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Novelties in acute myeloid leukemia: from biology to clinical applications

Edited by
Nico Gagelmann, Carmelo Gurnari, Luca Vincenzo Cappelli and Hassan Awada

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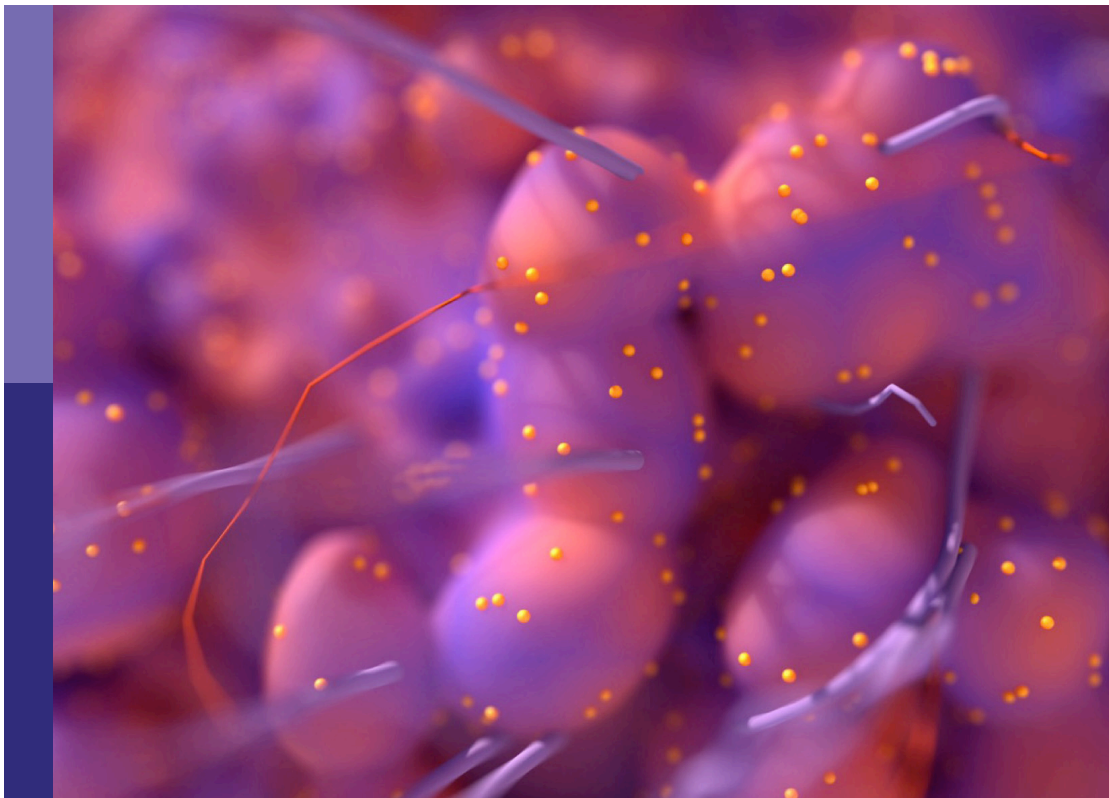

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Novelties in acute myeloid leukemia: from biology to clinical applications

Topic editors

Nico Gagelmann — University Medical Center Hamburg-Eppendorf, Germany
Carmelo Gurnari — Cleveland Clinic, United States
Luca Vincenzo Cappelli — Sapienza University of Rome, Italy
Hassan Awada — University at Buffalo, United States

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EDITED AND REVIEWED BY
Alessandro Isidori,
AORMN Hospital, Italy

*CORRESPONDENCE
Luca Vincenzo Cappelli
cappelli@bce.uniroma1.it
Carmelo Gurnari
carmelogurnari31@gmail.com

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Editorial: Novelties in acute myeloid leukemia: from biology to clinical applications

Luca Vincenzo Cappelli[1]*, Nico Gagelmann[2], Hassan Awada[3] and Carmelo Gurnari[4,5]*

[1]Department of Translational and Precision Medicine, Sapienza University of Rome, Rome, Italy, [2]Department of Stem Cell Transplantation, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [3]Roswell Park Comprehensive Cancer Center, Buffalo, NY, United States, [4]Department of Biomedicine and Prevention, University of Rome Tor Vergata, Rome, Italy, [5]Department of Translational Hematology and Oncology Research, Cleveland Clinic, Taussig Cancer Institute, Cleveland, OH, United States

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Editorial on the Research Topic
Novelties in acute myeloid leukemia: from biology to clinical applications

In the last decade, tremendous advances have revolutionized the biological landscape of acute myeloid leukemia (AML). The application of sophisticated molecular biology tools has unveiled a plethora of (epi)genomic and transcriptomic alterations, some of which have been recently recognized in pre-leukemic conditions (e.g. clonal hematopoiesis of indeterminate potential - CHIP (1)) with implications on AML ontogenesis and prognosis, paving the way for targeted approaches. The most recent diagnostic and prognostic schemes have included these latest advances (2), further improving the clinical management of AML patients.

The present Research Topic has provided a glimpse on some of these aspects, including: 1) new insights on AML biology, with the identification of novel transcriptomic signatures affecting prognosis, mechanistic description of pathogenic mutations (i.e. RAS) and oncogenic fusions (SET-CAN/NUP214) and the recent discoveries on leukemia stem cells (LSCs) at single-cell resolution; 2) new therapeutic perspectives in either induction (lower-dose chemotherapy + venetoclax or alternative chemotherapies for mixed phenotype acute leukemia – MPAL) or relapsed/refractory (R/R) AML (venetoclax + hypomethylating agents – HMA, targeted agent Selinexor, CAR-T cells). As a result, a balanced selection of articles spanning from AML biology and novel prognostic indicators to innovative therapeutic strategies has been selected for the readers.

Regarding AML biology, Villar et al. analyzed the transcriptome of 224 AML patients > 65 years-old at diagnosis treated in the Spanish PETHEMA-FLUGAZA clinical trial in order to identify new prognostic biomarkers in this population. They identified a specific transcriptomic signature for high-risk patients, revealing that low expression of *B7H3* gene

with high expression of *BANP* gene identifies a subset with a more favorable prognosis surviving more than 12 months. This result was further validated in the BEAT AML cohort.

Zhang et al. focused on autophagy, since beside being known as a natural cellular response to a wide spectrum of cellular processes, it is debated whether it might have a pathogenic role in leukemias and particularly in AML. The authors found >100 differentially expressed genes (DEGs) related to autophagy between AML and healthy controls. Next, they selected 12 of these genes and developed a prognostic model predictive for survival of AML patients in both TCGA data and independent AML cohorts from GEO databases.

Wan et al. in a first-time approach based on the differential expression of lysosome-related genes in AML identified two different subtypes: cluster1 showed longer overall survival (OS) and stronger immune infiltration compared to cluster2. The pivotal differential genes between the two clusters are *SYK*, whose pro-carcinogenic mechanism relies in the promotion of AML cell survival and drug resistance (3), and *TLR4* which is thought to modulate lysosomal function and is in turn degraded by lysosome themselves (4). Finally, a prognostic model consisting of six genes identified patients in a low-risk group who survived significantly longer than those in the high-risk group and had higher immune infiltration and stronger response to immunotherapy.

Further, Song et al. unravel the role of SET-CAN/NUP214 fusion in leukemia. This aberration mainly occurs in T-cell acute lymphoblastic leukemia (T-ALL) patients, but it has also been reported in other leukemias including AML, MPAL and B-ALL. Leukemias bearing this fusion often share common immunophenotypic markers such as: CD7, cCD3, CD34, CD33 and CD13. This supports a model where the transformation of SET-CAN/NUP214+ leukemia may occur in the early stage of myeloid or lymphocyte differentiation, and it may be related to the inhibition of differentiation of primitive progenitor cells by the fusion gene. Patients with SET-CAN/NUP214 fusion usually exhibit resistance to chemotherapy, including glucocorticoids in the early stages of induction therapy, however the overall CR rate is not affected.

In another original paper, Liang et al. focus on the pathogenic role of RAS mutations in AML, and their impact on cell metabolism. In fact, RAS gene mutations are prevalent in AML, and the RAS signaling pathway is closely related to many metabolic pathways. By using a Ba/F3 cell line model transduced with $NRAS^{Q61K}$ and $KRAS^{G12V}$ mutations, the authors conducted a DEG analysis between mutant and wild-type cell lines. They found 1899 DEGs, of which 1089 were related to metabolic pathways, particularly the *DGKzeta* and *PLA2G4A* genes in the glycerophospholipid metabolism pathway were significantly upregulated. These findings may contribute to new precision therapy strategies and the development of new therapeutic drugs for AML.

In a comprehensive review, Zhou et al. provide a detailed summary of single-cell sequencing strategies in AML. These techniques have revolutionized our understanding of AML pathogenesis by enabling high-resolution interrogation of the cellular heterogeneity in the AML ecosystem. The authors focus on the identification of different leukemia stem cells (LSCs), T-cell subpopulations displaying exhausted phenotypes permissive towards AML, and pinpoint novel actionable liabilities. Such targets include: LGALS1 (promoter of resistance to therapy), CD52 and CD47 (expressed on the quiescent LSCs), CSF1R and CD86 (highly expressed on LSCs). These latter two have been proved to be effective CAR-T targets in preclinical evaluation (5).

On the clinical side, this Research Topic provides both Original and Review articles highlighting different therapeutic strategies in AML. Several authors introduce novel combinations on real-life patients cohorts. Zhang et al. present a different induction strategy involving 3 days of cyclophosphamide and cytarabine plus low dose venetoclax in 25 newly diagnosed AML patients, achieving a 92% CR/Cri rate (all MRD-) and 79% overall survival at 12 months.

The Polish group of Karasek et al. elaborate on a first-line induction strategy using CLAG-M combination for mixed phenotype acute leukemia (MPAL) patients, an interesting approach for a disease still judged "orphan" due to its lineage ambiguity and elusive biology. The authors report an ORR of 73%, however responses need to be consolidated with allogeneic transplant to avoid relapses.

In the context of R/R AML, Chen et al. compared the outcome of AML patients relapsing after allogeneic transplant and treated with venetoclax + azacytidine (VEN+HMA) vs those undergoing intensive chemotherapy. ORR rates were not different between the two arms (60% vs 64%), leading to a median OS of 6.8 months for both arms. However, toxicity profile of VEN+HMA was more favorable, with fewer infections (17% vs 50%), thrombocytopenia (74% vs 95%) and acute graft-versus-host disease.

A Case Report by Sperotto et al. highlights the efficacy of CPX-351 (liposomal cytarabine and daunorubicin) as a salvage chemotherapy in a patient who developed a secondary AML (t-AML) 15 years after treatment for acute promyelocytic leukemia (all-trans-retinoic acid and chemotherapy). The patient achieved a complete remission, underwent an allogeneic transplant and was alive after 2 years of follow-up.

Despite these and other therapeutic interventions, several AML patients still fail and/or relapse after first-line chemotherapy; these are the ones experiencing the worst outcome. Identifying novel treatment options for this subgroup of patients is an unmet clinical need. Exportin-1 (XPO-1) is usually overexpressed in various tumors, including relapsed AML. Selinexor, an inhibitor of XPO1, effectively promotes nuclear retention and functional activation of tumor suppressor proteins, thereby inducing apoptosis in cancer cells. In their original Report, Zhang et al. describe a novel combination of Selinexor plus decitabine and half-dose CAG chemotherapy in an elderly patient with R/R AML, leading to complete remission and good tolerance.

Lastly, among the novel promising treatments, CAR-T cells are still under evaluation in AML but they might eventually take their

primal role as in other onco-hematological diseases. A Review on the role of CAR-T cells by Wei et al. in AML and T-ALL provides a great summary of the therapeutic efficacy of adoptive cell strategies in these two entities. CAR-T cells targeting CD5 and CD7 for T-ALL and CD123, CD33, and CLL1 for AML show promising efficacy and safety profiles in clinical trials.

Author contributions

LC: Conceptualization, Project administration, Writing – original draft, Writing – review & editing. NG: Writing – original draft, Writing – review & editing. HA: Writing – original draft, Writing – review & editing. CG: Project administration, Writing – original draft, Writing – review & editing, Conceptualization.

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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EDITED BY
Alessandro Isidori,
AORMN Hospital, Italy

REVIEWED BY
Giovanni Marconi,
Scientific Institute of Romagna for the Study and Treatment of Tumors (IRCCS), Italy
Depei Wu,
The First Affiliated Hospital of Soochow University, China

*CORRESPONDENCE
Felipe Prósper
fprosper@unav.es
Pau Montesinos
montesinos_pau@gva.es

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share senior authorship

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The transcriptomic landscape of elderly acute myeloid leukemia identifies *B7H3* and *BANP* as a favorable signature in high-risk patients

Sara Villar[1,2†], Beñat Ariceta[2,3,4†], Xabier Agirre[2,4], Aura Daniela Urribarri[5], Rosa Ayala[6], David Martínez-Cuadrón[7], Juan Miguel Bergua[8], Susana Vives[9], Lorenzo Algarra[10], Mar Tormo[11], Pilar Martínez[6], Josefina Serrano[12], Catia Simoes[4], Pilar Herrera[13], Maria José Calasanz[2,3], Ana Alfonso-Piérola[1,2], Bruno Paiva[1,2,3], Joaquín Martínez-López[6], Jesús F. San Miguel[1,2], Felipe Prósper[1,2*‡] and Pau Montesinos[7*‡]

[1]Servicio de Hematología y Terapia Celular, Clínica Universidad de Navarra, Instituto de Investigación Sanitaria de Navarra (IDISNA), Pamplona, Spain, [2]CIBERONC Centro de Investigación Biomédica en Red de Cáncer, Pamplona, Spain, [3]Centro de Investigación Médica Aplicada (CIMA) LAB Diagnostics, Universidad de Navarra, Pamplona, Spain, [4]Program of Hematology-Oncology, CIMA, Universidad de Navarra, Pamplona, Spain, [5]Navarrabiomed, Fundación Miguel Servet, Pamplona, Spain, [6]Hospital Universitario 12 de octubre, Madrid, Spain, [7]Hospital Universitario y Politécnico la Fe, Valencia, Spain, [8]Hospital San Pedro de Alcántara, Cáceres, Spain, [9]ICO Badalona-Hospital Germans Trias i Pujol, Badalona, Spain, [10]Hospital General de Albacete, Albacete, Spain, [11]Hospital Clínico Universitario de Valencia, Valencia, Spain, [12]Hospital Universitario Reina Sofía, Córdoba, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain, [13]Hospital Universitario Ramón y Cajal, Madrid, Spain

Acute myeloid leukemia (AML) in the elderly remains a clinical challenge, with a five-year overall survival rate below 10%. The current ELN 2017 genetic risk classification considers cytogenetic and mutational characteristics to stratify fit AML patients into different prognostic groups. However, this classification is not validated for elderly patients treated with a non-intensive approach, and its performance may be suboptimal in this context. Indeed, the transcriptomic landscape of AML in the elderly has been less explored and it might help stratify this group of patients. In the current study, we analyzed the transcriptome of 224 AML patients > 65 years-old at diagnosis treated in the Spanish PETHEMA-FLUGAZA clinical trial in order to identify new prognostic biomarkers in this population. We identified a specific transcriptomic signature for high-risk patients with mutated *TP53* or complex karyotype, revealing that low expression of *B7H3* gene with high expression of *BANP* gene identifies a subset of high-risk AML patients surviving more than 12 months. This result was further validated in the BEAT AML cohort. This unique signature highlights

the potential of transcriptomics to identify prognostic biomarkers in in elderly AML.

KEYWORDS

acute myeloid leukemia, elderly, transcriptomics, biomarkers, prognosis

Introduction

Acute myeloid leukemia (AML) remains a disease of the elderly, with the median age at diagnosis of 70 years old. While young and fit patients with AML may receive an intensive approach with chemotherapy and hematopoietic stem cell transplantation (HSCT) (1, 2) as consolidation, older or frail patients do not benefit from this strategy and receive less intensive and unfrequently curative approaches (3–5).

Current risk stratification in AML patients is based mainly on cytogenetics and the presence of common genetic aberrations (*NPM1*, *FLT3-ITD*, *CEBPA*, *RUNX1*, *ASXL1*, and *TP53* mutations) best exemplified in the ELN risk classification system (1). This classification has been validated for young and older but fit patients treated with intensive chemotherapy (1, 6) and defines 3 prognostic groups based exclusively on genetic data. In this context core binding factor (CBF) leukemias, *NPM1* and biallelic *CEPBA* mutations are considered of good prognosis, while complex or monosomal karyotype, *TP53*, *FLT3-ITD*, *RUNX1* and *ASXL1* mutations, other recurrent translocations and *KMT2A* rearrangements confer a poor prognosis. However, this classification is not validated for elderly patients treated with a non-intensive approach, and its performance seems to be suboptimal in this context (7).

In addition to molecular and clinical characteristics, alternative biomarker panels such as other somatic mutations and gene expression profiling have been proposed to refine risk classification in AML patients (8–12), providing models with a prognostic value. Clinical implementation of an improved AML risk classification model has the potential to aid in clinical decision-making including the indication of HSCT for patients with intermediate and adverse risk. However, the outcome of patients with specific cytogenetic and molecular abnormalities such as *TP53* mutations or complex karyotype is still disappointing, especially when both characteristics are present in the same patient, with virtually all patients relapsing soon after initial treatment (1, 13).

AML in the elderly remains a clinical challenge. On the one hand, comorbidities and general performance status are important factors limiting an intensive therapeutic approach, thus a careful multi-domain assessment should be ideally considered when deciding the best treatment option for an old patient with AML (14–16). On the other hand, the proportion of adverse genetic abnormalities such as high risk cytogenetics and *TP53* mutations is higher in the elderly (17, 18). Considering these clinical features, the prognosis of AML in elderly patients remains dismal, with a five-year overall survival rate below 10% (19, 20).

In the current study, we analyzed the transcriptome of 224 newly diagnosed elderly AML patients treated in the Spanish PETHEMA-FLUGAZA clinical trial, with the aim to define new prognostic groups in this population. The detailed results of treatment schedules, clinical outcomes with minimal residual disease (MRD) data, and genomic landscape of PETHEMA-FLUGAZA patients have been previously published (21–23).

Methods

Study design

The multicentric PETHEMA-FLUGAZA phase 3 clinical trial (NCT02319135) included a total of 283 elderly patients (> 65-year-old) diagnosed with *de novo* or secondary AML, who were randomized to receive FLUGA (n=141), consisting of 3 induction cycles with fludarabine and cytarabine followed by 6 consolidation cycles of reduced intensity FLUGA (riFLUGA), or AZA (n=142), 3 induction cycles with 5-azacitidine followed by 6 identical consolidation cycles (Figure S1A**).** Patients diagnosed with acute promyelocytic leukemia and ECOG ≥ 4 were excluded from the trial.

Clinical data was collected in a standardized form, from a total of 26 Spanish centers that participated in the PETHEMA-FLUGAZA trial. Cytogenetic analysis was locally performed. Regarding molecular landscape, *NPM1*, *FLT3-ITD* and *CEPBA* mutation assessment was locally performed when possible. However, wide mutational data was retrospectively analyzed in a central laboratory with a myeloid NGS platform (Hospital Universitario 12 de Octubre, Madrid) (23) (Figure 1A**).** This clinical trial was conducted in accordance with the Declaration of Helsinki. Written informed consent was provided by all patients.

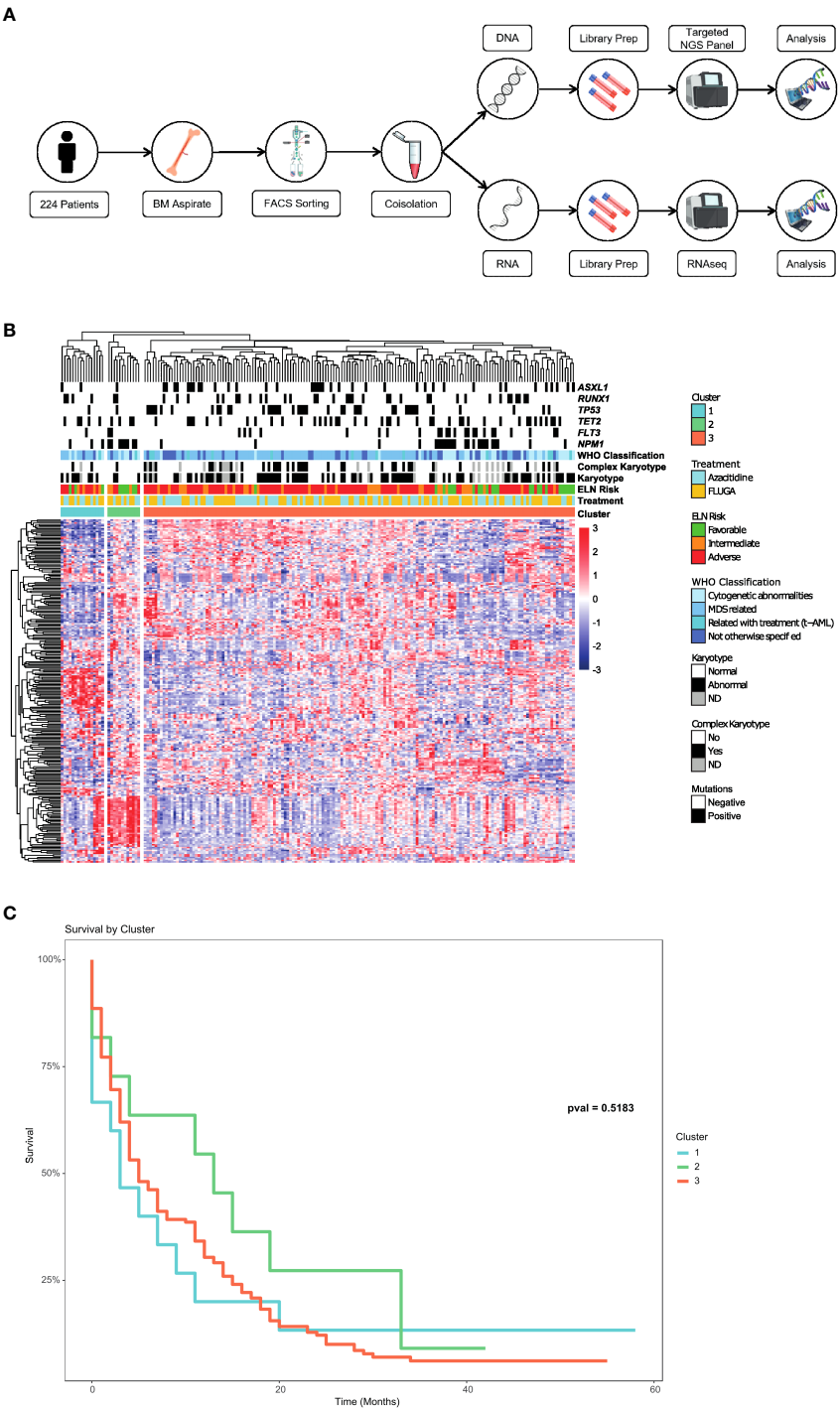

FIGURE 1
Identification of a long survival group beyond 12 months in elderly AML patients. **(A)** Scheme of the process carried out to obtain the RNAseq and mutation data through a myeloid NGS panel in AML samples of the PETHEMA-FLUGAZA clinical trial. **(B)** Unsupervised hierarchical clustering of the entire AML patient cohort using full transcriptional profiling, identifying 3 different groups. **(C)** Overall survival analysis of the 3 different AML groups based on the transcriptional profile.

Sample preparation and RNA sequencing

Bone marrow (BM) samples at diagnosis were characterized by multidimensional flow cytometry (MFC) in a central laboratory (CIMA, Centre for Applied Medical Research), and leukemic cells were purified by FACS. Co-Isolation protocol was performed to obtain DNA and RNA. Poly-A RNA was captured for further RNAseq protocol, while DNA was obtained from poly-A capture supernatant using SPRIselect beads (Figure 1A**).**

RNAseq was performed following MARS-seq protocol adapted for bulk RNAseq (24, 25) with minor modifications. Poly-A RNA was reverse-transcribed using poly-dT oligos carrying a 7 nt-index. Pooled samples were subjected to linear amplification by IVT. Resulting aRNA was fragmented and dephosphorylated. Ligation of partial Illumina adaptor sequences (24) was followed by a second reverse-transcription reaction. Full Illumina adaptor sequences were added during final library amplification. RNAseq libraries quantification was done with Qubit 3.0 Fluorometer (Life Technologies), and size profiles were examined using Agilent's 4200 TapeStation System. Libraries were sequenced in an Illumina NextSeq 500 at a sequence depth of 10 million reads per sample. Raw reads were demultiplexed according to manufacturer's instructions using bcl2fastq2 (v.2.20.0). Sequencing reads were aligned using STAR alignment tool (26) against hg38 reference genome. Counts were obtained using featureCounts from Rsubread R package, using ENSEMBL gene annotation (version 92). We used R (version 4.0.0) to perform hierarchical clustering and survival analysis. Results were visualized using R (Figure 1A).

Statistical analysis

Overall survival (OS) was defined as the time since enrollment until death from any cause. Univariate and multivariate Cox regression analyses were calculated using R (version 4.0.0; The R Foundation, Vienna, Austria). Continuous variables are presented as means and standard deviations or as medians with ranges. Categorical variables are represented by frequencies and percentages. For all analyses, the P values were 2 tailed, and $P < 0.05$ was considered statistically significant. For the multivariate analysis, median values of Counts per Million (CPMs) were calculated for each gene, and then factorized as Low (*Gene* Low) or High (*Gene* High) according to the median value.

Results

Baseline characteristics of patients included in the study are summarized in Supplementary Table 1. RNA sequencing (RNAseq) was performed on FACS sorted purified BM blasts obtained at diagnosis in 224 AML patients out of the 283 patients enrolled in the PETHEMA-FLUGAZA clinical trial (21) (112 of each arm) (Figure 1A**).** A total of 59 patients were excluded because of sample unavailability at diagnosis, assay failure, and/or bad sample quality. The median age at diagnosis was 75 years old. Median OS of the 224 patients was 5 months. Detailed treatment design, mutational landscape, ELN distribution and overall survival of patients are shown in Figures S1A–D.

Unsupervised hierarchical clustering identified 3 different groups based on the transcriptional profile (Figure 1B). There was no association between these transcriptional profiles and mutations in AML related genes, cytogenetics or ELN genetic risk categories such as the presence of *NPM1*, *FLT3-ITD*, *TP53*, *RUNX1* or *ASXL1* mutations. Survival analysis of the 3 transcriptomic groups did not show any differences, even though a trend to a better OS was identified for group 2 (Figure 1C**).**

Despite the dismal OS of this cohort, a group of elderly AML patients surviving beyond 12 months was identified (n=76). These long-term survivors were not characterized by a distinctive mutational or cytogenetic profile, and therefore we examined if there was a specific transcriptional signature associated with this group of patients. A differential expression analysis between patients surviving more or less than 12 months did not show any specific transcriptional signature either in the whole group or according to the treatment arm (Figure S2).

We next analyzed the transcriptomic profiles for these long-term survivors according to the different genetic groups such as *FLT3-ITD*, *NPM1*, *TP53*, *RUNX1*, *TET2*, *IDH1/2* mutations and complex karyotype. Patients with mutations in *NPM1*, *RUNX1, IDH1/2* or *TET2* did not show a transcriptional profile associated with long-term survivors (Figure S3). However, a specific transcriptional profile was identified in long-term survivors with complex karyotype, *TP53* or *FLT3-ITD* mutations (Figure 2). When we focused at the differentially expressed genes between long-term survivors in the *FLT3-ITD*, *TP53* mutated and complex karyotype groups, we found that *TP53* mutated and complex karyotype groups showed most of the differentially expressed genes, 77 and 1099 respectively (Figure 3A). In this context, we focused our analysis in *TP53* mutated and complex karyotype patients. We found 56 differentially expressed genes shared in both groups of patients, out of which 15 genes (*CPXM1, CLDN15, B7H3, RN7SL2, BANP, ATP2A1, ZNF182, NID1, BDH1, TREM1, CAV2, BAALC-AS2, CATSPERD, PIP4K2B*, and *PASK*) were significantly associated with overall survival of AML patients on the univariate analysis (Figure 3B). Enrichment analysis was performed with those 15 genes in order to find altered pathways (Figure 3C and Supplementary Table 2).

On the multivariate analysis including other adverse category mutations such as *RUNX1*, *ASXL1*, and *FLT3-ITDmt*

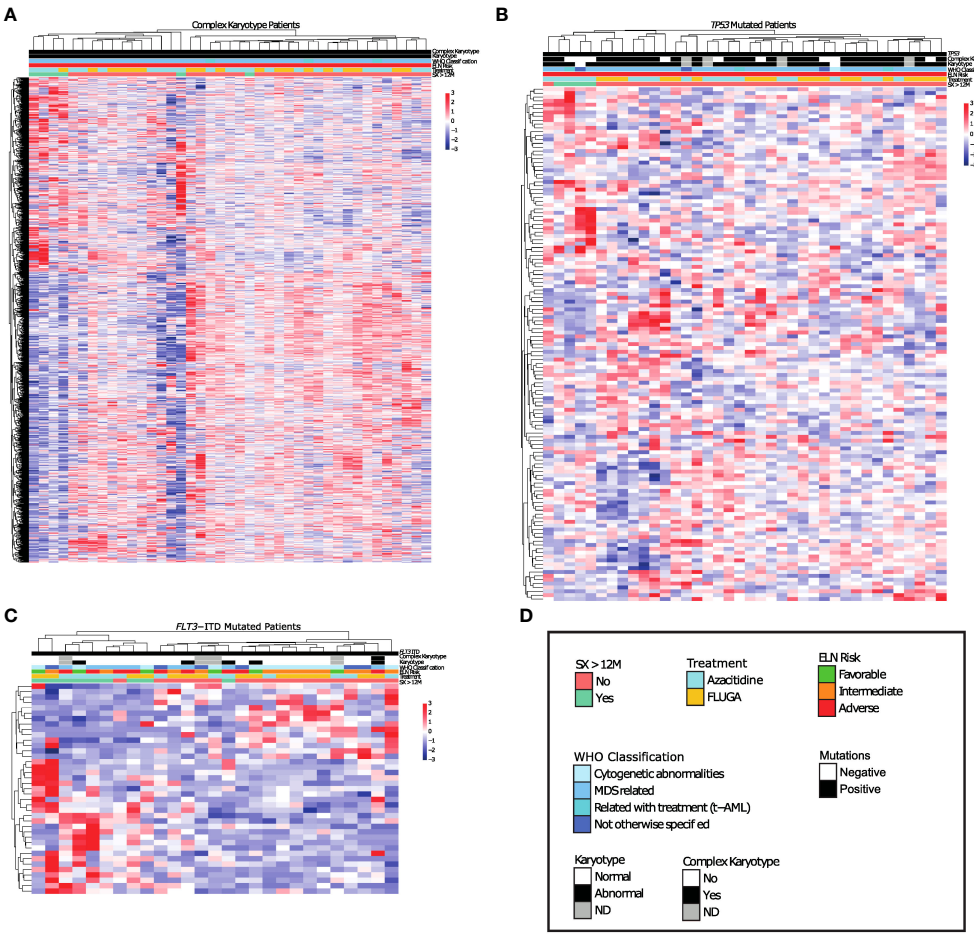

FIGURE 2
Long survival AML patients with complex Karyotype or *TP53* mutations show differential transcriptomic profile respect to non-long survival AML patients with these same genetic alterations. **(A-C)** Differential expression analysis between long survival AML patients and non-long survival AML patients with **(A)** Complex Karyotype, **(B)** *TP53* mutations. **(C)** FLT3-ITD mutations. **(D)** Legend.

with *NPM1wt*, as well as treatment arm, only the expression of *B7H3* and *BANP* was significantly associated with OS (Figure 3D). In fact, the expression of these 2 genes stratified patients with mutated *TP53* or complex karyotype into 3 groups with a different survival: patients with low expression of *B7H3* (CPM expression < 1.56 CPM) and high expression of *BANP* (CPM expression > 4.14 CPM) (*B7H3lo/BANPhi*) translated into a significantly better survival, whereas the opposite signature displayed a very short overall survival (*B7H3hi/BANPlo*) (MOS 1 month vs 14 months, $p < 0.001$). Patients with concordant expression profile (*B7H3lo/BANPlo*), (*B7H3hi/BANPhi*), presented with an intermediate prognosis (median OS 3.6 and 3.4 months respectively) (Figure 4A). Baseline characteristics of the three prognosis groups are summarized in Supplementary Table 3. Even though treatment arm did not have an impact in the multivariate analysis, (Figure 3D) we decided to confirm the prognostic value of our signature by taking each treatment arm separately, (Figure S4), confirming the same prognostic stratification. Finally, the prognostic value of the expression of *B7H3* and *BANP* was validated using the BeatAML independent cohort of AML patients (27) (Figure 4B), which includes also elderly patients intensively treated. Thus, low expression of *B7H3* (CPM expression, < 1.67 CPM) and high expression of *BANP* (CPM expression > 3.96 CPM) seem to identify a subset of patients with better outcome in the classical high-risk group of *TP53* mutated or complex karyotype elderly patients, including old AML patients treated with intensive chemotherapy.

Discussion

AML in the elderly remains a clinical challenge. Currently in the clinical setting, the WHO and ELN risk stratification guidelines combine cytogenetic abnormalities and genetic

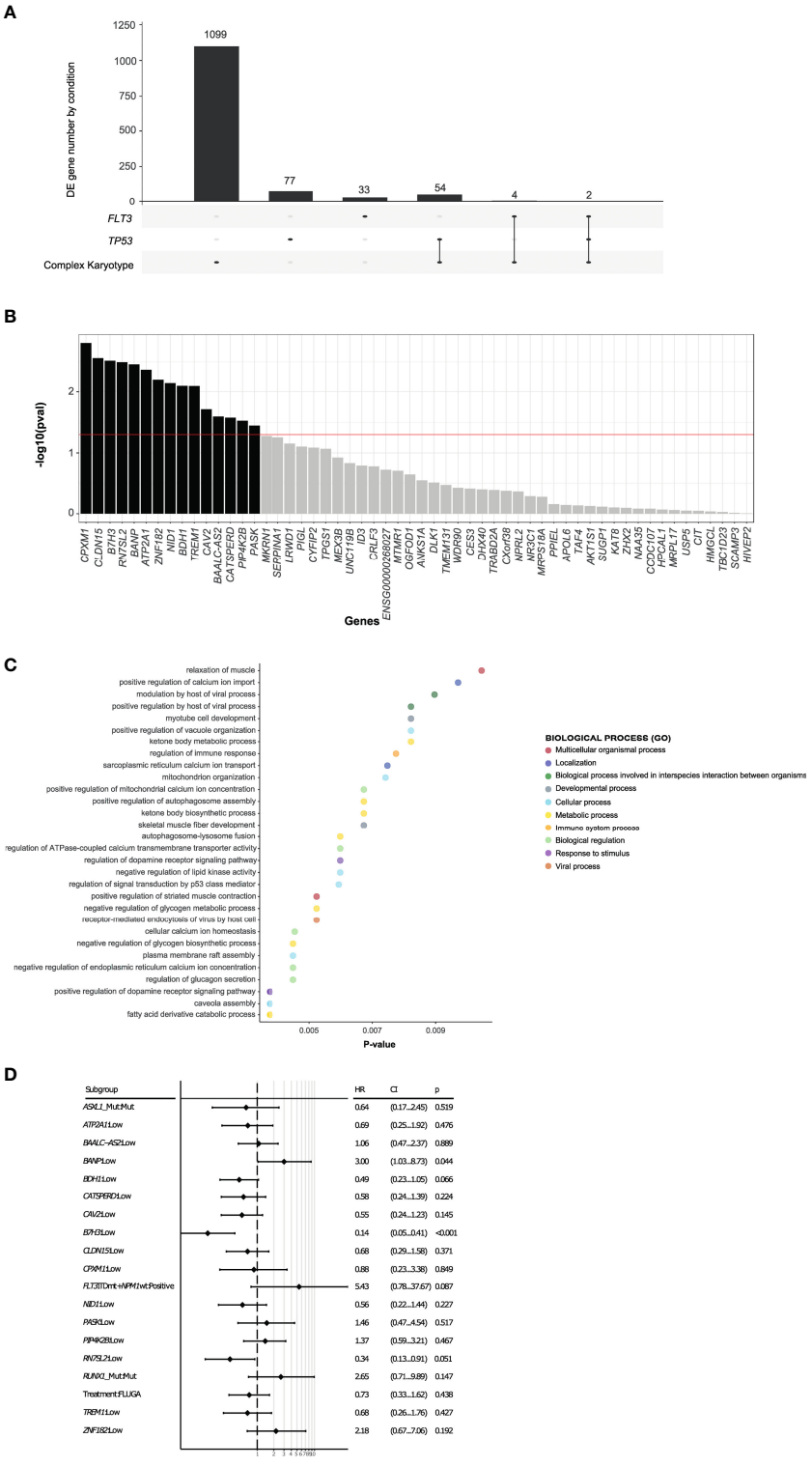

FIGURE 3
Expression of *B7H3 and BANP* genes are associated with overall survival of AML patients. **(A)** Differentially expressed genes between long-term survivors in the complex karyotype, *TP53* mutated and FLT3-*ITD* groups. **(B)** 15 differentially expressed genes shared by *TP53* mutated and complex karyotype long survival AML patients with impact on the univariate survival analysis. **(C)** GO study of the 15 differentially expressed genes in long survival AML patients with TP53 mutated or complex karyotype, with impact on the univariate survival analysis. **(D)** Multivariate analysis including differentially expressed 15 genes and ELN adverse category mutations such as *RUNX1*, *ASXL1, FLT3-ITD* with *NPM1 wt*.

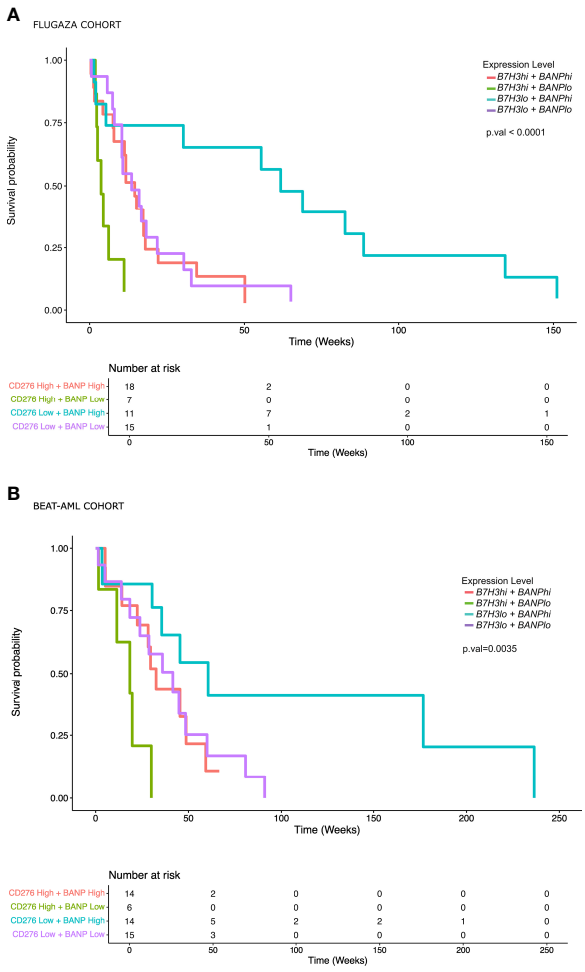

FIGURE 4
AML patients with low expression of *B7H3* and high expression of *BANP* show a significantly better overall survival. **(A)** Overall survival analysis in AML patients with *TP53* mutated or complex karyotype included in the PETHEMA-FLUGAZA trial using the expression of *B7H3* and *BANP* genes. **(B)** Overall survival analysis in AML patients included in BeatAML cohort using the expression of *B7H3* and *BANP* genes.

mutations to establish optimal therapies for patients with AML. The development of novel RNA sequencing based prognostic scores for AML (28), including the integration of mutational and gene-expression data, have been found to add prognostic value to the current European Leukemia Net (ELN) risk classification as well as to identify new genomic subtypes. However, there is a need to identify patients that despite their general poor prognosis may experience a longer survival and/or can benefit from specific therapy.

In this study, exploiting RNAseq data obtained from a group of 224 AML patients homogeneously treated, we aimed to identify transcriptional biomarkers to identify patients with a different prognosis within ELN genetic risk groups. Although the unsupervised hierarchical clustering of the whole cohort identified 3 different transcriptional profiles, there were no association with classical AML related mutational and cytogenetic data. These findings might be consistent with the already well stablished heterogeneous nature of AML (10, 29). The lack of significant differences in survival between the 3 subgroups might be related with the small size of some of the transcriptional subgroups.

However, our results identified a unique transcriptomic signature in the typically adverse group with *TP53* mutation or complex karyotype, based on the expression of *B7H3* and *BANP* genes. This high risk group of patients have commonly a short survival of less than 12 months (30), and significantly worse if they are old or unfit (31). Conversely, we demonstrate that high risk AML patients with low expression of *B7H3* together with high expression of *BANP* gene display a significantly better overall survival than the whole group. This signature might modify the negative prognostic impact of TP53 or complex karyotype in AML patients.

Beside their role as biomarkers, both genes identified have been implicated in the pathogenesis of AML. *B7H3*, a transmembrane protein type I located in chromosome 15, is an immune checkpoint from the B7 family. Previous studies have identified high expression of *B7H3* as an adverse factor in multiple tumors, including AML (32, 33), having an *immunological function*, acting essentially as a coinhibitory immune checkpoint with an important role in immune editing and immune evasion. Prior studies have shown that *B7H3* generates an immunosuppressive tumor microenvironment, thus favoring immune surveillance evasion and promoting tumor progression (34). However, in recent years, *non-immunological functions* of *B7H3* seem to be even more important than the immunological ones for tumor aggressiveness. *B7H3* regulates migration, invasion and adhesion (34, 35), as well as promoting apoptosis resistance and chemoresistance in models of colorectal and breast cancer (33). Specifically in AML three different studies have addressed the implications of expression of *B7H3* (32, 36, 37). In the most comprehensive and integrative study including 625 patients with AML, they found that *B7H3* expression was essentially regulated by DNA methylation, and it was associated with old age, *TP53* mutations, and a poor outcome in four independent datasets. In line with these findings, another study conducted by Zhang, W (38) showed that *B7H3* knockdown in an AML cell line significantly decreased cell growth and enhanced chemosensitivity. We found a favorable outcome for high-risk patients with low *B7H3* expression, regardless of treatment arm. This is consistent with previously described functional implications of *B7H3*. AML patients with low *B7H3* expression

could reflect a group with more active antitumor immunity, less aggressive AML cell properties and a more favorable chemosensitivity profile. Taken together, all these findings might explain why these initially high-risk patients do generally better in our cohort and enhance the potential of *B7H3* as a prognostic biomarker and possibly as a therapeutic target in high-risk AML.

BANP (BTG3 associated nuclear protein) is a nuclear matrix attachment region binding protein (MARBP) essential for nuclear matrix binding that has been implicated in cancer. MARBPs facilitate a correct chromatin assembly necessary for the normal gene replication and transcription (39). Thus, perturbations in these proteins might lead to an incorrect chromatin folding and aberrant replication and transcriptional programs, promoting genomic instability and oncogenesis (40, 41). Kaul et al. conducted the first study in mouse melanoma cells (42), showing that ectopic expression of *SMAR1* (murine homolog of *BANP*) promoted cell arrest. Subsequent studies have been carried out mainly in breast cancer models (43, 44). *BANP* exerts its antitumor activity through the modulation of crucial transcription factors such as p53 and NFκβ (45). These interactions take place through the formation of complexes with histone deacetylase (HDAC1). In addition, *BANP* regulates the TGFβ pathway by inducing the expression of SMAD7, an inhibitory SMAD that negatively regulates the TGFβ pathway. These studies reflect the important role of *BANP* as a tumor suppressor gene in cancer and may be consistent with our findings in which higher levels of *BANP* expression were associated with a favorable outcome in high-risk AML patients. In that sense, therapeutic approaches addressing the stabilization of *BANP* expression may be warranted.

In conclusion, we performed RNAseq in 224 elderly AML patients homogeneously treated, with the aim to define new prognostic groups. We identified the expression of *B7H3* and *BANP* genes as unique transcriptomic biomarkers, revealing a long survival group within *TP53* mutated or complex karyotype AML patients. As a potential limitation of our study, we acknowledge that *TP53* mutation assessment was performed with NGS, therefore information regarding *multihit* TP53 mutation was not available. According to our findings, *B7H3lo/BANPhi* patients have a clinical course more similar to a low-risk genetic group, and this signature might reduce the negative prognostic impact of TP53 or complex karyotype in AML patients. These two genes might serve as prognostic biomarkers and functional studies should address its utility as therapeutic targets in AML.

Data availability statement

The data presented in the study are deposited in Gene Expression Omnibus repository, with accession number: GSE208218.

Ethics statement

The studies involving human participants were reviewed and approved by PETHEMA. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SaV and BA wrote the manuscript. FP, XA, BP and AA-P revised and modified the manuscript. BA and SaV performed statistical analysis. DM-C, JB, SuV, LA, MT, PiM, JS, CS, PH, MC, AA-P, BP, JM-L and PaM provided study material and/or patients. AU and SaV prepared RNA-Seq libraries. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Publisher's note

Supplementary material

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SUPPLEMENTARY FIGURE 1
Detailed treatment design and mutational landscape of patients. **(A)** Scheme showing the two arms of treatment for AML patients in the clinical trial, FLUGA treatment arm (top) or AZA treatment arm (bottom). **(B)** Mutational landscape of AML patients included in the FLUGAZA clinical trial.

SUPPLEMENTARY FIGURE 2
AML patients surviving more than 12 months do not display any specific transcriptional signature. **(A-C)** Differential expression analysis between AML patients surviving more or less than 12 months **(A)** in the whole group, **(B)** in the AZA treatment arm or **(C)** in the FLUGA treatment arm. SX: more than 12 months of survival.

SUPPLEMENTARY FIGURE 3
Long survival AML patients with mutations in *NPM1*, *IDH1/2*, *TET2* or *RUNX1* do not show any differential transcriptomic profile respect to non-long survival AML patients with these same genetic alterations. **(A-D)** Differential expression analysis between long survival AML patients and non-long survival AML patients with **(A)** *NPM1* mutations, **(B)** *RUNX1* mutations, **(C)** *IDH1/2* mutations or **(D)** *TET2* mutations.

SUPPLEMENTARY FIGURE 4
Overall survival analysis in AML patients with *TP53* mutated or complex karyotype included in the PETHEMA-FLUGAZA trial using the expression of *B7H3* and *BANP* genes according to their treatment arm. **(A)** AZA arm. **(B)** FLUGA arm.

