate of neoadjuvant chemotherapy for colorectal cancer was about 40%, while ORR of neoadjuvant immunotherapy was 86.1% in our study. MPR of neoadjuvant immunotherapy was 58% in our study. Compared with the clinical statistics of neoadjuvant chemotherapy and neoadjuvant chemoradiotherapy, ORR and pCR rate of neoadjuvant immunotherapy were significantly improved. In the Rahma OE study, it was further confirmed that neoadjuvant immunotherapy had higher pCR rate and R0 resection rate than neoadjuvant CRT group. Although many meetings and case reports also had affirmed the clinical effect of neoadjuvant immunotherapy, there were little clinical controlled studies for the comparison between neoadjuvant chemoradiotherapy and neoadjuvant immunotherapy (33, 34). Due to the limited data we collected, there may be some bias in the final results. There are many ongoing clinical trials (clinical controlled studies about neoadjuvant chemoradiotherapy and neoadjuvant immunotherapy), we expected to the announcement of the final results, which can provide more recommendations for clinical treatment. R0-resection rate and anus preserving rate were 98.5% and 69.8% respectively, while cCR rate was 14.1%. The results of study indicated that neoadjuvant immunotherapy could improve tumor regression and pathological remission.

dMMR/MSI-H versus pMMR/MSS (pCRs and MPR)

dMMR/MSI-H status was a unique, biomarker-selected type of colorectal cancer and it accounted for approximately 12% to 15% of all colorectal cancer patients. It is more prevalent in the right colon with poorly differentiated or mucinous adenocarcinoma, while only 2% of rectal cancer patients have dMMR/MSI-H status (35). Al-Sukhni et al. reported that the pCR rate of rectal cancer in the pMMR/MSS group and dMMR/MSI-H group were 8.9% and 5.9% after neoadjuvant chemoradiotherapy respectively (36). In our study, pCR and MPR rate in the dMMR/MSI-H group were 63.9% and 80.3% after neoadjuvant immunotherapy, respectively. While pCR and MPR rate in the pMMR/MSS group were 32.7% and 51.2% after neoadjuvant immunotherapy, respectively. Some studies reported that dMMR/MSI-H colorectal cancer patients were mostly insensitive to neoadjuvant chemo(radio)therapy (37). dMMR/MSI-H colorectal cancer has higher TMB (tumor mutation burden), and there are a large number of immune cells in tumor tissue, which is more suitable for immunotherapy (38). Based on above results, we speculated that neoadjuvant immunotherapy could be applied to non-metastatic colorectal cancer patients, especially for dMMR/MSI-H non-metastatic colorectal cancer, and it could improve pCR and MPR rates in the non-metastatic colorectal cancer patients.

dMMR/MSI-H versus pMMR/MSS (immunotherapy alone vs immunotherapy +nC(R)T)

Three articles reported the clinical data of neoadjuvant immunotherapy alone, while six articles reported the clinical data of neoadjuvant immunotherapy combined with neoadjuvant chemo (radio)therapy (Supplementary Material 9). The MPR and pCR rates with the dMMR/MSI-H status were 81.3% and 65.3% in the neoadjuvant immunotherapy alone group, respectively. While the MPR and pCR rate with the pMMR/MSS status were 27% and 13.5% in the neoadjuvant immunotherapy alone group, respectively. The MPR and pCR rates with the dMMR/MSI-H were 86.6% and 80% in the neoadjuvant immunotherapy combined with neoadjuvant chemo (radio)therapy group, respectively. While the MPR and pCR rates with the pMMR/MSS status were 68.1% and 34.9% in the neoadjuvant immunotherapy combined with neoadjuvant chemo(radio)therapy group, respectively. Based on the above results, we speculated that non-metastatic colorectal cancer with the dMMR/MSI-H status is

more likely to benefit from immunotherapy, neoadjuvant immunotherapy combined with neoadjuvant chemo(radio)therapy could achieve better clinical results than neoadjuvant immunotherapy alone. Several ongoing clinical trials are immunotherapy combined with neoadjuvant chemo(radio)therapy. We expected that the results of the ongoing clinical researches could find out the direction of immunotherapy treatment mode and suitable population (39).

Neoadjuvant immunotherapy for rectal and colon cancer

Among the included articles for the neoadjuvant immunotherapy of colorectal cancer, there were several related studies about rectal cancer. We used the available data to perform statistical analysis. The rate of pCRs and MPR were 33.9% and 49.5% respectively, while R0-resection and anus preserving rate were 96.5% and 69.8% respectively (Table 4). In our study, the pCRs rate of neoadjuvant immunotherapy (33.9%) for rectal cancer was higher than the pCR rate (5-15%) of neoadjuvant chemo(radio)therapy. R0-resection, anus preserving rate and other index of neoadjuvant immunotherapy were similar with neoadjuvant chemo(radio)therapy for rectal cancer. Therefore, on the premise of timely controlling the adverse events of neoadjuvant immunotherapy, we speculated that neoadjuvant immunotherapy could improve the pCR rate and pathological response.

However, there are little related studies about colon cancer among the included articles. We can not collect available data for statistical analysis. NICHE study focused on early-stage colon cancer, the rate of pCRs and MPR were 40% and 65.7% respectively. In our study, the pCRs rate of neoadjuvant immunotherapy (40%) for colon cancer was also higher than the pCR rate (5-15%) of neoadjuvant chemo(radio) therapy. However, due to little literatures of colon cancer for neoadjuvant immunotherapy, the results of NICHE study could provide some reference for clinical work. More studies are needed to clarify the clinical effect of neoadjuvant immunotherapy for non-metastatic colorectal cancer.