SUPPLEMENTARY TABLE 1
Baseline characteristics of patients included in the FLUGAZA-PETHEMA clinical trial in whom RNASeq was performed.

SUPPLEMENTARY TABLE 2
Gene ontology pathways and biological processes associated with the 15 differentially expressed genes with survival impact on the univariate analysis in mutated *TP53* or complex karyotype groups.

SUPPLEMENTARY TABLE 3
Baseline characteristics of 51 patients of the cohort with the selected genetic signature according to the obtained prognosis group.

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EDITED BY
John Maher,
King's College London,
United Kingdom

REVIEWED BY
Pooria Safarzadeh Kozani,
Tarbiat Modares University, Iran
Jeffrey J. Pu,
University of Arizona, United States

*CORRESPONDENCE
Xudong Zhao
zhaoxudong@wchscu.cn
Liqun Zou
zouliqun1971@wchscu.cn

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Chimeric antigen receptor T-cell therapy for T-ALL and AML

Wenwen Wei[1,2], Dong Yang[1], Xi Chen[3], Dandan Liang[1], Liqun Zou[2*] and Xudong Zhao[1*]

[1]Laboratory of Animal Tumor Models, Frontiers Science Center for Disease-Related Molecular Network, State Key Laboratory of Biotherapy and Cancer Center, National Clinical Research Center for Geriatrics, West China Hospital of Sichuan University, Chengdu, China, [2]Department of Medical Oncology of Cancer Center, West China Hospital of Sichuan University, Chengdu, China, [3]Department of Radiotherapy, Cancer Center, West China Hospital of Sichuan University, Chengdu, China

Non-B-cell acute leukemia is a term that encompasses T-cell acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML). Currently, the therapeutic effectiveness of existing treatments for refractory or relapsed (R/R) non-B-cell acute leukemia is limited. In such situations, chimeric antigen receptor (CAR)-T cell therapy may be a promising approach to treat non-B-cell acute leukemia, given its promising results in B-cell acute lymphoblastic leukemia (B-ALL). Nevertheless, fratricide, malignant contamination, T cell aplasia for T-ALL, and specific antigen selection and complex microenvironment for AML remain significant challenges in the implementation of CAR-T therapy for T-ALL and AML patients in the clinic. Therefore, designs of CAR-T cells targeting CD5 and CD7 for T-ALL and CD123, CD33, and CLL1 for AML show promising efficacy and safety profiles in clinical trials. In this review, we summarize the characteristics of non-B-cell acute leukemia, the development of CARs, the CAR targets, and their efficacy for treating non-B-cell acute leukemia.

KEYWORDS

chimeric antigen receptor, T-ALL, AML, antigen, immunotherapy

Introduction

Clinical features, treatment, and prognosis of T-ALL

T-ALL is a highly invasive form of hematological malignancy that results from the malignant transformation of immature T-cell progenitors, characterized by active cell proliferation, high tumor burden, leucocyte count, extramedullary involvement, large thymic masses, and pleural effusions. T-ALL occurs in 10%-15% of pediatric and about 25% of adult ALL cases, respectively (1). Compared with B-ALL, T-ALL cases are generally diagnosed in older individuals, are biologically distinct to B-ALL and have different kinetic patterns of disease response. For example, most B-ALL originates from the pre-pro-B and pro-B-cell, while, in contrast, T-ALL originates from various stages of

T cells. In addition, T-ALL patients are generally more resistant to conventional chemotherapeutic drugs than patients with B-ALL. Notably, B-ALL has also been found to be associated with favorable (low-risk) genetic subtypes that inform reliable therapeutic implications and realistic prognostication of their condition in patients, thereby facilitating risk stratification and targeted therapy (2–6). However, given the greater genetic and cellular heterogeneity, such an approach is so far elusive for T-ALL, with current treatment approaches relying on multidrug combination followed by intensive consolidation and maintenance therapy, with central nervous system (CNS) prophylaxis given at intervals throughout treatment (7). Nevertheless, treatments for T-ALL show significant success, with 5-year survival rates of 80%-90% for pediatric cases, yet 30%-40% for adult cases (8, 9), but there are still 20% of T-ALL patients ultimately die because of relapsed or refractory disease. The development of radiotherapy, new drugs, and targeted therapies targeting CD19, CD20, and CD22, have altogether improved the clinical management of R/R B-ALL. In contrast, curative treatments for R/R T-ALL remain to be significantly found, with hematopoietic stem cell transplantation (HSCT) currently the only such approach.

Clinical features, treatment, and prognosis of AML

AML is a hematological malignancy formed by abnormal clonal proliferation of primitive myeloid cells, and characterized by the accumulation of deformed, immature, and nonfunctional myeloid cells in bone marrow and blood. The incidence of AML increases with age, accounting for 15%-20% of leukemia cases in childhood, and is the second most common form of leukemia in children while it is the most common adult acute leukemia (10). In most children, AML often occurs *de novo* while in adult, a major proportion of AML are generally preceded by myeloproliferative neoplasms (MPN) or myelodysplastic syndrome (MDS) (11, 12). Current strategies for the treatment of AML involve two-phase chemotherapy, including anthracycline- and cytarabine-based induction chemotherapy, wherein children also receive central nervous system (CNS) prophylaxis to prevent central nervous system relapse. Patients who achieve initial remission then receive consolidation/intensification therapy, including combination chemotherapy or HSCT. While the treatment for children with acute promyelocytic leukemia (APL) includes a third phase called maintenance, which gives lower dose treatment than those used during the induction and consolidation phases (13–15). After treatment with standard regimens, the long-term survival rate of AML approaches near 70% in children and 35-45% in adult patients under 60 years, compared with 10-15% for those over 60 years (16). For relapsed patients, the median survival is 6 months and approximately 10% of patients achieve long-term survival, relapse and associated complications are common causes of death in AML (17, 18). Most recently, targeted therapy, immunotherapy, and new drugs have provided more treatment options for AML, but the efficacy in R/R patients remains poor. It is an urgent need for more effective treatments to improve patient survival rates for AML.

The structure of CARs

CARs are various receptors that endow T cells with the capacity to recognize specific tumor antigens and induce cytotoxicity against malignant cells, based on their expression of such antigens (19). The basic structure of CARs consists of four components (as shown in Figure 1): (1) Antigen recognition domain. The antigen recognition domain is the extracellular domain of the CAR, which is essential for T-cell activation, recognition, and cytotoxicity. The most common extracellular antigen recognition domain is a single-chain variable fragment (scFv) composed of heavy (V_H) and light (V_L) chains derived from monoclonal antibodies. In addition, natural ligands or receptors, repeat proteins such as designed ankyrin-repeat proteins (DARPins) and variable lymphocyte receptors (VLRs) derived from the sea lamprey genome, T-cell receptor (TCR) variable fragments, multivalent binding domains, universal switchable recognition domains, single variable domain on a heavy chain such as nanobody (also referred as VHH) and peptide, as well as others, could be used to construct the CAR (20, 21). (2) Hinge domain. The hinge domain is an extracellular structure between the antigen recognition domain and the transmembrane domain. The length and composition of the hinge domain are known to affect the flexibility of the CAR, CAR expression, signal transduction, and epitope recognition (22, 23). Presently, the most commonly effective hinge domains for CAR design comprise amino acid sequences derived from CD8, CD28, IgG1, or IgG4 (24). (3) Transmembrane domain. The transmembrane domain anchors the CAR to the cell surface membrane and is frequently derived from type I proteins including CD3ζ, CD4, CD8α, and CD28. Different transmembrane domains influence the stability and function of CARs. For example, CD3ζ mediates CAR dimerization, and

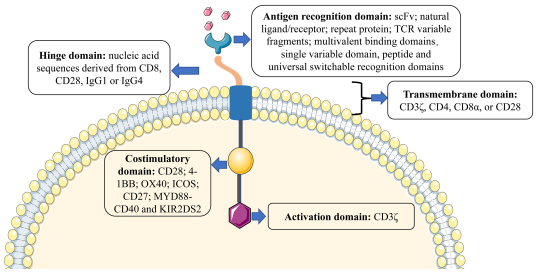

FIGURE 1
The basic structure of CARs.

the insertion of endogenous TCRs can promote CAR-mediated T-cell activation (25), but it is also less stable compared to the CD28 transmembrane domain (26). The transmembrane domain derived from CD8α induces less IFNγ and TNFα release than CD28 and is less sensitive to activation-induced cell death (AICD) (27). (4) Intracellular signal domain. This region is composed of a typical intracellular signal domain that includes an activation domain, as well as one or more costimulatory domains. Current CARs activate T-cells through the CD3ζ-derived immunoreceptor tyrosine-activated domain (24). However, the activation domain alone may not be enough to induce an effective response of CAR-T cells, since the persistence and activity of CAR-T cells *in vivo* remain limited (28). Costimulatory domains combined with the activation domain-bearing CD19-targeting CAR-T cells result in better persistence in B-cell malignancies (29). The two most common costimulatory domains, CD28 and 4-1BB (also known as CD137 or TNFRSF9) are used in most clinical trials and CD28-bearing and 4-1BB-bearing CD19 CAR have been approved by Food and Drug Administration (FDA) for B-cell malignancies and achieved promising clinical responses (30–35). Further, other costimulatory domains, such as OX40 (also known as CD134), ICOS (inducible T-cell costimulator), CD27, MYD88-CD40, and KIR2DS2 (killer cell immunoglobulin-like receptor 2DS2), that have demonstrated efficacy in preclinical models but have not yet been validated in clinical studies (36–40).

The evolution of CAR development

Since the first generation CAR was described in the late 1990s; four further generations of CARs have been developed, (summarised in Figure 2). The first generation CAR contained the CD3ζ intracellular signaling domain but without costimulatory domains, and it induced low interleukin (IL)-2 production and displayed inadequate proliferation and short lifespan *in vivo* (41). From this, the second generation CAR comprised an additional costimulatory domain, such as CD28, 4-1BB, or OX-40, and this led to enhanced proliferation, cytotoxicity, and persistence for CAR (42). The third generation CAR combined multiple costimulatory signaling domains. Although these represent a good safety profile in tumor therapy, their efficacy was not significantly improved compared with the second generation CAR (43). The fourth generation CAR refers to T-cells redirected for universal cytokine-mediated killing (TRUCKs), which added IL-12 based on the second generation CAR. In this design, IL-12 is expressed either constitutively or inducibly after CAR activation, which promotes the production and secretion of desired cytokines, as well as enhances cytotoxicity against tumor cells through multiple synergistic mechanisms (44, 45). The fifth generation CAR design involved the addition of a β-chain domain of the IL-2 receptor based on the second generation CAR, which comprises a binding site for the transcription factor STAT3. Antigen-specific activation of this receptor can trigger three signals: the TCR *via* CD3ζ domains, the costimulatory domain *via* the CD28 domain, and cytokine signaling *via* JAK-STAT, which act synergistically to activate and expand CAR-T cells (46, 47).

The advantage of CAR-T therapy

When compared with TCRs, CARs are major histocompatibility complex (MHC) independent and can recognize targeted antigens expressed on the cell surface (48, 49), which is in contrast to TCRs that only recognize natural

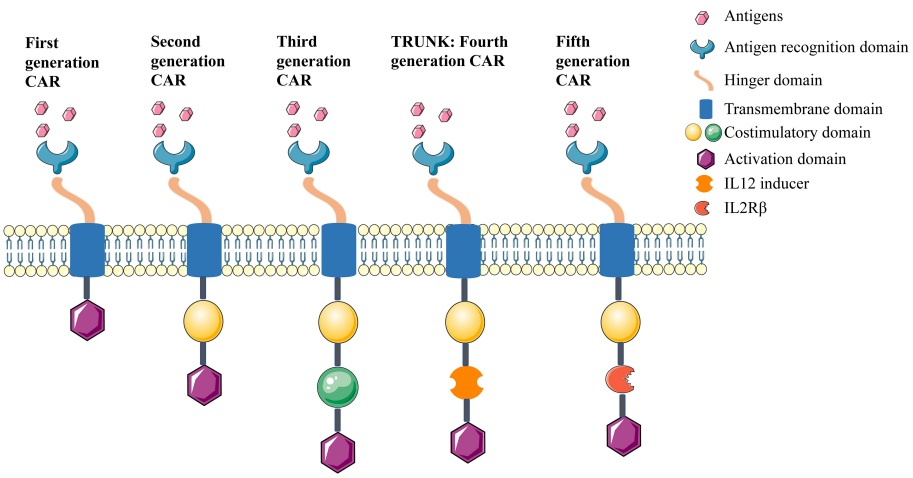

FIGURE 2
Five generation of CARs.

antigens presented by MHC (50). Thus, loss of MHC class I is recognized as the major mechanism of immune escape for tumor cells. As such, the characteristic of MHC independence makes CAR-T cells more applicable for tumor therapy (51). CAR-T cells eliminate tumor cells by recognizing tumor-specific antigens (TSA) on the surface of tumor cells, which has the advantage of minimizing damage to normal tissues (52, 53). In addition, tumor cells downregulate the expression of costimulatory molecules, and the intracellular structure of CAR contains a costimulatory domain that counteracts this effect, leading to improved therapeutic efficacy for treating tumors. It is noteworthy that CAR not only recognizes protein antigens but also recognizes carbohydrates and lipids antigens, thereby providing more design options for the preparation of effective CAR (54, 55).

CAR-T therapy for non-B-cell acute leukemia

The development of CAR-T therapy for non-B-cell acute leukemia faces some unique challenges, such as fratricide, malignant contamination, T-cell aplasia for T-ALL and antigen heterogeneity, and immunosuppressive environment for AML. Although there are obstacles in the development of CAR-T therapy for non-B-cell acute leukemia, some strategies have been developed to solve these problems, as shown in Figure 3. In this review, we focus on specific targets with promising efficacy and safety, which have been verified in preclinical or clinical trials.

Antigen targets of CAR-T therapy in T-ALL

CD5

CD5 is a surface marker of T-cell malignancies and is expressed in approximately 80%-95% of T-ALL or T lymphoblastic lymphoma (TLL) (56, 57). Typically, CD5 is also expressed on mature peripheral blood T cells, thymocytes, and some B-cell lymphocytes in healthy tissues, which lead to fratricide of CAR-T cells (58, 59). In 2015, Mamonkin et al. reported that CD5 CAR-T cells only exhibit partial fratricide, following which these could be expanded *in vitro*. The expanded CAR-T cells also maintained killing efficacy in T-ALL/TLL tumor cell lines (including Jurkat, CCRF-CEM, MOLT4, Hut78, and SupT1) and primary T-ALL cells *in vitro* and Jurkat and CCRF-CEM cell lines *in vivo*. Further investigation found that the incomplete fratricide of CD5 CAR-T cells was due to the internalization of surface CD5 molecules after ligand binding which, in turn, downregulated CD5 expression on the normal T-cell surface (60). To prevent fratricide completely, in 2017, Raikar et al. genetically knocked out CD5 expression on the surface of T cells with CRISPR-Cas9 genome editing, and CD5-edited effector T cells overcame the challenge of self-activation and fratricide, which demonstrating the feasibility for CD5 CAR-T therapy in T-cell malignancies (61). To prolong the persistence of CAR-T cells *in vivo*, in 2018, Mamonkin et al. designed a doxycycline (*Dox*) controlled Tet-Off system to inhibit CAR expression to prevent fratricide of 4-1BB CD5 CAR instead of CD28 CD5 CAR. In this study, CD5 expression in CAR-T cells occurred after *Dox* withdrawal, leading to improved and prolonged antitumor ability against CD5+ T-ALL cell lines Jurkat and CCRF-CEM *in vitro*, as well as the Jurkat mouse model *in vivo* (62). To explore the feasibility of CD5 CAR-T therapy as a bridge to HSCT in the clinic, in 2019, Hill et al. treated 9 heavily treated patients with autologous CD5 CAR-T cells, and 3/9 patients achieved CR, 4/9 patients obtained an objective response, and 2/9 patients relapsed at 6 weeks and 7 months post-infusion, all side-effects were manageable. These results proved that CD5 CAR-T cells could allow ineligible patients to proceed to HSCT with safety and clinical response for R/R CD5+ malignancies (63). Except for the fratricide, T-cell aplasia is also an important challenge for CD5 CAR. In 2020,

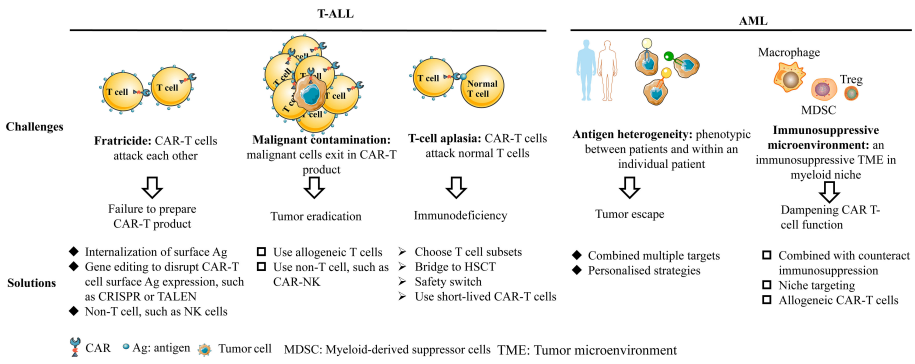

FIGURE 3
The challenges and solutions for T-ALL and AML.

Wada et al. used alemtuzumab, which targets CD52 as an inducible safety switch, to remove CAR-T cells from systemic blood circulation without affecting the anti-tumor efficacy in the mouse model, thus avoiding T-cell aplasia after therapy (64). T-cell malignancies with CNS infiltration always have poor outcomes and limited treatment options, IL-15 could strengthen the anti-tumor response. In 2021, Feng et al. modified a CD5-IL-15/IL15sushi CAR which secretes an IL-15/IL-15 complex, to explore its clinical effectiveness against one refractory T-cell lymphoma patient with CNS infiltration. This patient obtained a rapid ablation of the CNS lymphoblast and lymphoma and was accompanied by brief and transient T-cell aplasia (65). In another study, Dai et al. manufactured a new biepitopic CAR with fully human heavy-chain variables FHV_H3 and FHV_H1, which could bind different epitopes of CD5. As such, CD5KO FHV_H3/V_H1 CAR-T cells showed prolonged and sustained efficacy against CD5+ T-ALL cell lines, such as Jurkat, CCRF-CEM, MOLT4, SupT1 *in vitro*, and CCRF-CEM *in vivo*, with moderate cytokine production, proved that this new CD5 CAR deserved more exploration (66). Due to recurrence occurring in some patients after CAR-T therapy which targets single antigen CD5 or CD7 (63, 67), in 2022, Dai et al. designed a bispecific CAR that targeted CD5 and CD7 with a fully human variable heavy chain (FHV_H). The results showed that fratricide-resistant FHV_H-derived CD5/CD7 bispecific CAR-T cells showed potent antitumor activity to Jurkat, CCRF-CEM, MOLT-4, and SUP-T1 cell lines *in vitro* and CCRF-CEM T-ALL mouse model *in vivo*, it provided the possibility to the populations with antigen heterogeneous (68). Pan et al. explored the safety and efficacy of donor-derived CD5 CAR-T cells in CD7-negative relapsed patients after CD7 CAR therapy. The data showed that all five patients achieved CR at 1 month and no dose-limiting toxicities occurred. Donor-derived CD5 CAR showed promising clinical safety and response in R/R T-ALL, but the evaluation of durable remission and functional immune system reconstitution needs longer follow-ups (69). Possible approaches to overcome the challenges such as fratricide and T-cell aplasia include internalization of surface CD5, CRISPR-Cas9, or Tet-Off system technologies that could be used to deal with complete fratricide without affecting the efficacy of CAR-T cells, while alemtuzumab could be used as a safety switch to deplete CAR-T cells after therapy to prevent T-cell aplasia. Taken together, CD5 is a promising target for CD5+ hematological malignancies, there are 4 clinical trials about CD5 CARs that are being recruited, as shown in Table 1.

CD3

CD3 is a pan-T-cell surface antigen, expressed predominantly on all mature T-cells (70), however, the development of CAR-T targeting CD3 remains limited due to the complete fratricide of CAR-T cells. In 2018, Rasaiyaah et al. tried to disrupt endogenous TCRαβ/CD3 on T cells by adopting transcription activator-like effector nuclease (TALEN) to construct a CAR targeting CD3ϵ and CAR-T cells exhibited specific toxicity against the T-ALL cell line Jurkat *in vitro* and *in vivo* (71). However, since T-ALL and TLL cells derived from patients typically express cytoplasmic CD3 (cCD3) rather than membrane CD3 (mCD3) (72), the therapeutic effect of CAR targeting CD3 is limited in its clinical application. No clinical trials are currently documented in the literature.

CD7

CD7 is a 40-kD Ig superfamily member expressed on normal T and NK cells (73–75) and is expressed in over 95% of ALL and 30% of AML, as well as some lymphomas (56, 76–79). CAR-T cells targeting CD7 showed complete fratricide and could not be expanded, thereby resulting in limited studies of CAR targeting CD7 until 2017. Gomes-Silva et al. first knocked out CD7 expression on normal T cells by CRISPR–Cas9 pre-T-transfection so that CD7-knocked out CAR-T cells could be expanded, and these cells showed specific cytotoxicity to both CD7+ cell lines Jurkat, CCRF-CEM, MOLT-4, Hut78 and SupT1, also primary blasts, these provided a new insight for CD7 CAR (76). Png et al. reported a study that blocked CD7 in the ER/Golgi by using a protein expression blocker (PEBL) system, in which CD7 could not be expressed on the surface of T cells. This method alleviated fratricide without affecting the proliferation of CAR-T cells, while CD7 CAR-T cells showed strong antileukemia activity to CD7+ cell lines including Jurkat, CCRF-CEM, Loucy, MOLT4, and KG1a (80). Due to CD7 expression on both normal and malignant T cells, fratricide and malignant contamination occurred during CAR-T cell preparation. To solve both issues, allogeneic T cells may be another choice to prepare CAR-T cells. Then in 2018, Cooper et al. knocked out CD7 and the T-cell receptor alpha chain (TRAC) of T cells by CRISPR–Cas9, and CD7 CAR-T cells showed antileukemia efficacy against T-ALL cell lines MOLT-3,

TABLE 1 Recruiting for CD5 CAR clinical trials.

NCT Number	Interventions	First posted	Phase	Locations
NCT03081910	Autologous CD5.CAR/28 zeta CAR T	16-Mar-17	I	United States
NCT04594135	anti-CD5 CAR T	20-Oct-20	I	China
NCT05032599	Donor-derived CD5 CAR T	2-Sep-21	I	China
NCT05487495	Donor-derived CD5 CAR T (CT125B)	4-Aug-22	I	China

MOLT-4, HSB-2, and CCRF-CEM without graft-versus-host-disease (GVHD) (81). In 2020, Zhang et al. explored the antitumor activity and toxicity of CD7 CAR in clinical trials for the first time. The author reported that 2 of 3 enrolled R/R ALL/TLL patients obtained complete remission (CR) with minimal residual disease (MRD) negative at day 28 post-infusion. At the same time, cytokine release syndrome (CRS) occurred which was controllable. Nevertheless, this study showed the significant potential value for the clinical application of CD7 CAR (82). To address CRS after CAR-T cell therapy, in 2021, Li et al. treated 2 T-ALL patients with "off-the-shelf" allogeneic CD7 CAR-T cells combined with ruxolitinib. Strong CAR-T-cell expansion and rapid tumor cell clearance were detected after CAR-T cell infusion, and both patients achieved CR with MRD negative. While, both patients developed grade 3 CRS, but it was manageable with co-treatment of ruxolitinib (83). Yang et al. conducted a phase I clinical trial of CD7 CAR-T cell therapy for 14 R/R T-ALL. The data showed that 13/14 (92.9%) patients achieved MRD negative CR at day 28 post-infusion and all patients experienced mild CRS (grade<2) (84). For T-ALL patients, there may be not enough normal T cells for CAR-T cells preparation, Pan et al. used donor-derived CD7 CAR-T cells to explore the efficacy and safety in R/R T-ALL, 18/20 patients achieved CR with 7 patients proceeding to HSCT and with a manageable safety profile (67). Furthermore, CAR-T/NK targeting CD7 will clear CD7-positive normal T/NK cells *in vivo*, HSCT is usually required after CAR-T therapy; otherwise, the treatment causes severe immunodeficiency and potentially life-threatening infection by pathogenic microorganisms. Kim et al. knocked out CD7 in hematopoietic stem cells (HSCs) and found that these CD7-KO HSCs differentiated into CD7-negative T-cells and NK cells that exhibited effector functions after transplantation into mice. This study suggests that such an approach could resolve T-cell immunodeficiency caused by CD7-CAR therapy, altogether providing a potential new approach for the development of CD7 CAR (85). In 2022, Dai et al. reported a case that haploidentical CD7 CAR-T cells induced remission in an 11-year-old TP53 mutated R/R ETP-ALL/LBL patient, and grade 3 CRS and macrophage activation syndrome were observed but manageable (86). Due to the GVHD occurring in some patients after donor-derived CD7 CAR-T cell therapy, Zhao et al. treated five R/R T-ALL/LBL with autologous CAR-T cell therapy. The results showed that 4/5 patients achieved CR at day 30 post-infusion without neurotoxicity, GVHD, or infection (87). Due to 3-10% CD7- T cells exiting in peripheral blood, Lu et al. obtained naturally selected 7CAR (NS7CAR) T cells, which are a subtype of CD7 negative T-cells that survived from fratricide. The NS7CAR T cells comprised a higher proportion of CAR+ cells and CD8+ central memory T cells while maintaining similar therapeutic activity to cell line CCRF-CEM *in vitro* and *in vivo* when compared with CD7 knocked-out 7CAR T cells. Most importantly, a study of 14 R/R T-ALL and 6 T-LBL patients who received NS7CAR T therapy found that 19/20 patients achieved MRD CR in bone marrow at day 28, all with manageable side effects (88). In another study, Freiwan et al. sorted CD7- T cells from PBMC before transducing them with CD7 CAR, the CD7-CAR-T cells contained more CD4+ memory phenotype and have a robust antitumor activity to CD7+ cell lines CCRF-CEM and MOLT3 *in vitro* and eliminated CCRF-CEM cells in the mouse model, as well as bypass fratricide (89). Li et al. combined donor-derived CD7 CAR-T therapy with allogeneic HSCT for a 3-year-old hepatitis B-positive T-ALL patient. The patient had CR at seven months post-infusion and the copy number of hepatitis B virus continuously decreased during treatment (90). Hu et al. resisted fratricide, GVHD, and allogeneic rejection in healthy donor-derived CD7 CAR (RD13-01) by genetic modifications. Twelve patients were recruited in the phase I clinical trial, and the data showed that 81.8% of patients achieved objective responses and 63.6% of patients received CR with no dose-limiting toxicity, GVHD, neurotoxicity, or severe CRS (91). CD7 CAR showed satisfied efficacy and safety in various clinical trials, gene editing technologies such as CRISPR-Cas9, TALEN, or PEBL, and recently reported natural selection could be applied to overcome fratricide, while HSCT could be applied in combination with CAR to address potential T-cell aplasia, and CRS could be controlled by ruxolitinib. Taken together, CD7 CAR is a promising therapeutic target for R/R T-cell malignancies. Currently, there are 23 clinical trials of CD7 CAR recruiting to explore its therapeutic effects in T-cell malignancies, as shown in Table 2.

In addition to CARs targeting T-cell pan-antigens, some antigens are only expressed on a subset of T cells, which can avoid complete fratricide during CAR-T preparation and T-cell immunodeficiency during therapy.

CD4

CD4 is expressed in most TLLs and some T-ALLs and its expression is restricted to the hematopoietic compartment but it is not expressed in HSCs. As the target of CAR, it can potentially reduce the off-target side effects for non-hematological tissues. But the persistence of CD4 CAR T cells after removal of tumor cells can lead to aplasia of CD4 positive T cells and cause HIV/AIDS-like syndrome. In 2016, Ma et al. utilized CAMPATH (alemtuzumab) as a natural safety switch to deplete CD4 CAR T cells post-therapy in mice. They found that this approach was effective to kill Jurkat cells *in vitro* and *in vivo* with minimal toxic side effects from loss of CD4+ T-cells (92). CD4 is expressed on a subset of normal T cells, which could prevent complete fratricide and be used to kill CD4 high-expressed malignant T cells. Three CD4 CARs are currently being recruited to explore clinical efficacy in T-ALL, as shown in Table 3.

TABLE 2 Recruiting CD7 CAR clinical trials.

NCT Number	Interventions	First posted	Phase	Location
NCT04033302	CD7 CAR gene-engineered T	26-Jul-19	I/II	China
NCT05127135	Allogeneic CART7	19-Nov-21	I	China
NCT04702841	CD7 CAR-γδ T	11-Jan-21	I	China
NCT04620655	RD13-01 cells	9-Nov-20	NA	China
NCT04480788	CD7-CART	21-Jul-20	I	China
NCT04916860	Senl-T7	8-Jan-21	NA	China
NCT04689659	Donor-derived CD7 CAR-T	30-Dec-20	II	China
NCT04264078	CD7 UCAR-T	11-Feb-20	I	China
NCT04785833	autologous CD7 CAR-T	8-Mar-21	NA	China
NCT04823091	CAR7-T Cells	30-Mar-21	I	China
NCT04840875	Autologous CD7 CAR-T	12-Apr-21	I	China
NCT04938115	CD7 CAR-T	24-Jun-21	NA	China
NCT04762485	Humanized CD7 CAR-T	21-Feb-21	I/II	China
NCT03690011	Autologous CD7.CAR/28zeta CAR-T	1-Oct-18	I	United States
NCT05043571	anti-CD7 CAR-T	14-Sep-21	I	Singapore
NCT05170568	PA3-17 CAR-T	28-Dec-21	I	China
NCT04984356	WU- CART-007	30-Jan-21	I/II	United States
NCT04934774	Non-gene edited CD7 CAR-T	22-Jan-21	I	China
NCT04004637	CD7 CAR-T	2-Jul-19	I	China
NCT04599556	anti-CD7 CAR-T	22-Oct-22	I/II	China
NCT05212584	CD7CAR-T	28-Jan-22	I	China
NCT05290155	Anti-CD7 CAR-T	22-Mar-22	I	China
NCT05398614	Senl 101	1-Jun-22	I	China

"NA" means "Not Applicable".

CD1a

The expression of CD1a is largely restricted to developing cortical thymocytes, and neither CD34+ progenitor cells nor T-cells are expressed during ontogeny. In contrast, however, CD1a is only expressed in cortical T-ALL (coT-ALL) and its expression persists in relapsed patients. Diego et al. found that CD1a CAR-T cells are fratricide-resistant and showed good efficacy against CD1a+ Jurkat and MOLT4 cell lines and primary coT-ALL cells *in vitro* and Jurkat T-ALL mouse models *in vivo* (93). Although CD1a CAR could be used to treat coT-ALL, some limitations exist to CD1a CAR in clinical use, such as that only a minority of cases of T-ALL express CD1a. Furthermore, CD1a is associated with relatively a favorable prognosis, and CD1a+ patients rarely relapsed or are refractory to treatment.

Others, such as CCR4 (C–C chemokine receptor type 4) and TRBC (T-cell receptor beta constant), are also expressed in some T-ALL and can be potential targets for T-ALL immunotherapy (94, 95).

Antigen targets of CAR-T therapy in AML

CD123

One potential target for AML is CD123, an IL-3 receptor alpha chain that acts as a high-affinity receptor for stem cell factor (SCF) and is expressed at low levels in early hematopoietic cells, such as hematopoietic stem/progenitor cells (HSPCs) (96). CD123 is expressed in approximately 97% of AML patients and is overexpressed in 45% of patients, and at low levels by HSPCs, monocytes, a subset of dendritic cells, and endothelial cells (97–99). As early as 2013, Tettamanti et al. designed CD123 CAR-infected cytokine-induced killer cells (CIKs). These CIKs

TABLE 3 Recruiting CD4 CAR clinical trials.

NCT Number	Interventions	First posted	Phase	Locations
NCT04162340	CD4 CAR T	14-Nov-19	I	China
NCT04219319	LCAR-T2C CAR T	7-Jan-20	I	China
NCT04973527	LCAR-T2C CAR T	22-Jul-21	I	China

showed efficacy against CD123+ THP1 cell line and primary AML blasts, with minimal effects on healthy monocytes and endothelial cells with low expression of CD123, which proves the feasibility of CD123 as a therapeutic target (100). In 2014, Mardiros et al. demonstrated that CD123 CAR-T cells were effective in eliminating CD123+ LCL, KG-1a cell lines, and primary AML blasts without eliminating granulocyte/macrophage and erythroid colony formation *in vitro*. Notably, patients-derived CAR-T cells could eliminate autologous AML blasts *in vitro (*101). Gill et al. found that CD123 CAR was efficient in clearing human primary leukemia cells, meanwhile inducing myeloablation in xenograft mouse models, which suggests that CD123 CAR-based myeloablation may be used to bridge HSCT (102). Due to CD123 and CD33, both are expressed on AML cells and normal HSPCs, Pizzitola et al. modified CKI cells with the CD123 and CD33 CAR to compare their efficacy and safety. Both these CAR-T cells efficiently eliminated primary human AML KG-1a cells in mice, but only CD123 CAR showed limited killing efficacy on normal HSPCs compared to CD33 CAR (103). In 2015, Luio et al. first treated one relapsed AML-M2 (FLT3/ITD+), male patient with apoptosis-inducible CD123 CAR, and the patient subsequently achieved partial remission within 20 days. Although CRS occurred on day 4, it was effectively controlled with a single dose of Tocilizumab (104). To address myeloablation after CD123 CAR-T therapy in AML, Tasian et al. compared three CAR-T-cell clearance strategies: (1) transiently active anti-CD123 messenger RNA electroporated CAR T cells (RNA-CART123); (2) T-cell clearance with alemtuzumab after CD123 CAR-T-cell therapy; and (3) T cell ablation with rituximab to CD20-coexpressing CART123(CART123-CD20) after therapy. The author found that CD123 CAR-T cells showed strong antitumor activity to MOLM14 cell line and primary AML blasts in mouse xenograft models, and all these three approaches could efficiently deplete CAR-T cells without affecting antileukemic effects. Notably, the ablation of CAR-T cells allowed subsequent HSCT rescue in normal hematopoiesis xenograft models (105). Thus, strategies for posttreatment CAR-T cell clearance may be effective to alleviate detrimental side effects, such as myeloablation. Further to this area of investigation, Arcangeli et al. designed a rational mutation in the anti-CD123 CAR antigen binding domain to reduce the binding affinity of CAR, which could minimize the toxicity and side effects against normal tissues with low expression of CD123 without affecting the antileukemic activity to tumor cells; altogether demonstrating the manageable safety of CD123 CAR in AML (106). The same year (2017), Budde et al. reported in an abstract form that a Phase I dose escalation clinical trial, which evaluated the efficacy and safety of CD123 CAR in 6 patients with refractory AML after treatment with autologous HSCT (alloHSCT). In this study, 4 of 6 patients achieved CR, and 2 patients reported reduced blast counts but did not achieve remission after a single or second infusion of CAR-T cells. Notably, all side effects were reversible and controllable (107). Meanwhile, to ablate CAR-T cells after therapy in patients, Cummins et al. evaluated the safety of CD123 CAR in R/R AML patients with mRNA electroporate technology. CART123 cells did not expand successfully *in vivo*, and all 5 patients treated with mRNA CART123 eventually developed clinical progression (108). To improve the efficacy and persistence of CAR-T cells *in vivo*, in 2018, Mu et al. developed CD123 CAR-expressing IL-15. The results showed that genetically engineered CD123 CAR improved the anti-AML activity against CD123+ cell lines *in vitro* and eliminated primary AML cells *in vivo (*109). Loff et al. redirected CD123 CAR-T cells using a switch-controllable universal CAR T platform (UniCAR) based on two major elements: a non-reactive inducible CAR and a soluble targeting module (TM) enabling UniCAR-T reactivity in an antigen-specific manner. UniCAR T 123 exhibited potent cytotoxic activity against patient derived CD123+ leukemia cells *in vitro* and *in vivo*. Notably, in this study, the activation, cytolytic activity, and cytokine release profiles for UniCAR T123 were all tightly controlled. Compared with traditional CD123 CAR-T cells, UNICAR T 123 cells can additionally distinguish malignant leukemia cells with high CD123 expression from healthy tissues with low CD123 expression, features that further improve the safety of CD123 CAR (110). A phase I clinical trial of UNICAR-CD123-CAR is currently in progress (111). In 2019, Qin et al. developed a simple and highly selective D-domain, which was derived from *de novo*-designed α-helical bundle-α3D, to target CD123 for a unique CD123 CAR. The work revealed that CD123 CAR composed of D-domain mediated efficiently mediated T-cell activation and cytotoxicity to MOLM14, IM9, KG-1a, and NALM6 cell lines, and induced complete and durable remission in two AML xenograft mouse models. This work supports the development of multifunctional CARs through such an approach (112). Yao et al. used donor-derived CD123 CAR T cells as a conditioning regimen for haploidentical HSCT (haploid-HSCT) in a patient with FUS-ERG+ AML and found that CD123 CAR-T cells reduced chemotherapy-resistance blasts without affecting donor chimerism and myeloid implantation (113). To enhance the anti-tumor function of CD123 CAR-T cells, in 2020, You et al. combined decitabine treatment with CD123 CAR for AML. The results showed that decitabine enhances the anti-leukemia efficacy of CD123 CAR-T cells to THP1 cell line *in vitro* and *in vivo*, alongside CD123 CAR-T cells differentiate into naive and memory phenotypes (114). In 2021, UniCAR-T-123 with the targeting module TM123 was used to treat 3 R/R AML patients. 1 patient showed partial remission and 2 patients showed CR and adverse events were generally mild (115). This clinical trial is still ongoing. To minimize the side effect on normal hematopoietic progenitor cells and prolong the persistence of CD123 CAR-T cells, Khawanky et al. found that demethylating therapy could increase the CD123 expression on leukemia cells

and increase CTLA4- CD123 CAR-T cell proportion and showed superior cytotoxicity against AML cells, accompanied by higher TNFα production in leukemia-bearing mice (116). Due to CD123 could distinguish HSC from leukemia stem cells (LSCs), to eliminate LSCs and preserve normal HSC, in 2022, TALEN gene-editing technology was used to produce a TCRαβ negative allogeneic CD123 CAR (UCART123), which preferentially eliminates primary AML than normal cells with modest toxicity *in vitro (*117). While CAR targeting CD123 has shown efficacy and safety in preclinical and some clinical trials, but myeloablation occur after CAR-T therapy. Therefore, some strategies such as transiently active messenger RNA for CAR, alemtuzumab or rituximab treatment, and UniCAR may be utilized to ablate CAR-T cells *in vitro* and *in vivo*. The depletion of CAR-T cells after therapy requires further investigation in clinical studies. Nevertheless, the safety and efficacy profiles of CAR therapy targeting CD123 are reflected in the finding that 7 clinical trials are recruiting to explore its feasibility to treat AML, as shown in Table 4.

CD33

CD33 is a transmembrane receptor of the sialic-acid-binding immunoglobulin-like lectin (SIGLEC) family and is expressed on myeloid cells ranging from progenitors to well-differentiated cells, including neutral granulocytes, monocytes, and tissue-resident macrophages (118). However, in pathological states, CD33 is expressed in approximately 80-90% of AML patients and may also be expressed on LSCs (119–122). In 2010, to improve the effector functions of CIK cells, Marin et al. transduced anti-CD33-ζ and anti-CD33-CD28-OX40-ζ CARs into CIK cells and found that the CD33 CAR enhanced the anti-leukemic functions of CIK cells against HL60 and KG-1a cell lines and primary AML blasts (123). In 2012, to decrease tumor escape, Dutour et al. transduced human Epstein Barr virus (EBV)-specific cytotoxic T cells with CD33 CAR, and the CAR-T cells displayed EBV and HLA-unrestricted bispecificity *in vitro* and anti-AML tumor activity in CD33+ human AML-bearing mice without irreversibly disrupting the formation of CD34(+) hematopoietic progenitor clones (124). In 2014, Pizzitola compared the cytotoxicity of CD123 and CD33 CAR on AML, and find that no difference in anti-leukemic activity, yet CD33 CAR appeared to have stronger cytotoxicity on normal HSPCs compared with CD123 CAR (103). In 2015, Wang et al. reported an autologous CD33 CAR in one R/R AML patient, with no uncontrollable clinical toxicities, but with subsequent disease progression at 9 weeks post-T-cells infusion (125). Kenderian and colleagues used the scFv of *gemtuzumab ozogamicin* (clone My96) to develop a CD33 CAR (CART33). CART33 exhibited significant cytotoxicity against the MOLM14 cell line *in vitro* and eradication of leukemia in AML xenograft, yet a reduction of myeloid progenitors in xenograft models was also observed. Thus, the author prepared transient CART33 cells by expressing modified mRNA, which exhibited potent but self-limited activity against AML (126). To minimize the myelosuppression after CAR-T therapy, in 2016, Minagawa et al. designed Caspase9-CAR CD33T cells inducibly selected by ΔCD19, which could specifically lyse CD33+ MV4-11 tumor cells and primary leukemic blasts *in vitro*, following which CAR-T cells were largely eliminated by suicide gene activation (127). In 2018, Kim et al. proved that CD33 KO HSPC showed normal engraftment and differentiation in the mouse model, and autologous rhesus macaques CD33 KO HSPC showing normal myeloid function *in vivo*. Most importantly, CD33 CAR-T cells showed efficient elimination of leukemia, while CD33-deficient cells were spared without myelotoxicity, as observed in human xenograft models (128). Li et al. demonstrated that 4-1BB as a costimulator could endow CAR-T cells with increased central memory and prolonged survival for maximum efficacy, as compared to CD28, and both CD28 and 4-1BB (129). Due to PI3K pathway being activated in CD33 CAR-T cells, to increase the persistence of CAR-T cells *in vivo*, Zhang et al. used PI3K inhibitors to modulate the differentiation of CD33 CAR-T cells. The data showed that it maintained CAR-T cells at a lower differentiated state without affecting CAR-T-cell expansion (130). Schneider et al. constructed a humanized CAR33VH CAR, comprised of a human heavy-chain variable fragment, that exhibited antitumor activity to CD33 high expressed MOLM14 and HL60 cell lines and eliminated tumors in a MOLT-14 AML mouse model (131). In 2021, Tambaro and colleagues conducted a Phase I clinical trial to evaluate the efficacy and safety of CD33 CAR-T in R/R AML patients. It was reported that three patients received one low dose of CAR-T

TABLE 4 Recruiting CD123 CAR clinical trials.

NCT Number	Interventions	First posted	Phase	Locations
NCT03190278	Allogeneic UCART123v1.2	16-Jun-17	I	United States
NCT04010877	CLL-1, CD33 and/or CD123-specific CAR gene-engineered T cells	8-Jul-19	I/II	China
NCT04265963	CD123 CAR-T cells	12-Feb-20	I/II	China
NCT04272125	CD123 CAR-T cells	17-Feb-20	I/II	China
NCT04230265	UniCAR02-T	18-Jan-20	I	Germany
NCT04318678	CD123-CAR T	24-Mar-20	I	United States
NCT04678336	CART123 cells	21-Dec-20	I	United States

cells, and biologic activity was observed by associated symptoms and increased cytokine levels, however, an anti-leukemic response was not documented, and no dose-limiting toxicities were observed (132). To explore the impact of costimulatory domains on the CD33 CAR-T cells, Qin et al. developed six CD33-targeted CARs with one of three scFv of clinically tested CD33 antibodies, paired with CD28 or 4-1BB costimulatory domains. These six CARs exhibited cytotoxicity against CD33+ AML cell lines *in vitro* and *in vivo* and showed strong anti-leukemia activity against patient-derived xenograft (PDX) derived from pediatric AML patients. Furthermore, CD28-based CD33 CAR-T cells exhibited superior anti-leukemia compared with 4-1BB, and the safety and efficacy in patients were evaluated in a phase I clinical trial (133). In 2022, Liu et al. proved that the third generation CD33 CAR-T showed stronger vitality, proliferation ability, and stronger cytotoxicity than the second generation CAR. Notably, the third generation CD33 CAR-T preferentially killed leukemia cells while sparing CD33-deficient HSPCs (134). CD33 CAR showed efficient anti-AML activity *in vitro*, different costimulators, different generation CAR structures, and PI3K inhibitors may affect the anti-tumor activity, proliferation, and persistence of CD33 CAR-T cells. The efficacy of CD33 CAR in patients did not show promising results in some previous studies, more efforts are needed to improve the anti-tumor response of CD33 CAR. 6 clinical trials are recruited to explore the efficacy and safety of CD33 CAR in AML patients, as shown in Table 5.

CLL-1

C-type lectin-like molecule-1 (CLL-1) is reportedly expressed in more than 80% of AML blasts, and LSCs. Its expression is restricted to the myeloid lineage and absent in normal CD34+CD38- HSCs. Significantly, CLL1 is present on a small subset of chemotherapy-resistant LSCs (135–137), suggesting that it may be a potential target for therapeutic intervention. In 2017, Tashiro et al. first designed a CLL1 CAR with specific killing efficacy on CLL1+ HL60 and THP1 cell lines and primary AML blasts *in vitro*, as well as exhibited anti-leukemia activity in an HL60-AML xenograft mouse model. Notably, CLL1 CAR-T cells eliminated mature normal myeloid cells yet selectively spared healthy HSCs, to allow immune recovery after therapy (138). Laborda and colleagues designed an scFv-based CLL1 CAR that showed cytotoxic activity against HL60 and MOLM14 AML cell lines and patient-derived AML blasts *in vitro*, as well as clearance against tumor cells in mouse xenografts, while without damaging healthy HSCs. Compared with CAR designs utilizing CD8 as a hinge domain, IgG4 induced higher cytotoxicity in cell lines (139). In 2018, Wang et al. constructed a new CLL1 CAR using scFv from C57BL/6 mouse-derived CLL antibody, which has good anti-leukemia activity to U937 cell line and primary AML blasts *in vitro* and eliminated human AML in xenograft mouse models without targeting normal HSCs (140). Liu et al. first explored the safety and efficacy of CLL1 CAR in patient. A compound CAR targeting CLL1 and CD33 was constructed and allennimumab was used to clear CAR-T cells after tumor eradication. A 6-year-old patient with a complex karyotype of FLT3-ITD mutation received two split doses of CAR-T cells and achieved CR on day 19, followed by HSCT (141). In 2019, Atilla and colleagues explored the impact of different combinations of spacers, transmembrane, and intracellular signaling domains to CLL-1 CAR. By comparing their proliferation, functional persistence, and antitumor activity *in vitro* and *in vivo*, the data showed that CD28z CAR with a short hinge region or with a CD8 intracellular domain is better than others for CLL1 CAR (142). In 2020, Zhang et al. reported the case of one 10-year-old patient with secondary AML treated with CLL CAR-T cells. This patient finally achieved morphological, immunophenotypic, and molecular CR over 10 months, which provides a new treatment option for secondary AML (143). Atilla et al. demonstrated that CLL-1 CAR T cells with additional transgenic IL15 supplementation, and combined with a TNFα blocker antibody as well as activation of caspase-9 control switcher increased expansion, persistence, and anti-leukemia of CLL1 CAR-T cells in PDX and HL60 xenograft mouse models while avoided excessive cytokine production (144). PD-1 expression was increased after the activation of CAR-T cells and caused T cells exhaustion, to evaluate the efficacy of combination of PD-1 silencing and CLL-1 CAR-T therapy, in 2021, Lin et al. designed a PD-1-silenced CLL-1 CAR and reported that PD-1 silencing enhanced the cytotoxicity of CLL-1 CAR (145). Similar to this, Zhang et al. designed a CLL-1 CAR based on the apoptosis-inducing gene FKBP-Caspase9 and evaluated the efficacy and safety in four R/R

TABLE 5 Recruiting CD33 CAR clinical trials.

NCT Number	Interventions	First posted	Phase	Locations
NCT03795779	CLL1-CD33 CAR T cells	8-Jan-19	I	China
NCT04010877	CLL-1, CD33 and/or CD123 CAR T cells	8-Jul-19	I/II	China
NCT03971799	CD33 CAR T cells	3-Jan-19	I/II	United States
NCT04835519	Functionally enhanced CD33 CAR T cells	8-Apr-21	I/II	China
NCT05248685	Dual CD33/CLL1 CAR T cells	21-Feb-22	I	China
NCT03927261	PRGN-3006 T cells	25-Apr-19	I	United States

AML patients. The author reported that three patients achieved CR with MRD negativity, while the fourth survived for 5 months, with manageable side effects (146). CLL1 CAR showed promising anti-tumor efficiency in pre-clinical experiments and anti-AML response in AML patients and selectively spared normal HSCs. There are 8 clinical trials for CLL-1 CAR currently recruiting, as shown in Table 6.

CD70

CD70 is a tumor necrosis factor (TNF) receptor ligand and is proven to be absent in normal tissues and hematopoietic cells, but highly expressed on most AML blasts and AML stem/progenitor cells (147, 148). In 2017, Riether et al. reported that CD70/CD27 signaling promotes blast stemness, and blocking CD70/CD27 by mAb could prolong survival in murine AML xenografts, representing that CD70/CD27 is a promising therapeutic strategy for AML (149). CD19 CAR-T cell therapy has presented revolutionary progression in CD19+ hematological malignancies, but some patients reccurence due to the exiting of CD19- tumor cells (150). Therefore, it is necessary to develop alternative antigens to avoid antigen escape. In 2021, Deng et al. designed L/H and H/L svFv-based CD70 CAR, truncated CD27-based CD70 CAR and anti-CD19 CAR as controls. The results showed that anti-CD70 (H/L) effectively killed CD19+ and CD19- Raji cells *in vitro* and in NSG xenograft mouse models, altogether providing a new therapeutic option for patients who have CD19- recurrence (151). Sauer et al. compared a panel of scFv-based CD70 CARs with the same scFv and different size and flexibility of the extracellular spacer, different transmembrane, and different costimulatory domains to CD27-based CD70 CAR. The results showed that the ligand CD27-based CD70 CAR presents superior proliferation and antitumor activity against AML cell lines Molm-13, THP-1, and IMS-M2 *in vitro* and Molm-13 AML mouse xenografts and primary AML *in vivo* (152). In 2022, Leick et al. used azacitidine to increase antigen density of CD7 in tumor cells and designed a CD8 hinger and transmembrane-modified CD27-based CD70 CAR to mitigate cleavage of the extracellular portion of CD27, altogether could enhance avidity and expansion of CD70 CAR-T cells and lead to more potent activity *in vivo* (153). The safety and efficacy of CD70 CAR in patients need more exploration in the clinical trial. There are only one clinical trial about CD70 CAR-T in AML currently recruiting as shown in Table 7.

In addition, some antigens such as CD38, FLT3 et al., can be potential targets for AML CAR-T therapy. The recruiting clinical trials are shown in Table 8.

Summary

Compared with B-ALL, T-ALL and AML are forms of leukemia that display more complex morphological features and are associated with poor prognosis. Furthermore, T-ALL and AML are associated with fewer treatment options after relapse or refractory. Given the significant success of CAR-T-cell therapy in B-cell malignancies, a similar approach for non-B-cell acute leukemia appears to represent a promising direction for the development of improved treatments. Although CAR-T therapy for non-B-cell leukemia still faces great challenges, researchers are already exploring multiple therapeutic targets, with promising results in preclinical and

TABLE 6 Recruiting CLL1 CAR clinical trials.

NCT Number	Interventions	First posted	Phase	Locations
NCT04884984	anti-CLL1 CAR T cells	13-May-21	I/II	China
NCT03795779	CLL1-CD33 cCAR T cells	8-Jan-19	I	China
NCT04010877	CLL-1, CD33 and/or CD123 CAR T cells	8-Jul-19	I/II	China
NCT04219163	CLL-1 CAR T cells	6-Jan-20	I	United States
NCT04789408	KITE-222	9-Mar-21	I	United States
NCT04923919	Anti-CLL1 CAR T cells	1-Jun-21	I	China
NCT05248685	Dual CD33/CLL1 CAR T cells	21-Feb-22	I	China
NCT05252572	CLL1 CAR T cells	23-Feb-22	I	China

TABLE 7 Recruiting CD70 CAR clinical trials.

NCT Number	Interventions	First posted	Phase	Locations
NCT004662294	CD70 CAR T-cells	10-Dec-20	I	China

TABLE 8 Recruiting other CAR clinical trials for AML.

NCT Number	Interventions	First posted	Phase	Locations
NCT05023707	Anti-FLT3 CAR-T	26-Aug-21	I/II	China
NCT05432401	FLT3 CAR-T	27-Jun-22	I	China
NCT05017883	FLT3 CAR-T	24-Aug-21	NA	China
NCT05488132	Anti-siglec-6 CAR-T	4-Aug-22	I/II	China
NCT04692948	CD276 CAR-T	5-Jan-21	NA	China
NCT04169022	IL1RAP CAR-T	19-Nov-19	NA	France
NCT04351022	CD38 CAR-T	17-Apr-20	I/II	China
NCT04662294	CD70 CAR T	10-Dec-20	I	China
NCT04803929	Anti-ILT3 CAR-T	18-Mar-21	I	China

clinical studies, such as with CD7, CD5, CD4, and other targets in T-ALL that overcome CAR-T-cell fratricide, tumor cell contamination and T-cell immunodeficiency. Furthermore, targets such as CD123, CD33, CLL1, and CD70 also show great promise for the treatment of AML. Taken altogether, the collective efforts of researchers and clinicians to develop CARs and deliver them in current clinical trials will fulfill the promise to find effective treatments for non-B-cell leukemia.

Author contributions

WW drafted the original manuscript and designed the figures. DY, XC and DL edited the manuscript. LZ and XZ reviewed, revised, and supervised the work.

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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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EDITED BY
Ran Gao,
Chinese Academy of Medical Sciences and Peking Union Medical College, China

REVIEWED BY
Sudjit Luanpitpong,
Mahidol University, Thailand
Xiaoguang Liu,
University of Texas MD Anderson Cancer Center, United States

*CORRESPONDENCE
Yan-Qiu Han
qyh1016@sina.com

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Identification of autophagy-associated genes and prognostic implications in adults with acute myeloid leukemia by integrated bioinformatics analysis

Jing Zhang [1,2], Ying-Jun Wang [2,3] and Yan-Qiu Han [1,2,3]*

[1]Department of Hematology, the Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China, [2]National Clinical Research Center for Hematologic diseases, the First Affiliated Hospital of Soochow University, Suzhou, China, [3]Department of Laboratory Medicine, the Affiliated Hospital of Inner Mongolia Medical University, Hohhot, China

Acute myeloid leukemia (AML) is one of the most common malignant blood neoplasma in adults. The prominent disease heterogeneity makes it challenging to foresee patient survival. Autophagy, a highly conserved degradative process, played indispensable and context-dependent roles in AML. However, it remains elusive whether autophagy-associated stratification could accurately predict prognosis of AML patients. Here, we developed a prognostic model based on autophagy-associated genes, and constructed scoring systems that help to predicte the survival of AML patients in both TCGA data and independent AML cohorts. The Nomogram model also confirmed the autophagy-associated model by showing the high concordance between observed and predicted survivals. Additionally, pathway enrichment analysis and protein-protein interaction network unveiled functional signaling pathways that were associated with autophagy. Altogether, we constructed the autophagy-associated prognostic model that might be likely to predict outcome for AML patients, providing insights into the biological risk stratification strategies and potential therapeutic targets.

KEYWORDS

acute myeloid leukemia, autophagy, prognosis, biomarker, bioinforamtics

Introduction

Acute myeloid leukemia (AML), the most common acute leukemia in adults, is a heterogeneous group of leukemias characterized by aberrant clone transformation of hematopoietic precursors through the acquisition of chromosomal arrangements and abnormal gene expression patterns, exhibiting partial or complete arrest of maturation in the bone marrow, peripheral blood or other tissues (1). With application and refinement of the detection methodology such as chromosome banding, fluorescence *in situ* hybridization/ chromosomal painting and the next generation sequencing, there have been incremental understanding of abnormal genetic and molecular alterations in the pathogenesis of AML (2).With these efforts, it is gradually accepted that AML is rather an umbrella diagnosis that comprises diverse subtypes with different prognostic and predictive markers, which are recommended for distinguished classification criteria and require selective and possible targeted therapies (3, 4). However, approximately half of AML patients lack predicable or prognostic biomarker and widely variable transcriptome data and the overall prognosis remains dismal [5-year overall survival 28.7%] (5), highlighting the need for identifying novel genetic and molecular predictors.

Autophagy is a critical intracellular degradative process, leading to the turnover of cellular material and providing macromolecular precursors (6). Dysfunctional autophagy has been implicated linked to multiple disorders especially in cancer cells (7), but its biological roles vary a lot and the pathophysiologic mechanism has not yet been fully elucidated. As is mentioned, autophagy is required for hematopoietic stem cell (HSC) survival as well as normal hematopoiesis (8). Dysfunctional autophagy raises the occurrence of hematological malignant neoplasms especially in leukemia (9). However, the exact role of autophagy in leukemogenesis remains debatable, in that it appears to be both leukemia-promoting and -suppressive. Some thought that reduced level of autophagy-related genes might be beneficial for AML cells due to decreased autophagic flux with accumulation of impaired mitochondrial within leukemic cells. For example, a body of evidence suggested that key autophagy genes such as *ULK1*, *ATG3*, *ATG4D* and *ATG5* were significantly downregulated in primary AML cells compared to normal granulocytes (10). Lower expression of *Beclin-1*, *LC3*, *UVRAG*, *Rubicon* and *NBR1* were identified in the high-risk AML patient group with higher white blood cell(WBC) counts and worse overall survival (11). Marine studies demonstrated that inhibition of autophagy by deletion of *Atg5* or *Atg7* prolonged survival in leukemic mice and decreased functional leukemia-initial cells (LICs) (12). However, others found that the autophagy flux was significantly higher in AML patients with TP53 mutations and inactivation of the autophagy triggered a p53-dependent increase in apoptosis in AML $CD34^+$ cells (13). Nguye et al. reported loss of the autophagy receptor p62 deteriorated the expansion and colony-forming ability and impaired leukemia progression in murine models (14). Moreover, autophagy could lower the risk of myelodysplastic syndromes (MDS) progression to AML by suppress ROS levels (15).

Given the above, it is reasonable to believe that autophagy participates in the initiation and progression of AML due to its diverse roles. A deep understanding of autophagy in AML might contribute to identifying novel biomarkers in terms of diagnosis, risk stratification, prognosis as well as potential therapeutic targets. Given the functional role and therapeutic potential of autophagy in AML, we screened 12 key genes from autophagy-associated genes, and constructed risk scores that significantly predicted survival outcomes of AML patients in the study. The scoring system was also validated in an independent AML cohort. Moreover, we explored the biological pathways in that autophagy-associated genes are mainly involved. Overall, our study illustrates that the autophagy-associated model which might provide previously unrecognized risk stratification options for AML patients, shedding novel insights on potential personalized therapeutic strategies.

Methods

Selecting autophagy-associated signature

Autophagy-associated genes were collected by retrieving the GeneCards website (https://www.genecards.org/) using the term "autophagy". Relevance scores denote the correlation between autophagy activity and individual genes. A total of 117 autophagy-associated genes were identified with relevance score at the criteria of $|\log FC| \geq 1$ and P-value< 0.05.

Data collection

The gene expression matrix and corresponding clinical parameters of AML patients were collected from TCGA database, consisting of 200 adult patients and 51normal controls. The validation cohorts were downloaded from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/), under the accession of GSE23143 (16).

Hierarchical clustering analysis and principal component analysis

Hierarchical clustering analysis of the Euclidean distance of all collected autophagy-associated genes was used to investigate

the subtypes of AML. The discrimination and accuracy of the subtypes of AML patients were further evaluated by principle component analysis (PCA).

Construction of prognostic model based on autophagy-associated genes

Differential expression analyses were used to filter autophagy-associated genes that were also significantly differentially expressed between the high-risk groups and low-risk groups. Count matrices were loaded in R package "limma" (version 3.38)[PMID: 28367255]. Significant genes were selected at the criteria of |log2 fold change | > 1 and false discovery rate (FDR)< 0.05. In total, 6061 genes were kept for the following analyses.

The optimal autophagy-associated prognostic model was constructed by multivariable Cox regression method with the least absolute shrinkage and selection operator (LASSO) algorithm in the R package "glmnet" (version 2.0-18) (17). Using the 10-fold cross-validation, the best lambda that achieved the best model performance was selected. The risk score was calculated using the formula below.

$$risk\ \ score = \sum_{j=1}^{n} coef\ \ (j) * Expr(j)$$

Coef(j) denotes the coefficient of j gene in the Cox model, and Expr(j) represents the expression levels of autophagy-related gene j. The median risk score was selected as the cutoff to separate AML patients into high-risk and low-risk groups. The same method was applied in another independent AML cohort (GSE23143), to further evaluate the predictive power of the Cox model that was trained in TCGA data.

The time-dependent receiver operating characteristic curve (ROC) was used to estimate the sensitivity and specificity in the R package "survival ROC" (version 1.0.3) (18). The area under the curve (AUCs) estimated the prognostic accuracy for 1-, 3-, and 5-year overall survival respectively, to evaluate the predictive power of survival prediction using the selected 12 autophagy-related genes. Kaplan-Meier survival curve analysis was performed and visualized by R package "survival" (version 3.1-12). Log-rank test was applied to check the significant differences between high-risk and low-risk groups. Multivariate Cox regression and univariate Cox regression were applied to investigate the associations between genes within the 12 genes and overall survival.

Pathway enrichment analysis and regulatory network

Pathway enrichment analyses in Gene Ontology (GO) databases, including biological process, cellular component, and molecular function, were performed and visualized in the R package "clusterProfiler" (version 3.10.1) (19). These analyses were also conducted in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/). Significant pathways were identified as these with adjusted p values less than 0.05.

Protein-protein interaction networks were built and visualized on STRING website (https://string-db.org/), with all autophagy-associated genes.

Construction of nomogram model

Individual genes within 12 selected autophagy-associated genes were used to build a nomogram, using the R package "survival" and "rms" (version 6.0-1). Calibration curves were plotted to evaluate the concordance between actual survival and predicted survival for 6 months, 1 year, and 3 years. The concordance index (C-index) was used to measure the model performance for predicting prognosis.

Statistical analysis

All the statistics were conducted in the R software (version 3.5.2). The Wilcoxon test was applied to compare two groups with nonnormally distributed data. The Kruskal-Wallis tests were used for comparing more than two groups with nonnormally distributed data. Correlation coefficients were assessed by Spearman or Pearson correlations. Statistical significance in survival analysis was determined by the log-rank test. Significant p values were denoted as follows: ns ≥0.05, *<0.05, **<0.01, ***<0.001, and ****<0.0001. The statistical information for the experiments is detailed in the figure legends.

Results

Identification of prognostic signatures from autophagy-associated genes in AML

A total of 200 AML patients and 51 matched healthy controls (HC) were collected in TCGA database, and the autophagy-related genes were retrieved from Genotype-Tissue Expression database. Based on hieratical clustering of gene expressions of autophagy-related genes, the AML patients and HCs were divided into two different clusters (Figure 1A). Consistently, the principle component analysis also indicated that AML patients were separated from HCs clusters (Figure 1B). These results suggested that autophagy-related genes expressed differently between AML patients and HCs in general.

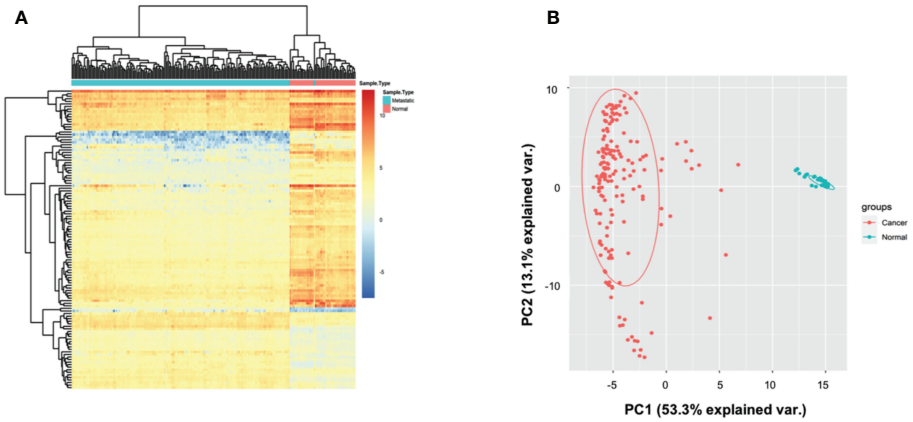


FIGURE 1
Autophagy-associated genes distinguished AML patients from controls. **(A)** Heatmap showing that the hieratical clustering of autophagy-associated genes separated AML patients from normal controls. **(B)** Scatterplot showing the gene expressions of autophagy-associated genes in AML patients are different from normal controls by PCA analysis.

To recognize the prognosis-related genes from the autophagy-associated genes, we identified 117 differentially expressed genes (DEGs) between AML patients and normal healthy controls, and then performed Cox regression model with LASSO algorithm. Using the best lambda parameters in the 10-fold validations, we finally selected 12 hub autophagy-associated genes, including *APOL1*, *BAG1*, *BAG3*, *BAX*, *CAPN10*, *DNAJB2*, *KLHL24*, *P4HB*, *RAC1*, *RAF1*, *SERPINA1*, and *SIRT1* (Figures 2A, B), which, as expected, were significantly differentially expressed in AML patients (Figure 2C). Univariate Cox analysis revealed that some of the 12 genes were correlated to the overall survival (OS) of AML patients. For instance, *KLHL24* has a hazard ratio of less than 1 in AML patients and was regarded as a protective gene (HR=0.71, 95% CI=0.53-0.94, P=0.016, Figure 2D), while *BAG3* was considered a risk gene (HR=1.23, 95% CI=1.08-1.40, P=0.002, Figure 2D). Multivariate Cox analyses were also conducted to confirm that *BAG3* was significantly correlated with worse overall survivals of AML patients (HR=1.201, 95% CI=1.021-1.412, P=0.027, Figure 2E). These analyses are consistent with the indispensable role of *BAG3* in cancer progression and tumor resistance to therapy (20).

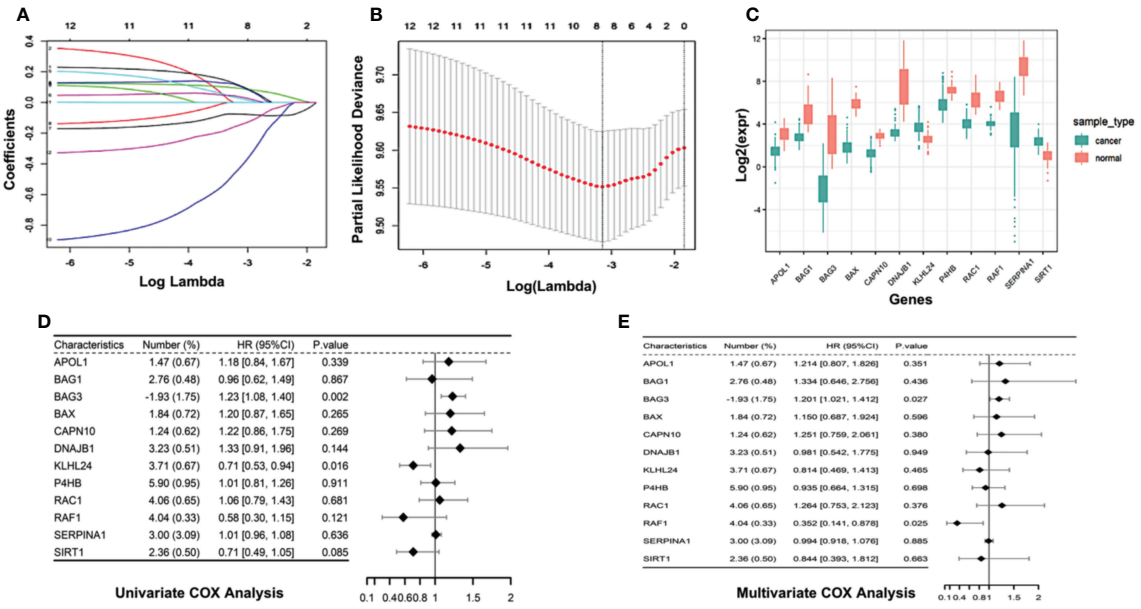
Reconstruction of prognostic evaluations for AML patients

We then calculated the risk score for individual AML patients based on the gene expression patterns and coefficients in LASSO model. Specifically, the risk score was the sum of gene expressions of the 12 autophagy-associated genes weighted by their corresponding coefficients in multivariable LASSO regression (Methods). The unsupervised hieratical clustering of gene expression profiles of 12 autophagy-associated genes exhibited that their expressions and the corresponding risk scores were confounded by age, gender and race in AML (Figure 3A). AML patients were then divided into the high-risk and low-risk groups based on the median value of risk scores (Figure 3B). Next, we evaluated the predictive power of the risk score in prognosis of AML patients. Compared with age and gender, risk scores indicated the highest hazard ratio with the OS of AML patients in the univariate COX regression model (HR=23, 95% CI=7.1-72, P<0.001, Figure 3C). These results revealed that the risk scores were an independent prognostic predictor in AML.

Evaluation of the prognostic model in AML

To evaluate the predictive power of the risk score in AML prognosis, we conducted Kaplan-Meier analyses, and found the AML patients with high-risk scores showed significantly worse overall survivals than patients with relatively low-risk scores in TCGA database (Figure 4A). The time-dependent ROC curve also revealed that the risk scores were capable to predict 6-month (Figure 4D), 1-year (Figure 4E) and 3-year (Figure 4F) survivals with area under curve more than 0.579 (AUC for 6 months, 0.579; AUC for 1 year, 0.729; AUC for 3 years, 0.803, Figure 4B).

Next, we developed a nomogram model of individual autophagy-associated genes to predict patients' survivals (Figure 4C). The calibration curve showed that the combination of these autophagy genes achieved better performance for AML patients' 6-month, 1-year prognosis, compared with each one alone (Figure 4D). These findings consistently revealed the success of 12 autophagy-related genes



Characteristics	Number (%)	HR (95%CI)	P.value
APOL1	1.47 (0.67)	1.18 [0.84, 1.67]	0.339
BAG1	2.76 (0.48)	0.96 [0.62, 1.49]	0.867
BAG3	-1.93 (1.75)	1.23 [1.08, 1.40]	0.002
BAX	1.84 (0.72)	1.20 [0.87, 1.65]	0.265
CAPN10	1.24 (0.62)	1.22 [0.86, 1.75]	0.269
DNAJB1	3.23 (0.51)	1.33 [0.91, 1.96]	0.144
KLHL24	3.71 (0.67)	0.71 [0.53, 0.94]	0.016
P4HB	5.90 (0.95)	1.01 [0.81, 1.26]	0.911
RAC1	4.06 (0.65)	1.06 [0.79, 1.43]	0.681
RAF1	4.04 (0.33)	0.58 [0.30, 1.15]	0.121
SERPINA1	3.00 (3.09)	1.01 [0.96, 1.08]	0.636
SIRT1	2.36 (0.50)	0.71 [0.49, 1.05]	0.085

Characteristics	Number (%)	HR (95%CI)	P.value
APOL1	1.47 (0.67)	1.214 [0.807, 1.826]	0.351
BAG1	2.76 (0.48)	1.334 [0.646, 2.756]	0.436
BAG3	-1.93 (1.75)	1.201 [1.021, 1.412]	0.027
BAX	1.84 (0.72)	1.150 [0.687, 1.924]	0.596
CAPN10	1.24 (0.62)	1.251 [0.759, 2.061]	0.380
DNAJB1	3.23 (0.51)	0.981 [0.542, 1.775]	0.949
KLHL24	3.71 (0.67)	0.814 [0.469, 1.413]	0.465
P4HB	5.90 (0.95)	0.935 [0.664, 1.315]	0.698
RAC1	4.06 (0.65)	1.264 [0.753, 2.123]	0.376
RAF1	4.04 (0.33)	0.352 [0.141, 0.878]	0.025
SERPINA1	3.00 (3.09)	0.994 [0.918, 1.076]	0.885
SIRT1	2.36 (0.50)	0.844 [0.393, 1.812]	0.663

FIGURE 2
Construction of autophagy-associated prognostic model in TCGA-AML cohort. **(A)** 10-fold cross-validation of tuning parameter selection in LASSO model using the TCGA- AML data. **(B)** LASSO coefficient of the selected 12 autophagy-associated genes. **(C)** Boxplot comparing the difference in gene expression of the selected 12 autophagy-associated genes between AML patients and normal controls. **(D, E)** Univariate Cox analysis **(D)** and multivariate Cox analysis **(E)** show the coefficients and corresponding p values of individual genes within 12 autophagy-associated genes.

in predicting patient survivals, suggesting the critical role of autophagy signaling in the development of AML progression.

Validating the performance of the autophagy-associated prognostic model

We further validated the prognostic model in independent dataset (GSE23143), including the 200 AML patients. Applied the same methods, we calculated the risk scores based on the 12 autophagy-associated genes, and then divided AML patients into two groups, including high-risk and low-risk groups. Consistent with the results in TCGA data (Figure 4A), we found the high-risk groups showed the significantly worse overall survivals for 6 months, 1 year, and 3 years (p-value = 0.035 for 6 months; p-value = 0.048 for 1 year; p-value = 0.007 for 3 years; Figures 5A-C). These results suggested the effectiveness of autophagy-associated model in predicting prognosis in AML cohorts.

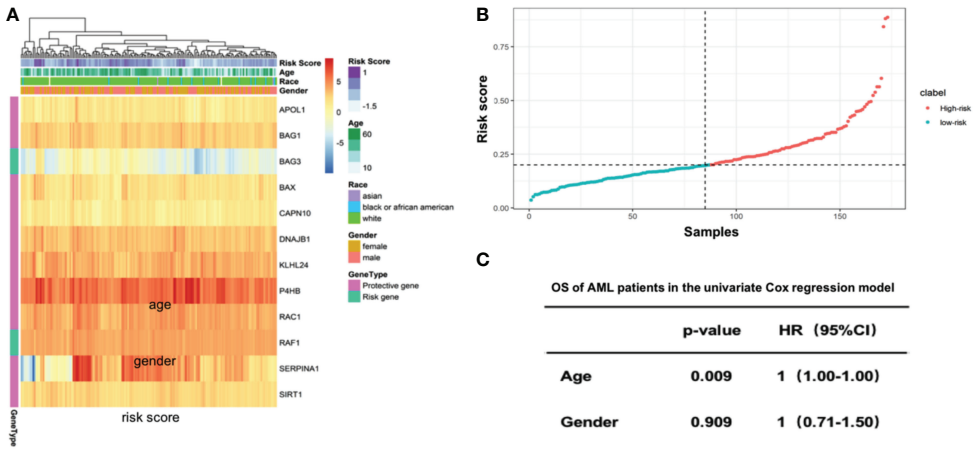
Pathway analyses showed the autophagy-related signaling pathways

In order to decipher the biological pathways related to autophagy-related risks, we viewed the 12 autophagy-associated genes as baits to find 106 most related neighbor genes in TCGA-AML cohort. Totally, 106 genes were identified as neighbor genes. Biological pathway analysis in GO database showed that cellular response to nutrients levels and response to starvations pathways were prominently related to the 12 genes, in addition to autophagy pathway (Figure 6A). Cell component analyses in GO databases showed consistently enriched in the autophagosome and autophagosome membrane (Figure 6B). Molecular function enrichment displayed several related functions, such as protein serine/threonine kinase activity, ubiquitin-like protein ligase binding and ubiquitin protein ligase binding (Figure 6C). These findings are also in line with the fact that the ubiquitin-proteasome system and autophagy are two major quality control systems responsible for protein degradation (21).

Pathway enrichment in KEGG database showed several disease-related pathways, including amyotrophic lateral sclerosis, shigellosis, and Kaposi sarcoma-associated herpesvirus infection (Figure 6D). The significant enrichments of disease pathways indicated that the 12 autophagy-associated genes might play an essential role in the development of these human diseases. The tight protein-protein interaction network of the 12 autophagy genes and their neighbor genes reflect their close relationships as well (Figure 6E).

Discussion

The genetic alterations in AML are highly heterogeneous and the manifestation of the disease is distinguishing in each



	p-value	HR (95%CI)
Age	0.009	1 (1.00-1.00)
Gender	0.909	1 (0.71-1.50)
Risk score	< 0.001	23 (7.10-72.00)

FIGURE 3
Risk score of 12 selected autophagy-associated genes is an independent predictor of patient survival in the AML cohort. **(A)** Heatmap showing the gene expression of the 12 selected autophagy-associated genes. Columns were annotated by patient race, gender and age. **(B)** Rank of risk scores in AML patients who were divided into high-risk and low-risk groups by the median value of risk score. **(C)** Multivariate Cox analysis shows the coefficients and corresponding p values in the model with risk score, age and gender as covariates.

patient. New therapeutic targeted drugs have shown promising effects but these are directed only to specific AML subgroups (22). There still remains around half of the patients who cannot be reasonably categorized due to a lack of prognostic biomarkers. Given this context, there is urgent need to better understand the molecular mechanisms pathogenesis involved in AML and novel classification systems are needed to improve the accuracy of predicting patients' prognosis.

In this study, we focused on the autophagy-associated genes due to their essential roles in leukemogenesis. We constructed a multivariate prognostic model and identified 12 key autophagy associated genes that indicated significant relevance with prognosis. The filtered 12 genes provided previously unrecognized stratification strategies for AML patients, and also potentially promising targets for AML treatments (23, 24).

We constructed the autophagy-based risk scores based on 12 LASSO regression-selected prognostic genes. The scoring systems are robust to predict the outcome of AML patients in both TCGA-AML cohorts and another independent cohort. Multivariate Cox analysis also revealed that the risk scores were an independent factor, but not the age and gender. These findings are consistent with previous studies that have reported autophagy was involved in cancer initiation *via* regulating many oncogenes and tumor suppressor genes (25–27). Altogether, our analysis suggested the undermined role of autophagy in AML development.

Among the selected 12 key genes, we identified several genes which have been reported to participate in tumor prognosis and tumorigenesis. For instance, Bcl2-associated athanogene-3 (BAG3), also known as CAIR-1(CAI-stressed-1), belongs to the family of co-chaperones interacted with the ATPase domain of the hear shock protein Hsp 70 *via* the structural domain known as BAG domain(110-124 amino acids) (28). *BAG3* gene expression is constitutive in normal cells such as the skeletal and heart muscles, while aberrant expressed *BAG3* is also found in neoplastic cell lines as well as primary AML and CML cell (29, 30). Studies has confirmed that overexpressed *BAG3* could reverse the pro-apoptotic effect of WT1 silencing and regulate the leukemia stem cell-supporting activity (31). Further evidence demonstrated that *BAG3* down-modulation resulted in a reduction of the anti-apoptotic protein level such as MCL1, BCL2 and BCL-XL, which are capable of regulating autophagy in AML cells (32). It is expected that *BAG3* will serve as a key player in leukemogenesis and potential therapeutic drug target (33–35). RAC1, a member of the Ras superfamily of small guanosine triphosphatases (GTPases), is capable to activate several signaling pathways and cytoskeletal arrangements, resulting in cell cycle progression, morphogenesis, migration as well as autophagy (36). Abnormal overexpression has been regularly reported in cancer. Early research indicated that inactivation of RAC1-GTPase suppressed migration and promoted drug induced apoptosis in KG-1 cells (37). Recent *in vitro* studies found that suppression of RAC with a RAC inhibitor (EHT-1864) could increase autophagy, apoptosis, cell cycle, modulation of p53 factor and inhibit the PI3K/AKT/mTOR signaling pathway in

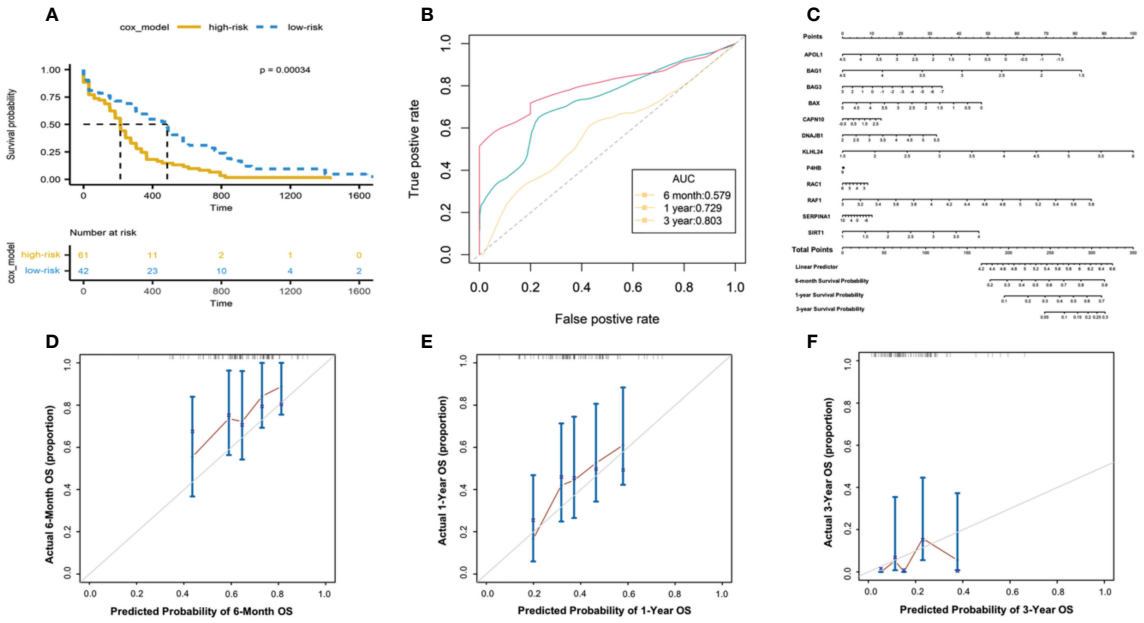


FIGURE 4
Evaluating the predictive power of risk scores in TCGA-AML cohorts. **(A)** Kaplan-Meier analysis based on the 12-gene signature in TCGA- AML cohort. Color denotes patients' stratifications based on risk scores. The table below shows the alive patients at individual time points. **(B)** The ROC curve showing the AUC of the predictions for 6-month, 1-year and 3-year survivals. **(C)** Nomogram constructed between the 12 autophagy-associated genes and 6-month, 1-year and 3-year survivals. The calibration plot of the nomogram showing the observed survivals and Nomogram-predicted survivals for 6-month **(D)**, 1-year **(E)** and 3-year **(F)** survivals.

AML cell lines (38). In fact, it has recently been reported that the combination of EHT-1846, venetoclax(BCL-2 inhibitor) and midostaurin(FLT3 inhibitor) could reverse midostaurin resistance in AML cells (39). Sirtuin 1, known as NAD-dependent deacetylase sirtuin-1, promoted cancer cell proliferation and metastasis *via* STAT3/MMP-13 signaling (40), which is also found participated in the abnormal metabolism pathways in AML. Recent study suggested that SIRT1 was a downstream factor of AdipoR1 and ANRIL in glucose metabolism and regulate AML cell survival (41). Apolipoprotein L1 (APOL1) functions as both extra- and intra-cellular regulators in host innate immunity and cellular homeostasis in the kidney (42). It is worth noting that these identified autophagy genes have not been fully recognized in the development of hematopoietic disorders especially in AML, which might provide novel promising molecular targets and help to predict the outcomes, monitor the minimal residue disease and find therapeutic targets in AML.

The protein-protein interaction analysis and pathway enrichment results demonstrate that autophagy is related to environmental stimulations. Similarly, autophagy can respond to a wide spectrum of cellular stresses, including nutrient

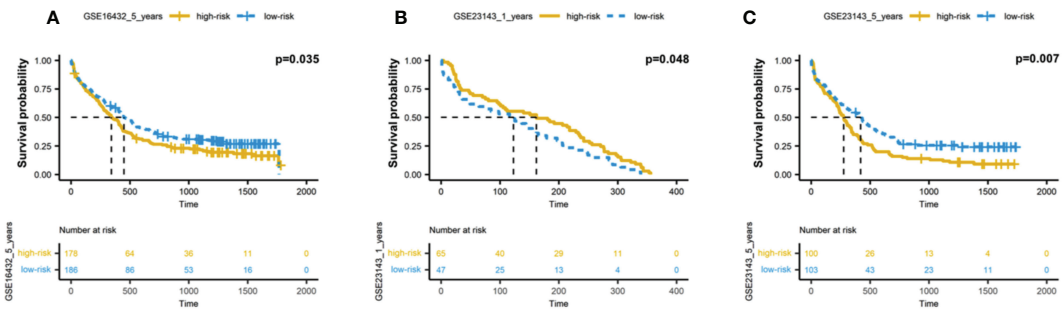


FIGURE 5
Validation of autophagy-associated prognostic model in independent AML cohort. **(A-C)** Kaplan-Meier analysis of 6-month **(A)**, 1-year **(B)** and 3-year **(C)** survivals based on the 12 autophagy-associated genes in GSE23143 cohort.

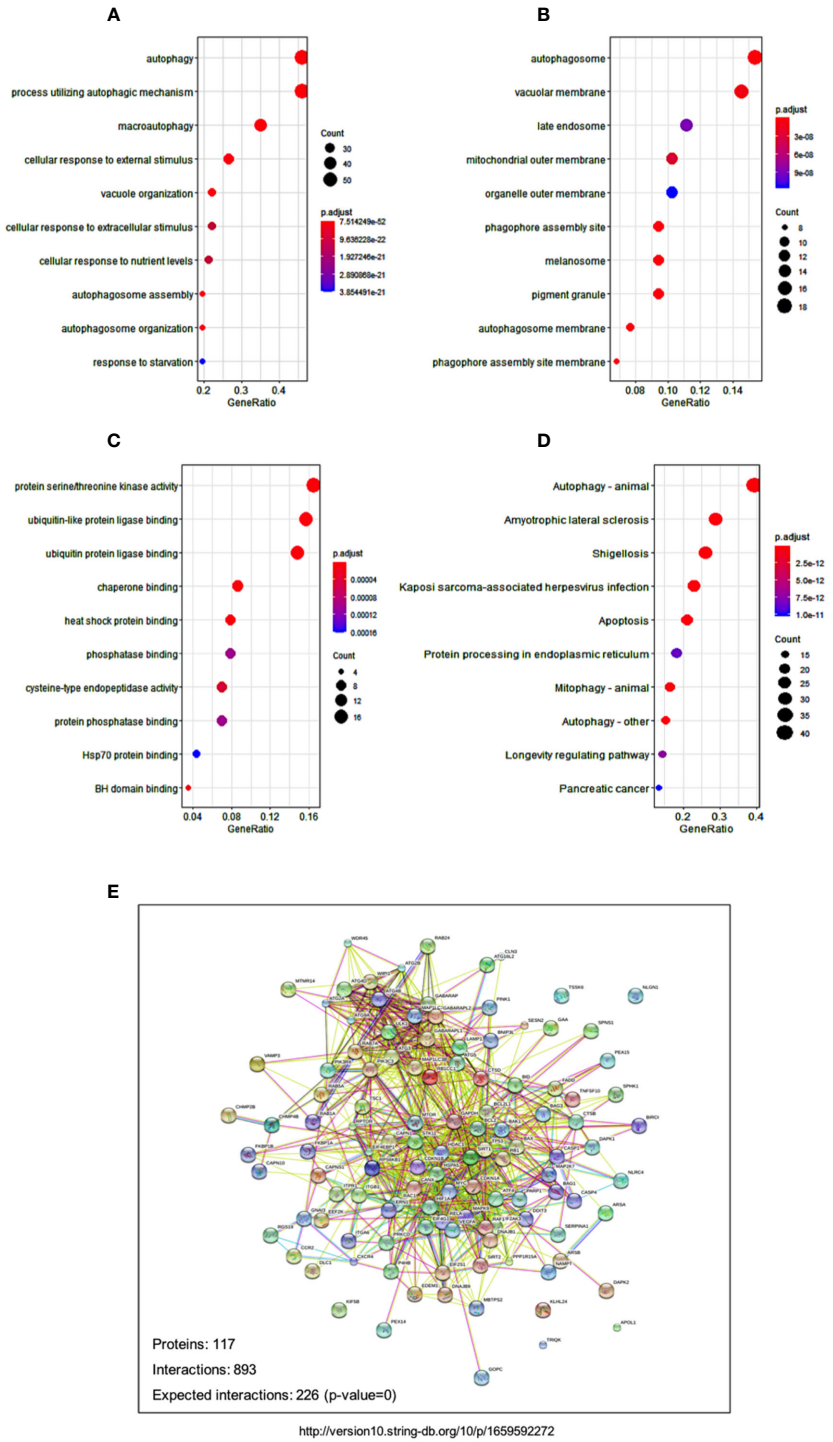


FIGURE 6
Signaling and networks related to autophagy-associated genes. **(A-C)** Dot plot reveals the GO enrichment of autophagy-associated genes, showing the significant biological pathways **(A)**, cellular components **(B)**, and molecular functions **(C)**, respectively. **(D)** Dot plot displays the significant pathways that were enriched in KEGG databases. Dot sizes denote the count number of enriched genes in the pathway, and dot color represents the adjusted p value of the enriched pathway. **(E)** Protein-protein interaction network shows the relationships among autophagy-associated proteins in the STRING database.

deprivation, hypoxia, and abnormal macromolecule accumulation (43, 44). Also, KEGG enrichment analysis revealed the significant enrichment of disease-related pathways, which could be partly explained by its essential functions for cell survival, bioenergetic homeostasis, and intracellular component degradation (45). Indeed, it is gradually recognized that autophagy might be responsible for tumorgenesis in multiple direct and indirect signaling ways. For example, aberrations in metabolic rewiring has been described in leukemogenesis due to dysfunctional autophagy in recent years (46). As our analysis aimed mainly to the autophagy, it is insufficient to fully display the pathways of autophagy in leukemia pathogenesis. Additionally, in different setting of chemotherapy, immunotherapy as well as hematopoietic stem cell transplantation, the role of the identified autophagy-genes in AML still remains largely unknown and more objective proof of this waits further experimental testing and detailed functional analyses.

Besides that, there are other limitations in our study. This study aimed to identify a prognostic autophagy-associated gene signature in patients with AML. However, our analysis was mainly based on TCGA and GEO databases which could have biased our conclusions. Considering this, more validations in larger clinical population groups are required to provide more applicable results and enhance the clinical application value as prognostic tools in the AML patients.

Taken together, we recognized a 12-autophagy-associated gene signature which might likely act as an independent predictor of prognosis based on multiple AML cohorts. A nomogram model and Cox regression analyses revealed the accuracy of the gene signature in predicting 6-month, 1-year and 3-year survival probability for individual AML patients. Pathway enrichment analyses demonstrated the potentially related biological pathways of autophagy. Our finding illustrates that the 12-autophagy gene signature would provide new insight into a better understanding of autophagy in AML. Although further validation is needed, we hope it will provide promising prognostic significance and potential therapeutic targets in AML treatment.

Data availability statement

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

Author contributions

JZ, YQH designed the project and supervised the typescript preparation. JZ and YJW performed the analyses and interpreted all the data. JZ prepared the figures and tables. YQH reviewed and revised 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.

Publisher's note

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EDITED BY
Jean El Cheikh,
American University of Beirut Medical Center, Lebanon

REVIEWED BY
Jiaqian Qi,
The First Affiliated Hospital of Soochow University, China
Tamim Alsuliman,
Hôpital Saint-Antoine, France

*CORRESPONDENCE
Xin Chen
chenxin@ihcams.ac.cn
Sizhou Feng
doctor_szhfeng@163.com
szfeng@ihcams.ac.cn

†These authors have contributed equally to this work

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Venetoclax plus hypomethylating agents versus intensive chemotherapy for hematological relapse of myeloid malignancies after allo-HSCT

Zhangjie Chen[1,2], Sisi Zhen[1,2], Tingting Zhang[1,2], Yuyan Shen[1,2], Aiming Pang[1,2], Donglin Yang[1,2], Rongli Zhang[1,2], Qiaoling Ma[1,2], Yi He[1,2], Jialin Wei[1,2], Weihua Zhai[1,2], Xin Chen[1,2†*], Erlie Jiang[1,2], Mingzhe Han[1,2] and Sizhou Feng[1,2†*]

[1]State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Haihe Laboratory of Cell Ecosystem, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China, [2]Tianjin Institutes of Health Science, Tianjin, China

Introduction: Since allogeneic stem cell transplantation (allo-HSCT) is considered one of the curative treatments for acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), hematological relapse following allo-HSCT remained a crucial concern for patients' survival.

Methods: We retrospectively compared patients who received venetoclax plus hypomethylating agents (VEN+HMA, n=23) or intensive chemotherapy (IC, n=42) for hematological relapse of myeloid malignancies after allo-HSCT. HMA selection included decitabine (n=2) and azacitidine (n=21), and combined donor lymphocyte infusion was administered to 21 and 42 patients in VEN+HMA and IC groups, respectively.