Neoadjuvant immunotherapy plan and tumor response to neoadjuvant immunotherapy

In the included literatures, the neoadjuvant immunotherapy regimens were mostly single PD-1 monoclonal antibody (ipilimumab, nivolumab or other PD-1 monoclonal antibody, 200mg, 2-6 cycles), while the regimen of the NICHE study was the combination of PD-1 monoclonal antibody (single ipilimumab 1 mg/kg and two nivolumab 3 mg/kg treatments). Neoadjuvant immunotherapy combined with neoadjuvant radiotherapy (SCRT or IMRT, 25-50.4 Gy) was widely used in rectal cancer, while neoadjuvant immunotherapy combined with neoadjuvant chemotherapy (FOLFOX or CAPOX) was widely used in colon cancer (Supplementary Material 7) Neoadjuvant immunotherapy could achieve effective clinical treatment effect for the dMMR/MSI-H non-metastatic colorectal cancer. Neoadjuvant immunotherapy combined with chemo(radio)therapy could be more helpful for for pMMR/MSS non-metastatic colorectal cancer. Based on the above results, the pCR rate, MPR rate, the proportion of III patients, T3-T4 patients and N1-N2 patients were significantly improved after neoadjuvant immunotherapy.

In Liu ZX 2022 et al, 26 patients with dMMR/MSI-H status were treated with neoadjuvant immunotherapy, the pCR rate was 57.7% (15/26) and the MPR rate was 65.4% (17/26). Among the 68 patients with pMMR/MSS status who received immunotherapy combined with neoadjuvant chemo(radio)therapy, the pCR rate was 35.3% (24/68), and the MPR rate was 58.8% (40/68). At present, whether neoadjuvant immunotherapy was suitable for neoadjuvant treatment of colorectal cancer, which neoadjuvant immunotherapy regimens was more suitable for colorectal cancer, whether neoadjuvant immunotherapy requires combined chemo(radio)therapy was still inconclusive, and further research was needed to explore.

Adverse events and postoperative complications

The most common immune-related adverse events of immune checkpoint inhibitors (ICPIs) were skin disease (44%-68%), followed by gastrointestinal reactions (5%-50%), abnormal liver function (incidence) and endocrine disorders (6%) (40). The included studies also made the similar conclusions, while most immune-related adverse events were mild events (I-II grade). The occurrence of adverse events could be related to the overactivation of T lymphocytes. Mild adverse events can be treated symptomatically, while severe adverse events require discontinuation of immunotherapy time, hormone replacement therapy and other treatment options (41). Therefore, the options of the suitable immunotherapy drug, dosage and administration time can effectively avoid the occurrence of adverse events. The adverse reactions of neoadjuvant chemo(radio)therapy are mainly leukopenia, elevated transaminases, gastrointertinal disorders. The adverse reactions of neoadjuvant chemo(radio)therapy are mostly mild reactions, and many patients could successfully complete neoadjuvant chemo (radio)therapy.

The incidence of postoperative complications was 19.3%, and most of the postoperative complications were grade I-II complications. Infection, intestinal obstruction and anastomotic stenosis were the main postoperative complications. All the patients with postoperative complications were discharged smoothly with conservative symptomatic treatment. Lupattelli et al. published a multicenter retrospective study in which 76 patients with locally advanced rectal cancer were given 45 Gy (25 times) in the pelvis, and the local tumor dose was increased to 52.5 to 57.5 Gy (25 times), and the recent results showed that the pCR rate was 27.8%, the incidence of grade 3-4 adverse reactions was 10.5%, and the incidence of surgical complications was 18.1% (42). Based on the above results, we speculated that neoadjuvant immunotherapy with or without neoadjuvant chemo(radio)therapy did not significantly increase postoperative complications, but many studies were still needed to confirm.

Limitations

The meta-analysis had several limitations. Firstly, neoadjuvant immunotherapy regimens were inconsistent among the included

articles, which could affect the results. Whether it should be combined with neoadjuvant chemo(radio)therapy and neoadjuvant chemo(radio) therapy regimens were inconsistent, which was also one of the limitations. Secondly, few related articles on neoadjuvant immunotherapy for colorectal cancer (most of which are single-arm studies with no control group) could be one of the limitations. Thirdly, limited patients and clinical data of neoadjuvant immunotherapy for non-metastatic colorectal cancer could affect the results.

Conclusion

By using the collected clinical data, we speculated that neoadjuvant immunotherapy could increase MPR and pCR rate, especially for dMMR/MSI-H status. Neoadjuvant immunotherapy combined with chemo(radio)therapy could enhance the therapeutic effect (MPR and pCR rate). Compared with previous clinical data of neoadjuvant chemo(radio)therapy, neoadjuvant immunotherapy did not increase the incidence of postoperative complications and adverse events. We expected the precise neoadjuvant immunotherapy regimens with or without chemo(radio)therapy would appear, which could reduce postoperative complications and adverse events, increase MPR, pCR rate and other outcomes. At the same time, we also looked forward to the emergence of more RCTs that can confirm the clinical effects of neoadjuvant immunotherapy for non-metastatic colorectal cancer. Neoadjuvant immunotherapy could be another treatment option for non-metastatic colorectal cancer treatment.

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

All authors participated in the study. LZ, G-YZ and F-JW performed the literature search and the acquisition of data. LZ and F-JW performed data analysis. X-QY and XL participated in the interpretation of data and paper writing. LZ and XL were responsible for the editing, revision and submission of the paper. All authors contributed to the article and approved the submitted version.

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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/fimmu.2023.1044353/full#supplementary-material

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