Results: Median age of all patients was 39 (16-64) years old. Overall response rates, including complete response (CR), CR with incomplete recovery of normal neutrophil or platelet counts (CRi) and partial response (PR), were not significantly different between VEN+HMA and IC groups (60.1% versus 64.3%, P=0.785). CR/CRi rate was 52.2% in VEN+HMA and 59.5% in IC group (P=0.567). The rate of relapse after response was 66.7% in VEN+HMA group and 40.7% in IC group (P=0.176). Median overall survival was 209.0 (95%CI 130.9-287.1) days for VEN+HMA group versus 211.0 (95%CI 28.7-393.3) days for IC group (P=0.491). The incidence of lung infection (17.4% versus 50.0%, P=0.010), thrombocytopenia (73.9% versus 95.2%, P=0.035) and acute graft-versus-host disease (aGvHD) (50.0% versus 13.0%, P=0.003) was significantly higher in IC group.

Discussion: In conclusion, VEN+HMA is not inferior to IC regimen in terms of improving response and survival, and is associated with a lower incidence of adverse events and aGvHD. However, further research is required to enhance long-term survival.

KEYWORDS

venetoclax, myeloid malignancy, acute myeloid leukemia, allo-HSCT, relapse

Introduction

As a curative therapies, allogeneic stem cell transplantation (allo-HSCT) plays a crucial role in treating acute myeloid leukemia (AML) and high high-risk myelodysplastic syndrome (MDS), particularly for prolonging relapse-free survival and overall survival in patients with intermediate- and poor-risk AML (1). However, up to half of the patients may experience post-transplantation relapse, depending on disease status and patients' characteristics (2, 3). Relapse often occurs during the 3-6 months following transplantation, with an overall survival of only 19% at 2 years (4). Intensive chemotherapy, donor lymphocyte infusion and second-HSCT have been utilized without significant success (5–8), indicating a need for further investigation of appropriate treatment protocols for relapse of myeloid malignancies after allo-HSCT.

BCL-2 and its inhibitors have been the subject of increasingly deepened hematological research, starting with the study of follicular lymphoma conducted by Fukuhara et al. (9) Venetoclax, the most clinically promising BCL-2 inhibitor, has been granted approval by FDA in combination with hypomethylating agents (HMA) for the treatment of newly diagnosed AML in patients not tolerant to intensive chemotherapy. Additionally, recent studies have demonstrated the impressive efficacy of venetoclax plus intensive chemotherapy for newly diagnosed and relapsed/refractory (R/R) AML (10, 11). The combination treatment of venetoclax and HMA in R/R AML patients has also been reported with varying remission rates and survival (12–15). However, its safety and effectiveness compared to other regimens in post-transplantation relapse has yet to be determined. In this retrospective study, we investigated 65 patients treated with either venetoclax plus hypomethylating agents (VEN+HMA) (n=23) or intensive chemotherapy (IC) (n=42) for hematological relapsed myeloid malignancies after allo-HSCT and compared response, survival, graft-versus-host disease (GvHD) and adverse events between the two regimens.

Methods

Patients

A retrospective analysis of clinical data was performed on 65 patients diagnosed with relapse of myeloid malignancy after allo-HSCT, who were treated at the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, between November 2013 and December 2022. The study included 23 patients who received VEN+HMA and 42 patients who received IC. Patients who were initially diagnosed with primary or secondary AML or MDS and experienced hematological relapse after allo-HSCT were included in the study, while patients with severe organic dysfunction were excluded. Risk stratification, diagnosis of relapse and response criteria were according to European Leukemia Network 2017 criteria (16). Overall response rate (ORR) was defined as CR+CRi +PR. MRD positivity is defined as >0.01% myeloid blasts detected by multiparameter flow cytometry or >0.001% leukemia-associated genes detected by RT-qPCR. This study was approved by the ethical committee of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, and informed consent forms were obtained from all patients.

Treatments and efficacy evaluation

Azacitidine (50mg/m^2/d for 5 days) was used as prophylactic therapy in 4 patients after allo-HSCT. All relapsed patients discontinued immunosuppressants after diagnosis. In VEN+HMA group, venetoclax was gradually increased to a maximal dose of 400mg/d in 3 days and each treatment cycle was 14-28 days. Combined hypomethylating agents include azacitidine (75mg/m^2/d for 7 days) or decitabine (20mg/m^2/d for 5 days). Furthermore, eleven patients in VEN+HMA group received low-dose cytarabine (20 mg/m^2 twice daily) for 14 days. Patients in IC group received CLAG or FLAG (cladribine 5mg/m^2/day or fludarabine 30mg/m^2 plus cytarabine 1-2g/m^2/day plus G-CSF 5 ug/kg for 5 days) or IDAC, including cytarabine 1 g/m^2/q12h plus mitoxantrone 8-10 mg/m^2/d or idarubicin 8-12 mg/m^2/d or daunorubicin 45-60 mg/m^2/d or amsacrine 100 mg/m^2/d for 3 days. Previous unsuccessful regimens were avoided in the selection of IC regimens. Donor lymphocyte infusion (DLI) was obtained from previously cryopreserved donor graft or donor's peripheral blood. Concomitant DLI infusion was administered in 63 patients, and calcineurin inhibitor was administered in patients receiving DLI from haploidentical donors or matched unrelated donors (MUD) to prevent GvHD. GvHD prophylaxis was identical between the two groups. Median mononuclear cells and median CD34$^+$ cells each dose were 2.13 (1.22-4.00) *10^8/kg and 0.60(0.08-2.12)*10^6/kg in VEN+HMA group, and were 2.76 (0.96-8.33) *10^8/kg (P=0.144) and 0.65(0.17-4.27)*10^6/kg (P=0.442) in IC group. Bone marrow aspiration was performed after each treatment course and then continued monthly to evaluate efficacy in patients achieving complete response (CR)/CR with incomplete recovery of normal neutrophil or platelet counts (CRi). Overall survival (OS) was recorded from initiation of venetoclax or IC to last follow-up or death. Relapse-free survival (RFS) was defined as time from CR/CRi to the date of hematologic relapse or last follow-up. And Data cutoff date was January 31th, 2023. Treatment-related mortality (TRM) was defined as death not directly caused by relapse.

Adverse events and GvHD

During treatment session, blood routine examination, kidney and hepatic functions were monitored in all patients. Patients with neutropenic fever underwent blood culture for pathogenic microorganisms, chest imaging examination and antimicrobial therapy. Adverse events were evaluated according to CTCAE v5.0. Acute GVHD (aGVHD) and chronic GvHD (cGVHD) were diagnosed according to Glucksberg (17) and NIH (18) criteria, respectively.

Statistical analysis

The statistical analysis was performed using IBM SPSS (v.26) and R programming language (v 4.21). Quantitative variables were expressed as median (range), categorical variables were presented as rate and percentage. Mann-Whitney U test was performed for non-normally distributed quantitative data, Chi-square test and Fisher exact probability test were used for comparison of categorical variables. Survival analysis was conducted using Kaplan-Meier method and compared using log-rank test. Univariable and multivariable analyses were calculated *via* Cox proportional hazards regression model. Co-variables were selected using a stepwise forward procedure, and clinical factors with a P<0.1 in univariable analysis were selected to fit the multivariable model. A P<0.05 was considered statistically significant.

Results

Primary disease status, treatment and transplantation

Patient information is summarized in Table 1. No significant differences were observed between VEN+HMA and IC groups concerning age, gender, initial disease types, risk stratification, therapies before allo-HSCT, donor types and disease status at transplantation. Patients in VEN+HMA group carried FLT3-ITD (n=7), RUNX1 (n=2) and c-KIT (n=1) mutations, while those receiving IC regimen had FLT3-ITD (n=3), TP53 (n=4), ASXL1 (n=3), GATA2 (n=2) and c-KIT (n=2) mutations. Additionally, complex karyotypes were presented in 1 patient in VEN+HMA group and 6 patients in IC groups. All patients received

TABLE 1 Baseline patients and transplantation characteristics.

Item	VEN+HMA (n=23), n (%)	IC (n=42), n (%)	P value
Age (years), median (range)	39 (16-60)	39.6 (16-64)	0.842
Gender			0.725
Male	11 (47.8%)	22 (52.4%)	
Female	12 (52.2%)	20 (47.6%)	
Initial disease			0.289
Primary AML	19 (82.6%)	28 (66.7%)	
Secondary AML	1 (4.3%)	7 (16.7%)	
MDS	3 (13.0%)	7 (16.7%)	
MDS-MLD	3 (13.0%)	0 (0)	
MDS-EB-2	0 (0)	7 (16.7%)	
ECOG score			0.306
0	12 (55.0%)	11 (26.2%)	
1	5 (21.7%)	14 (33.3%)	
2	3 (13.0%)	3 (7.1%)	
NA	2 (8.7%)	14 (33.3%)	
Median (range)	0 (0-2)	1 (0-2)	0.337
ELN 2017 risk stratification			0.436
Favorable	1 (4.3%)	5 (11.9%)	
Intermediate	15 (65.2%)	20 (47.6%)	
Adverse	6 (26.1%)	12 (28.6%)	
NA	1 (4.3%)	5 (11.9%)	
Pre-transplant treatment			
Intensive chemotherapy	20 (87.0%)	34 (81.0%)	0.786
Decitabine exposure	5 (21.7%)	7 (16.7%)	0.865
Azacitidine exposure	6 (26.1%)	5 (11.9%)	0.266
Venetoclax exposure	3 (13.0%)	0 (0)	0.075
Median lines of therapies (range)	3 (0-5)	3 (0-6)	0.615
Time from diagnosis to transplant (days), median (range)	167 (24-343)	170.5 (41-801)	0.661
Disease status at transplant			0.286
CR/CRi	16 (69.1%)	28 (66.7%)	0.811
MRD-	12 (52.2%)	16 (38.1%)	0.273
PR	0 (0)	4 (9.5%)	
NR	4 (17.4%)	3 (7.1%)	
MDS	3 (13.0%)	7 (16.7%)	
Donor type			0.771
Haploidentical donor	10 (43.5%)	15 (35.7%)	0.538
MSD	11 (47.8%)	24 (57.1%)	0.471
MUD	2 (8.7%)	3 (7.1%)	0.793

VEN, venetoclax; HMA, hypomethylating agent; IC, intensive chemotherapy; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; MDS-MLD, MDS with multilineage dysplasia; MDS-EB, MDS with excess blasts; ECOG, Eastern Cooperative Oncology Group; ELN, European leukemia network; CR, complete remission; CRi, CR with incomplete hematologic recovery; MRD, minimal residual disease; PR, partial response; NR no response; MSD, matched sibling donor; MUD, matched unrelated donor.

myeloablative conditioning and GvHD prophylaxis before allo-HSCT. The majority of patients in both VEN+HMA (n=11) and IC (n=24, P=0.471) groups used matched-sibling donors (MSD). One patient in VEN+HMA group and 3 patients in IC group received azacitidine maintenance after transplantation.

Relapse and treatment

Relapse and treatment information is displayed in Table 2. One patient in VEN+HMA group suffered from skin involvement and received radiation therapy. In IC group, orbital chloroma (n=1), invasion of skin (n=2), ribs (n=1), lymph nodes (n=1) and vertebras (n=2) were observed. Two patients were treated with radiotherapy and four patients with either skin or vertebras invasion presented with concurrent bone marrow relapse. Notably, 17.4% of relapsed patients (n=4) in VEN+HMA group suffered from concomitant GVHD or lung infection (aGvHD=1, cGVHD=1, pneumocystis pneumonia =1, mycoplasma pneumonia with decreased oxygen saturation=1), while only 9.5% patients in IC group (n=4, P=0.597) had similar diseases (aGvHD=2, cGvHD=1, pulmonary mycosis=1). In VEN+HMA group, four patients used VEN +HMA as second (n=3) or third line (n=1) treatment, two of whom received previous IC regimen without response and switched to venetoclax-based regimen. Twenty-one patients received azacitidine and 2 patients used decitabine in combination with venetoclax. In addition, 11 patients in VEN +HMA group received 14-day low-dose cytarabine. In IC group, IC was the first-line treatment in 37 patients, second-line in 4 patients and third-line in 1 patient. IDAC (n=15), FLAG (n=16) and CLAG (n=11) were used. IDAC treatment included cytarabine combined with mitoxantrone (n=7) or idarubicin (n=4) or daunorubicin (n=3) or amsacrine (n=1).

Efficacy and survival

Treatment efficacy was shown in Table 3. All treatment responses were achieved in one cycle. Patients who did not respond, but were medically fit and willing to receive further therapies, were switched to a different regimen. In VEN+HMA group, twelve patients (52.2%) achieved CR/CRi (CR=2, CRi=10), with 4 patients (17.4%) reaching MRD negativity. However, eight of the 12 CR/CRi patients (66.7%) relapsed later. One of the 2 patients who failed prior IC achieved CRi, MRD+. Of the eight CR/CRi patients who continued with venetoclax-based treatment, one proceeded to second allo-HSCT and was alive until last follow-up. The other 4 responders all relapsed and were either treated successfully with FLAG (n=1) or died (n=3). Of the 11 non-responders, five switched to intensive (n=2) or low-dose chemotherapy (n=3), and allo-HSCT was performed in 1 NR patient, who later died of relapse. In the IC group, twenty-five (59.5%) patients achieved CR/CRi (CR=8, CRi=17), and 12 patients (28.6%) achieved MRD negativity. Eleven of the 25 patients (40.7%) who responded later relapsed. Eleven responders continued treatment with azacitidine (n=4), venetoclax (n=3), or DLI (n=4), and 6 of 17 non-responders were treated with azacitidine (n=1), DLI (n=4) or intensive chemotherapy (n=1). No statistical significance was observed between two groups regarding response, relapse after response, treatment-related mortality and early mortality.

Kaplan-meier survival analysis showed that achieving CR/CRi significantly improved patients' prognosis (median OS 524 days in

TABLE 2 Relapse and treatment information.

Item	VEN+HMA (n=23), n (%)	IC (n=42), n (%)	P value
Relapse type, n (%)			0.293
Bone marrow only	22 (95.7%)	35 (83.3%)	
Extramedullary +/- BM relapse	1 (4.3%)	7 (16.7%)	
Relapse within 1 years after transplantation	12 (52.2%)	27 (64.3%)	0.341
Concomitant disease at relapse	4 (17.4%)	4 (9.5%)	0.597
Active GvHD	2 (8.7%)	3 (7.1%)	0.793
Lung infection	2 (8.7%)	1 (2.3%)	0.588
BM blasts at relapse, median (range)	21.0 (1.5-90.0) %	20.5 (0.5-91.5) %	0.429
Hemogram at relapse, median (range)			
Median WBC, 10^{12}/L	2.9 (0.9-49.6)	3.9 (1.3-97.6)	0.424
Median hemoglobin, 10^{9}/L	105 (49-141)	115 (61-156)	0.131
Median platelet, 10^{9}/L	69.5 (10-192)	57.5 (3-205)	0.625
Post-relapse treatment before HMA+venetoclax, n (%)			
IC exposure	2 (8.7%)	1 (2.3%)	0.588
AZA exposure	2 (8.7%)	1 (2.3%)	0.588
DAC exposure	1 (4.3%)	2 (4.7%)	0.588
DLI	2 (8.7%)	3 (7.1%)	0.793
Median lines of therapies, median (range)	0 (0-2)	0 (0-2)	0.466
Median time from relapse to venetoclax+HMA/IC (days), median (range)	6 (0-178)	4 (0-104)	0.525
Concomitant DLI, n (%)	21 (91.3%)	42 (100.0%)	0.122

VEN, venetoclax; HMA, hypomethylating agent; IC, intensive chemotherapy; GvHD, graft-versus-host disease; BM, bone marrow; WBC, white blood cell; AZA, azacitidine; DAC, decitabine; DLI, donor lymphocyte infusion.

TABLE 3 Clinical outcomes.

Items	VEN+HMA (n=23), n (%)	IC (n=42), n (%)	P value
Reponse status, n (%)			
ORR	14 (60.1%)	27 (64.3%)	0.785
CR	2 (8.7%)	8 (19.0%)	0.455
CRi	10 (43.5%)	17 (40.5%)	0.814
MRD- in CR/CRi	4 (17.4%)	12 (28.6%)	0.317
PR	2 (8.7%)	2 (4.8%)	0.927
NR	9 (39.1%)	15 (35.7%)	0.785
Time to response (days), median (range)	39 (14-55)	32.5 (14-71)	0.334
Relapse after response, n (%)	8/12 (66.7%)	11/27 (40.7%)	0.176
Duration of response (days), median (range)	131 (27-394)	181 (39-1231)	0.520
Mortality, n (%)			
Day-30 mortality	1 (4.3%)	1 (2.4%)	1.000
Day-60 mortality	3 (13.0%)	6 (14.3%)	0.813
Day-90 mortality	4 (17.4%)	11 (26.2%)	0.421
Treatment-related mortality	1 (4.3%)	9 (21.4%)	0.143

VEN, venetoclax; HMA, hypomethylating agent; IC, intensive chemotherapy; ORR, overall response rate (CR+CRi+PR); CR, complete remission; CRi, CR with incomplete hematologic recovery; MRD, minimal residual disease; PR, partial response; NR no response.

CR/CRi versus 130 days in PR/NR, P=0.004) (Figure 1A). Patients reaching MRD negativity also had significantly prolonged median OS (742 days in MRD negativity versus 169 days in MRD positivity, P=0.014) (Figure 1B). The median time of post-transplantation follow-up was not significant different (614 days in VEN+HMA group versus 377 days in IC group, P=0.347). Median OS was 209 days for VEN+HMA group and 211 days for IC group (P=0.491) (Figure 1C). In VEN+HMA group, ten patients died due to no response to regimen (n=8), relapse after CR/CRi (n=1) or severe pneumonia (n=1). In IC group, lack of response and relapse led to the death of 10 and 9 patients, respectively, and 8 patients died of infection (n=4) or GvHD (n=1) or multiorgan failure (n=3).

Clinical factors for survival and subgroup analysis

The univariable and multivariable analysis using Cox proportional hazards regression model (Table 4) revealed that certain characteristics of patients' initial diseases, including age, baseline ECOG score and adverse mutations did not significantly impact survival. TP53 mutation (HR=3.077 (95%CI 1.055-8.972), P=0.040), Grade III/IV aGvHD after treatment (HR=4.011 (95%CI 1.689-9.525), P=0.002) and time from allo-HSCT to relapse>1 year (HR=0.214 (95%CI 0.093-0.491), P<0.001) were found to have significant effects on survival in univariable analysis. Furthermore, multivariable analysis confirmed that late-onset relapse (HR=0.083 (95%CI 0.020-0.339), P=0.001) and treatment-induced grade III/IV aGvHD (HR=3.534 (95%CI 1.141-10.953), P=0.029) significantly impacted survival. In addition, multivariable analysis identified male gender (HR=4.406 (95% CI 1.599-12.140), P=0.004), FLT3-ITD mutation (HR=3.523 (95% CI 1.091-11.376), P=0.035), concomitant pulmonary infection (HR=4.060 (95% CI 1.027-16.056), P=0.046) and WBC>10,000/microL at relapse (HR=4.720 (95%CI 1.561-14.271), P=0.006) as posing significant risks. The subgroup analysis of survival was displayed in Figure 2, demonstrating the positive trending effect of VEN+HMA regimen in multiple subgroups, with significance observed in patients with Hgb < 110g/L at relapse.

Adverse events and GvHD

Detailed information of adverse events and GvHD is shown in Table 5. All patients experienced grade 3-5 adverse events during their initial course of treatment. Thrombocytopenia was the most common event in both treatment groups, but the incidence was significantly higher in IC group than in VEN+HMA group (95.2% versus 73.9%, P=0.035). Pneumonia was the most common infection, with a significant higher incidence rate in IC group (50.0% versus 17.4%, P=0.010). The incidence of bacteremia was comparable between VEN +HMA group (17.4%) and the IC group (21.4%, P=0.948), and sepsis occurred in 4.3% and 4.8% patients, respectively (P=0.588). No cases of tumor lysis syndrome, patient intolerance or medication reduction were recorded, except the reduction of venetoclax to 100mg when combined with azoles. Of the 11 patients receiving further venetoclax therapy in VEN+HMA (n=8) and IC group (n=3), grade 3-5 adverse events were observed, including thrombocytopenia (n=5), neutropenia (n=3), upper respiratory infection (n=1), urinary tract infection (n=1), elevated aminotransferase (n=1).

After treatment of relapse, aGvHD incidence was significantly lower in VEN+HMA group (13.0% versus 50.0% in IC group, P=0.003). Grade III/IV aGvHD was observed in one patient (4.3%) in the VEN+HMA group and five patients (11.9%) in the IC group (P=0.577). Among patients with concomitant aGvHD at relapse in VEN+HMA (n=1) and IC group (n=2), one patient in each group suffered aGvHD progression. The disease severity of the 2 patients with concomitant cGvHD did not progress during treatment.

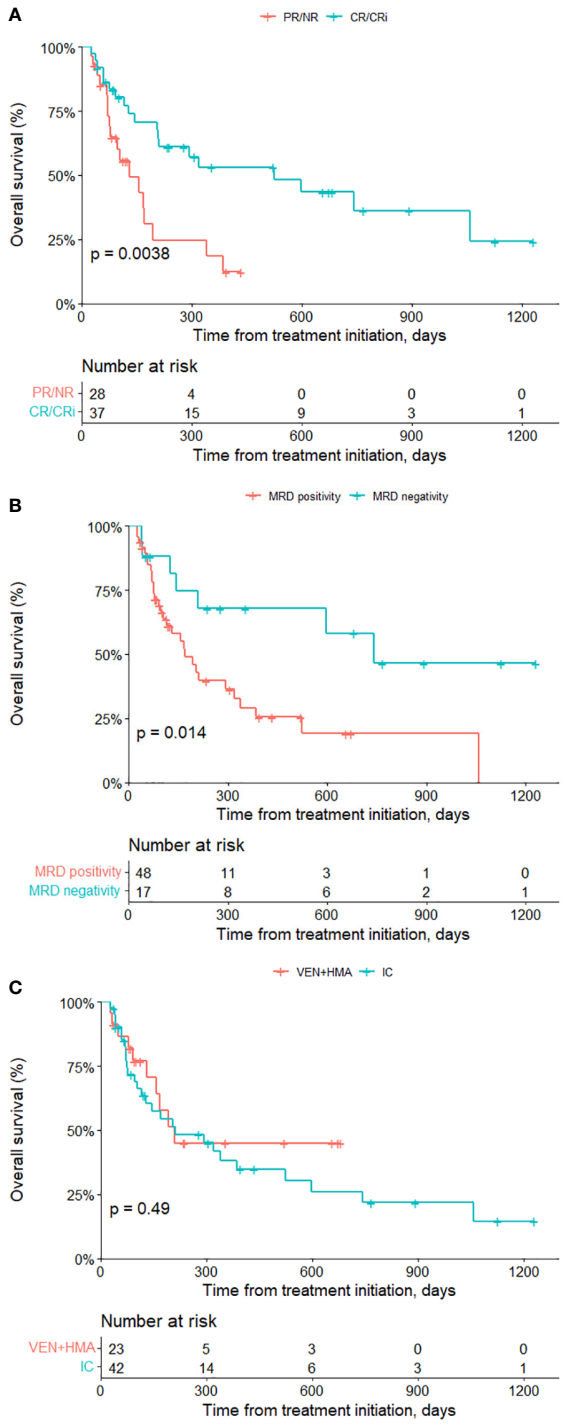

FIGURE 1
Survival analysis of all patients. Overall survival of patients achieving CR/CRi versus non-CR/CRi **(A)**, MRD negativity versus MRD positivity **(B)** and receiving venetoclax-based treatment vs. IC treatment **(C)**. VEN, venetoclax; HMA, hypomethylating agent; IC intensive chemotherapy.

Discussion

Allo-HSCT is considered as one of the curative treatments for high-risk AML and MDS. Despite this, relapse after transplantation remains a significant challenge. Currently available treatments, including intensive chemotherapy, DLI, etc., were partially hindered by poor efficacy and toxicity (2, 4, 19). Previous researches on IC treatment for post-transplantation AML relapse have demonstrated CR rates from 13% to 71% and 1-year OS from 25% to 34.4% (20). A recent study including 175 patients showed a remission rate of 36% and median OS of 188 days, while early mortality within 28 days occurred in 12% patients (21). The promising efficacy of venetoclax-based treatment in newly-diagnosed AML also promoted its use in R/R AML and post-transplantation relapse of myeloid malignancies. A retrospective study analyzed the efficacy of venetoclax-combined and IC regimens in R/R AML, clinical outcomes of VEN and IC groups were 59.3% and 44.4% for ORR rate (P=0.081) and 8.9 months and 12.4 months for median OS (P=0.724), revealing the comparable remission and survival provided by venetoclax (22). In contrast, two other researches showed venetoclax-based regimen can achieve significantly improved response and OS in R/R AML compared to IC treatment (23, 24). Venetoclax combination therapy for relapse of myeloid malignancies after transplantation has been reported with a CR/CRi rate ranging from 26.9% to 47.1% and a median OS from 3.4 to 9.5 months (25–28). However, these studies lack a comparison of venetoclax versus other regimens. To address this gap, we conducted this study to compare efficacy and adverse events of different salvage regimens in 65 patients with post-transplantation relapse of myeloid malignancies. Patients included received VEN+HMA (n=23) or IC treatment (n=42).

Patients' characteristics prior to hematological relapse did not significantly differ between the two groups, CR/CRi rates were 52.2% and 59.5% for VEN+HMA and IC groups (P=0.567) and MRD negativity rates were 17.4% and 28.6%, respectively (P=0.317). However, lung infection (17.4% versus 50.0%, P=0.010), thrombocytopenia (73.9% versus 95.2%, P=0.035) and aGvHD (13.0% versus 50.0%, P=0.003) occurred significantly more frequent in IC group. Median OS was 209.0 days in VEN+HMA group versus 211.0 days in IC group (P=0.491). Although VEN +HMA achieved noninferior response and fewer adverse events, significantly improved survival was not demonstrated in OS, early mortality rate and most subgroup analyses. Patients in our study would switch to another regimen after failing the first course of venetoclax. However, previous researches have indicated the significance of multiple cycles of venetoclax treatment, as a portion of patients may reach remission after several cycles (26, 28). In addition, an increasing number of studies have emphasized the efficacy and tolerability of venetoclax maintenance therapy (29–31). Although Kaplan-Meier (median OS not reached versus 157 days, P=0.007) and univariate analysis (HR=0.184 (95%CI 0.047-0.713), P=0.014) both revealed that patients receiving continued venetoclax achieved prolonged survival versus those without maintenance therapy, the significance could be biased since patients with better physical condition were more likely to receive further treatment. Therefore, we could only speculate that the lack of continued venetoclax treatment in our study may have partially contributed to suboptimal survival.

The study found that only one patient in VEN+HMA group and 3 patients in IC group received prophylactic azacitidine maintenance, and none experienced aGvHD after relapse and

TABLE 4 Prognostic factors for overall survival using univariable and multivariable analysis .

Items	Univariable analysis		Multivariable analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Age ≥40	1.000 (0.973-1.027) 1.081 (0.566-2.064)	0.982 0.814		
Gender (male vs. female)	1.852 (0.951-3.607)	0.070	**4.406 (1.599-12.140)**	**0.004**
Baseline ECOG score (2-3 vs. 0-1)	2.328 (0.781-6.941)	0.129		
Adverse ELN risk stratification **FLT3-ITD mutation** **TP53 mutation** **ASLX1** **GATA2**	1.915 (0.943-3.890) 2.345 (0.937-5.864) **3.077 (1.055-8.972)** 1.301 (0.306-5.522) 1.584 (0.375-6.693)	0.072 0.068 **0.040** 0.722 0.531	2.469 (0.904-6.745) **3.523 (1.091-11.376)** 0.849 (0.216-3.333)	0.078 **0.035** 0.814
VEN-based treatment vs. IC	0.773 (0.370-1.613)	0.493		
GvHD at any time **Grade III/IV aGvHD after treatment**	1.057 (0.520-2.150) **4.011 (1.689-9.525)**	0.878 **0.002**	**3.534 (1.141-10.953)**	**0.029**
Time from allo-HSCT to relapse>1 year **GvHD at relapse** **Pulmonary infection at relapse** **BM blasts at first relapse** **BM blasts>20% at relapse**	**0.214 (0.093-0.491)** 1.415 (0.431-4.650) 3.407 (1.024-11.334) 1.003 (0.991-1.015) 1.733 (0.882-3.407)	**<0.001** 0.567 0.046 0.679 0.111	**0.083 (0.020-0.339)** **4.060 (1.027-16.056)**	**0.001** **0.046**
WBC at relapse **WBC>10,000/microL**	1.002 (0.985-1.020) 2.054 (0.921-4.579)	0.796 0.078	**4.720 (1.561-14.271)**	**0.006**
Hgb at relapse **Hgb<110g/L**	0.993 (0.980-1.005) 1.386 (0.679-2.831)	0.260 0.370		
PLT at relapse **PLT<100,000/microL**	0.998 (0.992-1.003) 1.450 (0.697-3.016)	0.399 0.320		
Concomitant DLI **Previous HMA after relapse**	1.493 (0.204-10.947) 0.873 (0.266-2.862)	0.693 0.823		

HR, hazard ratio; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group; VEN, venetoclax; IC, intensive chemotherapy; GvHD, graft-versus-host disease; DLI, donor lymphocyte infusion; aGvHD, acute graft-versus-host disease; allo-HSCT, allogeneic hematopoietic stem cell transplantation; BM, bone marrow; PLT, platelet; HMA, hypomethylating agent.

treatment. The patient in VEN+HMA group suffered disease progression, whereas 3 patients in IC group all reached CRi, but 2 of them later relapsed. Univariable cox analysis did not show difference in terms of HMA prophylaxis (HR=0.772 (95% CI 0.185-3.225), P=0.723). Besides the fact results based on limited data may not accurately assess effects, previous research suggested that regular maintenance therapy could be necessary to improve survival (31, 32). Additionally, some researches (28, 33–35) revealed negative impacts of previous HMA on VEN+HMA efficacy, while other studies (14, 15) did not. In the VEN+HMA group, none of the patients with prior HMA exposure as maintenance or pre-emptive treatment achieved CR/CRi, compared to 50.0% (10/20) of those without HMA exposure. But univariable analysis did not demonstrate any significant impact of prior HMA exposure or the usage of VEN+HMA as a first-line therapy on survival.

The role of DLI and GvHD on survival also remained controversial. Previous research has produced conflicting results, with some studies indicating a positive effect of DLI and GvHD on disease remission and survival (27), while others showing no such benefits (25, 28). Our study examined the association between concomitant DLI or GvHD and patient outcomes and found no significant improvement in survival with either factor. Nevertheless, we did observe that grade III/IV aGvHD after treatment prognosticated significantly poorer survival, particularly in IC group (HR=6.547 (95% CI 2.201-19.474), P=0.001). In addition, grade III/IV aGvHD occurred with no significant difference in MSD (2/35, 5.7%) and non-MSD recipients (4/30, 13.3%, P=0.530), indicating the importance of immunosuppressants in reducing severe aGvHD in haploidentical or MUD recipients. Our study also found that relapse combined with pulmonary infection increased risks in patients treated with VEN+HMA (HR=16.598 (95%CI 2.298-119.915), P=0.005). Therefore, we recommend initiating VEN-based regimens in relapsed patients without concomitant infection. Additionally, VEN-treated patients may be more tolerant to treatment-induced GvHD than those receiving IC.

Adverse genetic abnormalities are strongly associated with R/R AML and lead to worse survival (36–38). In this study, ELN adverse stratification only showed a trend towards reducing survival (HR=2.469 (95% CI 0.904-6.745), P=0.078). Larger studies (22, 38) with more cases of R/R AML patients have shown significant impact of ELN risk stratification on survival. However, its effect has not been clearly established in patients with post-transplantation relapse. In addition, detecting new mutations at relapse and reassessing ELN risk at that time point might more accurately indicate patients' survival. Nevertheless, due to lack of genetic testing for every patient at relapse, we were not able to

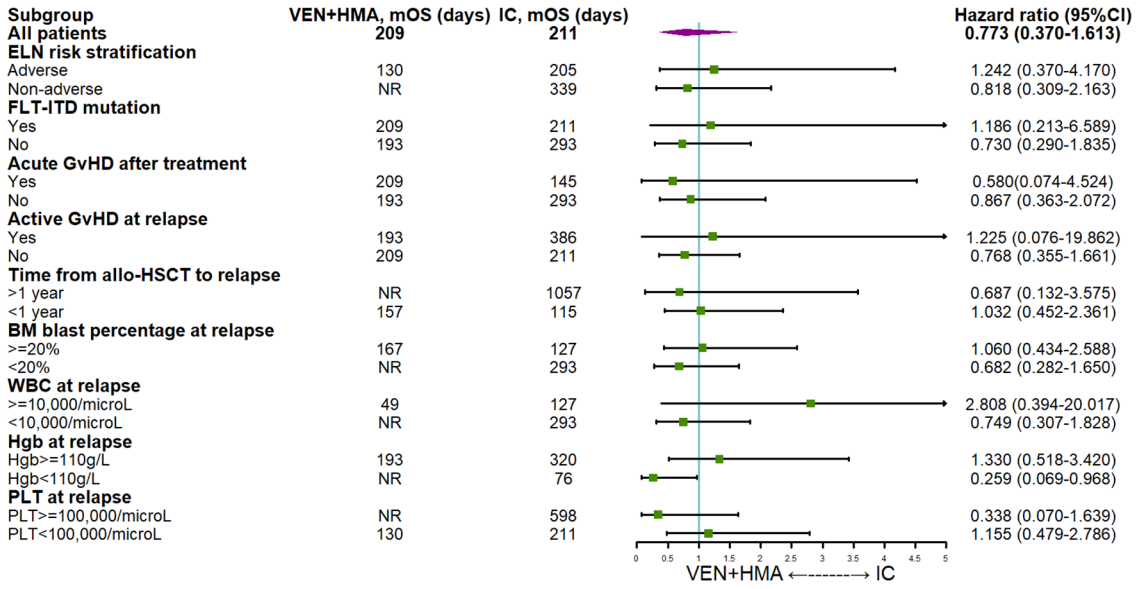

FIGURE 2
Subgroup analysis of survival VEN, venetoclax; HMA hypomethylating agent; IC, intensive chemotherapy; ELN, European leukemia network; DLI, donor lymphocyte infusion; GvHD, graft-versus-host disease; aGvHD, acute graft-versus-host disease; allo-HSCT, allogeneic hematopoietic stem cell transplantation; BM, bone marrow; WBC, white blood cell; HgB, hemoglobin; PLT, platelet.

TABLE 5 Adverse Events and GvHD.

Events	VEN+HMA (n=23), n (%)	IC (n=42), n (%)	P value
Grade 3-5 adverse events (CTCAE v5.0)	23 (100.0%)	42 (100.0%)	1.000
Infection			
Sepsis	1 (4.3%)	2 (4.8%)	0.588
Lung infection	4 (17.4%)	21 (50.0%)	**0.010**
Upper respiratory infection	1 (4.3%)	4 (9.5%)	0.793
Laryngitis	2 (8.7%)	1 (2.4%)	0.588
Gum infection	1 (4.3%)	7 (16.7%)	0.293
Oral mucositis	1 (4.3%)	5 (11.9%)	0.577
Anal mucositis	3 (13.0%)	7 (16.7%)	0.978
Intestine infection	0 (0)	7 (16.7%)	0.098
Abdominal infetion	0 (0)	2 (4.8%)	0.536
Skin infection	0 (0)	2 (4.8%)	0.536
Anemia	11 (47.8%)	27 (64.3%)	0.198
Neutropenia	17 (73.9%)	38 (90.5%)	0.158
Thrombocytopenia	17 (73.9%)	40 (95.2%)	**0.035**
Elevated aminotransferase	0 (0)	8 (19.0%)	0.105
Acute GvHD after treatment	3 (13.0%)	21 (50.0%)	**0.003**
Grade III/IV aGvHD	1 (4.3%)	5 (11.9%)	0.577
Intestine	1 (4.3%)	11 (26.2%)	0.066
Stage 3-4	1 (4.3%)	6 (14.3%)	0.414
Skin	3 (13.0%)	7 (16.7%)	0.978
Stage 3-4	3 (13.0%)	0 (0)	0.075
Liver	0 (0)	19 (45.2%)	**<0.001**
Stage 3-4	0 (0)	4 (9.5%)	0.323

VEN, venetoclax; HMA, hypomethylating agent; IC, intensive chemotherapy; CTCAE, Common Terminology Criteria for Adverse Events; GvHD, graft-versus-host disease; aGvHD, acute graft-versus-host disease; cGvHD, chronic graft-versus-host disease.

demonstrate this speculation. Furthermore, multivariable analysis revealed FLT3-ITD mutation significantly influence survival, which is consistent with other research findings (22, 39). TP53 mutation also showed such significance in univariate analysis, supporting conclusion from other articles (40, 41).

In conclusion, this retrospective study demonstrated that compared to intensive chemotherapy, venetoclax plus hypomethylating agents is an effective and safe regimen for hematological relapse of myeloid malignancies after allo-HSTC. Nevertheless, prospective researches and clinical trials are necessary

to verify results, and more detailed exploration is required for maintenance therapy in responders.

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 ethical committee of the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SF and XC designed the study and revised the manuscrpt. ZC analyzed the data and wrote the manuscript. SZ, TZ, YS colleted the data. AP, DY, RZ, QM, YH, JW, WZ, XC, EJ, MH, SF provided patients to study. 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.

Publisher's note

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EDITED BY
Anna Maria Testi,
Sapienza University of Rome, Italy

REVIEWED BY
Weiming Li,
Huazhong University of Science and Technology, China
Xiao-Dong Mo,
Peking University People's Hospital, China

*CORRESPONDENCE
Qingguo Liu
liuqingguo@ihcams.ac.cn

†These authors have contributed equally to this work and share first authorship

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Venetoclax plus cyclophosphamide and cytarabine as induction regimen for adult acute myeloid leukemia

Baohang Zhang[1,2†], Qingguo Liu[1,2*†], Junfan Li[1,2], Yimin Hu[1,2], Xin Zhao[1,2], Pingping Huang[1,2], Shangzhu Li[1,2] and Ying Wang[1,2]

[1]National Clinical Research Center for Blood Diseases, State Key Laboratory of Experimental Hematology, Haihe Laboratory of Cell Ecosystem, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin, China, [2]Tianjin Institutes of Health Science, Tianjin, China

Background: The efficacy of induction chemotherapy (IC) for acute myeloid leukemia (AML) has improved significantly with the application of targeting drugs. Our previous study showed that a 4-day IC regimen of cyclophosphamide (CTX) and Ara-C [CA (4 + 3)] achieved similar complete remission (CR) rate (80%) compared with the traditional 7-day regimen, and the survival rate appeared to be better.

Methods: In this pilot study, we further shortened the CA regimen to 3 days, added low-dose venetoclax (VEN, 200 mg/day) (VCA), and reported the efficacy and safety here.

Results: Twenty-five newly diagnosed adult AML patients were enrolled in this study and evaluated for the remission rate after one cycle of the VCA regimen. The CR/Cri was 92%, and all these patients had undetectable minimal residual disease (MRD^-). The estimated overall survival at 12 months was 79.3%. The median time for both platelet recovery and absolute neutrophil count recovery was 16 days, faster than that of traditional IC. Compared with the previous CA (4 + 3) regimen, a higher CR rate (92% vs. 80%, $P < 0.01$) and a deeper degree of remission (CR_{MRD-} rate, 92% vs. 45%, $P < 0.01$) were found in the VCA group.

Conclusions: This study showed that the 3-day CTX and Ara-C regimen is highly effective in newly diagnosed AML patients, and the addition of VEN to the CA regimen achieves higher and deeper one-course remission.

KEYWORDS

venetoclax, cyclophosphamide, acute myeloid leukemia, induction chemotherapy, cytarabine

Introduction

Acute myeloid leukemia (AML) is a malignant disorder of hematopoietic stem cells. It accounts for approximately 80% of adult acute leukemia. Optimization of induction chemotherapy (IC), consolidation chemotherapy, or intensive chemotherapy to enhance the clearance of leukemia cells has the potential to improve survival. So far, anthracycline combined with cytarabine (Ara-C) is the first-line IC regimen for AML, and the complete remission (CR) rate after one or two courses of treatment is up to 80% (1, 2). Venetoclax (VEN), a selective BCL-2 inhibitor, may partly overcome the difficulty of treatment caused by the genetic heterogeneity of AML and improve the CR rate. The combination of VEN with hypomethylating agents or low-dose Ara-C in older or unfit newly diagnosed AML has shown significant improvement in overall survival (OS) (3–5). Furthermore, VEN combined with cytotoxic drugs as IC showed that CR rates could exceed 90% (6). These studies suggest that the combined chemotherapy regimens with VEN have a synergistic function.

We previously reported a 4-day IC regimen in AML that includes 4-day cyclophosphamide (CTX) and 3-day Ara-C [CA (4 + 3)] (7). The CR rate was 80%. Among the patients who completed three courses of consolidation chemotherapy, the actual 5-year disease-free survival (DFS) rate was 64%. Since the addition of VEN to IC may improve the CR rate, we further explored a 3-day CA regimen combined with a 7-day VEN (VCA) to explore whether the CR rate could be increased and preliminarily evaluated the survival rate. Correspondingly, a comparison between VCA and historical CA (4 + 3) was reported here.

Methods

Patients and study design

This study was carried out in the Institute of Hematology and Blood Diseases Hospital, CAMS & PUMC, between April 2021 and July 2022. Patients with newly diagnosed AML [defined by the World Health Organization (8)] were enrolled and classified into three risk groups according to the 2017 European Leukemia Net (ELN) criteria (9). The primary endpoint was CR rate, including CR and CR with incomplete blood count recovery (CRi) according to the modified International Working Group criteria (10). The secondary endpoints included overall survival (OS), minimal residual disease (MRD), response rates, event-free survival (EFS), disease-free survival (DFS), durable remissions, and adverse events. The historical CA (4 + 3) set was used for control. This study was approved by the Ethical Committee of the Institute of Hematology and Blood Diseases Hospital. Informed consent was obtained from the patients and their legal guardians in accordance with the Declaration of Helsinki.

Treatment

A combined regimen of VEN, CTX, and Ara-C was used as induction chemotherapy. VEN was given orally at the dosage of 200 mg per day from day 1 to day 7. Ara-C (1 g/m^2) was administered intravenously every 12 h from day 1 to day 3. CTX was administered at 20 mg/kg/day from day 1 to day 3. Posaconazole was used concomitantly to prevent invasive fungal infections and act synergistically with VEN. Patients with intermediate or poor prognosis were recommended for allogeneic hematopoietic stem cell transplantation (allo-HSCT) once the first CR was achieved. Patients unable to perform allo-HSCT were given consolidation therapy according to the NCCN guidelines. Patients remained on study for OS assessment and follow-up even if they accepted other kinds of treatment.

Safety assessment

Adverse events (AEs) were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0 (https://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_50). Treatment-emergent AEs, including clinical tumor lysis syndrome (TLS), were defined as those that occurred between the first dose of the study drug and 30 days after the last dose of the study drug. Clinical and laboratory TLS was defined according to the criterion reported by Howard et al. (11).

Efficacy

Response assessment was performed between 28 and 35 days after chemotherapy, including bone marrow morphology, cytogenetics, and genetic detection. Flow cytometry was used to quantify the MRD of the marrow. Responses were defined according to the European Leukemia Net recommendations (9): CR as <5% of bone marrow blasts with normal peripheral blood counts (neutrophils ≥ 1.0×10^9/L, platelets ≥ 100×10^9/L) and CR with incomplete hematologic recovery (CRi, neutrophils < 1.0×10^9/L, and/or platelets < 100×10^9/L). Neutrophil recovery was defined as days from the start of induction therapy to neutrophil count recovered to >0.5×10^9/L. Platelet recovery was defined as days from the start of chemotherapy to platelets recovered to >20×10^9/L for twice evaluation without platelet transfusion.

Statistical analysis

SPSS (version 25.0, Chicago, IL, USA) was used for the statistical analysis. Comparisons between categorical variables were performed with the χ^2 test or Fisher's exact test. The differences between continuous variables were compared using the *t*-test or the Mann–Whitney *U* test. Overall survival was

evaluated by the Kaplan–Meier method, and the statistical differences between the two groups were evaluated using the log-rank test. P <0.05 was defined as statistically significant.

Results

Patients' characteristics

Twenty-five patients at a median age of 47.4 (range 27–68) years were enrolled in this study, consisting of 16 men (64%) and 9 women (36%). The clinical characteristics of the patients are shown in Table 1. All patients were diagnosed with *de novo* AML. Twenty-three patients had complications at admission. Infections were the most common, including six pulmonary infections (two hemoptysis), three invasive fungal infections, four pharyngeal or gingival infections, and three neutropenic fevers. Other comorbidities included cardiac insufficiency, vomiting, hypokalemia, and hypoproteinemia.

The ELN risk stratification showed favorable prognosis in 44% of patients, intermediate prognosis in 32% of patients, and adverse prognosis in 24% of patients. All patients had gene mutations related to AML at diagnosis detected by next-generation sequencing (NGS) (Figure 1). Recurrent mutations in *RUNX1/RUNXT1*, *CBFβ-MYH11*, *NPM1*, and *CEBPA* were found in 11 patients. Three of them also carried mutations in *TP53*, *ASXL1*, and/or *RUNX1*. Another patient had mutations in *TP53*, *ASXL1*, or *KMT2A*. Mutations in the class II gene *WT1* were found in 18 patients (76%).

TABLE 1 Clinical characteristics of the patients.

Characteristic	VCA (n = 25)	CA (4 + 3) (n = 20)	P-value
Age (years)			0.72
Median (range)	47.4 (27-68)	50 (24-69)	
Gender			0.94
Male (%)	16 (64)	13 (65)	
Female (%)	9 (36)	7 (35)	
AML type			
De novo	25 (100)	20 (100)	
Secondary	0 (0)	0 (0)	
ECOG performance status			0.14
0	5	6	
1	13	11	
2	4	3	
3	2	0	
4	1	0	
Blast percentage			0.23
Median (range)	64.4 (21-96.5)	55.7 (22.5-88.5)	
Fever/infection (%)	13 (52)	12 (60)	0.12
Classification (%)			0.18
NOS	6 (24)	7 (35)	
RUNX1-RUNX1T1	4 (16)	2 (10)	
CBFB-MYH11	1 (4)	1 (5)	
GATA2, MECOM (EVI1)	0 (0)	1 (5)	
NPM1	1 (4)	5 (25)	
CEBPA	5 (20)	1 (5)	
MLLT3-KMT2A	2 (8)	0 (0)	
KMT2A rearranged	4 (16)	2 (10)	
Myelodysplasia-related change	2 (8)	1 (5)	
Prior HMA treatment	0 (0)	0 (0)	
European Leukemia Network risk (%)			0.59
Favorable	11 (44)	9 (45)	
Intermediate	8 (32)	4 (20)	
Adverse	6 (24)	7 (35)	

Efficacy

Two patients were discharged shortly after chemotherapy due to financial reasons although their hematopoiesis had not yet recovered, and they were confirmed to have died at a later follow-up. Therefore, these two patients were evaluated as having no remission. The other 23 patients all achieved CR (including one CRi) after one course of VCA regimen and the CR rate was 92%. All those 23 patients showed MRD negativity, including the one with CRi (Table 2).

Compared with the previous CA (4 + 3) regimen, patients who received VCA achieved a higher CR_{MRD-} rate (92% vs. 45%, P < 0.01). The time required for platelet recovery (≥20 × 10^9/L) and neutrophil recovery (≥0.5 × 10^9/L) was assessed among patients who achieved CR/CRi. The median time for both platelet recovery and neutrophil recovery was 16 (9–20) days, and it had no difference compared with that of the CA (4 + 3) group. The plasma concentration of VEN was detected in five patients and the mean level was 1,880 ng/ml.

Survival

The last follow-up of the patients in the VCA group was in December 2022, and the median time of follow-up was 18.6 (1–20.3) months. Two patients died 1 month after IC. Three patients with adverse prognosis stratification relapsed (12%, 3/25) and finally died of the disease. Another patient died of infection during consolidation therapy. Three patients accepted allo-HSCT. By the end of follow-up, 19 patients were alive and remained CR. The median duration of OS was not reached. The estimated OS at 12 months was 79.3%.

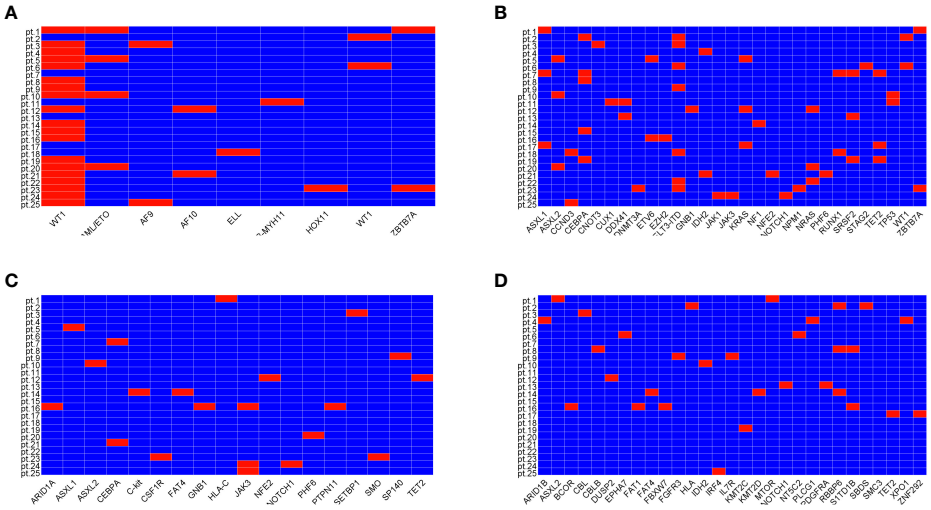

FIGURE 1
Mutations of genes related to diseases. Mutations of class II genes **(A)**, mutations of class I genes highly related to disease **(B)**, mutation of class I genes moderately related to disease **(C)**, and mutations of class I genes with unknown correlation with disease **(D)**.

The overall survival rate of VCA and CA (4 + 3) is shown in Figure 2. We further combined the data of patients in the VCA group and the CA (4 + 3) group to analyze the predictors for OS. Multivariate analysis showed that VCA regimen use (odds ratio 0.08; 95% CI, 0.01 to 0.59, $P = 0.013$), younger age (odds ratio 1.08; 95% CI, 1.01 to 1.16, $P = 0.025$), and the ELN risk stratification of favorable prognosis (odds ratio 0.05; 95% CI, 0 to 0.6, $P = 0.018$) were associated with better survival.

Safety

A summary of treatment-related AEs in patients who received the VCA regimen is shown in Table 2. Patients who received VCA had a lower count of white blood cells compared with those who received CA (4 + 3) (0.02×10^9/L vs. 0.11×10^9/L, $P = 0.002$). However, the infection rate and infection severity were similar between the two groups. The most common origins of infections

TABLE 2 Efficacy and adverse events between VCA and CA (4 + 3) regimen.

	VCA	CA (4 + 3)	*P*-value
Induction response (%)			<0.01
Morphologic CR	23 (92%)	16 (80%)	
MRD negative	23 (92%)	9 (45%)	
Induction failure	2 (8%)	4 (20%)	
Hematologic AEs [d, M (range)]			
The nadir of WBC ($\times 10^9$/L)	0.02 (0.01-0.51)	0.11 (0.01-1.05)	0.002
The day of ANC recovery (> 0.5×10^9/L)	16 (9-20)	17 (10-20)	0.20
The day of PLT recovery (> 20×10^9/L)	16 (9-20)	16 (12-34)	0.97
Non-hematologic AEs (%)			
Angina	1 (5.3)	1 (5)	
Upper respiratory tract infection	1 (5.3)	3 (15)	
Pulmonary infections	4 (21.1)	10 (50)	
Intestinal infections	12 (63.2)	11 (55)	
Rash	0 (0)	1 (5)	
Oral ulcer	1 (5.3)	1 (5)	

WBC, white blood cell; PLT, platelet; ANC, absolute neutrophil count; AEs, adverse events.

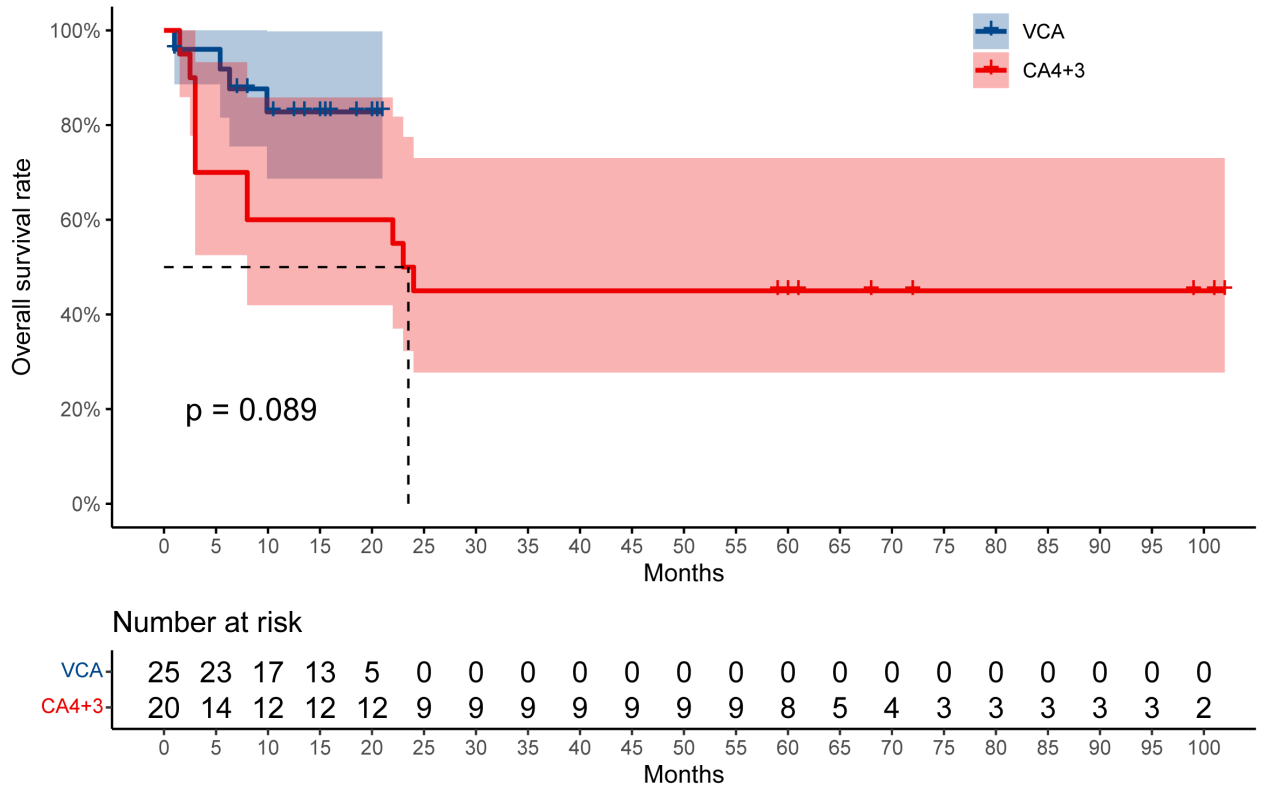

FIGURE 2
The overall survival rate between VCA and CA (4 + 3).

were the intestines and lungs. Laboratory-defined TLS was found in one patient (elevations of potassium, phosphorus, and uric acid) without clinical symptoms.

Discussion

Previous studies of VEN have shown significant improvement in OS when combined with hypomethylating agents (HMAs). A 25% reduction in the risk of death was reported with the combination of VEN, showing that VEN plus LDAC was associated with improvement in median OS (7.2 vs. 4.1 months) (12). Higher response rates and OS were reported using VEN and azacitidine (AZA) than AZA alone (response rate, 66.4% vs. 28.3%; OS, 14.7 vs. 9.6 months) (13). VEN combined with intensive chemotherapy such as CLIA and FLAG-IDA for newly diagnosed AML or high-risk myelodysplastic syndrome also showed encouraging results (3, 14, 15). In our study, the median OS was 23.5 months in the CA (4 + 3) regimen, and it was not reached in the VCA regimen (P = 0.089), demonstrating improved survival. The estimated OS at 12 months of the VCA regimen is 79.3%. This cohort of the study showed that the addition of VEN to the CA regimen leads to a one-course CR rate of up to 92% in adult AML, higher than that of the previous CA (4 + 3) regimen, supporting the role of VEN in improving the inducible remission rate.

A meta-analysis suggested that MRD negativity is associated with better DFS and OS in AML patients (16). Patients who achieved morphological remission but had detectable minimal diseases (MRD^+) are at high risk of relapse (17). For patients under 40 years old, consolidation therapy containing high-dose Ara-C reduces relapse rate (18). In the current study, all the patients with CR/CRi achieved MRD negativity (100%). Compared with the one-course MRD-negative rate of 45% in the historical CA (4 + 3) group, patients who received VCA achieved deeper remission regardless of their prognostic stratification. Our results herald the ability of VEN in clearing leukemic cells (19). However, the sample size of this study is small, and further prospective study with a large sample size is required to validate the results. Nevertheless, this study showed that the combination of low-dose VEN with CA achieved deeper remission in newly diagnosed AML.

The hematologic toxicity of IC is highly associated with the duration of agranulocytosis which most likely causes life-threatening infections. The median time to ANC recovery of the VCA regimen was 16 days, much shorter than 29 days in traditional induction chemotherapy (20, 21). This result may be attributed to the short chemotherapy duration of CTX and Ara-C (3 days) and

the low total dose but high blood concentration of VEN. VEN is a substrate of cytochrome P450 (CYP) 3A enzyme (CYP3A4). Posaconazole, which is used to prevent invasive fungal infections, also functions as a potent inhibitor of CYP3A4. Hence, the concomitant use of posaconazole and VEN can increase the blood concentration of VEN. The total dosage of VEN in our study is 1,400 mg, much lower than that used in previous reports (4,800, 5,600, 2,800, and 2,700 mg, respectively) (3, 6, 14, 15), but the blood concentration is high. Thus, the fast ANC recovery of VCA results in a lower percentage of severe infection and death caused by infections.

In conclusion, we demonstrate that the VCA regimen could achieve a high CR and CR_{MRD-} rate and long-term survival than traditional IC regimens in newly diagnosed AML. Patients also benefit from the shorter ANC recovery time. A prospective study with a large sample size is required to validate the results.

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 approved by the Ethical Committee of the Institute of Hematology and Blood Diseases Hospital. Informed consent was obtained from the patients and/or their legal guardians in accordance with the Declaration of Helsinki.

Author contributions

QL designed the study, enrolled the patients, analyzed the data, and wrote the manuscript. BZ performed the statistical analysis and drafted the manuscript. JL, YH, PH, and SL enrolled the patients and edited the manuscript. XZ and YW analyzed the data and 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.

Publisher's note

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EDITED BY
Massimiliano Bonifacio,
University of Verona, Italy

REVIEWED BY
Jessica Heath,
University of Vermont Children's Hospital, United States
Yifan Pang,
Levine Cancer Institute, United States

*CORRESPONDENCE
Huibo Li
lihuibo-1985@163.com
Shengjin Fan
fansjhmu@163.com

†These authors have contributed equally to this work

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SET-CAN/NUP214 fusion gene in leukemia: general features and clinical advances

Jingyu Song[1], Huibo Li[1*†] and Shengjin Fan[1,2*†]

[1]Department of Hematology, The First Affiliated Hospital, Harbin Medical University, Harbin, China, [2]NHC Key Laboratory of Cell Transplantation, The First Affiliated Hospital, Harbin Medical University, Harbin, China

SET-CAN/NUP214 fusion is a recurrent event commonly observed in adult male patients diagnosed with T-cell acute lymphoblastic leukemia (T-ALL) and has occasionally been reported in other diseases such as acute myeloid leukemia (AML), myeloid sarcoma (MS), acute undifferentiated leukemia (AUL), chronic myeloid leukemia (CML) and B-cell acute lymphoblastic leukemia (B-ALL). This fusion gene is derived from chromosome del(9)(q34.11;q34.13) or t(9;9)(q34;q34) and may have an inhibitory effect on primitive progenitor differentiation. The prognosis of the reported patients is varied, with these patients often show resistance to chemotherapy regimens that include high doses of glucocorticoids. The optional treatment has not been determined, more cases need to be accumulated and evaluated. The scope of this review is to summarize the general features and prognostic significance in leukemia associated with the *SET-CAN/NUP214* fusion gene and to discuss the methods of detection and treatment, aiming at providing some useful references for relevant researchers in the field of blood tumor.

KEYWORDS

SET-CAN/NUP214 fusion gene, leukemia, T-cell acute lymphoblastic leukemia (T-ALL), acute myeloid leukemia (AML), molecular anomaly, treatment, prognosis

1 Introduction

Leukemia is a malignant clonal disease originating from hematopoietic stem and progenitor cells. Leukemia cells with proliferation and survival advantages proliferate and accumulate uncontrollably in the body, gradually replacing normal hematopoiesis and invading other organs and systems, resulting in a series of symptoms such as anemia, hemorrhage, infection and immersion. According to the degree of differentiation and maturation of leukemia cells and the natural course of disease, leukemia can be roughly divided into two categories: acute leukemia and chronic leukemia, and then divided into myelogenic/myeloid and lymphocytic/lymphoblastic according to the cell of origin.

SET-CAN/NUP214 fusion gene is formed by del(9)(q34.11;q34.13) or t(9;9)(q34;q34) and has been identified in the LOUCY cell line of T -ALL and the MEGAL cell line of AML (1, 2). In 1992, Von Lindern et al. first identified the *SET-CAN/NUP214* fusion gene in a

case of acute undifferentiated leukemia (AUL). Since then, with the development of detection technology and the deepening understanding of leukemia, subsequent cases of AML, MS, AUL, CML, and B-ALL have also been found (3–6). Overall, the disease experienced by most patients carrying *SET-CAN/NUP214* is T-ALL.

The NUP214 protein, also known as CAN, is a nucleoporin with FG repeats rich in phenylalanine-glycine. The *NUP214* gene is located on band 9q34.1 and it has a total of 36 exons numerically labeled from 1 to 36 (Figure 1). Chromosome abnormality involving *NUP214* occur repeatedly in leukemia, in addition to the *SET-CAN/NUP214* reviewed here, other chromosome abnormalities were found such as *DEK-NUP214*, *SQSTM1-NUP214* and *NUP214-ABL1*. *DEK-NUP214* [t (6;9)(p22;q34)] was associated with AML, *NUP214-ABL1* was identified in T-ALL patients, the rarest leukemia NUP214 fusion protein is SQSTM1-NUP214: to date, only two cases have been reported, one in ALL and the other in AML. The structure of the *SQSTM1-NUP214* fusion gene consists of five exons located at the N-terminus of the *SQSTM1* gene fused to a portion of the C-terminus of *NUP214*, including its last 14 FG repeats (7). In eukaryotic cells, nucleo-cytoplasmic transport plays an important role in maintaining the normal function and integrity of cells (8). Molecules with a molecular mass greater than 40kDa cannot move across the nuclear membrane by simple diffusion, but require to be facilitated by nuclear transporter receptors (NTRs) with the help of nuclear pore complexes (NPCs) embedded within the nuclear membrane (9–11). NUP214 interacts with NTRs via the FG repeat region in the cytoplasmic filaments of the nuclear pore complexes (NPCs) to control macromolecule trafficking (12). NUP214 has been shown to interact with exportin-1 (XPO1) and nuclear RNA export factor 1 (NXF1) of NTRs, which are highly mobile in cells (13) and play an important role in the response to NUP214 by nuclear export sequences (NES) protein; Furthermore, NUP214 fusion proteins such as SET-CAN/NUP214 and DEK-NUP214, reduce the mobility of XPO1 and lead to the accumulation of XPO1 cargo within the nucleus, impair nuclear output by sequestering XPO1 in the nucleus, interfere with nuclear-cytoplasmic transport of macromolecules, and potentially affect the transcriptional regulatory function of the NF-κB pathway (14), leading to various blood diseases (15). Moreover, genomic knockout of *NUP214* led to embryonic lethality in mice (1).

SET, also referred to as TATA box binding protein-associated factor 1 (*TAF1*). *SET* is a component of the histone acetyltransferase inhibitor (Inhat), which has been reported to be a putative oncogene involved in transcription by regulating chromatin organization (16). *SET* encodes a protein which can exert an inhibitory effect on apoptosis induced by cytotoxic T lymphocytes (4). In eukaryotic cells, the occurrence of selective splicing in the first two exons of the *TAF1* gene results in the formation of two forms of *SET* expression: the two heterodimeric forms, *TAF1-α* and *TAF1-β* (1). Whereas in *SET-CAN/NUP214*, only the *TAF1-β* isoform is present (17). The structure of SET/TAF1-β consists of three parts: an N-terminal dimerization domain, a central "Earmuff" domain named for its headphone-like structure, and an acidic and negatively charged C-terminal domain (Figure 1). *SET/TAF-Iβ* has a variety of different activities, such as inhibiting phosphatase 2A activity, inducing cell transformation and differentiation, and transferring histones to naked DNA. The structural and negative regulatory functions may be related to glucocorticoid resistance (16, 18, 19).

SET-CAN/NUP214 fusion gene encodes a protein containing an almost complete portion of SET fused to the carboxy-terminal two-thirds of CAN, which is a rare gene rearrangement occurs primarily in hematological malignancies (3). The appearance of the fusion gene may be the result of prior cancer therapy, but it may also occur *de novo*.

SET-CAN/NUP214 positive patients often show resistance to chemotherapy including glucocorticoids, but the mechanism is not

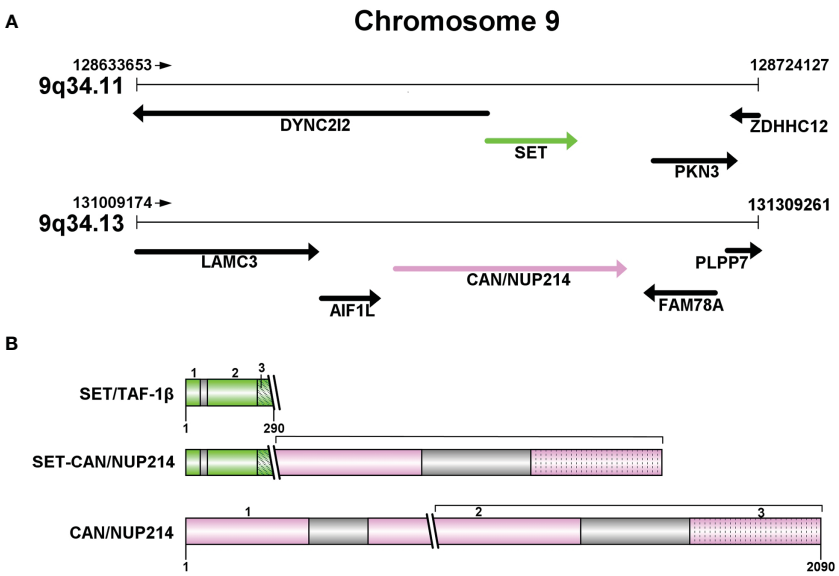

FIGURE 1
(A) Partial structure of chromosome 9 long arm (9q34): SET at 9q34.11 and CAN/NUP214 at 9q34.13. **(B)** Protein structures of SET/TAF-1β, CAN/NUP214 and SET-CAN/NUP214. SET/TAF-1β: 1-3: N-terminal dimerization domain; "Earmuff" domain; acidic and negatively charged C-terminal domain. CAN/NUP214: 1-3: β-propeller; coiled-coil region; FG repeats C-terminal region.

completely clear. The optional treatment has not been determined, previous studies have adopted different treatment options with varying prognoses for patients. Some previous studies have shown that *SET-CAN/NUP214* fusion gene positive patients have a worse prognosis (3, 7, 20), while clinical studies have shown that there is no significant difference in 3-year event-free survival (EFS) and overall survival (OS) between patients with *SET-CAN/NUP214* fusion gene positive and *SET-CAN/NUP214* negative patients (21, 22). Conventional techniques such as chromosomal karyotype analysis may have limitations in detecting patients with *SET-CAN/NUP214*. Due to the emergence of more advanced detection techniques such as fluorescence in situ hybridization (FISH), previously challenging fusion genes like *SET-CAN/NUP214* can now be detected with increasing frequency. This necessitates more precise disease classification and optimization of therapeutic regimens. Research shows that HSCT can improve the prognosis, the level of *SET-CAN/NUP214* after transplantation can predict recurrence to a certain extent (23), new methods such as CAR-T may be effective for patients and further research is needed (24).

In this review, we summarized the general features and clinical advances of *SET-CAN/NUP214* fusion gene in leukemia.

2 Materials and methods

2.1 Literature search

The cases and literature cited and included in this review were retrieved by Jingyu Song and his colleagues using PubMed, Web of Science, Google Scholar, and metstr databases or websites.

The whole screening process is shown in Figure 2. First we exhaustively searched the literature through the databases or websites, and in this step of the search we disregarded the country of publication and time constraints of the literature in order to obtain more comprehensive results. After the search was completed, we performed the exclusion of duplicates and initial screening. Next, by scanning the full-text content, we screened the literature based on its content and excluded incomplete and missing information, leaving behind content that (1) contained complete information and data (2) related to clinical cases, basic research, or reviews of *SET-CAN/NUP214*.

After completing the screening, we proceeded to the integration of viewpoints and statistics of cases.

2.2 Data analysis

We analyzed the statistical case data by SPSS software and performed survival analysis using Kaplan-Meier survival curves.

3 General features of the patient

In the 2022 international consensus classification of acute lymphoblastic leukemia/lymphoma, *SET-CAN/NUP214* fusion gene positive has been listed as a subtype of the *HOXA* gene family in the latest eight temporary entities (25). *SET-CAN/NUP214* fusion gene is rare in leukemia patients, and there is no prospective clinical study for such patients. Relevant articles focus on case reports and mechanism studies. This section provides an overview of the general features of patients.

According to a statistic in 2016, a total of 42 *SET-CAN/NUP214* positive patients were reported up to that year, including T-ALL(38/42,90.5%), AUL(2/42,4.8%), AML(1/42;2.4%) and B-ALL(1/42;2.4%) (4), another study involving 59 T-ALL patients showed that about 10.3% of T-ALL patients carried *SET-CAN/NUP214* fusion gene (20), Ben Abdelli et al. reported that the positive rate of *SET-CAN/NUP214* fusion gene in 196 patients with T-ALL was about 5.6% (21), in 2022, Yan C and others first reported two CML patients with positive *SET-CAN/NUP214* fusion gene (7). The data revealed that although *SET-CAN/NUP214* fusion gene occurs in various types of leukemia, it mainly occurs in T-ALL. This review compiled relevant literature containing more complete patient characteristics published since the emergence of the first *SET-CAN/NUP214* fusion gene positive case to date, some articles were not included due to lack of patient information, a total of 81 patients' information was collected, the overall statistical characteristics of the patients are listed in Table 1, and detailed information on the individual characteristics of the patients are listed in Table 2. Among the 81 patients in Table 2, there are 57(57/81, 70.4%) patients with T-ALL, which is much higher than other types, consistent with the conclusion that the fusion gene is more likely to occur in T-ALL.

Among the fusion gene positive patients counted in this review, there are 59 male and 22 female patients, respectively, with the proportion of male patients reaching more than 70%, suggesting that the *SET-CAN/NUP214* fusion gene is more likely to occur in male patients. The number of fusion gene positive T-ALL patients included 40 males and 17 females, with the proportion of males reaching 70.2%. Although there were fewer cases of other types of leukemia, there were still significantly more males than females, which suggests that the type of leukemia in fusion gene positive patients may not be an influencing factor in the proportion of males and females in the disease (4, 7, 20, 21).

There is a large difference in the age of patients at initial diagnosis, the youngest patient is only 8 years old (T-ALL), the oldest patient is 58 years old (T-ALL), the average age is 30.2 years old and the patients are distributed in all age groups (6, 17, 24, 26, 35, 41). Relatively speaking, the probability of fusion gene positive in adult leukemia patients is higher (40). Two CML patients with *SET-CAN/NUP214* fusion gene positive were 37 and 42 years old, far from the average age of fusion gene positive patients. However, due to the small number of cases and the older age of CML patients, the relationship between age and fusion gene could not be established.

In previous cases, the patients with fusion gene positive leukemia did not show symptoms different from those with fusion gene negative leukemia, and most remained symptomatic with classic anemia, fever, and lower sternal segment tenderness. However, liver and spleen enlargement, lymph node enlargement, mediastinal involvement, as well as tumor bulk and rapid growth were more common than in fusion gene negative patients (5, 29, 34, 42).

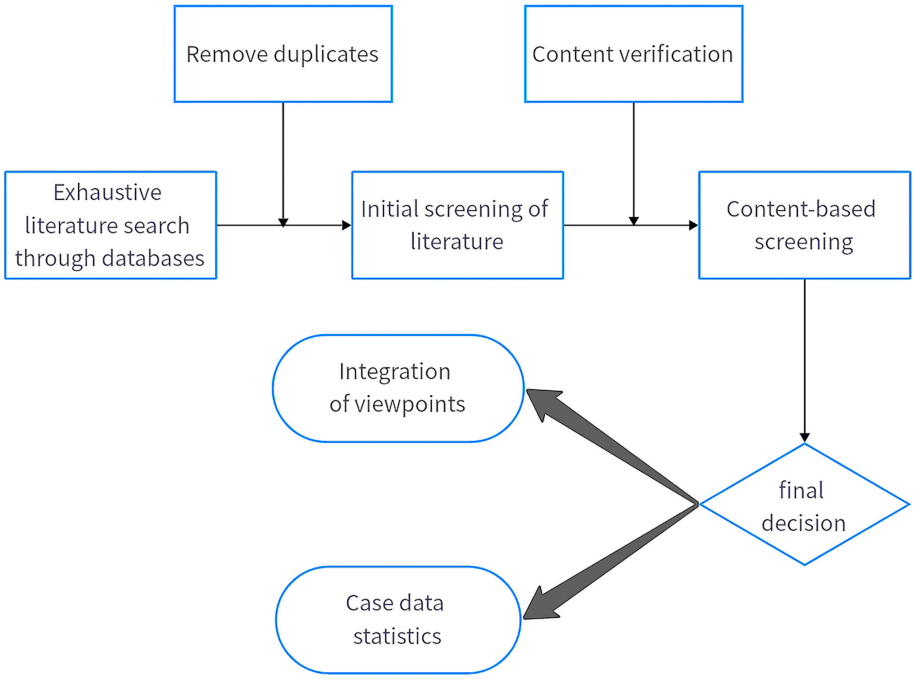

FIGURE 2
Flowchart for searching and selecting literature.

Some patients came to see doctors because of liver and spleen enlargement and related symptoms caused by mediastinal mass. Sang-Guk Lee et al. described a 28-year-old patient who complained of dyspnea and chest pain. Physical examination found that multiple lymph nodes in the neck were swollen. Chest CT showed that mediastinal mass compressed the main pulmonary artery with pleural effusion and splenomegaly. Finally, the patient was diagnosed as *SET-CAN/NUP214* positive T-ALL (34). Song Y et al. (43) also confirmed that patients often have extramedullary infiltration at the onset of the disease, including areas such as the skin, liver and breast. According to the statistics, the median WBC count of the patients was 18.0×10^9/L. Based on the collected patient information, the highest WBC count was 604.4×10^9/L (T-ALL) and this patient died 5 months after diagnosis. The median percentage of leukemic blasts in the bone marrow was high (82.0-97.0%), probably reflecting the high proliferation status of fusion gene positive patients (4, 24).

Patients with fusion gene positive may have normal chromosome karyotype or complex karyotype, the existence of a complex karyotype may mask the presence of the fusion gene (34, 44). As a molecular abnormality with low frequency, this is also the reason why *SET-CAN/NUP214* patients were not widely concerned at first.

In terms of immunophenotype, the fusion gene positive leukemia cells showed characteristics of extreme immaturity. Flow cytometry showed that their most frequent immunophenotype was CD7, except for the two CML cases mentioned previously (7), only one T-ALL patient and one AML patient reported by Zhang H (6) and Rosati R (29) did not detect CD7+. CD7 was highly frequent in *SET-CAN/NUP214* fusion gene positive leukemia, and the other immunophenotypes with higher frequency were cCD3, CD34, CD33 and CD13. The immunophenotypic results suggest that the transformation of fusion gene positive leukemia may occur in the early stage of myeloid or T-lymphocyte differentiation, and it may be related to the inhibition of differentiation of primitive progenitor cells by the fusion gene (6, 7, 35, 45, 46).

Generally, myeloid markers such as CD13 and CD33 are only expressed in about 19% of T-ALL cases. The reason why the fusion gene induces myeloid marker expression remains to be further investigated.

4 Molecular anomaly in SET-CAN/NUP214

SET-CAN/NUP214 fusion gene impairs the process of hematopoietic differentiation, but it alone is not sufficient to induce leukemia. Additional chromosomal aberrations and molecular events are required to mediate the development of leukemia. Understanding the process is greatly helpful for understanding the disease.

SET-CAN/NUP214 fusion gene may contribute to leukemia through direct and indirect effects. Saito S et al. (47) developed transgenic mice expressing *SET-CAN/NUP214*, which is active in different groups of hematopoietic cell groups, and the transgenic mice carrying *SET-CAN/NUP214* gradually developed symptoms such as anemia, thrombocytopenia and splenomegaly, so that within 6 months, a considerable number of transgenic mice died successively, the course and characteristics of the lesions are more similar to those of leukemias, and the characterization of bone

TABLE 1 Patient characteristic statistics.

Characteristics	Statistical overview
Age (year,range)	
average age	30.2 (8-58)
median age	29.0 (8-58)
Sex (n,%)	
male	59/81 (72.8%)
female	22/81 (27.2%)
Average WBC ($\times10^9$/L)	65.6
Subtype (n,%)	
T-ALL	57/81 (70.4%)
AML	12/81 (14.8%)
B-ALL	4/81 (4.9%)
MPAL	3/81 (3.7%)
AUL	2/81 (2.5%)
CML	2/81 (2.5%)
MS	1/81 (1.2%)
Treatment (n,%)	
Chemotherapy	20/69 (29.0%)
Transplant	49/69 (71.0%)
Clinical outcome (n,%)	
CR	52/69 (75.4%)
Relapse	28/69 (40.6%)
Death	30/69 (43.5%)

marrow cells in mice during the course of the disease showed that the *SET-CAN/NUP214* fusion gene increased the number of immature cells and impaired the hematopoietic differentiation of erythroid, granulocytic, and megakaryocytic lineages (47).

Previous studies have shown that the fusion gene impairs the process of hematopoietic differentiation, but cannot induce the occurrence of leukemia alone. *HOXA* upregulation may be the key mechanism and play an intermediary role. *HOX* genes is a kind of gene that specially regulates biological form in organisms. The expression of *HOX* gene in various organisms is similar, and its sequence is related to its action sequence and action position. Human *HOX* gene can be divided into four gene clusters: *HOXA*, *HOXB*, *HOXC*, and *HOXD*, which are located on different chromosomes respectively. The DNA sequence of these gene family members is similar to the protein sequence transcribed. Quantitative and comparative analysis of bone marrow samples from *SET-CAN/NUP214* positive patients during initial diagnosis and morphological remission using RT-PCR and other detection methods showed that the expression level of *HOXA9* and *HOXA10* at initial diagnosis was 3.53 and 4.15 times higher than that during morphological remission, while the expression level of *HOXA5* was similar (34). Sang-Guk Lee and others found that the up-regulation of *HOXA* gene was caused by the interaction of *SET-CAN/NUP214* fusion gene with *XPO1*, *hDOT1L* and *HOXA* promoters. The fusion genes with similar mechanism include *CALM-AF10* and *MLL-AF10* (33), which can lead to the H3K79 hypermethylation of *HOXA* genes and mediate the occurrence of leukemia. Hypermethylation and subsequent upregulation of *HOXA* genes play an important role in the pathogenesis of leukemia with positive fusion gene (48). Gorello P et al. detected the overexpression of *HOXA7*, *HOXA9* and *HOXA10 in the* fusion gene positive patients selected from 256 ALL patients (7, 42). 17 fusion gene positive patients were detected by FISH analysis,

TABLE 2 Characteristics of SET-CAN/NUP214 positive patients reported in the literature.

Case no.	Diagnosis	Year	Ref.	Sex	Age (y)	WBC($\times10^9$/L)	Immunophenotype/ Flow cytometry				
							CD7	CD33	CD34	CD13	cCD3
1	AUL	1992	Von Lindern (17)	Male	19.0	/	+	+	–	–	–
2	AUL	2010	Kim.J (26)	Male	40.0	53	+	+	–	–	+
3	MS	2020	Zhang.H (6)	Female	32.0	4.15	+	+	–	–	–
4	MPAL	2020	Li MY (27)	Male	29.0	0.56	+	+	+	–	+
5	MPAL	2021	Chen SM (28)	Female	22.0	/	/	/	/	/	/
6	MPAL	2021	Chen SM (28)	Male	34.0	/	/	/	/	/	/
7	AML	2007	Rosati R (29)	Male	35.0	40	–	+	+	+	–
8	AML	2019	Jeong IH (30)	Male	46.0	17.1	+	+	+	–	–
9	AML	2020	Zhang.H (6)	Male	24.0	11.41	+	+	+	+	–
10	AML	2021	Zheng YZ (31)	Male	12.0	231.8	+	+	+	+	–
11	AML	2021	Zheng YZ (31)	Male	10.0	38.75	+	+	+	+	–
12	AML	2021	Chen SM (28)	Male	20.0	/	/	/	/	/	/

(Continued)

TABLE 2 Continued

Case no.	Diagnosis	Year	Ref.	Sex	Age (y)	WBC(x10^9/L)	Immunophenotype/ Flow cytometry				
							CD7	CD33	CD34	CD13	cCD3
13	AML	2021	Chen SM (28)	Male	32.0	/	/	/	/	/	/
14	AML	2021	Chen SM (28)	Male	26.0	/	/	/	/	/	/
15	AML	2021	Chen SM (28)	Male	12.0	/	/	/	/	/	/
16	AML	2021	Chen SM (28)	Female	46.0	/	/	/	/	/	/
17	AML	2021	Chen SM (28)	Male	38.0	/	/	/	/	/	/
18	AML	2021	Chen SM (28)	Male	50.0	/	/	/	/	/	/
19	CML	2022	Chen Y (5)	Male	42.0	/	–	+	+	+	–
20	CML	2022	Chen Y (5)	Female	37.0	283.5	–	+	+	+	–
21	B-ALL	2010	Nowak NJ (32)	Female	42.0	/	/	/	/	/	/
22	B-ALL	2014	Hong HZ (4)	Male	19.0	217.0	+	+	+	+	–
23	B-ALL	2021	Chen SM (28)	Male	18.0	/	/	/	/	/	/
24	B-ALL	2021	Chen SM (28)	Male	22.0	/	/	/	/	/	/
25	T-ALL	2008	Van Vlierberghe P (33)	Female	15.3	213.0	/	/	/	/	/
26	T-ALL	2008	Van Vlierberghe P (33)	Female	10.6	142.0	/	/	/	/	/
27	T-ALL	2008	Van Vlierberghe P (33)	Female	17.1	15	/	/	/	/	/
28	T-ALL	2010	Gorello P (7)	Male	38.0	24	/	/	/	/	/
29	T-ALL	2010	Gorello P (7)	Male	19.0	3.28	/	/	/	/	/
30	T-ALL	2010	Gorello P (7)	Male	47.0	/	/	/	/	/	/
31	T-ALL	2010	Gorello P (7)	Female	27.0	/	/	/	/	/	/
32	T-ALL	2010	Gorello P (7)	Male	19.0	/	/	/	/	/	/
33	T-ALL	2010	Gorello P (7)	Male	18.0	/	/	/	/	/	/
34	T-ALL	2010	Gorello P (7)	Male	23.0	/	/	/	/	/	/
35	T-ALL	2011	Lee SG (34)	Male	28.0	37.3	+	+	+	–	–
36	T-ALL	2011	Chae H (35)	Female	55.0	24.43	+	+	+	+	+
37	T-ALL	2011	Chae H (35)	Female	32.0	18.04	+	+	+	+	+
38	T-ALL	2011	Chae H (35)	Male	32.0	39.06	+	+	+	–	+
39	T-ALL	2011	Chae H (35)	Male	20.0	5.07	+	+	+	–	+
40	T-ALL	2011	Li WJ (36)	Female	12.0	1.5	+	+	+	+	+
41	T-ALL	2011	Li WJ (36)	Male	11.0	6.4	+	+	–	–	+
42	T-ALL	2011	Li WJ (36)	Male	8.0	99.6	+	–	+	–	+
43	T-ALL	2012	Dai HP (20)	Male	20.0	34.1	+	+	+	+	+
44	T-ALL	2012	Dai HP (20)	Female	56.0	6.8	+	+	+	–	+
45	T-ALL	2012	Dai HP (20)	Female	23.0	2.6	+	+	+	–	+
46	T-ALL	2012	Dai HP (20)	Male	27.0	/	+	+	+	+	+
47	T-ALL	2012	Dai HP (20)	Male	45.0	33.3	+	+	+	–	+
48	T-ALL	2012	Dai HP (20)	Male	23.0	15.1	+	+	+	–	+
49	T-ALL	2012	Lee EY (37)	Female	43.0	60.6	+	+	+	+	+

(Continued)

TABLE 2 Continued

Case no.	Diagnosis	Year	Ref.	Sex	Age (y)	WBC(x10^9/L)	Immunophenotype/ Flow cytometry				
							CD7	CD33	CD34	CD13	cCD3
50	T-ALL	2014	Ben (21)	Male	34.0	30.4	+	+	+	–	+
51	T-ALL	2014	Ben (21)	Female	37.0	8.6	+	–	+	–	+
52	T-ALL	2014	Ben (21)	Male	29.0	10.1	+	+	+	+	+
53	T-ALL	2014	Ben (21)	Male	41.0	18.4	+	+	+	–	+
54	T-ALL	2014	Ben (21)	Male	23.0	604.4	+	–	–	–	+
55	T-ALL	2014	Ben (21)	Male	30.0	24.9	+	–	–	–	+
56	T-ALL	2014	Ben (21)	Male	36.0	181.8	+	+	+	–	+
57	T-ALL	2014	Ben (21)	Male	45.0	50.8	+	–	–	–	+
58	T-ALL	2014	Ben (21)	Male	38.0	2.8	+	+	+	–	+
59	T-ALL	2014	Ben (21)	Male	28.0	41.8	+	+	+	–	+
60	T-ALL	2014	Ben (21)	Male	20.0	30.9	+	–	–	–	+
61	T-ALL	2015	Prokopiou C (38)	Female	48.0	/	+	–	+	–	+
62	T-ALL	2015	Prokopiou C (38)	Male	45.0	/	+	+	+	–	–
63	T-ALL	2019	Yang Q (3)	Male	26.0	12.3	+	–	–	–	–
64	T-ALL	2019	Yang Q (3)	Male	51.0	109.1	+	+	–	–	–
65	T-ALL	2019	Yang Q (3)	Male	37.0	131.5	+	+	+	–	–
66	T-ALL	2020	Zhang.H (6)	Male	21.0	37.16	–	–	–	–	–
67	T-ALL	2021	Xianying Xu (39)	Female	44.0	21.1	+	–	+	–	+
68	T-ALL	2021	Na Lin (40)	Female	15.0	23.5	+	–	+	–	+
69	T-ALL	2021	Chen SM (28)	Male	58.0	/	/	/	/	/	/
70	T-ALL	2021	Chen SM (28)	Female	27.0	/	/	/	/	/	/
71	T-ALL	2021	Chen SM (28)	Male	37.0	/	/	/	/	/	/
72	T-ALL	2021	Chen SM (28)	Male	27.0	/	/	/	/	/	/
73	T-ALL	2021	Chen SM (28)	Female	16.0	/	/	/	/	/	/
74	T-ALL	2021	Chen SM (28)	Male	36.0	/	/	/	/	/	/
75	T-ALL	2021	Chen SM (28)	Male	40.0	/	/	/	/	/	/
76	T-ALL	2021	Chen SM (28)	Male	41.0	/	/	/	/	/	/
77	T-ALL	2021	Chen SM (28)	Female	34.0	/	/	/	/	/	/
78	T-ALL	2021	Chen SM (28)	Male	15.0	/	/	/	/	/	/
79	T-ALL	2021	Chen SM (28)	Male	12.0	/	/	/	/	/	/
80	T-ALL	2021	Chen SM (28)	Male	42.0	/	/	/	/	/	/
81	T-ALL	2021	Chen SM (28)	Male	36.0	/	/	/	/	/	/

AUL, Acute undifferentiated leukemia; ALL, Acute lymphoblastic leukemia; T-ALL, T-cell ALL; B-ALL, B-cell ALL; AML, Acute myeloid leukemia; MS, Myeloid sarcoma; MPAL, Mixed phenotype acute leukemia; CML, Chronic myeloid leukemia; Immunophenotype positive: +; Immunophenotype negative: -; unknown:/

and all patients(17/17, 100%) had overexpression of *HOXA* gene. There were also studies that summarized the up-regulation of *HOXA* gene with the positive expression of several *NUP214* fusion gene subtypes. *SET-NUP214*, *DEK-NUP214* and *SQSTM1-NUP214* have the same characteristics, which can lead to the up-regulation of *HOXA3*, *HOXA5*, *HOXA7*, *HOXA9*, *HOXA10* and *HOXB* in the *HOX* family (2, 33, 49, 50). These studies also confirmed the relationship between *HOXA* and *SET-CAN/NUP214* fusion gene.

Na Lin et al. (40) evaluated common recurrent mutations in *SET-CAN/NUP214* positive T-ALL patients through next-

generation sequencing. The results showed that mutations were more common in *NOTCH1*(23/31,74.2%), *PHF6*(11/21,52.38%), *KRAS*(6/14,42.86%), *JAK3*(4/12,33.33%), *CCND3*(3/12,25%), *JAK1* (3/15,20%), *STAT5B*(2/10,20%), *DNM2*(2/10,20%) and *EED*(2/10,20%), these are common recurrent mutations in *SET-CAN/NUP214* positive patients in T-ALL and ETP-ALL. The patients with fusion gene positive are accompanied by more molecular events than those with fusion gene negative. These complex molecular events may promote adverse reactions to induction therapy, and may also be one of the factors of poor prognosis (51). As the total number of cases remains low, these issues remain to be explored.

The protein encoded by *NOTCH* gene is a highly conserved cell surface receptor, which can regulate the development of a variety of biological cells. *NOTCH* signaling can affect a series of normal life processes of cells, including the differentiation of pluripotent progenitor cells, cell apoptosis, proliferation and cell boundary formation. The abnormality of *NOTCH* signaling is related to esophageal cancer, gastric cancer, leukemia and other diseases, Among them, abnormal *NOTCH1* is most often detected in tumor diseases.

The activation mutation of *NOTCH1* or the inactivation mutation of *NOTCH1* negative regulatory factor(*FBXW7*) can be found in about 60% of T-ALL cases. However, the proportion of *NOTCH1* mutation seems to be higher in *SET-CAN/NUP214* positive leukemia patients. A gene sequencing of 6 *SET-CAN/NUP214* positive T-ALL patients by Dai HP et al.(Jiangsu Institute of Hematology, China) showed most T-ALL patients with positive fusion gene have *NOTCH1* mutations(5/6,83.3%) and *PHF6* mutations(4/6,66.7%) (20). The next-generation sequencing of patients by Na Lin et al. (40) showed that the proportion of *NOTCH1* mutations in 31 patients reached 74.2%, similarly, the results of the test performed by Wang Q et al. (52) on the association between 96 fusion gene positive patients and mutations such as *NOTCH*, *JAK1* and others demonstrated a possible positive correlation between *NOTCH1* mutations and fusion gene positivity.

The mutations of *NOTCH1*, *PHF6* and *JAK1* are closely linked in the process of leukemia, which may be the secondary genetic alterations of *SET-CAN/NUP214* fusion gene. *PHF6* is a tumor suppressor gene with transcriptional regulation linked to the X sex chromosome in the nucleus. Tumorigenic mutations have a higher incidence rate in T-ALL and can also be seen in AML, most of them occur in male patients. *JAK1* plays a key role in initiating reactions related to a variety of major cytokine receptor families. It appears in about 20% of adult T-ALL patients, generally indicating poor prognosis. If the patients with positive fusion gene have co-mutation of *NOTCH1* and *PHF6*, they are more likely to have symptoms such as splenomegaly and lymph node enlargement (2, 22, 52–54). In addition, the existence of *SET-CAN/NUP214* fusion gene is related to the up-regulation of the expression level of lymphoblastic leukemia-associated hematopoietic regulator 1 (*LYL1*) and myocyte enhancer 2C(*MEF2C*) genes (22). Contrary to the common mutations such as *NOTCH1*, *PHF6* and *JAK1*, the overexpression of *CALM-AF10*, *SIL-TAL*, *TLX1* or *TLX3* is mutually exclusive with the existence of *SET-CAN/NUP214* fusion gene. A gene test of 11 fusion gene positive T-ALL patients by Ben et al (21). showed that none of the 11 patients expressed *CALM-AF10*, *SIL-TAL*, *TLX1* or *TLX3*(0/11,0%).

In the process of leukemogenesis mediated by *SET-CAN/NUP214* fusion gene, it is generally accepted that additional chromosomal aberrations also play a role. Chae H et al. (35) reported del (12)(p13)/ETV6 in 3 of 4 patients, while Ben et al. (21) found this aberration numerous times in their cases. Similarly, the patients in the reports also presented del (6) (q21q23) and del (11) (q22q23) chromosomal aberrations (51, 55). The recurrent chromosomal aberrations in the rare fusion gene positive patients are intriguing and worth pondering.

5 Treatment and prognosis of patients

5.1 Prognosis of leukemia patients with SET-CAN/NUP214 fusion gene

The prognosis of patients with positive *SET-CAN/NUP214* fusion gene is different. Most studies consider that the prognosis is poor. The prognosis of patients may vary due to leukemia classification, concomitant molecular events, treatment plan and the age stage. Patients generally showed delayed response and drug resistance to chemotherapy including glucocorticoids, but studies showed that this drug resistance might not have a negative impact on clinical outcomes (21). Yang Q et al. demonstrated that the prognosis of T-ALL patients with *SET-CAN/NUP214* was quite poor, their treatment of three patients with fusion gene positive showed that none of the three patients achieved complete remission(CR) during chemotherapy, and all of them were infected by drug-resistant bacteria such as Candida tropicalis and Pseudomonas aeruginosa. Because of the disease progress and the inability to control the concurrent infection, two patients died during chemotherapy (3). Gorello P et al. also found that the prognosis of fusion gene positive patients was poor. In this study, 6 of the 7 patients received treatment, of which 4 patients died 12 to 24 months after treatment. The main causes of death were refractory disease and leukemia recurrence (7). The treatment results of 6 patients by Dai HP et al. showed that 4 of the 6 patients had recurrence (the median recurrence time was only 7.8 months), and 3 of them died (20). There are also studies show that the positive fusion gene has no effect on the clinical outcome of patients. In the study of Ben et al., the difference between the 3-year total survival rate(3y OS) and event-free survival rate(3y EFS) of fusion gene positive patients and fusion gene negative patients is not statistically significant (3y OS:73% vs 68%; 3y EFS:45% vs 59%) (21, 24), while in the study of Chen B et al, the 3-year overall survival rate(3y OS) and event-free survival rate(3y EFS) of 8 fusion gene positive patients were 87.5% and 70% respectively (22). It can be seen that the outcomes of patients in different clinical trials vary greatly, and finding more effective treatment methods may be beneficial to patients.

5.2 Studies on the causes of corticosteroid resistance

Patients with positive *SET-CAN/NUP214* fusion gene usually exhibit general resistance to chemotherapy regimens including glucocorticoids in the early stages of induction therapy. Although patients have a delayed response to chemotherapy, the overall CR rate is not affected (40).

The relevant research evaluated patients based on *in vitro* drug sensitivity screening, monitoring of blasts during induction and MRD results after induction. Compared with the patients with negative fusion gene, the rate of corticosteroid resistance in patients with positive fusion gene(91% of patients had corticosteroid resistance, while the data of patients with negative fusion gene was only 44%) and the rate of early chemotherapy resistance (nearly 100% of patients had early chemotherapy resistance, and only 44% of patients with negative fusion gene) were significantly higher (3, 34, 39, 40).

The anti-inflammatory, immunosuppressive and proapoptotic effects of glucocorticoids play an important role in the treatment of various inflammatory, autoimmune and tumor diseases. In the treatment of leukemia, glucocorticoids are involved in various chemotherapy regimens, especially for ALL. Corticosteroid therapy induced GR target gene transcription is also one of the reference treatment options for ALL (56, 57). The powerful role of glucocorticoids is based on the ubiquitous glucocorticoid receptors(GR) in human cells (58), ligands activate GR and bind with glucocorticoid response elements(GREs) in the nucleus. The transcription process starts under the mediation of "coactivators" such as steroid receptor coactivator 1 (*SRC1*) and glucocorticoid receptor interaction protein 1 (*GRIP1*). Under pathological conditions, *SET* is fused with *CAN/NUP214*, and the *SET* subtype mainly exists in *SET-CAN/NUP214* is *TAF1-β*. *TAF1-β* serves as a component in the INHAT complex, which interacts with a variety of trans-acting factors through *TAF1-β* to inhibit the transcriptional activity of multiple transcription factors and nuclear receptors. Due to this mechanism, Takamasa Ichijo et al. reported that the potential cause of glucocorticoid resistance in patients with positive *SET-CAN/NUP214* fusion gene is the co-precipitation of SET-CAN/NUP214 fusion protein and glucocorticoid response element, which inhibits the transcription activity of glucocorticoid receptor and histone acetylation (56, 59). The *in vitro* experimental data reported by Yang Q and others also believe that the lack of histone acetylation regulation mediated by *SET-CAN/NUP214* may be the cause of glucocorticoid resistance in many patients (3).

Even though nearly 100% of *SET-CAN/NUP214* fusion gene positive patients exhibit resistance during the early stages of chemotherapy, studies have shown a high complete response rate (26 of 36 patients,72.22%) (40, 60). The CR rate of the 69 patients counted in Table 1 is also relatively high, reaching 75.4% (52/69). The drug resistance situation and mechanism of the patients still need further research, which may be helpful for the selection of chemotherapy regimen.

5.3 Chemotherapy and transplantation

The optional treatment method of *SET-CAN/NUP214* fusion gene positive leukemia has not been determined. We present patients with clear treatment methods and outcome information reported so far in Table 3 for reference. Analyzing the treatment methods and prognosis of previous cases may provide guidance for the establishment of treatment strategies for such patients.

The *SET-CAN/NUP214* fusion gene is mainly found in T-ALL patients. Table 3 contains 49 T-ALL patients, of which 18 patients received chemotherapy and 31 patients received transplantation. Among the patients receiving chemotherapy, 7 patients survived, 11 patients died, and 9 patients relapsed; Among the patients receiving transplantation, 18 patients survived, 13 patients died and 13 patients relapsed.

Most patients developed drug resistance at the initial stage of chemotherapy, but 35 T-ALL patients finally achieved complete remission (CR, 35/49, 71.4%), which was similar to the complete remission rate suggested in previous studies (72.22%) (40, 60). Yang Q et al. (3) reported that CLAG chemotherapy combined with asparaginase might be a potential treatment option for adult *SET-CAN/NUP214* fusion gene positive T-ALL patients. They implemented VICP chemotherapy for the first two patients (No.52-53) in the case, but the effect was not obvious. The patients eventually died because of the disease progress and uncontrollable infection of drug-resistant bacteria, for the third patient(No.54), the CLAG chemotherapy regimen combined with asparaginase was used. Surprisingly, the patient's condition was quickly controlled. Na Lin et al. (40) conducted a drug sensitivity screening tests on the leukemic cells of a refractory fusion gene positive T-ALL patient (No.57) with up to 165 drugs, suggesting that the DAE protocol of "AML like treatment" (daunorubicin+cytarabine+etoposide) showed the highest inhibition rate *in vitro*. At the same time, they suggested that the induction treatment could adopt a 28-day course of chemotherapy such as used in GRAALL 2003 or 2005. The reason why such "AML like treatment" is effective for patients with fusion gene positive may be related to the frequent occurrence of markers such as CD33 and CD34. Carfilzomib may have a strong inhibitory effect on leukemic cells with positive fusion gene. It can mediate the production of reactive oxygen species as an inducer and synergistically enhance the cytotoxicity of dexamethasone. It is worth noting that in the drug sensitivity screening test, the inhibition rate of single drug treatment of carfilzomib is 37.57%, which shows that carfilzomib may also have potential benefits for patients with refractory *SET-CAN/NUP214* fusion gene positive T-ALL (40, 61, 62). Unfortunately, carfilzomib is not currently available in China.

In the treatment of fusion gene positive patients, transplantation may benefit more. A literature based comparison of the treatment methods of patients shows that the average survival time of the chemotherapy group was 22.5 months, the average survival time of the transplantation group was 50.1 months, the average survival time of the chemotherapy group was less than half of that in the transplantation group (24). The statistical analysis shows that hematopoietic stem cell transplantation (HSCT) can significantly improve the survival rate of patients, we can consider that only chemotherapy for patients with fusion gene positive is not enough. The total 3-year overall survival rate (3y OS) of the 9 patients with fusion gene positive T-ALL who received allogeneic hematopoietic stem cell transplantation was 73% (21), which is similar to the outcome of the patients with fusion gene negative after allogeneic

TABLE 3 Treatment and outcome of patients.

Case no.	Diagnosis	Year	Ref./ Year	Treatment	Outcome
1	AUL	40	Kim.J (26) 2010	cytosine arabinoside, idarubicin	CR, alive 7 months and lost to follow-up
2	MS	32	Zhang.H (6) 2020	idarubicin, cytarabine homoharringtonine	Myelosoppression with a rapidly increased pericardial effusion
3	MPAL	29	Li MY (27) 2020	idarubicin, vincristine, dexamethasone, hyper-CVAD-A regimen, hyper-CVAD-B regimen, HSCT,CAR-T	Chemotherapy achieved CR, HSCT,CAR-T,relapse alive>42 months
4	MPAL	22	Chen SM (28) 2021	CODLP or VPIA(vincristine + prednisone + daunorubicin + cytarabine) HSCT	CR,HSCT alive>42 months
5	MPAL	34	Chen SM (28) 2021	CODLP or VPIA(vincristine + prednisone + daunorubicin + cytarabine) HSCT	CR,HSCT alive>24 months
6	AML	35	Rosati R (29) 2007	daunorubicin, cytosine arabinoside HSCT	CR,HSCT still alive
7	AML	46	Jeong IH (30) 2019	idarubicin and cytosine arabinoside,HSCT	CR,HSCT still alive
8	AML	24	Zhang.H (6) 2020	daunorubicin,cytarabine HSCT	CR,HSCT alive>8 months
9	AML	12	Zheng YZ (31) 2021	Cytarabine,FLAG-IDA allo-HSCT	CR,HSCT relapse,died +16.5months
10	AML	10	Zheng YZ (31) 2021	FLAG-IDA allo-HSCT	CR,HSCT alive>27 months
11	AML	20	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>34 months
12	AML	32	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>40 months
13	AML	26	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse alive>90 months
14	AML	12	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>32 months
15	AML	46	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>41 months
16	AML	38	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +45months
17	AML	50	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +25months
18	CML	42	Chen Y (5) 2022	Imatinib, dasatinib, decitabine, venetoclax, ponatinib HSCT	partial response, HSCT alive>95.7 month

(Continued)

TABLE 3 Continued

Case no.	Diagnosis	Year	Ref./ Year	Treatment	Outcome
19	CML	37	Chen Y (5) 2022	Imatinib, dasatinib Idarubicin, cytarabine	Increased after two years of treatment with Imatinib, change to dasatinib, idarubicin and cytarabine relapse,died+36 months
20	B-ALL	18	Chen SM (28) 2023	combination chemotherapy HSCT	HSCT died +9months
21	B-ALL	22	Chen SM (28) 2023	combination chemotherapy HSCT	CR,HSCT relapse, died +15months
22	T-ALL	38	Gorello P (7) 2010	combination chemotherapy ASCT	CR, ASCT alive>29 months
23	T-ALL	19	Gorello P (7) 2010	/	CR, SCT relapse, died +23months
24	T-ALL	27	Gorello P (7) 2010	/	drug resistance died +12months
25	T-ALL	19	Gorello P (7) 2010	/	CR alive>3 months
26	T-ALL	18	Gorello P (7) 2010	/	CR relapse, died +24months
27	T-ALL	23	Gorello P (7) 2010	combination chemotherapy ASCT	CR, ASCT relapse, died +17 months
28	T-ALL	55	Chae H (35) 2011	/	relapse alive>31 months
29	T-ALL	32	Chae H (35) 2011	/	relapse died +42 months
30	T-ALL	32	Chae H (35) 2011	/	relapse died +21 months
31	T-ALL	20	Chae H (35) 2011	HSCT	HSCT alive>41 months
32	T-ALL	12	Li WJ (36) 2011	allo-HSCT	Allo-HSCT relapse, alive
33	T-ALL	11	Li WJ (36) 2011	combination chemotherapy	died +10 months
34	T-ALL	8	Li WJ (36) 2011	combination chemotherapy	CR, alive
35	T-ALL	20	Dai HP (20) 2012	combination chemotherapy	CR, relapse died +9months
36	T-ALL	23	Dai HP (20) 2012	combination chemotherapy	CR relapse, alive>18months

(Continued)

TABLE 3 Continued

Case no.	Diagnosis	Year	Ref./ Year	Treatment	Outcome
37	T-ALL	27	Dai HP (20) 2012	combination chemotherapy	CR relapse, died +15months
38	T-ALL	45	Dai HP (20) 2012	combination chemotherapy	CR relapse, died +30months
39	T-ALL	34	Ben (21) 2014	GRAALL trail	CR, SCT relapse, died +49months
40	T-ALL	37	Ben (21) 2014	GRAALL trail	CR, SCT alive>64months
41	T-ALL	29	Ben (21) 2014	GRAALL trail	CR, SCT relapse, alive>44months
42	T-ALL	41	Ben (21) 2014	GRAALL trail	CR, SCT alive>46months
43	T-ALL	23	Ben (21) 2014	GRAALL trail	died +5months
44	T-ALL	30	Ben (21) 2014	GRAALL trail	CR, SCT relapse, alive>66months
45	T-ALL	36	Ben (21) 2014	GRAALL trail	CR, SCT alive>24months
46	T-ALL	45	Ben (21) 2014	GRAALL trail	CR alive>33months
47	T-ALL	38	Ben (21) 2014	GRAALL trail	SCT died +9months
48	T-ALL	28	Ben (21) 2014	GRAALL trail	CR, SCT alive>30months
49	T-ALL	20	Ben (21) 2014	GRAALL trail	CR, SCT alive>28months
50	T-ALL	48	Prokopiou C (38) 2015	combination chemotherapy	ASCT died +12months
51	T-ALL	45	Prokopiou C (38) 2015	combination chemotherapy	ASCT died +6months
52	T-ALL	26	Yang Q (3) 2019	VICP	died of infection +15days
53	T-ALL	51	Yang Q (3) 2019	VICP, mitoxantroned, etoposide, cytarabine	died of infection +37days
54	T-ALL	37	Yang Q (3) 2019	CALGB9111, CLAG, asparaginase	alive>10months
55	T-ALL	21	Zhang.H (6) 2020	VICP, hyper-CVAD-B, MTX, cladribine,decitabine, HSCT	CR, HSCT alive>14months
56	T-ALL	44	Xianying Xu (39) 2021	VDCLP, CAM(cyclophosphamide, cytosine arabinoside, 6-mercaptopurine), chidamide	CR, but the disease progressed again within a month

(Continued)

TABLE 3 Continued

Case no.	Diagnosis	Year	Ref./ Year	Treatment	Outcome
57	T-ALL	15	Na Lin (40) 2021	VICLP, methotrexate, pegaspargase, DAE, EAD, Hypr-CVAD-A/B, HSCT	CR, HSCT alive>16months
58	T-ALL	58	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>35 months
59	T-ALL	27	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +24months
60	T-ALL	37	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>59 months
61	T-ALL	27	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +26months
62	T-ALL	16	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>41 months
63	T-ALL	36	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +15months
64	T-ALL	40	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +18months
65	T-ALL	41	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT died +22months
66	T-ALL	34	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>51 months
67	T-ALL	15	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +12months
68	T-ALL	12	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, died +12months
69	T-ALL	42	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT alive>29 months
70	T-ALL	36	Chen SM (28) 2021	combination chemotherapy HSCT	CR,HSCT relapse, alive>14 months

hematopoietic stem cell transplantation. This suggests that transplantation can significantly improve the prognosis of patients. It may be a good choice to complete the transplantation at the right time in the first CR.

In this review, we screened 46 effective cases from 49 T-ALL patients in Table 3 (excluding No. 32, No. 34 and No. 56), 30 patients received transplantation, of which 13 died with a median survival of 49 months, 16 patients received chemotherapy, of which 11 died with a median survival of 20 months. The difference between the two groups was tested to be statistically significant (P=0.012). We listed the Kaplan-Meier survival curves of the patients in Figure 3, and it is clear that for T-ALL patients, transplantation can significantly improve the survival status and prolong the overall survival.

CAR-T may play a role in acute leukemia patients with positive fusion gene. The expression frequency of CD7 in previous cases is close to 100%. Research shows that CD7 may play a role in promoting chemoresistance and accelerating disease progression in leukemia (63, 64). Gomes-silva et al. (65) demonstrated that CAR-T targeting CD7 can delay disease progression and prolong patient survival in the mouse model. In the MPAL case reported by Li MY et al. (27) (no. 3),

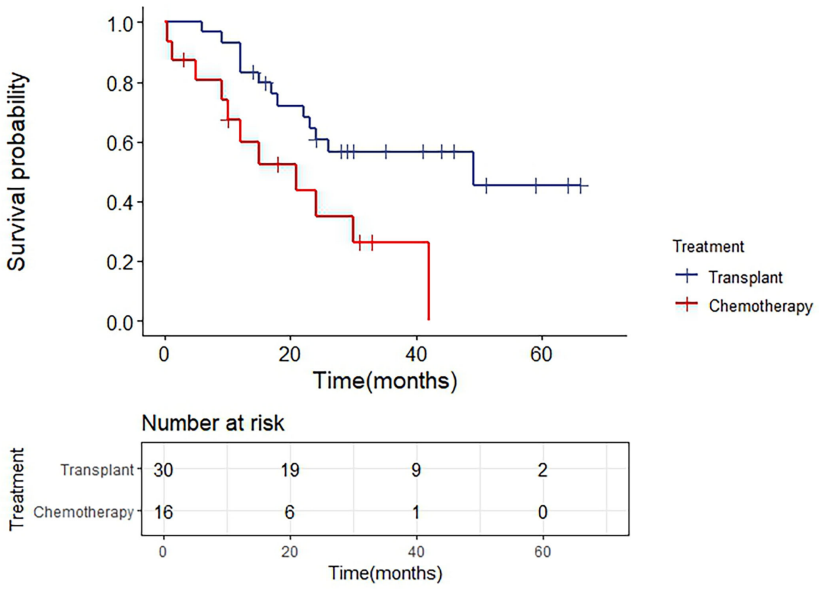

FIGURE 3
Survival analysis of SET-CAN/NUP214 fusion gene positive T-ALL patients.

they performed two times of CAR-T cell infusion treatment on the patients who had relapsed after HSCT, which significantly improved the patient's condition. By the end of follow-up, the patients had survived for more than 42 months. It suggests the application prospect of CAR-T technology in the treatment of fusion gene positive leukemia, which is worthy of further exploration and research.

In this review, 12 patients(12/70) with fusion gene positive AML were included, and the number was second only to T-ALL. Although the chemotherapy regimen of 12 patients was not the same, they all achieved complete remission(CR, 12/12, 100%), all patients received HSCT. Finally, 9 patients survived, 3 patients died and 4 patients relapsed. Chen SM et al. (28) showed that the survival data of *SET-CAN/NUP214* fusion gene positive AML patients were similar to those of fusion gene negative patients.

To date, only four patients with fusion gene positive B-ALL have been reported. Similarly, their treatment process was very difficult. The two patients reported by Nowak NJ et al. (32) and Hong HZ et al. (4) were resistant to chemotherapy, and have not achieved complete remission. Unfortunately, the report didn't mentioned the follow-up of the two patients. One of the two patients(No.20-21) reported by Chen SM et al. (28) achieved complete remission, and both patients received HSCT, but they died of graft-versus-host disease (GVHD) and relapse respectively 9 and 15 months after transplantation. Although the sample of related B-ALL cases is small, we can still speculate that the patients with *SET-CAN/NUP214* fusion gene positive B-ALL may have poor prognosis.

Chen Y et al. (5) first reported two rare cases of *SET-CAN/NUP214* fusion gene positive CML in 2022 (No.18-19), the two patients detected *BCR-ABL1* and *SET-CAN/NUP214* fusion transcripts after 7 and 2 years of treatment with tyrosine kinase inhibitor (TKI), one patient (no.18) received chemotherapy and HSCT, and still survived up to the end of follow-up (95.7 months after initial diagnosis, 6.5 months after transplantation), the other patient (No.19) gave up treatment and died 36 months after the initial diagnosis. Retrospective analysis of samples from two patients showed that *SET-CAN/NUP214* fusion transcript was present at the initial diagnosis, but not during TKI treatment. The disease progression of CML is slow and typically categorized into three phases. The chronic phase (CP) is often asymptomatic but may include mild fatigue, emaciation, and splenomegaly on physical examination. The accelerated phase (AP) is characterized by fever, progressive splenomegaly, and the appearance of additional chromosomal abnormalities. The acute transformation stage (BP) is marked by the continued deterioration of symptoms and signs. Additional chromosome abnormalities play an important role in the deterioration of CML in chronic phase (CP) and accelerated phase (AP), *SET-CAN/NUP214* fusion gene may be used as the main clone in CML to promote disease transformation, and its combination with *BCR-ABL1* accelerates disease progression. Similar to the treatment of other fusion genes in CML cases, high intensity TKI chemotherapy and HSCT may be more effective for these patients (5, 66).

SET-CAN/NUP214 fusion gene has also been found in AUL, MS and MPAL. The incidence of AUL is relatively rare. It is considered to be the result of clone expansion and maturation stagnation of undifferentiated hematopoietic cells, and does not express myeloid or lymphoid specific antigen. MS is a limited tumor formed by the proliferation and infiltration of myeloid primitive cells or immature myeloid cells outside the marrow. It may occur in association with various myeloproliferative disorders or in isolation. The lesions are mostly located in a single site, and sometimes multifocal or multiorgan involvement is present (67, 68). In this review, a case of *SET-CAN/NUP214* fusion gene positive MS patient (No.2) was included. During the

treatment, the patient also suffered from bone marrow compression and pericardial effusion. The incidence of MPAL in acute leukemia is relatively low, accounting for only 2-5% of acute leukemia cases. At present, MPAL lacks a unified treatment option, and the prognosis of patients is usually worse than AML or ALL (69). Li MY et al. (27) treated a 29-year-old *SET-CAN/NUP214* fusion gene positive MPAL patient identified by them (Table 3, No.3) with induction and consolidation therapy leading to CR and transplanted the patient, but the patient relapsed six months later, followed by a lymphocyte consumption program based on fludarabine (30 mg/m^2, 1-3days) and cyclophosphamide (300 mg/m^2, 1-3days) and CAR-T cell therapy. The patient ultimately survived greater than 42 months. Chen SM et al. (28) used the treatment regimen CODLP or VPIA (vincristine+prednisone+daunorubicin+cytarabine) and transplantation for two patients(Table 3, No.4-5) with positive *SET-CAN/NUP214* fusion gene positive MPAL who were 22 years old and 34 years old. Both patients ultimately survived to the end of the follow-up period(survival>42 months and>24 months).

5.4 Prognosis prediction based on the expression level of SET-CAN/NUP214 fusion gene

Among the 70 patients counted in Table 3, 28 patients relapsed and 30 patients died. Relapse and death are common clinical outcomes in *SET-CAN/NUP214* fusion gene positive leukemia. We need to monitor the prognosis of patients with some indicators and detection methods, so as to better evaluate the condition of patients and timely intervene.

Current research shows that the detection of *SET-CAN/NUP214* fusion gene may be a minor residual disease (MRD) with early recurrence, or an early indicator of poor prognosis (24). Chen SM et al. carried out a long-term continuous monitoring of *SET-CAN/NUP214* gene transcript level in 24 patients, and learned that the expression level of fusion gene was lower than 0.001% continuously, which was a sign of good prognosis. The median time of morphological relapse in patients with expression level higher than 0.001% was only 5 months. Gao MG et al. (41) studied the prognostic significance of fusion gene expression level before and after allogeneic hematopoietic stem cell transplantation for patients. The expression level of fusion gene after transplantation is higher than 0.02%, which is an effective indicator of patients' relapse. Monitoring the expression level of *SET-CAN/NUP214* fusion gene through RQ-PCR is more sensitive than flow cytometry (FCM), its sensitivity for detection of various genetic abnormalities and mutation types can reach 10^{-5}, whereas the sensitivity of FCM is usually at 10^{-4} (70). 4 of the 5 patients with relapse after transplantation have *SET-CAN/NUP214*+ before relapse, and their FCM detection results are negative. Previous studies also emphasized the significance of MRD monitoring in transplantation. Positive MRD before transplantation may indicate poor prognosis after transplantation (70, 71).

6 Conclusion

In summary, *SET-CAN/NUP214* fusion gene is relatively rare in leukemia and mainly occurs in adult male T-ALL patients. It has also been reported in AUL, MS, MPAL, AML, CML and B-ALL. Patients are generally resistant to chemotherapy, and the prognosis in different diseases may be different. The clinical symptoms of positive and negative fusion gene patients are relatively similar, and the common immunephenotypes are CD7, cCD3, CD34, CD33 and CD13. The karyotypes may be normal or complex, the concomitant molecular events can become the influencing factors of disease progression and prognosis. HSCT can significantly improve the survival rate of patients, CAR-T is also a potential treatment method. RQ-PCR is an effective monitoring method, and the monitoring of fusion gene may be more sensitive than FCM. Prognosis prediction and recurrence intervention based on the expression level of *SET-CAN/NUP214* fusion gene can improve the treatment effect. Further research is needed to evaluate the role of *SET-CAN/NUP214* fusion gene in leukemia.

Author contributions

JS: Data curation, Investigation, Methodology, Software, Visualization, Writing – original draft. HL: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. SF: Project administration, Supervision, Writing – review & editing.

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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.

Publisher's note

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EDITED BY
Carmelo Gurnari,
Cleveland Clinic, United States

REVIEWED BY
Alankrita Taneja,
University at Buffalo, United States
Luca Vincenzo Cappelli,
Sapienza University of Rome, Italy

*CORRESPONDENCE
Chun Chen
chenchun@mail.sysu.edu.cn

†These authors have contributed equally to this work and share first authorship

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Mutations of RAS genes identified in acute myeloid leukemia affect glycerophospholipid metabolism pathway

Tianqi Liang[1†], Yanxiang Kong[2†], Hongman Xue[1], Wenqing Wang[1], Chunmou Li[1] and Chun Chen[1*]

[1]Pediatric Hematology Laboratory, Division of Hematology/Oncology, Department of Pediatrics, The Seventh Affiliated Hospital of Sun Yat-Sen University, Shenzhen, Guangdong, China, [2]Department of Reproductive Medicine, The Seventh Affiliated Hospital, Sun Yat-sen University, Shenzhen, Guangdong, China

Background: Acute myeloid leukemia (AML) is a malignant disease originating from myeloid hematopoietic stem cells. Recent studies have shown that certain gene mutations promote tumor cell survival and affect the prognosis of patients by affecting metabolic mechanisms in tumor cells. *RAS* gene mutations are prevalent in AML, and the *RAS* signaling pathway is closely related to many metabolic pathways. However, the effects of different *RAS* gene mutations on AML cell metabolism are unclear.

Objectives: The main purpose of this study was to explore the effect of *RAS* gene mutation on the metabolic pathway of tumor cells.

Methods: In this study, we first used a retrovirus carrying a mutant gene to prepare Ba/F3 cell lines with *RAS* gene mutations, and then compared full-transcriptome data of Ba/F3 cells before and after *RAS* gene mutation and found that differentially expressed genes after $NRAS^{Q61K}$ and $KRAS^{G12V}$ mutation.

Results: We found a total of 1899 differentially expressed genes after $NRAS^{Q61K}$ and $KRAS^{G12V}$ mutation. 1089 of these genes were involved in metabolic processes, of which 167 genes were enriched in metabolism-related pathways. In metabolism-related pathways, differential genes were associated with the lipid metabolism pathway. Moreover, by comparing groups, we found that the expression of the *DGKzeta* and *PLA2G4A* genes in the glycerophospholipid metabolism pathway was significantly upregulated.

Conclusion: In conclusion, our study revealed that *RAS* gene mutation is closely related to the glycerophospholipid metabolism pathway in Ba/F3 cells, which may contribute to new precision therapy strategies and the development and application of new therapeutic drugs for AML.

KEYWORDS

acute myeloid leukemia, NRAS Q61K, KRAS G12V, glycerophospholipid metabolism, DGKzeta, PLA2G4A

1 Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy originating from hematopoietic stem cells and is characterized by clonal proliferation and abnormal differentiation of myeloid cells in bone marrow and peripheral blood (1). AML is the most common leukemia in adults and the second most common acute leukemia in children, with high mortality and low overall survival in both adults and children (2). AML is accompanied by many kinds of cytogenetic abnormalities, and different cytogenetic abnormalities can significantly affect prognosis in AML (3). During AML pathogenesis, metabolic mechanisms are altered to meet the high demands of metabolic models established by cloning malignant tumor cells (4). By using different sources of nutrients for energy and biomass supply, AML cells exhibit metabolic plasticity and rapidly outcompete normal hematopoietic cells, leading to their high involvement in disease progression and resistance to treatment (5). The *RAS* oncogene has been identified as a key factor in the regulation of cell proliferation induced by retroviruses (6). The RAS protein encoded by this gene is a specialized guanine nucleotide-binding and hydrolyzing molecule that belongs to the small G-protein superfamily (7). Mutant Ras proteins differentially activate the RAF/MEK/ERK kinase cascade and other noncanonical downstream signaling molecules, which are closely related to tumorigenesis (8). In addition, studies have shown that the RAS protein family can significantly affect the metabolism of tumor cells and exert a significant impact on the metabolism of various organic compounds in tumor cells (9). Statistical analyses revealed a high incidence of *RAS* gene mutations in AML, especially in children (10). However, whether *RAS* gene mutations affect the metabolism of AML cells remains unclear.

In our study, by comparing changes in the transcriptome before and after *RAS* gene mutation, we identified key pathways and genes related to cell metabolism that are affected by *RAS* gene mutation, which may lead to the identification of new targets and strategies for the treatment of AML.

2 Materials and methods

2.1 Cell culture

Ba/F3 is an IL-3 dependent mouse pre B-cell line. Because it can survive independently of IL-3 after the introduction of a driving mutant gene, it has been used as a common tool to study the role of secondary mutant genes (11). In the published literature, Ba/F3 cell line was also used as a model cell to study AML (12–14). In our study, Ba/F3 cells were maintained in RPMI (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS, FBS-S500), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, Thermo Fisher Scientific, USA) and 1 ng/ml IL-3 (PeproTech, USA) at 37°C in a humidified atmosphere containing 5% CO_2.

2.2 Generation of RAS gene-mutated Ba/F3 cells

Following the experimental method described by Chen et al (15), we used a retrovirus carrying a mutant gene to prepare Ba/F3 cell lines with *RAS* gene mutations. Retroviruses carrying the pMSCV-IRES-GFP plasmid vector harboring full-length KRAS-G12V and NRAS-Q61K were used, along with the pVSV-G plasmid, to transfect GP2-293 cells (16, 17). Recombinant retroviruses were isolated by centrifugation at 20000×g for 2 h, and these viruses were used to infect Ba/F3 cells in the presence of 5 μg/ml polybrene (Sigma–ldrich, USA) under centrifugation at 1800×g for 2 h at room temperature. Infected Ba/F3 cells were cultured in RPMI with 10% FBS in the presence of IL-3 for 24 h and then seeded in semisolid medium containing RPMI, 10% FBS, and 1% methylcellulose but not IL-3. Single colonies were selected after 8-10 days in culture and expanded in IL-3-free liquid medium. Ba/F3 cells successfully producing RAS gene mutations can grow independently of IL3.

2.3 Transcriptome analysis

After cultivation and further amplification, stable Ba/F3 parental, $KRAS^{G12V}$ and $NRAS^{Q61K}$ cell lines were obtained. The cells were collected in lyophilization tubes and frozen in liquid nitrogen for 10 minutes. Raw data and normalized gene expression data are deposited in the sequence read archive database under accession numbers PRJNA1006527. The isolation of RNA and next-generation sequencing were performed by Beijing Genomics Institute (Beijing, China). Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, Venn diagram and heatmap analyses were performed with OmicShare tools, a free online platform used for data analysis (https://www.omicshare.com/tools/).

2.4 Statistical analysis

Data visualization and statistical analysis were carried out using GraphPad Prism 8.0 software (GraphPad Software Inc., CA, USA). Differences between experimental groups were analyzed for significance by unpaired Student's t test. A P value <0.05 was considered significant.

3 Results

3.1 Mutations in the RAS gene significantly affect metabolic pathways in Ba/F3 cells

We performed transcriptome analysis in 3 strains of cell lines, including the Ba/F3 parental strain, Ba/F3 $KRAS^{G12V}$ strain and Ba/F3 $NRAS^{Q61K}$ strain. Flow cytometry showed that $KRAS^{G12V}$ and

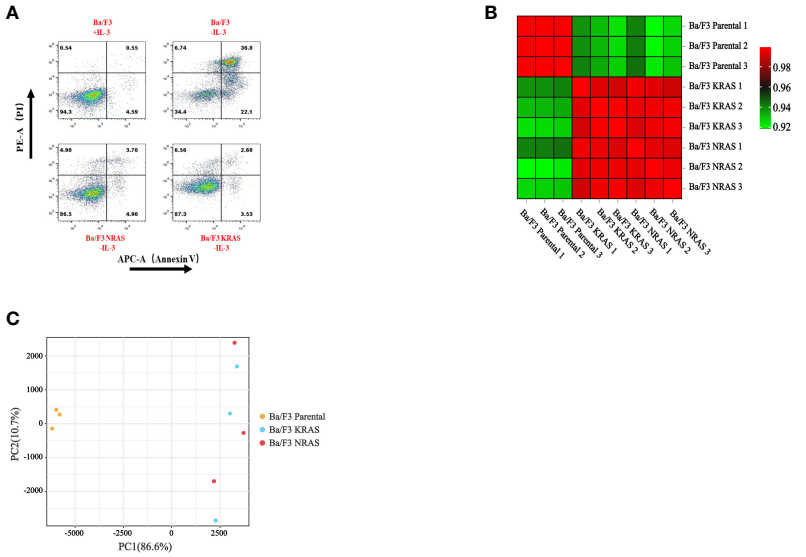

FIGURE 1
Changes in cellular characteristics after RAS gene mutation. **(A)** After the removal of IL-3, apoptosis occurred in wild-type Ba/F3 cells, and Ba/F3 cells with RAS gene mutations continued to grow. The heatmap **(B)** and principal component analysis **(C)** were used to analyze the intergroup variability before and after RAS gene mutation.

$NRAS^{Q61K}$ mutant cell lines were successfully prepared. (Figure 1A). Heatmap analysis and principal component analysis revealed large intergroup variability and small intragroup variability before and after *RAS* gene mutation (Figures 1B, C). These data indicate an ideal cell line model for our transcriptome analysis. To explore the effect of *RAS* gene mutation, we analyzed whole-genome and full-transcriptome sequencing data of the Ba/F3 parental, $KRAS^{G12V}$ and $NRAS^{Q61K}$ cell lines. A volcano map shows that many genes were differentially expressed before and after the induction of the $KRAS^{G12V}$ and $NRAS^{Q61K}$ mutants. After $KRAS^{G12V}$ induction, 963 genes were upregulated, and 1216 genes were downregulated. In addition, after $NRAS^{Q61K}$ induction, 979 genes were upregulated, and 1310 genes were downregulated (Figures 2A–C; Supplementary Tables S1–S4). According to a Venn analysis, there were 1899 common differentially expressed genes (Figure 2D; Supplementary Table S5).

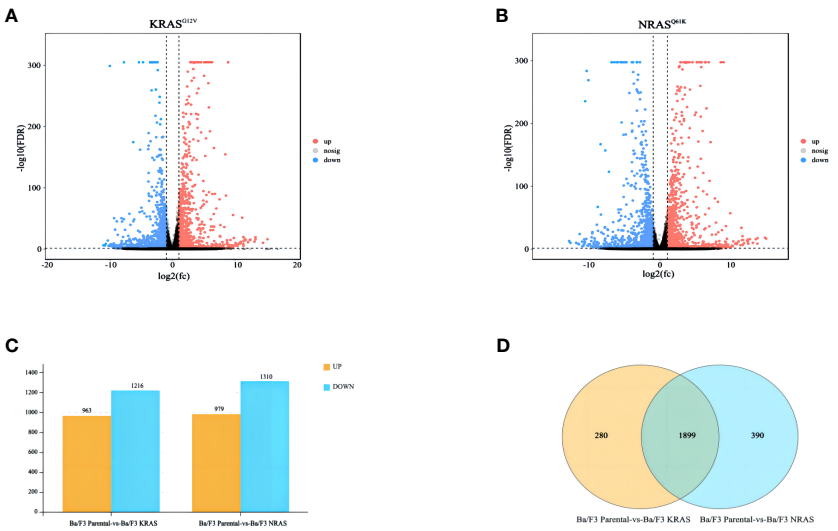

FIGURE 2
Differentially expressed genes after RAS gene mutation. **(A)** Differentially expressed genes after $KRAS^{G12V}$ mutation. **(B)** Differentially expressed genes after $NRAS^{Q61K}$ mutation. **(C)** Bar charts showing the number of significantly different genes between the two groups (FDR < 0.05, multiple differences greater than or equal to 2). **(D)** Venn diagram showing common differentially expressed genes.

3.2 Genes after KRAS^{G12V} and NRAS^{Q61K} induction mainly affect the metabolism-related pathways of Ba/F3 cells

We analyzed the differentially expressed genes after induction of KRAS^{G12V} and NRAS^{Q61K} via Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. Finally, we found that a total of 1089 genes were involved in metabolic processes (Supplementary Table S6), of which 167 genes were enriched in metabolism-related pathways (P<0.05) (Figures 3A, B) (Supplementary Table S7).

3.3 Metabolism-related genes mainly affected glycerophospholipid metabolism after KRAS^{G12V} and NRAS^{Q61K} induction

A total of 167 genes related to metabolic pathways were identified in Ba/F3 cells with *RAS* gene mutations. The GO analysis showed that these genes mainly affected the small molecule metabolic process in cells. To identify a specific metabolic pathway, we carried out a KEGG analysis. The results showed that these genes were enriched in multiple metabolic pathways, of which 12 genes were enriched in the glycerophospholipid metabolism pathway (P<0.05) (Figures 4A, B; Supplementary Table S8).

3.4 *DGKzeta* and *PLA2G4A* were key genes in the glycerophospholipid metabolism of Ba/F3 cells with RAS mutations

There are 12 genes involved in the regulation of glycerophospholipid metabolism, and the heatmap shows the differences in their expression among the Ba/F3 parental group, KRAS^{G12V} group and NRAS^{Q61K} group (Figure 5A). Through Venn analysis, 2 of the 12 genes involved in glycerophospholipid metabolism were found to be significantly upregulated and coexpressed in the KRAS^{G12V} and NRAS^{Q61K} mutant cell lines (FPKM>100) (Figure 5B). Gene expression analysis showed that the *DGK**zeta*** and *PLA2G4A* genes were increased significantly in both the KRAS^{G12V} and NRAS^{Q61K} mutant cell lines, and a significant difference was found between the Ba/F3 parental groups (P<0.05) (Figures 5C, D).

4 Discussion

Studies have shown that the original metabolic patterns in tumor cells change to meet the increased bioenergetic and biosynthetic demand during tumorigenesis and progression and to mitigate oxidative stress during the proliferation and survival of tumor cells (18). Studies on the metabolic mechanisms of tumor cells are helpful to explore the occurrence, progression, diagnosis and treatment of tumors. It has been proven that the fatty acids produced by lipid decomposition enter the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) metabolic pathway after oxidation by mitochondrial β-oxidation, thus producing ATP and NADPH to provide energy (5). More importantly, some special lipids produced by lipid metabolism can be used as essential lipid signaling molecules to regulate the biological processes of tumor cells. Meanwhile, the two upregulated genes *DGKzeta* and *PLA2G4A* found in our study are involved in lipid signal regulation, suggesting that RAS gene mutations in AML may have biological effects by affecting lipid signals.

Diacylglycerol (DAG) is a key secondary lipid messenger in signal transduction downstream of many receptors and plays an important role in driving adaptive and innate immune cell activation, proliferation, migration and effector functions (19). Diacylglycerol kinases (DGKs) can regulate the DAG signaling pathway by phosphorylating DAG and converting it into phosphatidic acid (PA) (20). DGK has 10 different isoforms, which are composed of five different classes of DGKs, each of

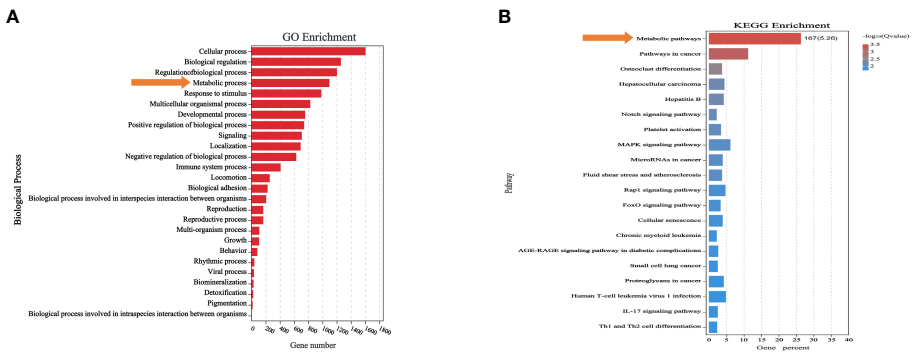

FIGURE 3
Differentially expressed genes were associated with metabolic processes and pathways. **(A)** Biological process significantly affected metabolic process. **(B)** Differentially expressed genes after RAS mutation were found to mainly affect metabolic pathways.

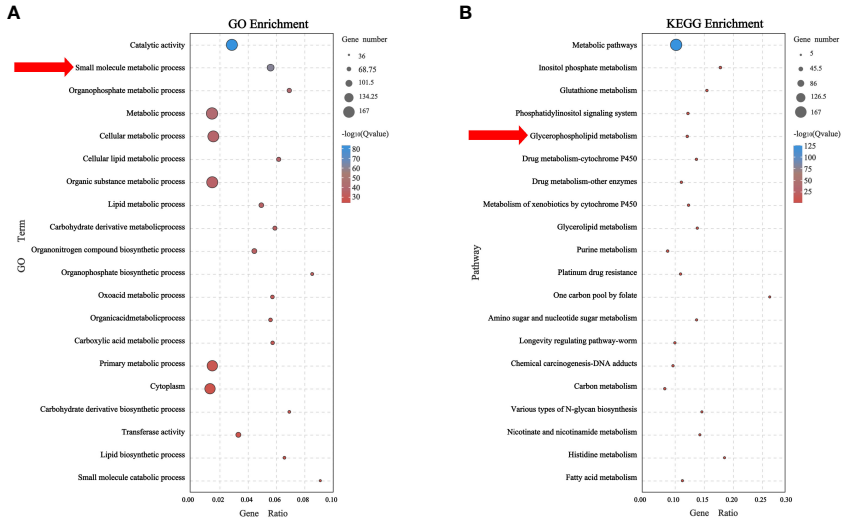

FIGURE 4
Metabolic-related genes mainly affect glycerophospholipid metabolism **(A)** Biological process of related genes affected small molecule metabolic process. **(B)** Related genes were found to mainly affect glycerophospholipid metabolism by KEGG enrichment analysis.

which regulates different cellular functions according to its different structure and location in different cells. Studies have confirmed that DGKα is highly expressed in several refractory cancer cells, such as melanoma, hepatocellular carcinoma and glioblastoma. It can slow tumor cell apoptosis and promote cell proliferation (21). As an isoform of DGKα, DGKzeta is highly expressed in lymphoid tissues (22), which affects tumor cell apoptosis and cell cycle arrest. In human AML HL-60 cells, knockout of DGKzeta can induce apoptosis and G2/M phase arrest through the MAPK/survivin/caspase pathway (23). Our study found that the expression of DGKzeta was significantly upregulated after RAS gene mutation, indicating that DGKzeta may be the key factor affecting the regulation of AML cell proliferation after RAS gene mutation. DGKzeta has a negative regulatory effect on T cells (19), which can suppress the development of natural regulatory T cells and predominantly mediates Ras and Akt signaling downstream of the TCR (24). Interestingly, DGKzeta expression was also significantly upregulated after RAS gene mutation in our study, and whether it affects the immune escape of tumor cells needs to be further studied.

Phospholipase A2 enzymes (PLA2s) are the key enzymes of phospholipase metabolism. According to their location in the body, substrate specificity and differences in physiologic function, PLA2s can be divided into six subfamilies. Its function is to hydrolyze the sn-2 acyl bond of glycerol phospholipids (GPLs), release lysophospholipids (LPLs) and generate free fatty acids (25). These fatty acids are important energy sources for AML cells. PLA2G4A

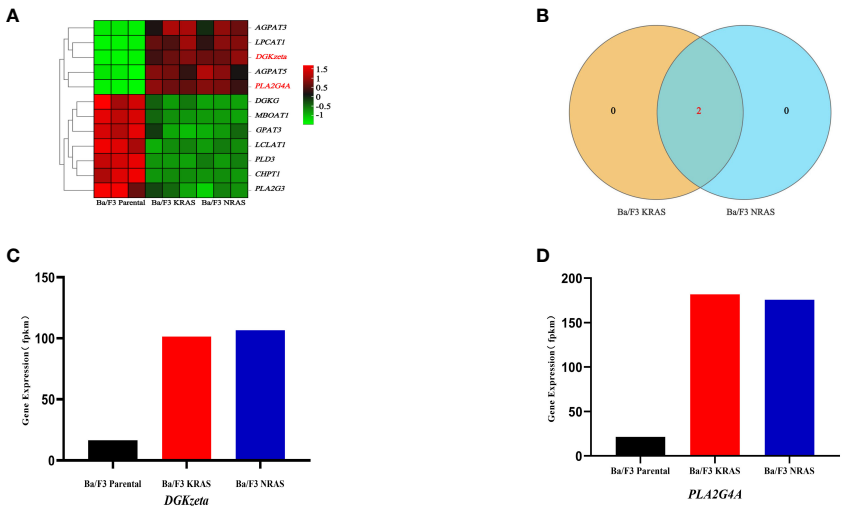

FIGURE 5
DGK*zeta* and *PLA2G4A* were candidate genes in glycerophospholipid metabolism. **(A)** The heatmap shows the expression of genes in different strains of cell lines. **(B)** The shared key genes were found by Venn analysis (FPKM>100). **(C, D)** Expression of key genes related to glycerophospholipid metabolism was detected by RNA-seq.

(cPLA2-IVA) belongs to a kind of cPLA2. In tumor cells, its activation is mainly regulated by the MAPK signaling pathway, and it is a key enzyme in AA metabolism (26). Overexpression of PLA2 can increase the release of AA and enhance the protumoral effects mediated by eicosanoids in promoting tumor survival, proliferation, antiapoptosis, transformation and metastasis (27). Studies have shown that cPLA2 plays a carcinogenic role in most cancers except colon cancer (28). Downregulation or deletion of cPLA2 can significantly inhibit the formation of small intestinal tumors induced by Apc(Min) and lung tumors induced by urethane (29, 30). Moreover, the inactivation of cPLA2 inhibits the occurrence of liver cancer (31) and the formation of prostate tumors (32). Using weighted gene coexpression network analysis to analyze the RNA sequencing data and clinicopathological characteristics of large samples of AML patients, it was found that the high expression of PLA2G4A was related to adverse overall survival (33). It was also found that PLA2G4A can be used as an independent prognostic marker in some specific types of AML. For example, in non-M3/nucleophosmin (NPM1) wild-type AML, patients with high expression of PLA2G4A had a significantly shorter overall survival rate. Moreover, some proteins with well-characterized oncogenic properties in AML, such as RUVBL2, CAP1, STAT3 and MYCBP, can physically interact with PLA2G4A (34). It has also been found that the high expression of PLA2G4A in FLT3-mutated AML is not only an indicator of poor prognosis but also related to drug resistance to tyrosine kinase inhibitors and changes in the tumor microenvironment of AML (35). Our study found that the expression of PLA2G4A was significantly upregulated after RAS gene mutation, which may be a potential therapeutic target for the treatment of AML with RAS gene mutation.

In conclusion, our study revealed that RAS gene mutations may affect cell metabolism. This effect may be achieved by altering the glycerophospholipid metabolism pathway. Among these candidate genes, *DGKzeta* and *PLA2G4A* were identified as key to cell metabolism. These results may provide a new strategy and therapeutic target for AML therapy with RAS gene mutations.

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 below: https://www.ncbi.nlm.nih.gov/, PRJNA1006527.

Author contributions

TL: Methodology, Writing – original draft. YK: Writing – original draft. HX: Data curation, Writing – review & editing. WW: Data curation, Writing – review & editing. CL: Data curation, Writing – review & editing. CC: Project administration, Writing – original draft.

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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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Anna Maria Testi,
Sapienza University of Rome, Italy

REVIEWED BY
Nico Gagelmann,
University Medical Center Hamburg-Eppendorf, Germany
Hassan Awada,
University at Buffalo, United States
Marco Antonacci,
Sapienza University of Rome, Italy

*CORRESPONDENCE
Alessandra Sperotto
alessandra.sperotto@iov.veneto.it

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CPX-351 and allogeneic stem cell transplant for a therapy-related acute myeloid leukemia that developed after treatment of acute promyelocytic leukemia: a case report and review of the literature

Alessandra Sperotto[1]*, Maria Teresa Lupo Stanghellini[2], Jacopo Peccatori[2], Roberta De Marchi[1], Simona Piemontese[2], Giulia Ciotti[1], Marco Basso[1], Elisabetta Pierdomenico[1], Paolo Fiore[2], Fabio Ciceri[2] and Michele Gottardi[1]

[1]Onco Hematology, Department of Oncology, Veneto Institute of Oncology, Istituto Oncologico Veneto-Istituto di Ricerca e Cura a Carattere Scientifico (IOV-IRCCS), Castelfranco Veneto, Italy, [2]Hematology and Hematopoietic Stem Cell Transplantation Unit, Istituto di Ricerca e Cura a Carattere Scientifico (IRCCS) San Raffaele Scientific Institute, Milano, Italy

Therapy-related myeloid neoplasms (t-MNs), which develop after cytotoxic, radiation, or immunosuppressive therapy for an unrelated disease, account for 7%–8% of acute myeloid leukemia (AML). Worse outcomes and consequently shortened survival are associated with t-MNs as compared with *de novo* AML. Therapy-related MNs are being reported with increasing frequency in successfully treated acute promyelocytic leukemia (APL), in particular, before the introduction of all-*trans* retinoic acid (ATRA) plus arsenic trioxide (ATO). Considering the high curability of APL, t-MNs represent one of the prognosis-limiting factors in this setting of leukemia. We report our experience with a patient who developed t-AML 15 years after treatment for APL. Treatment included three cycles of chemotherapy with CPX-351 (Vyxeos, Jazz Pharmaceuticals) followed, as in remission, by an allogeneic hematopoietic stem cell transplant. A review of available literature was also included.

KEYWORDS

acute promyelocytic leukemia, therapy-related myeloid neoplasm, allogeneic hematopoietic stem cell transplantation, CPX-351, acute myeloid leukemia

Introduction

Therapy-related myeloid neoplasms (t-MNs), including therapy-related myelodysplasia (t-MDS) and acute myeloid leukemia (t-AML), have been extensively reported after cytotoxic therapy or immunosuppressive treatment for solid tumors, lymphomas, or autoimmune disorders, more rarely after treatment for acute myeloid leukemia (1).

Traditionally subgrouped according to the previous exposure to alkylating agents, topoisomerase II inhibitors, or radiotherapy (RT), more recently, t-MN development has been associated with new agents belonging to different classes of chemotherapy (CHT) drugs, such as poly(ADP-ribose) polymerase inhibitors or purine analogs (2, 3).

Moreover, recent advances in deep sequencing techniques have significantly improved the knowledge of t-MNs over the last years, changing some of the classical views.

Acute promyelocytic leukemia (APL) is characterized by the translocations that fuse the *PML* gene on chromosome 15 to the *RARalpha* gene on chromosome 17 [t(15;17)], leading to a *PML–RARalpha* fusion gene; other peculiarities are the morphology of blast cells and a specific coagulopathy. Thanks to the advent of all-*trans* retinoic acid (ATRA) combined with anthracycline-based chemotherapy (4, 5) and/or arsenic trioxide (ATO), a cure rate higher than 70% has been achieved, even in relapsed patients (6–8). Thereby, the number of long-term survivors of this disease has increased over time. Consequently, more patients will be at risk of late complications related to antileukemic treatment.

Regarding t-MNs occurring after treatment for APL, sporadic cases have been reported in the literature, while only three major studies have assessed the incidence of t-MNs, ranging from 0.97% to 6.5% (4, 5). Moreover, only one of those studies calculated the cumulative incidence of a competing risk at a given time, resulting in approximately 2.2% at 6 years (9).

Survival in t-MNs is poor. In addition to the biology of t-MNs, the patient's previous disease history and remission status at t-MN diagnosis are significant factors contributing to unfavorable outcomes. Also, t-MNs secondary to APL are usually difficult to treat, representing one of the prognosis-limiting factors for the curable APL disease.

We report a patient who developed t-AML 15 years after completion of maintenance therapy according to the GIMEMA AIDA2000 protocol for a previous APL still in molecular remission. A comprehensive review of the literature of previously published cases is also included.

Case report

A 46-year-old man presented in January 2005 with fatigue, dyspnea, and a history of bleeding tendency. Coagulation tests showed disseminated intravascular coagulation, and peripheral blood cell count was as follows: hemoglobin 90 g/L, white cells 66.000 × 10^9/L (with 60% hypergranular promyelocytes), and platelets 12.000 × 10^9/L. Bone marrow revealed 70% hypergranular promyelocytes, with the characteristics t(15;17)(q22;q21) in all metaphases examined; molecular biology studies (performed by reverse transcription–polymerase chain reaction (RT-PCR)) confirmed the presence of *PML/RARα* gene rearrangement type bcr3. A diagnosis of high-risk hypergranular APL was made. Next-generation sequencing (NGS) analysis was not performed at diagnosis of APL.

The patient was treated according to the GIMEMA AIDA2000 protocol, receiving induction treatment with oral ATRA (45 mg/m^2 per day for a total of 45 days) and four doses of intravenous idarubicin (12 mg/m^2 on days 2, 4, 6, and 8): a complete molecular remission was achieved on day 38. Consolidation (according to a risk-adapted strategy) consisted of three courses, as follows: one course with intravenous cytosine arabinoside (Ara-C) (1 g/m^2 on days 1, 2, 3, and 4) plus idarubicin (15 mg/m^2 on days 1, 2, 3, and 4) plus oral ATRA (45 mg/m^2 per day for 15 days); then, intravenous mitoxantrone (10 mg/m^2 on days 1, 2, 3, 4, and 5) plus etoposide (100 mg/m^2 on days 1, 2, 3, 4, and 5) plus oral ATRA (45 mg/m^2 per day for 15 days); finally, intravenous idarubicin (12 mg/m^2 on days 1) plus Ara-C (150 mg/m^2 every 8 hours on days 1, 2, 3, 4, and 5) plus 6-thioguanine (70 mg/m^2 every 8 hours on days 1, 2, 3, 4, and 5) plus oral ATRA (45 mg/m^2 per day for 15 days).

Then, as in molecular remission, maintenance therapy was started, consisting of intramuscular methotrexate (15 mg/m^2) plus oral 6-mercaptopurine (50 mg/m^2) alternating with oral ATRA (45 mg/m^2 per day for 15 days) every 3 months for a total of 2 years.

Annual cytogenetic and molecular analyses were performed until December 2015, confirming molecular remission. From January 2018 to February 2020, the patient stopped his annual follow-ups. In March 2020, blood cell count revealed mild anemia (hemoglobin 120 g/L) and thrombocytopenia (platelets 111.000 × 10^9/L). Bone marrow analysis, performed in May 2020, confirmed molecular remission with initial cytological signs of dysplasia. Blood cell count remained stable until May 2022, when a morphological analysis of peripheral blood detected almost 10% blast cells. Bone marrow aspiration was hypercellular, showing 60% blast cells and red-cell line hyperplasia with multiple dyserythropoietic changes in erythroblasts (megaloblastic features, abnormal mitosis, and lobulated nuclei). Cytogenetic analysis revealed a complex karyotype (47, XY, +8, −2, −5, ins(mar;9)(?;q)?, del(12)(p13), +mar, inc), without t(15;17)(q22;q21). The molecular biology study was negative for *PML/RARα* gene rearrangement and positive for *WT1* gene hyperexpression and *KIT-D816V* exon 17 mutation.

NGS analysis, performed using second-generation sequencing technology on an Illumina MiSeq System (Illumina, San Diego, CA, USA) high-throughput sequencing platform, showed TP53 positivity with a variant allele frequency (VAF) of 78.0%.

Treatment with CPX-351 (Vyxeos, Jazz Pharmaceuticals, Dublin, Ireland; a liposomal encapsulation of cytarabine and daunorubicin in a synergistic 5:1 drug ratio) was started on June 2022—when the patient was 63 years old. CPX-351 has a specific indication for newly diagnosed s-AML, including t-AML, and the

choice of CPX-351 was also linked to the age of the patient, good performance status, and time to previous treatment.

Before starting treatment, the patient had a normal echocardiogram [left ventricular ejection fraction (LVEF) 68%] and spirometry (diffusing capacity of the lungs for carbon monoxide (DLCO) 85%) and was considered fit for an intensive chemotherapy program. A total of three cycles of CPX-351 were administered (first and second induction and then consolidation), all well tolerated.

Cytofluorimetric remission but not a complete clearance of *WT1* gene hyperexpression (Figure 1) was obtained after the first CPX-351 cycle and then maintained during the other two cycles.

In October 2022, as still in cytofluorimetric remission but with *WT1* over conventional threshold limits (Figure 1), an allogeneic hematopoietic stem cell transplant from an unrelated donor was performed. The patient was 64 years old at transplant with a hematopoietic cell transplant-specific comorbidity index (HCT-CI) score of 3 (previous leukemia) (10).

The conditioning regimen consisted of treosulfan i.v. plus fludarabine i.v.; graft versus host disease (GVHD) prophylaxis consisted of sirolimus, mycophenolate, and post-transplant cyclophosphamide. Allogeneic peripheral blood stem cells were infused on October 15, 2022.

The patient developed acute and then chronic skin GVHD, treated and resolved by steroid and extracorporeal photopheresis. Immunosuppressive treatment was completely withdrawn in March 2023. At the last follow-up—August 31, 2023—the patient was alive, with a mild chronic GVHD (mouth and skin), and in molecular remission with a full donor chimerism.

Review of the literature

A total of 57 t-MN cases secondary to APL treatment were reported in the literature from 1992 to 2010: 44 (77.0%) patients were diagnosed with t-MDS and 13 (23.0%) with t-AML. After 2010, no other t-MN cases secondary to APL treatment were reported in the literature.

The main characteristics of the 57 patients are listed in Table 1 (t-MDS) and Table 2 (t-AML); the median age at diagnosis of APL was 51.5 years (8–73).

Table 3 summarizes the clinical and treatment characteristics of the whole population.

In all 57 patients, RT-PCR monitoring and/or cytogenetic analysis indicated molecular remission of APL at diagnosis of t-MNs. Overall, the median time from the achievement of remission to diagnosis of t-MN was 42.5 months (4–168).

No significant statistical difference between t-MDS and t-AML was observed in the time from the first complete response (CR) to the development of t-MNs (t-MDS: 39.5 months (4–168) *vs.* t-AML: 43 months (17–54), p = 0.07).

Using conventional karyotyping or fluorescent *in situ* hybridization, cytogenetic characterization was successful in 52 (91.0%) of 57 patients and was abnormal in all except three cases, with complex karyotypes (≥three independent abnormalities) observed in 24 (42.0%) patients (Table 3).

Treatment and clinical course of t-MN

Except for three patients for whom treatment was not included in the report, in all the other 54 patients, therapy for t-MN consisted of only supportive therapy in 16 patients (29.5%) (15 MDS and one AML); all of them died after a median of 9 months from t-MN diagnosis (range, 1 to 39 months). The majority of patients (33%–61.0%) were treated with conventional chemotherapy (in one patient, an autologous stem cell transplant was performed after induction treatment): for two patients, follow-up was not available, and all the others died of progressive disease.

Allogeneic stem cell transplant (alloHSCT) was performed in 15 (29.5%) patients (three patients up-front and 11 patients after induction chemotherapy): four transplanted patients lived more than 12 months from transplant, but follow-up was not subsequently updated, while all the others died due to transplant-related mortality (five patients) or progressive disease (six patients) within 12 months from reinfusion (Table 3).

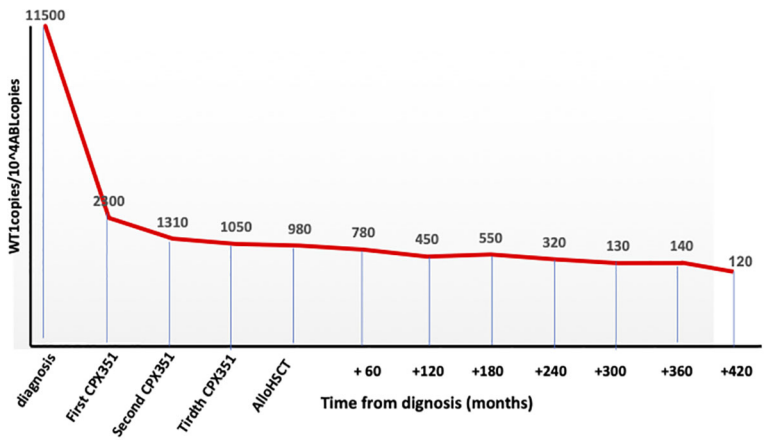

FIGURE 1
WT1 clearance during the whole program.

TABLE 1 Main characteristics and clinical course of patients developing t-MDS after APL therapy.

Ref. n.	Karyotype at APL diagnosis	Therapy for APL				Time to t-MDS (months)	PML-RARα at t-MDS (RT-PCR)	Karyotype at t-MDS diagnosis	Time to t-AML (months)	Therapy for t-AML
		ATRA	Anthracycline	Etoposide	Alkylating agent					
(11)	t (15;17) (q22;q21)		#	#		33	Negative	t (7;21) (q31;q22)	11	ATRA +CHT
(12)	t (15;17) (q22;q21)		#		#	24	Negative	45,XX,dic (5;17) (q11;p11/43,idem, −7, −20	2	NA
(13)	t (15;17) (q22;q21)	#	#			34	NA	45, XX, −7, der (7)del (7) (p10), del (7) (q21)	No t-AML	CHT
(14)	t (15;17) (q22;q21)	#	#	#		25	Negative	47, XY, +8	10	CHT +autoHSCT
(15)	t (15;17) (q22;q21)		#	#		84	NA	43, XX, del (5) (q15), −7, add (9) (q34), −18, −21	No t-AML	NA
(16)	t (15;17) (q22;q21)	#	#	#		26	Negative	−5, add (6)/ (p23-25), +8, add (17) (p23)	4	CHT
(17)	t (15;17) (q22;q21)	#	#			32	Negative	45, XX, −5, add (17) (p11.2), del3 (p23p25), −5, der (6), t (6;15) (p21;q15), −15, add17 (p13)	No t-AML	AlloHSCT upfront
(18)	t (15;17) (q22;q21)	#	#			35	Negative	45, XX, del (4) (q31), −5, add (5) (q35), −7, der (17)t (17);?(p11);?,-18, +mar1, +marX2[cp21]/46,XX (4)	No t-AML	CHT +alloHSCT
(4)	t (15;17) (q22;q21)	#	#			43	Negative	45, XX, −7	18	Supportive therapy
(4)	t (15;17) (q22;q21)	#	#			46	Negative	Failure	1	Supportive therapy
(5)	46, XX, del (3) (q24,q26), del (5) (q23q32), t (7;11) (p11;p12), t (15;17) (q22;q21)	#	#			32	Negative	45, XX, del (5) (q21q34), −7	7	CHT
(5)	46, XY, del (9) (q21q31), t (15;17) (q22;q210	#	#			111	Negative	45, XY, −5, der (7)t (7;20) (q11;p?orq)?,der (10)t (7;10;20) (q3?;q2?;p?orq)?,-13, der (17)t (10;17) (q2?;p11),-20,del (20) (q11),+mar1,+mar3/47,idem,del (X) (q26), der (1) (1);?(p36);?,+8,+mar2	No t-AML	Supportive therapy
(5)	failure	#	#			74	Negative	45, XY, −8, t (8;11) (q32;q21)	18	Supportive therapy
(5)	t (15;17) (q22;q21)	#	#			47	Negative	45, XY, t (3;17) (p11;q11),del (5) (q13q33), del (6) (p22),-17	No t-AML	CHT
(19)	t (15;17) (q22;q21), inv (6) (p24q13)	#	#			4	Negative	44, X, −Y, −7	6	Supportive therapy
(19)	t (15;17) (q22;q21)	#	#			20	Negative	46, XX, del (5) (q13,q33)	No t-AML	Supportive therapy

(Continued)

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TABLE 1 Continued

Ref. n.	Karyotype at APL diagnosis	Therapy for APL				Time to t-MDS (months)	PML-RARα at t-MDS (RT-PCR)	Karyotype at t-MDS diagnosis	Time to t-AML (months)	Therapy for t-AML
		ATRA	Anthracycline	Etoposide	Alkylating agent					
(20)	t (15;17) (q22;q21)	#	#			168	Negative	47, XY, +1, i (1) (q10) (21)/46,XY (4)	No t-AML	Supportive therapy
(22)	t (15;17) (q22;q21)	#	#			18	Negative	46, X, del (X) (q22q28),t (2;11) (q37;q23),del (7) (q22q36)	No t-AML	alloHSCT upfront
(9)	48, XY, t (15;17) (q22; q21), +21,+mar	#	#			52	Negative	45, XY, −7	19	Azacytidine than CHT
(9)	Failure	#	#			62	Negative	45, XX, del (5) (q13;q32), add (10) (p15),der (11) (q)?,add (12) (p13),add (12) (q)?,-13,-18,+mar	6	CHT +alloHSCT
(9)	Failure	#	#			23	Negative	Failure	No t-AML	CHT +alloHSCT
(9)	t (15;17) (q22;q21)	#	#			48	Negative	46, XX, del (7) (q23), del (5), iso (17q)	6	Supportive therapy
(9)	t (15;17) (q22;q21)	#	#			23	Negative	46, XX, del (7q) (q23), t (2;11) (q37;q23),del (X) (q22)	9	CHT +alloHSCT
(9)	t (15;17) (q22;q21)	#	#			44	Negative	44,XY,del (5) (q13q33),-7,-18,add (20) (q13.3), add (11) (p11.2)	4	CHT
(9)	t (15;17) (q22;q21)	#	#			33	Negative	44, YX, −5,add (12) (p13),add (7) (q32),-19	6	Supportive therapy
(9)	t (15;17) (q22;q21), add (7q)	#	#			45	Negative	45, XX, −7, t (12;18) (p12;q21)	18	CHT +alloHSCT
(9)	t (15;17) (q22;q21)	#	#			56	Negative	Failure	No t-AML	Supportive therapy
(9)	Failure	#	#			41	Negative	46, XX, −7	13	CHT +alloHSCT
(23)	t (15;17) (q22;q21)	#	#	#	#	38	Negative	45, XY, −7	NA	NA
(24)	47, XX, +8, t (15;17) (q22;q21)	#	#			29	NA	45, XX, −5, −7, +11	No t-AML	ATRA
(4)	t (15;17) (q22;q21)	#	#	#	#	48	Negative	del (5) (q)?	2	Supportive therapy
(4)	t (15;17) (q22;q21), add (7q)	#	#	#	#	24	Negative	46, XX	5	AlloHSCT upfront

(Continued)

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TABLE 1 Continued

Ref. n.	Karyotype at APL diagnosis	Therapy for APL				Time to t-MDS (months)	PML-RARα at t-MDS (RT-PCR)	Karyotype at t-MDS diagnosis	Time to t-AML (months)	Therapy for t-AML
		ATRA	Anthracycline	Etoposide	Alkylating agent					
(5)	t (15;17) (q22;q21)	#	#			13	Negative	46, XX, del (5) (q22q34), t (15;21) (p11;q21), −17, +mar	No t-AML	Supportive therapy
(5)	t (15;17) (q22;q21)	#	#		#	46	NA	46, XY, del (5) (q12q35), add (11) (q23), dup (12) (q12q22), −17, −18, −22	1	Supportive therapy
(25)	46, XX	#	#	#		NA	Negative	45, XX, −7/46,idem,+21 RUNX1D171N; NRASG12V	t-AML	CHT
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	45, XY, −7/46, XY RUNX1D171G	t-AML	CHT
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	46,XY, t (7;15) (q11;q11), der (12)t (12;17) (p11;q21), t (16;21) (q24;q22), add (17) (q11), add (19) (p13), del (21) (q21), 46,idem,der (18)t (15,18) (q11;p11)/46,XY RUNX1MTG16	t-AML	CHT
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	46, XX, t (6;11) (q21;q23) MLL-FOXO3	t-AML	CHT
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	46, XY, aad (2) (p23), inv (5) (p11q23),add (11) (q23)/46, idem,inv (2) (p23q11)/47,idem,+13 RUNX1S295fsX571	t-AML	CHT
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	45, XY,-7, RUNX1G172W	t-AML	CHT
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	46,XY,t (11;16) (q23;p13.3) MLL-CBP; FLT3ITD	t-AML	CHT +alloHSCT
(25)	t (15;17) (q22;q21),	#	#	#		NA	Negative	46, XY CEBPAQ305P	t-AML	CHT
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	46, XX, del (20) (q11)	No t-AML	Supportive therapy
(25)	t (15;17) (q22;q21)	#	#	#		NA	Negative	46, XX, del (20) (q1)?	No t-AML	Supportive therapy

APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; autoHSCT, autologous hematopoietic stem cell transplant; alloHSCT, allogeneic hematopoietic stem cell transplant; CHT, chemotherapy; NA, not available; t-AML, therapy-related acute myeloid leukemia; t-MDS, therapy-related myelodysplastic syndrome.
#=yes; ?= symbols of cytogenetic.

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TABLE 2 Main characteristics and clinical course of patients developing t-AML after APL therapy.

Ref. n.	Karyotype at APL diagnosis	Therapy for APL				Time to t-AML (months)	PML-RARα at t-MDS (RT-PCR)	Karyotype at t-AML diagnosis	Therapy for t-AML
		ATRA	Anthracycline	Etoposide	Alkylating agent				
(26)	NA		#	#		43	NA	t(3;21)(q26;q22), der(4)t(4);?(q27);?, der(7)t(4;7)(q27;q22), der(16)t(16);?(p11);?	ATRA
(27)	t(15;17)(q22;q21)		#	#	#	43	NA	t(10;11)(q23;p15)	CHT
(28)	t(15;17)(q22;q21)	#	#	#	#	34	NA	45, XY, −7	CHT
(29)	t(15;17)(q22;q21)	#	#	#		49	Negative	t(10;11)(p14;q21)	CHT +alloHSCT
(30)	Failure	#	#			12	Negative	46,XX,t(8;16)(p11.2;p13.3), inv(11)(p15q22-q23)(31);47,idem,+i(8)(q10) (9)	CHT
(9)	Failure	#	#			39	Negative	46, XX	Supportive therapy
(9)	t(15;17)(q22;q21)	#	#			43	Negative	45, XX, −5, add(17)	CHT
(9)	t(15;17)(q22;q21)	#	#			54	Negative	55, X, der(Y), t(Y;10)(p11;q11),add(1p),+4,+9,+11,+17,-18,+20,+21,add(22q),+3mar	CHT +alloHSCT
(9)	t(15;17)(q22;q21)	#	#			24	Negative	46, XY, t(9;11)(p22;q23)	CHT +alloHSCT
(9)	Failure	#	#			42	Negative	failure	CHT +alloHSCT
(9)	t(15;17)(q22;q21)	#	#			52	Negative	46, X, −Y, +8	CHT +alloHSCT
(9)	Failure	#	#			17	Negative	Failure	CHT
(25)	t(15;17)(q22;q21)	#	#	#		80.4	Negative	46, XY, add(13)(q32)-	CHT

APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; autoHSCT, autologous hematopoietic stem cell transplant; alloHSCT, allogeneic hematopoietic stem cell transplant; CHT, chemotherapy; NA, not available; t-AML, therapy-related acute myeloid leukemia; t-MDS, therapy-related myelodysplastic syndrome.
#=yes; ?= symbols of cytogenetic.

Discussion

Therapy-related AML after APL treatment is a relatively infrequent (<7.0%) and late complication bearing a poor prognosis (4, 5).

Incidences reported in the largest studies ranged between 0.97% (European APL study: a series of 617 patients with a median follow-up of 51 months) and 6.5% (Italian study of 46 patients: follow-up not reported) (4, 5). Because the risk of developing t-AML continues for many years after the end of treatment, the PETHEMA group evaluated the cumulative incidence of t-AML in patients enrolled in three consecutive trials (LPA96, LPA99, and LPA2005): 918 patients were observed for a median of 77 months with a cumulative incidence of t-AML of 2.2% at 6 years, not comparable with the crude incidence of the other two studies (9).

The introduction of ATO in combination with ATRA had further reduced the incidence of t-AML in the APL setting, as reported by the Italian-German APL0406 study, where, with a follow-up of 6 years; no t-MN cases were observed in ATRA/ATO group patients *vs.* 1.5% in those treated with the AIDA regimen (10). Similar results have been reported from the AML17 trial (National Cancer Research Institute): with a follow-up of 5.7

TABLE 3 Main characteristics: treatment and outcome of the whole population.

N. patients	
• t-MDS • t-AML	44 (77.0%) 12 (23.0%)
Sex	
• Male • Female	28 (49.0%) 29 (51.0%)
Median age, years (range) at diagnosis of APL **Median age, years (range) at diagnosis of t-MNs**	51.5 (8.0–73.0) 55.2 (26.0–78.0)
Median time to t-MDS, months (range)—44 patients **Median time from t-MDS to t-AML, months (range)—28 patients** **Median time to t-AML, months (range)—13 patents**	39.5 (4.0–168.0) 6.5 (1.0–19.0) 43.0 (17.0–54.0)
Cytogenetic at diagnosis of t-MNs	52 (91.0%) patients
Normal −5/del(5q) −7/del(7) Complex 21q22 11q23	3 (5.5%) 18 (31.5%) 21 (37.0%) 24 (42.0%) 9 (15.5%) 8 (14.0%)
Treatment for APL	
Anthracycline Etoposide 6-Mercaptopurine plus mitoxantrone 6-thioguanine Alkylating agent (autoHSCT)	57 (100%) 22 (38.5%) 42 (73.5%) 8 (14.0%) 7 (12.0%)
Treatment for t-MNs	
Supportive therapy ATRA alone Conventional CHT AlloHSCT NA	16 (29.5%) 2 (3.5%) 33 (61.0%) 15 (29.5%) 3 (5.0%)

APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; autoHSCT, autologous hematopoietic stem cell transplant; alloHSCT, allogeneic hematopoietic stem cell transplant; CHT, chemotherapy; NA, not available; t-AML, therapy-related acute myeloid leukemia; t-MDS, therapy-related myelodysplastic syndrome.

years, no t-AMLs were observed in the ATRA/ATO group *vs.* 1.0% in the AIDA group (32).

Cytogenetic abnormalities have been largely described in patients with t-AML: a decreased prevalence of normal karyotype (<30.0%) and a prevalence of complex or unbalanced karyotypes with chromosomal deletions as compared with the *de novo* AML were reported (2, 21, 33–35). The combination of multiple chemo-immunotherapy agents with different mechanisms of action makes it difficult to ascribe the mutagenic potential to a single drug. Traditionally, recurrent translocations as t(15;17), t(8;21), inv(16), t(15;17), and 11q23 abnormalities were associated with topoisomerase II inhibitors, and t-AML usually developed after a latency time of 1 to 3 years (36–38). Very complex karyotypes (>5 simultaneous chromosomal abnormalities) and deletions of chromosomes 5 and 7 were usually associated with alkylating agents or radiotherapy, occurring after a latency of 5 to 7 years (2, 39, 40).

Therapy-related MDS is usually characterized by very complex karyotypes and consequently by a poor and very poor cytogenetic risk (40). Therefore, according to the Revised International Prognostic Scoring System (IPSS-R), a high prevalence of high- and very-high-risk subgroups was expected in the t-MDS setting. The IPSS-R is applicable to t-MDS and *de novo* MDS and reliably predicts AML transformation. As reported in the literature, 28 of the 44 (63.5%) t-MDS cases that developed after APL treatment subsequently progressed to t-AML at a median time of 6.5 months (1–19) (Tables 1, 3).

The molecular characteristics of MNs have been extensively analyzed in recent years: in more than 95% of AML and MDS, somatic mutations have been detected, without significant difference in the overall number of mutations in secondary *vs. de novo* subtypes. Moreover, none of the genes were exclusively mutated in t-AML. Mutations in RNA-splicing genes, epigenetic regulators genes, or cohesin complex genes were more than 90.0% specific for the diagnosis of s-AML (39, 41) and were present in only 30.0% of t-AML.

In the 57 t-MN cases that developed after APL treatment reported in the literature, in addition to an anthracycline (all the 57 patients), 22 (38.5%) patients also received etoposide, 42 (73.5%) received 6-mercaptopurine plus mitoxantrone as maintenance treatment, and eight (14.0%) received 6-thioguanine. Only seven patients (12.0%) received an alkylating agent as a part of the conditioning regimen for autologous stem cell transplantation.

Concerning cytogenetic analysis, in the 57 cases reported in the literature (Table 3), balanced translocations that involved 21q22 and 11q23 (typical breakpoints observed in t-AML occurring after administration of topoisomerase II inhibitors) were detected in nine (15.5%) and eight (14.0%) patients, respectively (Table 3). Moreover, 18 (31.5%) patients had −5/del(5q), and 21 (37.0%) had −7/del(7) abnormalities. Complex karyotypes (≥three independent abnormalities) were revealed in 24 (42.0%) patients (Table 3).

No NGS analysis was performed in the 57 t-MN cases reported in the literature, while in 11 patients (19.5%), molecular analysis by RT-PCR was reported (ref (25), Tables 1, 2).

Our patient was extensively studied by RT-PCR and NGS at diagnosis of t-AML, confirming the absence of *PML/RARα* gene rearrangement and presence of *KIT-D816V* exon −17 mutation and TP53 gene mutation, with a VAF of 78.0%.

In an independent series, mutations of *TP53* were reported in 30.0% to 47.0% of cases of t-MNs, resulting in the single most frequent molecular abnormality in this setting associated with complex karyotype in almost 80.0% of cases (39, 42–44). Lindsley et al. showed that TP53 mutations define a specific subgroup of t-AML, which differs from other AMLs like s-AML, in terms of younger age, lower recurrent driver mutations, more cytogenetic abnormalities, and poor prognosis with a reduced probability of achieving response after conventional treatment (39).

KIT mutations are detected in approximately 4%–6% of adult patients with *de novo* AML (45, 46) and 20%–40% of adult patients with *de novo* core-binding factor (*CBF*) leukemia (47–51). Three mutational hot spots (exon 8, exon 10–11, and exon 17) have been identified in the *KIT* gene (37, 52–54). Of these, exon 17 (detected in our patient) represents the site of *KIT* mutations most strongly associated with poor prognosis.

As KIT mutations have been reported mostly in *CBF*-AML, most studies on *KIT* mutations have been limited to *CBF*-AML, with few studies investigating *KIT* mutations in t-MNs. Schnittger et al. performed a large-scale study involving almost 2,000 unselected patients with AML: among 125 t-AML patients of the series, *KIT* mutation was detected in only one patient, who also presented t(8;21) translocation (54). Another study on 140 patients with t-MNs reported two cases with *KIT-D816V* mutation, one of which had t(8;21) (55).

KIT and *TP53* mutations were not detected together in any of the cases reported in the literature. Survival in t-MNs is poor when compared with that in other leukemia subtypes: until recent years, patients with t-MNs have been conventionally excluded from many clinical trials. This is particularly relevant in patients with previous APL, which is now considered a curable disease in many patients. New drugs with specific activity on secondary leukemia (including t-AML), targeting pathogenic mutations or interfering with immune mechanisms, are or will be available in the future. Our patient was treated with CPX-351 (Vyxeos, Jazz Pharmaceuticals): up to now, no other cases treated with CPX-351 and allogeneic stem cell transplant for a t-AML that developed after treatment according to GIMEMA AIDA2000 protocol have been reported in the literature.

The risk of anthracycline-induced heart failure increases as the cumulative dose administered increases: 3%–5% at 400 mg/m^2 and as high as 18%–48% at 700 mg/m^2 (56). However, there is a different level of risk for each patient scheduled for anthracycline therapy: patients younger than 5 years or older than 65 years, with prior or concurrent chest irradiation, pre-existing heart disease, or already known cardiovascular risk factors, have an increased risk of cardiotoxicity.

Our patient was 46 years old when he was treated according to GIMEMA AIDA2000 protocol: the anthracycline cumulative dose administered (as by protocol) was 600 mg/m^2, and no concomitant cardiovascular risk factors were present at diagnosis, but unfortunately, LVEF before treatment was not available.

Before starting treatment for t-AML, our patient was 63 years old, without cardiac dysfunction (LVEF 68%), hypertension, or other cardiovascular risk factors.

As mentioned, CPX-351 is a liposomal encapsulation of cytarabine and daunorubicin: in the heart, liposomes cannot get out of the vascular space because capillaries have tight junctions. As the tendency to accumulate in the heart cells is limited, this may reduce the risk of cardiotoxicity. On the contrary, the liposomes reach high concentrations in the tumor site, leaving the circulatory system where tumor growth damages the capillaries (56).

In our patient, no cardiac dysfunction or other cardiovascular diseases were developed during the treatment for t-AML (from induction to transplant).

Of the 57 t-MN patients reported in the literature (Table 3), 15 underwent allogeneic stem cell transplant; no details about disease status at transplant and at last follow-up were reported, particularly about the molecular response. In our patient, with a high-risk genetic profile (*TP53* and *KIT-D816V* exon −17 mutation), a molecular response was achieved with a transplant procedure and confirmed at the last follow-up. Of course, a longer follow-up would be needed for overall response and chronic GVHD assessment.

Considering the high curability of APL with excellent complete remission and long-term survival rates, it is necessary to try to reduce the incidence of t-MNs with a risk-adapted strategy and use chemotherapy-free regimens like ATO/ATRA.

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

AS: Conceptualization, Writing – original draft, Writing – review & editing. MS: Investigation, Writing – review & editing. JP: Investigation, Writing – review & editing. RM: Investigation, Writing – review & editing. SP: Data curation, Writing – review & editing. GC: Data curation, Writing – review & editing. EP: Data curation, Writing – review & editing. PF: Data curation, Writing – review & editing. FC: Writing – review & editing. MG: Writing – review & editing. MB: Data curation.

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Conflict of interest

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EDITED BY
Luca Vincenzo Cappelli,
Sapienza University of Rome, Italy

REVIEWED BY
Nidhi Sharma,
The University of Chicago, United States
Enrico Attardi,
University of Rome Tor Vergata, Italy

*CORRESPONDENCE
Jianbiao Zhou
csizjb@nus.edu.sg

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Unveiling novel insights in acute myeloid leukemia through single-cell RNA sequencing

Jianbiao Zhou[1,2,3]* and Wee-Joo Chng[1,2,3,4]

[1]Cancer Science Institute of Singapore, Center for Translational Medicine, National University of Singapore, Singapore, Singapore, [2]Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, [3]NUS Center for Cancer Research, Center for Translational Medicine, Singapore, Singapore, [4]Department of Hematology-Oncology, National University Cancer Institute of Singapore (NCIS), The National University Health System (NUHS), Singapore, Singapore

Acute myeloid leukemia (AML) is a complex and heterogeneous group of aggressive hematopoietic stem cell disease. The presence of diverse and functionally distinct populations of leukemia cells within the same patient's bone marrow or blood poses a significant challenge in diagnosing and treating AML. A substantial proportion of AML patients demonstrate resistance to induction chemotherapy and a grim prognosis upon relapse. The rapid advance in next generation sequencing technologies, such as single-cell RNA-sequencing (scRNA-seq), has revolutionized our understanding of AML pathogenesis by enabling high-resolution interrogation of the cellular heterogeneity in the AML ecosystem, and their transcriptional signatures at a single-cell level. New studies have successfully characterized the inextricably intertwined interactions among AML cells, immune cells and bone marrow microenvironment and their contributions to the AML development, therapeutic resistance and relapse. These findings have deepened and broadened our understanding the complexity and heterogeneity of AML, which are difficult to detect with bulk RNA-seq. This review encapsulates the burgeoning body of knowledge generated through scRNA-seq, providing the novel insights and discoveries it has unveiled in AML biology. Furthermore, we discuss the potential implications of scRNA-seq in therapeutic opportunities, focusing on immunotherapy. Finally, we highlight the current limitations and future direction of scRNA-seq in the field.

KEYWORDS

acute myeloid leukemia (AML), single cell RNA-sequencing (scRNA-seq), intratumoral heterogeneity, leukemia stem cell (LSC), bone marrow microenvironment, immunotherapy, novel therapy

Background

Acute myeloid leukemia (AML) is a complex and aggressive group of hematopoietic stem cell disorders. It is characterized with excessive accumulation of immature myeloid blasts in the bone marrow (BM) and peripheral blood (PB) (1–3). The uncontrolled proliferation is driven by genetic mutations that affect signaling pathways regulating cell cycle progression and apoptosis. Mutations in genes such as *FMS-like tyrosine kinase 3* (*FLT3*), *Nucleophosmin* (*NPM1*), and other regulators of cell growth contribute to the dysregulation of these processes. In AML, there is a disruption in the normal process of hematopoietic cell differentiation. Immature myeloid blasts, which are the precursor cells to mature blood cells, fail to undergo proper maturation (4, 5). Instead, they become arrested at an early stage of development. This differentiation block is often associated with specific genetic mutations, such as those in transcription factors like *CEBPA*, *RUNX1* or genetic alterations affecting epigenetic regulation, including mutations in *TET2*, *DNMT3A* or isocitrate dehydrogenase 1 (*IDH1*)/*IDH2* (4, 6, 7).

The standard therapy for AML consists of induction with 7 days of cytarabine plus 3 days of an anthracycline (e.g., daunorubicin or idarubicin), followed by consolidation with additional chemotherapy or stem-cell transplantation (8, 9). This "7 + 3" regimen has yielded a 5-year survival of 20% to 35% in young patients and 10% in older patients (10–12). During the last 10 years, progress in the field has resulted in the creation of targeted agents tailored for specific mutations (1, 13). Many of these agents have obtained approval from the Food and Drug Administration and are under investigation in ongoing clinical trials (13–15). However, the effectiveness of these targeted drugs as monotherapies has been hindered by the development of drug resistance over time (16, 17). There are still subsets of patients with limited therapeutic options (18). Finding effective treatments for these groups remains a challenge. The presence of diverse and functionally distinct populations of leukemia cells within the same patient's BM or PB poses a significant challenge in diagnosing and treating AML (19, 20). A significant portion of patients with AML exhibit resistance to chemotherapy or targeted therapies, leading to refractory or relapsed disease with a worse prognosis (14, 21, 22).

Since the emergence of next-generation Sequencing (NGS) in 2005, a multitude of comprehensive bulk RNA-seq studies have provided further insight into the pathogenesis, molecular classification, characterization of recurrent mutations, and detection of minimal residual disease in AML (7, 23, 24). However, bulk RNA-seq measures the average gene expression from all cells in the sample, but fails to distinguish between individual cells within a population and lacks the ability to distinguish individual cell variations (25, 26). In contrast, single cell (sc) RNA-sequencing (scRNA-seq) allows the analysis of gene expression at the resolution of individual cells, providing insights into cell-to-cell variability (27, 28). scRNA-seq also facilitates the exploration of individual cell dynamics, including cell differentiation trajectories, a crucial aspect of both normal hematopoiesis and leukemogenesis research (29–31). The application of scRNA-seq has revolutionized our understanding of AML by enabling the dissection of cellular hierarchies, identification of rare cell populations, as well as novel cell types. scRNA-seq empowers researchers to uncover the dynamic transcriptional profiles of individual leukemic cells (32). The characterization of subpopulations with distinct molecular profiles and cellular functions has not only broadened our understanding of disease heterogeneity but has also provided potential targets for more precise therapeutic interventions (33).

Adult AML has a genomic and epigenetic profile distinct from that of pediatric AML. Adult AML often exhibits a higher frequency of some DNA mutations mentioned above. However, mutations of F*LT3-ITD*, *NPM1* are less commonly found and mutations of *TP53* and *DNMT3A* are almost absent in pediatric AML (34). On other side, somatic structural variants are approximate 10 times higher in pediatric AML as compared to adult AML (34). Therefore, they could be considered as two different disease entities. Here, we summarize findings from various RNA-seq studies in adult AML that dissected the cellular and molecular heterogeneity of the AML ecosystem, including leukemia stem cells (LSCs), immune cells and their interactions with AML bone marrow microenvironment. This review delves into the recent advancements in understanding the novel mechanisms of drug resistance and relapse in AML. We also highlight the transformative potential of single-cell analysis in the development of novel and personalized treatment strategies with the focus on immunotherapy for fighting AML.

Unmasking heterogeneity

van Galen and colleagues integrated single cell mutation detection and transcriptomes for 16 AML patients (35). Six types of AML cells resembled normal cell types along the differentiation axis from hematopoietic stem cell (HSC) to myeloid (HSC-like, progenitor-like, granulocyte-monocyte progenitor (GMP)-like, promonocyte-like, monocyte-like, and conventional dendritic cell (cDC)-like malignant cells) were classified (Figure 1A). Importantly, the relative abundances of these cell types were strikingly heterogeneous among tumors. Some AMLs consisted of major one or two cell types, while others contained a range of malignant cell types. Wu et al. used Microwell-seq and SMART-seq to analyze samples from 40 AML patients and identified a "cloud cluster" with no functional marker genes after compared with normal BM samples (36). Genetic network and correlation analysis showed that this "cloud cluster" resembles hematopoietic stem and progenitor cells (HSPCs). Gene expression profiling revealed upregulation of a spectrum of ribosomal protein (*RP*) genes in these AML progenitors. Despite of common features, AML progenitors could be divided into 16 sub-clusters and summarized into 4 main groups, including *RP* gene-High, neutrophil-like, monocyte-like, and myeloid cell-like. The number of cycling cells and activation of pathways were markedly different in some clusters, even within the same main group. Refractory-specific cluster cells overexpressed *MYC*, *SRC*, *RELA*, *MTOR* compared to non-refractory cluster cells in AML-M5 subtype (Figure 2A) (36).

Intratumoral heterogeneity has been dissected not only in same subtype based on AML morphology, but also in AML with same driving genetic lesion. *NPM1* is the most common mutated gene in

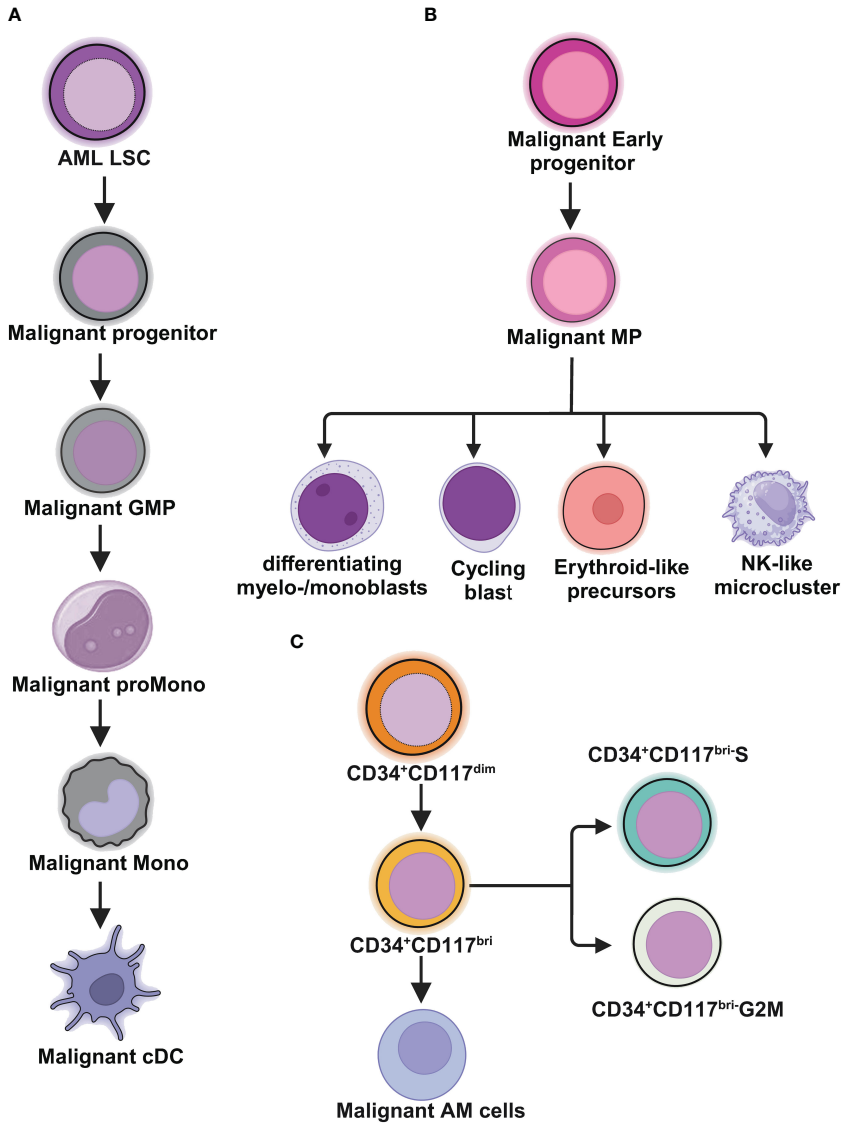

FIGURE 1
Schematic illustrations depict the leukemic spectrum at different stages of differentiation trajectory revealed by RNA-seq studies. **(A)** Unbiased single cell transcriptomic classifier identifies six types of AML cells mirroring the normal myeloid differentiation axis from hematopoietic stem cell (HSC) to myeloid (HSC-like, progenitor-like, granulocyte-monocyte progenitor (GMP)-like, promonocyte-like, monocyte-like, or conventional dendritic cell (cDC)-like malignant cells. The abundances of these 6 different stages of malignant cells vary significantly among AML patients. These abundances correlate closely with cell morphology and surface phenotypes. The scRNA-seq data reveal greater malignant cell diversity compared to flow cytometry-based estimates, highlighting the potential of scRNA-seq to provide more detailed information on AML cell types and differentiation states. **(B)** In *NPM1*^{mut} AML, at the peak of differentiation trajectory, malignant early progenitors differentiate into malignant myeloid progenitor (MP). These MP cells give rise to various malignant progeny cells, including differentiating myelo-/monoblasts, actively cycling blasts, erythroid-like precursors, and a microcluster with NK-like characteristics. **(C)** In AML with t(8;21), by integrating clinical immunophenotypic characterization, the scRNA-seq analysis delineates five distinct intrapatient leukemic cell clusters. At the top of the hierarchy are CD34^{+}CD117^{dim} cells, representing the earliest differentiation trajectory, followed by CD34^{+}CD117^{bri} blasts and abnormal myeloid cells with partial maturation (AM) at the end of differentiation axis. The CD34^{+}CD117^{bri} blasts can be further divided into CD34^{+}CD117^{bri}-S blasts and CD34^{+}CD117^{bri}-G2M blasts based on their cell cycle status inferred from their single cell transcriptomic profiles. This figure was created with BioRender.com.

AML, occurring in approximately 30% of adult AML. *NPM1* mutations result in the cytoplasmic localization of NPM1 (NPM1c) protein. In *NPM1*^{mut} AML progenitors, 15 distinct clusters in 6 major group were identified distributed across the AML landscape, including early progenitors, myeloid progenitors, erythroid-like precursors, actively cycling blasts, differentiating myelo-/monoblasts and a microcluster with NK-like characteristics (Figure 1B). These early progenitors expressed *CD34*, *CD99*, *HOPX* and *EGFL7*, the host gene for *miR-126* (Figure 2B) (37). The distribution of these clusters evidently varied among *NPM1*^{mut} patients. Some predominantly mapped to one or two groups, and others showed an extensive presentation across the whole landscape. Intrapatient cellular heterogeneity in t(8;21) AML has been explored at single cell level too (38). Combined with clinical immunophenotypic characterization, scRNA-seq analysis identified 5 distinct intrapatient leukemic cell

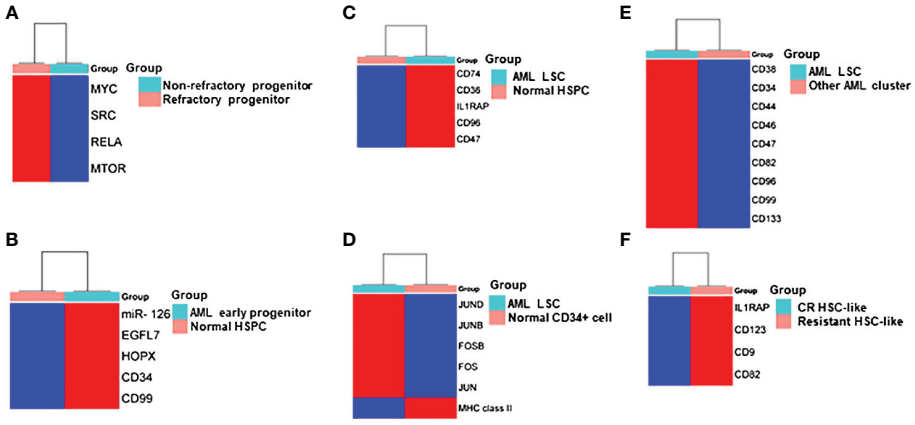

FIGURE 2
Heatmap Comparisons of differential gene expression determined by scRNA-seq in **(A)** refractory progenitor vs non-refractory progenitor from AML patients; **(B)** AML early progenitor vs normal HSPC; **(C)** AML LSC vs normal HSPC; **(D)** AML LSC vs normal CD34+ cell; **(E)** LSC vs other clusters from AML patients; **(F)** HSC-like cell from chemotherapy-resistant AML patients vs AML patients who achieved complete remission (CR). Red and blue shadings represent higher and lower relative expression levels, respectively. These heatmaps were constructed by using SRplot (https://www.bioinformatics.com.cn/plot).

clusters: $CD34^{+}CD117^{dim}$ blasts, $CD34^{+}CD117^{bri}$ blasts, $CD34^{+}CD117^{bri}$-S blasts, $CD34^{+}CD117^{bri}$-G2M blasts, and abnormal myeloid cells with partial maturation (AM) (Figure 1C). Transcriptomic profiling revealed well-defined gene signatures in these clusters, for example, cell migration and adhesion genes in $CD34^{+}CD117^{dim}$ blasts and cell cycle and DNA replication genes in $CD34^{+}CD117^{bri}$-S and $CD34^{+}CD117^{bri}$-G2M blasts (38). Similarly, combined whole-exome sequencing (WES) with scRNA-seq in longitudinal analysis of t(8;21) AML and *FLT3-ITD* AML revealed substantial heterogeneity both within and between blast cells of each patient and more heterogeneity among diagnosis-relapsed pairs (39). The most pronounced transcriptional variances were linked to large-scale copy number variations specific to each patient. Additionally, somatic variants, such as single nucleotide polymorphisms and small insertions and deletions contributed to further heterogeneity, highlighting distinct abundance and dynamics of AML clones unique to each patient (39). The key findings of some of these studies were summarized in Table 1.

Taken together, scRNA-seq technology has significantly advanced our understanding of AML by uncovering a spectrum of distinct clusters of leukemic cells at different stage of differentiation trajectory. The representation of these clusters is markedly varied among AML patients.

Characterizing leukemia stem cells

Differentiation block or maturation arrest is a key characteristic of AML disease, allowing AML blasts to proliferate continuously without undergoing the terminal differentiation and apoptosis process (44, 45). At the core of the blocked differentiation in AML are LSCs (46, 47). LSCs represent a subset of AML cells with a unique capability of initiating and maintaining a cellular hierarchy in AML (48). A large literature suggests that LSCs are responsible for the persistence of the disease and can be refractory to standard chemotherapy, resulting in relapse (47–50). Chemoresistance and relapse are the leading cause of AML-related deaths (51–53). scRNA-seq technology has added value in LSC biology by unveiling novel surface makers, and distinct transcriptomics.

The primitive AML cell types identified in the study of van Galen et al. expressed established LSC markers, such as *CD96*, *CD47*, *IL1RAP* and *CD36*, and the additional candidate *CD74* (Figures 2C, 3A) (35). CD96, a member of the Ig gene superfamily, is an LSC-specific marker in AML and is associated with dismal survival (54–57). CD74, also known as MHC HLA-DR gamma chain, plays an important role in AML cell survival in a network with LGALS3 (58). AML with more primitive LSCs inferring from higher HSC/Prog-like gene signature had significantly worse outcomes (35).

Integrated single cell transcriptomic data from all four patients described by Velten and colleagues (59), along with data from $CD34^{+}$ BM cells of a healthy individual, revealed a subpopulation of quiescent immature HSC-like leukemic cells. These LSCs have increased expression of AP1 transcription factors (*FOS*, *JUN*, *FOSB*, *JUNB*, *JUND*) and decreased *MHC class II* expression (Figure 2D) (59). Interestingly, CD96, a known LSC marker mentioned earlier, was upregulated in only one patient, suggesting that LSC might be patient-specific too. In normal karyotype AML (FAB AML-M4/5), a presumable LSC cluster highly expressing *CD34*, *CD38*, *CD96*, *CD46*, *CD47*, CD82, *CD44* and *CD133* was uncovered (Figure 2E) (40). However, due to the small sample size in these two studies (40, 59), further validation of these findings in a larger cohort of AML patients is necessary to determine their general applicability. In $NPM1^{mut}$ AML, scRNA-seq provided a higher resolution and further anatomized the miR-126^{high} LSCs into dormant and cycling sub-compartment. Exhibiting overexpression in AML, miR-126 restrains cell cycle progression, inhibits differentiation, and enhances self-renewal of

TABLE 1 Summary of some studies of scRNA-seq in AML.

Key finding	Sample size	scRNA-seq platform	Suppl. techniques	Reference
1. identify 15 distinct cell types in healthy bone marrow 2. Reveal six types of AML malignant cell along the hierarchy of myeloid cell differentiation 3. High HSC/Prog-like signals had worse outcome than those with GMP-like signals 4. Monocyte-like cells potently inhibited T-cell activation (immunosuppressive)	16 AML (35 BM samples) 5 HDs	Seq-well	1. Targeted DNA seq 2. Sc genotyping by short- read seq 3. Sc genotyping by nanopore seq	(35)
1. Normal cell type: lymphoid, erythroid, and myeloid lineages (6 types of neutrophils) 2. Identify 20 cell clusters in AML and Type I (short survival) and Type II AML 3. Patients with ribosomal protein (RP) high progenitor cells had a low remission rate	40 AML 3 HDs	Micro-well Seq	Long-read single-cell targeted SMRT seq	(36)
1. A generalized inflammatory and senescence-associated response induced by chemotherapy 2. Heterogeneity within progenitor AML cells: OxPhos^{high} vs miR-126^{high}OxPhos^{low} 3. miR-126^{high}OxPhos^{low} LSCs are more quiescent with stemness, associated with refractory and relapse in *NPM1* mutant AML.	20 AML	10X Genomics	1. Immunophenotyping 2. NPM1 Mutation Finder, NPM1-MF	(37)
1. Three distinct leukemic cell populations identified: CD34$^+$CD117^{dim} blasts, CD34$^+$CD117^{bri} blasts, and abnormal myeloid cells with partial maturation (AM). 2.CD34$^+$CD117^{dim} cells overexpress ell migration and adhesion genes, while CD34$^+$CD117^{bri} cells overexpress cell cycle and DNA replication genes. 3. CD34$^+$CD117^{dim} cells show higher LSC17 score compared to CD34$^+$CD117^{bri} cells 4. A high proportion of CD34$^+$CD117^{dim} cells in t(8;21) AML patients predicts inferior outcomes.	9 t(8;21)-AML	10X Genomics	Immunophenotyping	(38)
1. Pathway switch from AP1-regulated clone at diagnosis to mTOR-driven clone at relapse in *DNMT3A/FLT3-ITD* AML 2. Shared LSC signature between diagnosis and relapse in two ETO-AML cases 3. Tumor heterogeneity among patients with similar initiating mutations, also between each diagnosis-relapse pair	6 AML (diagnosis-relapse pair)	Single cell SORT-seq	1. WES 2. Fusion genes detection by RNA-seq	(39)
1. Identify18 clusters into 8 main cell populations 2. One LSC-like cluster with known LSC marker (CD34, CD96, CD133) and nontraditional LSC markers (CD38, CD46)	5 normal karyotype (NK) AML (M4/M5) 1 HD	10X Genomics	None	(40)
1. The fraction of progenitors is significantly higher in non-CR AML than CR AML, suggesting early hematopoiesis arrest in non-CR AML. 2. Distinct LSC markers uncovered in HSC-like cells from non-CR (CD9, CD82, CD123, IL1RAP)	13 AML (8 CR; 5 non-CR)	10X Genomics	None	(41)
1. CD99$^+$CD49d$^+$Galectin-1$^+$CD52$^+$ quiescent stem-like cells (QSCs) are involved in the chemoresistance and relapse of AML. 2. Interaction between QSCs and monocytes mediated by CD52-SIGLEC10 leads to immune suppression and poor outcomes. 3. LGALS1 is a promising target for refractory and relapsed AML.	10 AML (refractory and early relapsed)	10X Genomics	None	(42)
1. Inflammatory BM niche inhibits normal hematopoiesis, but not LT-HSCs and *NPM1* mutant leukemic cells. 2. Niche remodeling provides the competitive advantage of mutated cells over their normal and preleukemic counterparts, promoting leukemogenesis.	6 *NPM1*-mutant AML 4 HDs	10X Genomics	1. Immunophenotyping 2. Bulk RNA-seq 3. *NPM1* mutation identification by scRNA-seq	(43)

HDs: health donors; Suppl., Supplementary; WES, Whole exon sequencing; Sc, Single cell.

LSCs *in vivo* (60). The dormant miR-126^{high} LSCs had low expression of oxidative-phosphorylation (OxPhos) signatures and this miR-126^{high}OxPhos^{low} population was enriched after chemotherapy in AML patient-derived xenografts (PDX) model (Figure 3B). A miR-126^{high} gene signature derived from these AML xenografts could identify a subset of chemotherapy-resilient LSCs enriched in refractory and relapsed AML. This signature also predicts poor survival in patients with *NPM1*^{mut} (37). A subpopulation of CD34$^+$CD117^{dim} cells were located at the earliest stage of differentiation as shown by single cell trajectory analysis of t(8;21) AML (Figure 3C). Longitudinal scRNA-seq at different disease stages revealed CD34$^+$CD117^{dim} blasts expanded at post-relapse refractory stage after several cycles of chemotherapy (38). Importantly, higher percentage of CD34$^+$CD117^{dim} cells in

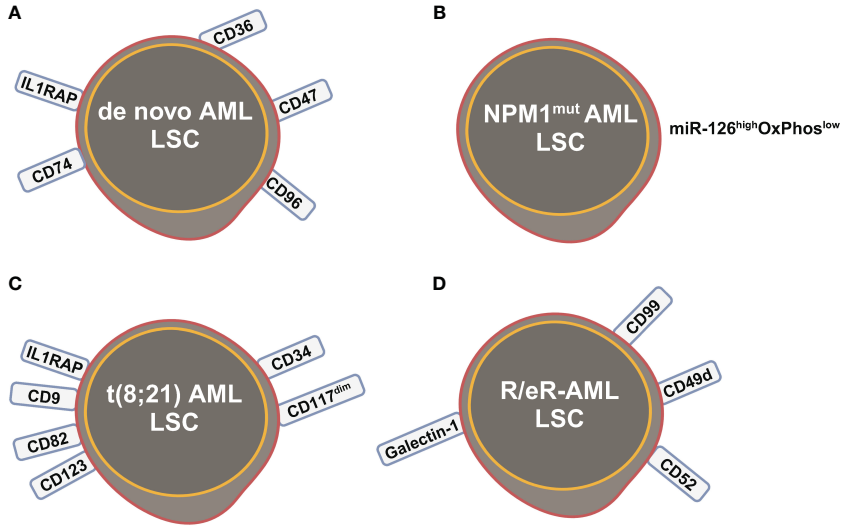

FIGURE 3
Leukemia stem cells (LSCs) identified by scRNA-seq studies in **(A)** *de novo* AML; **(B)** *NPM1*^{mut} AML; **(C)** t(8;21) AML; **(D)** refractory and early relapsed (R/eR) AML. This figure was created with BioRender.com.

AML patent with t(8;21) was an indicator of poor prognosis (38). Chemoresistant HSC-like cells in non-CR AML were found to express more LSC markers, including *CD9*, *CD82*, *CD123* (*IL3RA*), and *IL1RAP* than those from CR AML (Figure 2F) (41). CD9, enriched in the CD34^{+}CD38^{-} fraction of AML cells, is associated with chemoresistance (61, 62). However, the prognostic value of CD9 in AML remains elusive as contradictory results have been reported (62–64). CD82, also known as tetraspanin-27, is a member of the tetraspanin superfamily of cell surface proteins. CD82 plays multiple roles in promoting AML cell survival, adhesion, migration, resistance to Ara-C via activation of STAT5 pathway, PKCα and β1 integrin, N-cadherin (65, 66). CD123 has been well-studied in LSC biology, contributing to poor prognosis, high-risk, resistance to apoptosis and drug resistance of AML (67–69). CD123 serves a promising target for novel immunotherapies against AML and numerous clinical trials are currently ongoing (70–74).

Longitudinal scRNA-seq analyses of refractory and early relapsed AML (R/eR-AML) uncovered that, unlike proliferating stem/progenitor-like cells (PSPs), a distinct subpopulation identified as quiescent stem-like cells (QSCs) played a pivotal role in AML chemoresistance and led to adverse clinical outcomes. The QSCs had increased expression of *CD52* and *LGALS1* mRNA and a combination of cell surface markers: CD99^{+}CD49d^{+}CD52^{+}Galectin-1^{+} (Figure 3D). Chemotherapy induced reprogram of PSPs to obtain a QSC-like expression pattern in refractory AML, leading to accumulation of QSCs. The presence of QSCs at diagnosis could be associated with chemoresistance, and these cells were further enriched in the residual AML cells of refractory patients (42).

In summary, scRNA-seq has unveiled significant heterogeneity within the AML LSC population. There isn't a single uniform profile but rather distinct subpopulations of LSCs with varying gene expression patterns and functionalities. The identification of multiple LSC markers and diverse LSC gene expression patterns/signatures underscores the complexity and heterogeneity within these cell populations. Such diversity significantly influences their response to therapy and their refractory/relapse mechanisms.

Scrutinizing bone marrow microenvironment

The BM microenvironment (niche) is a highly complex network, consisting of a cellular compartment, an extracellular matrix, and a liquid compartment (75). Various cell types including hematopoietic and nonhematopoietic cells such as BM mesenchymal stromal cells (BMSC), osteoblasts, osteoclasts, adipocytes, fibroblasts, BM endothelial cells (BMEC), and effector immune cells, inhabit and interact within the cellular compartment alongside the extracellular environment. The liquid compartment contains a mixture of growth factors, cytokines and chemokines (76, 77). Mounting evidence suggests that BM microenvironment can influence and regulate functions of HSCs, orchestrating hematopoiesis. The role of the BM microenvironment in supporting AML cell survival, fostering resistance to conventional chemotherapy and targeted treatments, and ultimately contributing to disease relapse has garnered growing interest (77–79). ScRNA-seq allows the identification and characterization of individual cell types within this BM microenvironment, offering a detailed understanding of the BM atlas and its interactions with AML cells.

scRNA-seq studies have been performed to define a cellular taxonomy of the mouse BM microenvironment and its perturbation by malignancy and stress (80–82). The initial comprehensive atlas of the mouse BM microenvironment identified 17 distinct cellular subsets, including BMSCs, osteolineage cells (OLCs), pericytes, BMECs, chondrocytes, and fibroblasts (80). Their putative functions and developmental relationships have been annotated too. Several scRNA-seq studies revealed novel insights into the interactions between AML cells and BM niche.

Leptin receptor (LepR) is a transmembrane receptor protein involved in responding to the hormone leptin. LepR$^+$ cells are key components of the BM hematopoietic microenvironment, and involved in regulating hematopoiesis, bone formation and remodeling (83, 84). In the MLL-AF9 knockin leukemic model, LepR$^+$ BMSCs were found to exhibit downregulation of key HSC niche factors like *ANGPT1*, which acts as an agonist for TEK receptors present on BMECs and HSCs. Additionally, factors promoting lymphoid or myeloid differentiation, as well as HSC homing to the bone marrow, were also observed to be downregulated (80). *SPP1*, an osteoblastic maturation marker that acts as an inhibitor of HSC pool size and proliferation, was noted to be upregulated in AML. In addition, osteogenic differentiation blockage in LepR$^+$ BMSCs and OLCs could also be induced by leukemic cells, leading to remodeling and changes in bone composition. *WNT1-inducible-signaling pathway protein 2* (*WISP2*), known to inhibit MSCs ability to differentiate, was observed to be upregulated in all LepR+ BMSCs subsets as well as OLC progenitors, suggesting a compromised differentiation of LepR$^+$ BMSCs and OLCs in AML (80). Under stress conditions such as chemotherapy, significant alterations in niche components occur, including the adipocytic skewing of perivascular cells regulated by the activation of adipogenesis-related pathways, as well as a widespread reduction in gene expression linked to the osteolineage (81). Consequently, the transcriptional remodeling of nice altered the normal hematopoiesis process. For example, downregulation of vascular Notch delta-like ligands (encoded by *DLL1* and *DLL4* genes) prematurely turned on a myeloid transcriptional program in hematopoietic stem cells (81).

Results from scRNA-seq in AML patients and healthy donors are consistent with these findings in mouse (43). In human BM from healthy donors, LepR$^+$ BMSCs were identified as a key "communication hub", playing central roles in in the homeostatic regulation of HSC and BM niche cells (43). Subclustering analysis identified 4 types of BMSC, ie, BMSC-0-3, with different level of *LepR* expression. The BMSC-0 population expressed highest level of *LepR* gene and *CXCL12*, *KITLG*, *ANGPT1*, and *IL7*. These genes encode critical HSPC regulatory factors. Accordingly, BMSC-0 are predicted to have the strongest interactions across HSPC subsets, particularly long-term (LT)-HSCs (43). In *NPM1*^{mut} AML, BMSCs undergo inflammatory remodeling in which gene signatures reflecting inflammatory signaling, such as "TNFα signaling via NFκB", "IL2–STAT signaling", and "inflammatory response", are among the most upregulated. The inflammatory remodeling disrupted the homeostasis of BMSC subclusters, resulting in a significant decrease in the BMSC-0 cluster, which was expected to have the strongest interaction with HSPCs, alongside a concurrent nearly seven-fold increase in BMSC-2. In all cases of *NPM1*^{mut} AML, the BMSC-2 cluster consistently displayed a transcriptional profile characterized by the upregulation of genes associated with inflammation, as well as genes and signatures related to cell-extracellular matrix (ECM) remodeling. This indicates a significant expansion of an inflammatory subset, accompanied by a concurrent decline in the BMSC subset expected to sustain the normal maintenance of HSPCs (43). Collectively, LepR$^+$ BMSCs in AML undergo remodeling due to inflammatory activation. This results in a significant expansion of an inflammatory subset (BMSC-2) alongside a simultaneous reduction in the BMSC subset expected to sustain the maintenance of normal HSPCs (BMSC-0).

A profound shift in cell-cell interaction between hematopoietic BM niche and HSPCs has been found in AML patients when compared to healthy donors (85). At the time of diagnosis, there was a notable increase in the abundance of the most primitive myeloid progenitor population, HSC/multipotent progenitor cell (MPP). Following treatment and achieving remission, these proportions reverted to levels comparable to those observed in a healthy state (85). However, during relapse, there was a resurgence in the enrichment of myeloid progenitors. Predictive analyses unveiled interactions between HSPCs and other BM cell types, revealing a notable expansion of interactions in AML that promote HSPC-cell adhesion, immunosuppression, and cytokine signaling. Specifically in AML, integrin β1 (ITGB1, CD29) was predicted to form interactions with a broader spectrum of ligands, which promoted adhesion and survival of HSC and MPP (85). The transforming growth factor-β (TGF-β) signaling pathway plays a crucial role in regulating various cellular processes, including normal hematopoiesis, and its dysregulation has been implicated in AML (86, 87). The interaction between TGFβ1 and TGFBR2 was predicted to be widespread in AML when compared to healthy controls (85). Increased expression or secretion of TGFβ1 inhibits normal HSC proliferation and is linked to the quiescence state of LSC in AML (85–87). Additionally, ECM and cytokine production have been observed in AML too (85). However, caution should be exercised regarding these predicted interaction changes due to the lack of rigorously experimental validation in this study (85).

Taken together, these findings from scRNA-seq in the context of AML suggest that malignant cells can affect normal hematopoiesis by remodeling in BM microenvironment composition and alter the regulation of HSC niche factors in the stroma. This compromises the BM microenvironment to be less conducive towards normal hematopoietic cell production, but confers competitive advantage to AML cells, such as adhesion, survival, quiescence for the AML cells.

Divulging the immune system

Immune surveillance mechanisms, comprising adaptive and innate immune systems, are natural protectors in preventing hematological malignancies (88). A large body of pre-clinical and clinical studies indicates the substantial contribution of compromised immune surveillance mechanisms to the establishment of preleukemic states and their progression toward AML (89–92). Recent studies using scRNA-seq provide comprehensive view of immune escape strategies employed by AML cells to evade immune recognition, as well as AML cell-induced modifications of various immune cell populations, including T cells, natural killer (NK) cells, dendritic cells, and myeloid-derived suppressor cells (93–97).

The development of AML leads to profound alterations in the lymphoid lineage, including a significant reduction in the proportion of common lymphoid progenitors (CLPs) and their

offspring, such as pre-B cells, mature B cells, as well as, to a lesser extent, $CD4^+$ and $CD8^+$ naive T cells and $CD4^+$ memory T cells (35). Although both AML and healthy control samples have the same two T cell subsets, naive T cells and cytotoxic T cells (CTLs), and a related population of NK cells, their proportion and function differ. AML samples have comparatively fewer T cells and CTLs but relatively more regulatory T cells (T-regs) compared to healthy controls. Overall, these findings underscore long-term damage to the adaptive immune system and an immunosuppressive BM niche of AML (35). It has been recently showed that differentiated monocyte-like AML cells exhibit features of classical and non-classical monocytes, but lack cytotoxic signature genes (35). These differentiated monocyte-like AML cells can inhibit T cell activity, contributing compromised immune tumor surveillance in AML (35). Another observation that aligns with the immune suppressive state of AML disease is the enrichment of CD8+ memory T cells at relapse compared to diagnosis (94) (Figure 4A). This suggests that while cytotoxic cell numbers recover during remission, they are functionally ineffective to execute anti-AML immune responses, potentially contributing to relapse.

Several scRNA-seq studies, paired with single-cell T cell receptor (TCR) sequencing on T cell, have defined high-resolution atlas of different T cell populations with distinct functions in AML. In general, the clonotype size, denoted by the count of cells expressing identical TCR sequences, is highest in refractory/relapsed AML, followed by newly diagnosed AML and healthy donors, revealing that T cells are more clonal in the AML microenvironment (93, 94, 97). Five T cell phenotypes, including 2 conventional ($CD4^+$, $CD8^+$) and 3 unconventional, such as gamma-delta (γδ) cells, mucosal associated invariant T-cells (MAIT) cells, and all other (unconv T) cells were identified in AML BM samples. AML had lower CD4:CD8 ratio in BMs than that in healthy BMs (93) (Figure 4A). CD8+ T cells in AML could be further divided into 16 clusters, forming 6 major types, including naive, memory, effector memory, CTL, MAIT, and exhausted (94). The percentage of exhausted population was lowest in AML compared to other 20 types of tumors using the same exhaustion definition (98), which involves coexpression of *PDCD1*, *TOX*, *CXCL13*, *TIGIT*, *CTLA4*, *TNFRSF9*, *HAVCR2*, and *LAG3* (94) (Figure 4B). This finding of a lower abundance of $CD8^+$ T cells expressing canonical immune inhibitory–related markers in AML has important clinical significance, partially explaining the limited efficacy of immune checkpoint inhibitors observed in AML patients, in contrast to remarkable responses seen in some solid tumors. The $CD8^+$ effector T cells are markedly different between newly diagnosed and refractory/relapsed AML (Figures 4C, D). Refractory/relapsed AML shows relatively higher abundance of clusters enriched with expression of the inhibitory receptor *KLRG1*, a marker of antigen-experienced and senescent cells (99, 100), while newly diagnosed AML displays relative higher percentage of clusters expressing cell-migratory receptor *CXCR4* and AP-1 transcription factor *FOSB* (94). Thus, these heterogeneous clusters of $CD8^+$ effector T cells with different cellular states might contribute to refractory/relapsed disease.

In a study of analyzing normal karyotype AML (M4/M5), seven T cell clusters were discovered, including 4 clusters of $CD8^+$, 2 clusters of $CD4^+$ cells NFE2 cluster, $CD4^-CD8^-$ cluster, based on their gene expression characteristics. MAIT cells express a semi-invariant TCR called the TCRα7.2, which recognizes microbial-derived metabolites presented by the MHC-related protein 1 (MR1) (101, 102). The cluster, predominantly composed of MAIT cells (97), showed a higher proportion in refractory and relapsed cases compared to the other samples, implying a potential role of MAIT cells in the pathogenesis and disease progression of normal

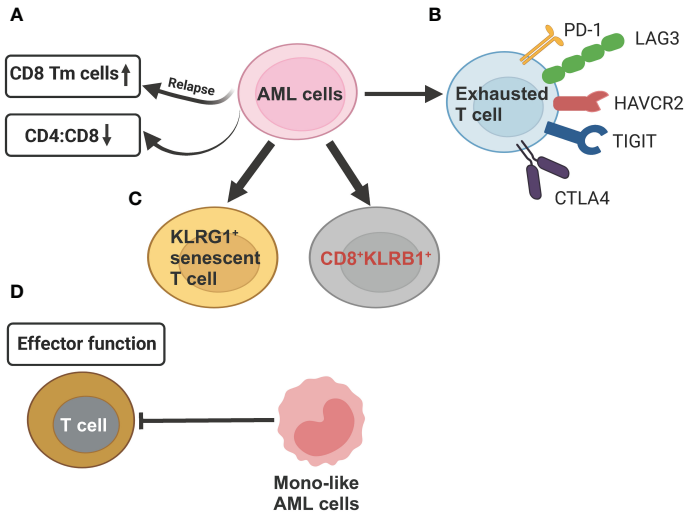

FIGURE 4
Schematic illustration summarizing the compromised T cell functions mechanisms exerted by AML cells revealed by scRNA-seq studies. **(A)** AML cells can reduce CD4:CD8 ratio as compared to healthy controls. In relapsed AML, there is an increase in $CD8^+$ memory T cells (Tm) when compared to newly diagnosed AML. **(B)** AML cells induce T-cell exhaustion by increasing expression of inhibitory T-cell receptors (PD-1, LAG3, HAVCR2, TGIT, CTLA4. **(C)** In relapsed AML, the expansion of $KLRG1^+$ senescent T cell and $CD8^+KLRB1^+$ T cells contribute to chemoresistance. **(D)** Differentiated monocyte-like AML cells hamper effector T cell function. This figure was created with BioRender.com.

karyotype AML (97). Another study of PB mononuclear cell (PBMC) samples in AML patients also found predominant MAIT cells in one of the $CD8^+$ T cell clusters (95). However, the clinical significance of MAIT cells was not explored in this study (95). Five subclusters of $CD4^+$ T cells and four subclusters of $CD8^+$ T cells were also defined based on transcriptomic signatures. While the proportion of cytotoxic $CD4^+$ effector T cells varied among AML patients, it was generally lower compared to healthy donors. This suggests that the reduced presence of these immune cells contributes to the progression of AML (95).

NK cells are a type of cytotoxic innate immune cells that produce inflammatory cytokines and chemokines (103). They play a crucial role in the immune response by lysing infected and cancer cells, including AML (104). NK cells are generally divided into 2 classic subsets, cytokine-producing $CD56^{dim}$ and cytolytic $CD56^{bright}$ NK cells (105). Recent single-cell transcriptomics study analyzing NK cells in PB from healthy donors revealed six subtypes of NK cells, including 3 well-defined subsets ($CD56^{bright}CD16^-$, $CD56^{dim}CD16^+CD57^-$, and $CD56^{dim}CD16^+CD57^+$) and 3 novel subsets (type I interferon–responding NK cells, cytokine-induced memory-like NK cells, and population with low-ribosomal expression) (96).

Comparative scRNA-seq studies have been performed on NK cells from healthy donors and AML BM samples to better understand NK cell dysfunction in AML. Crinier et al. identified three NK cell populations common in 8 healthy donors based on gene signature analysis. Among them, two populations resembled $CD56^{dim}CD16^-$ and $CD56^{bright}CD16^-$ NK cells, while the third cluster was constituted of a $CD56^{bright}$ tissue-resident NK cell population that resides in the spleen and is absent from the blood (106). In contrast, NK cells in AML patients were profoundly heterogeneous and exhibited patient-specific features. There was no distinct NK cell subset shared among AML patients, suggesting that the NK cells were impacted by AML cells in an individual fashion (106). Transcriptomic profiling demonstrated that NK cells from AML patients had higher expression of interferon-induced genes, while NK cells from healthy donors expressed higher level of NK cell effector molecules. CD160 is an important human NK cell activating receptor and its decreased expression is associated impaired NK cell function. AML patients with lower expression of CD160 have poorer survival than those with higher level of CD160 (106). In PBMC samples, the number of NK cells was lower in AML patients than that in healthy donors, indicating the AML-induced suppressive circumstance (95). However, they shared same NK cell expression makers and formed a single functional subtype. Differential gene expression analysis revealed downregulation of 10 transcription factors involved in homeostatic NK cell proliferation and survival, such as *CEBPD*, *KLF3*, *KLF2*, *USF2*, and *FOXP1*, in AML patients (95). A unique subset of NK cells characterized by the $CD56^{bright}CD16^{hi}$ phenotype, particularly in the hypomaturation stage, is prevalent in AML. The hypomaturation stage of these NK cells has been linked to decreased overall survival (OS) and event-free survival (EFS) in AML patients (107). In contrast to other NK cell populations, the $CD56^{bright}CD16^{hi}$ cells displayed distinct shifts in both phenotype and function, marked by remarkably low scores for activating and inhibitory receptors (108). Current NK cell-based therapies primarily aim to enhance NK cell activation and longevity, often overlooking the heterogeneity among cancer types and the suppressive influence of the tumor cells and tumor microenvironment on NK cell cytotoxic functions. The new knowledge of NK cell dysfunction gained from extensive scRNA-seq research is crucial for shaping future therapeutic strategies for AML and other cancers in general.

Expanding therapeutic opportunities

With a deeper understanding of the genetic and transcriptomic landscape of AML at the single-cell level, new cell type-specific targets can be identified and more tailored and effective treatments, such as targeted therapies or immunotherapies, can be developed.

As mentioned earlier, *CD52*, *LGALS1* and *CD47* are significantly elevated in QSCs cells compared to other cellular states (42). Additionally, LGALS1 expression is also found to be significantly increased in chemo-residual QSCs in refractory AML patients and in daunorubicin-resistant leukemia cell lines compared to sensitive cells (109). Furthermore, scRNA-seq comparison of $CD34^+$ BM cells at diagnosis from CR vs non-CR AML patients revealed that a cluster of GMP cells characterized by $CRIP1^{high}LGALS1^{high}S100As^{high}$ was significantly enriched in non-CR samples and associated with poor prognosis of AML (110). AML patients with higher expression of LGALS1 had a worse OS and EFS that those with lower LGALS1 expression in the TCGA cohort (42). Overall, these data demonstrate that LGALS1 may mediate the chemoresistance and represent a novel therapeutic target for resensitizing QSC LSCs to chemotherapy. The LGALS1 inhibitor, OTX008, has been shown to enhance the chemotherapy in AML cell lines, primary AML cells, cell lines, and to eliminate the chemoresistant QSCs in AML PDX models (42). Other markers specifically expressed on QSCs, such as CD52 and CD47 are potential targets for the development of small molecule inhibitors or immunotherapies.

Chimeric Antigen Receptor T-cell (CAR-T) therapy, involves genetically engineering T cells to express chimeric antigen receptors, enabling them to recognize and eliminate tumor cells (111). However, only limited efficacy of CAR-T therapy targeting CD33, CD123 in AML have been observed in clinical trials (112). The widespread occurrence of adverse events resulted from CAR-T therapies is the “on-target, off-tumor toxicity”, which arises in patients who have target antigen expressed on both tumors and healthy tissues (113). Unfortunately, frequently targeted antigens in AML CAR-T therapy, such as CD33 and CD123, found in around 80–90% and 70–80% of AML patients, respectively, are also detected on HSCs and normal myeloid progenitor cells (114). This lack of target specificity can lead to unintended toxicity, prolonged severe myelosuppression, and dependence on transfusions. scRNA-seq is a powerful tool for comparing the expression levels of antigens in malignant cells and non-malignant cells from a broad range of healthy tissues.

The expression patterns of common AML-related target antigens, including *CD33*, *CD123*, and *CLEC12A* (*CLL-1*, *CD371*), were investigated in normal tissues and organs (115). Targeting CD33,

CD123, and CLEC12A primarily affected $CD14^+$ monocytes, $CD16^+$ monocytes, and DC populations, with minimal impact on other hematopoietic lineages, such as B lineages, T lymphocytes, and NK cells. Notably, all these genes exhibited expression at the mRNA level in partial on HSPCs. A major concern arises from the higher frequency of *CLEC12A* in platelets at the mRNA level, and the abundant presence of *CD123* in multiple pancreatic cell types, as well as ECs across various organs, including cardiac, lung, skin, liver, and urinary bladder (115). *CD123* was also expressed in a small number of cardiac fibroblasts, aortic fibroblasts/smooth muscle cells/mesenchymal stem cells (MSCs), and lung epithelial cells (115). Thus, CD123 CAR-T can inadvertently injure these healthy cells, causing damage to endothelial cell and hematopoietic toxicity, including prolonged myelosuppression. Meanwhile, CD33 CAR-T treatment could eradicate skin Langerhans cells, potentially compromising the skin's defense against pathogenic microorganisms (115). Therefore, the widespread of "on-target, off-tumor toxicity" associated with these CAR-T therapies poses major limitations to their application in treating AML. The selection of the right target antigens for CAR-T immunotherapy in AML remains a significant challenge.

In attempt to search for novel CAR-T targets with minimal or none of "on-target, off-tumor toxicity" in AML, Gottschlich and colleagues utilized a comprehensive RNA-sequencing dataset comprising over 500,000 single cells from 15 AML patients and 9 healthy individuals (116). After a serial of stepwise filtering, colony-stimulating factor 1 receptor (CSF1R) and CD86 were identified as novel target antigens for CAR-T cell therapy in AML. The expressions of *CSF1R* and *CD86* were higher on malignant HSC-like and HSPC-like cells than on healthy controls. Furthermore, *CSF1R* and *CD86* were lack on T cells and had minimal expression on nine organs in healthy controls (116). Functional validation of these CAR-T cells demonstrated robust efficacy in both *in vitro* and *in vivo* AML models, with minimal off-target toxicity to relevant healthy tissues (116). These findings provide a compelling basis for advancing these CAR-T cells into further clinical development. The high-resolution, single-cell expression analysis offers an innovative strategy for identifying new CAR-T targets in AML. The ongoing clinical trials of these new CAR-T therapies give an opportunity for combating AML and improved outcomes for patients, especial for refractory and relapsed cases in the future.

Conclusion and perspective

The past few years have witnessed substantial progress in scRNA-seq study of AML. These advances signify the cellular heterogeneity within the AML ecosystem, a complexity previously overlooked in bulk RNA-seq analyses. The insights gained from scRNA-seq greatly enhance our understanding of LSC, compromised immune cells, altered BM microenvironment, and mechanisms of resistance to chemotherapy and relapse. Furthermore, they pave the way for new therapeutic avenues, such as the development of truly AML-specific immunotherapies and small molecular inhibitors for unique LSC subpopulation.

Despite these advancements, certain challenges and unresolved issues persist in the application of scRNA-seq in AML. Spatial transcriptomics technologies have emerged as powerful instruments in cancer research, providing valuable insights into the spatial organization of gene expression within intact tissue sections in the original physiological context (117, 118). However, current scRNA-seq methods cannot provide spatial information, leading to a loss of spatial dimension regarding how different cell types and subpopulations interact directly within the BM microenvironment. While this is not a concern in solid tumor samples, it is not applicable to formalin fixed paraffin embedded (FFPE) BM samples because the harsh decalcification procedure can result in severe degradation of RNA (119). A technical breakthrough is required to enable the utilization of FFPE BM samples in conjunction with spatial transcriptomics and single-cell technologies. This advancement would facilitate the construction of 3D spatial maps of gene expression, providing a visual depiction of interactions among AML cells, including LSCs, immune cells, and stromal components, within their natural BM environments. Another major challenge is the reproducibility and comparability of results across studies due to the different scRNA-seq platforms, variations in sequence depth and bioinformatic pipelines applied (120). Hence, future scRNA-seq studies could be enhanced through the standardization of protocols and methodologies, alongside the creation of robust computational tools and analytical frameworks, complemented by artificial intelligence technology.

Furthermore, there is a need for scRNA-seq in combination with other omics technologies, such as genomics, epigenomics, proteomics and metabolomics to provide a more holistic view of gene mutations, methylation, acetylation, chromatin remodeling, protein abundance and substrates and products of metabolism (121–126). For example, AML is known for its cellular heterogeneity, with different subpopulations of AML cells exhibiting diverse gene expression profiles and epigenetic states. Single-cell ATAC-seq (Assay for Transposase-Accessible Chromatin with high-throughput sequencing) enable the genome-wide mapping of accessible chromatin regions at the single-cell level (127). scRNA-seq combined with single-cell epigenomics techniques, such as scATAC-seq, can provide simultaneous profiling of gene expression and chromatin accessibility in rare AML populations like LSC, delineating epigenetic dysregulation and transcriptional regulatory networks. Single-cell proteomics techniques, such as mass cytometry (CyTOF) (128–130) or single-cell proteomic assays (131, 132), complement scRNA-seq by profiling the expression levels of proteins at the single-cell level. Likewise, integrating scRNA-seq with single-cell proteomics enables comprehensive assessment on how a single gene's protein levels track with its mRNA levels across individual AML cells, given the fact that genome-wide correlation between expression levels of mRNA and protein on bulk RNA-seq and proteomic studies are around 40% (133). Single-cell metabolomics using mass spectrometry allows for the simultaneous detection of a wide range of metabolites from individual cells (134, 135), Integration of scRNA-seq with single-cell metabolomics facilitates measurement of metabolite levels associated with key metabolic pathways, such as glycolysis, tricarboxylic acid cycle, and amino acid metabolism (136, 137), capturing both transcriptional signatures and metabolic profiles, within individual AML cells.

CRISPR/Cas9 technology enables the targeted manipulation of specific genes in a controlled manner (138). By combining scRNA-seq with CRISPR/Cas9, like Perturb-seq (139) and Cas13 RNA Perturb-seq (CaRPool-seq) (140), researchers can systematically perturb potentially important oncogenes and assess the effects on the transcriptome on single cell level (141). This approach aids in pinpointing crucial oncogenic drivers involved in both the onset and advancement of AML, thereby streamlining the development of targeted therapeutic approaches designed to eliminate LSC populations responsible for disease recurrence.

The established prognosis factors used in clinic decision are age, cytogenetic abnormalities, molecular mutations (e.g., *FLT3*, *NPM1*, *CEBPA*, *DNMT3A*, *IDH1*, *IDH2*, *TP53*), and the new 2022 edition of European LeukemiaNet (ELN) risk classification (Favorable prognosis, Intermediate prognosis and Adverse prognosis) based on the genetic alterations (8). One major limitation of current risk stratification is primarily derived from clinical studies comprising younger, fit patients with *de novo* AML who received intensive chemotherapy. However, the average age at diagnosis for adult AML is 68 years. Older adult AML patients often receive lower-intensity therapy instead of intensive therapy (142). Therefore, this limitation significantly restricts the applicability of these guidelines. scRNA-seq has the capability to capture rare cell subsets, such as AML LSC or therapy-resistant clones, which may have prognostic implications. Importantly, the application of scRNA-seq in prognosis is applicable to both young and old patients, as well as to patients who have received either intensive or low-intensity chemotherapy, targeted therapies, or immunotherapies. Hence, incorporating scRNA-seq data with established risk factors, such as age, cytogenetic abnormalities, and mutation status, could improve prognostic accuracy.

Furthermore, scRNA-seq can be leveraged to monitor the dynamic changes in the AML cell and immune cell populations during treatment, enabling real-time tracking of treatment response and the emergence of resistant clones. These data derived from scRNA-seq can be used to identify transcriptional signatures associated with drug sensitivity or resistance in AML cells. The signatures may serve as predictive biomarkers for treatment response, which can guide personalized treatment adjustments, potentially minimizing the risk of relapse and improving patient survival. However, the clinical implementation of scRNA-seq in AML faces several limitations and challenges. One of the primary concerns is the cost of scRNA-seq, which can be prohibitively expensive for routine clinical use, particularly with high volume of samples. Secondly, the bioinformatic analysis of scRNA-seq data is complex and requires fair amount of computational resources and dedicated bioinformaticians, which may not be readily available in some resource-limited hospitals.

Despite these limitations, the potential advantages of scRNA-seq in improving AML diagnosis, prognosis, and treatment are indisputable. Moving forward, we provide our view on the trajectory towards clinical application of scRNA-seq. As a relatively young, but fast-evolving field of single-cell field, ongoing advancements in sequencing technologies, bioinformatics tools, and protocol optimization are driving down the cost of scRNA-seq and improving its scalability. We foresee the sequencing costs continue to decrease and technologies become more user-friendly, the implementation of scRNA-seq in clinical settings is expected to increase. Clinical validation studies are essential for establishing the clinical utility of scRNA-seq in AML diagnosis, prognosis, and treatment. Larger cohorts of AML patients and prospective, longitudinal studies are needed to determine the clinical utility of scRNA-seq in prognosis assessment and personalized medicine for AML patients. Regulatory approval and standardization of scRNA-seq protocols are critical steps towards its widespread adoption in clinical practice. With continued innovation, cost reduction, and validation in clinical trials, we expect scRNA-seq holds great promise for clinic use in management of AML patients in the near future.

Collectively, the perspective for integrating scRNA-seq into basic and translational research in AML is optimistic. The new insights uncovered by the widespread use of scRNA-seq are crucial for the development of novel therapies, especially for refractory/relapsed AML and significantly improve the survival rate of AML patients.

Author contributions

JZ: Conceptualization, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing, Resources. W-JC: Writing – original draft, Writing – review & editing, Funding acquisition, Resources.

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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.

Publisher's note

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Glossary

AML	Acute myeloid leukemia
ATAC-seq	Assay for Transposase-Accessible Chromatin with high-throughput sequencing
B-ALL	B cell acute lymphoblastic leukemia
BM	Bone marrow
BMEC	BM endothelial cells
BMSCs	bone marrow mesenchymal stromal cells
CaRPool-seq	Cas13 RNA Perturb-seq
CAR-T	Chimeric antigen receptor T cell
cDC	Conventional dendritic cell
CSF1R	colony-stimulating factor 1 receptor
CLPs	common lymphoid progenitors
CTLs	cytotoxic T cells
DLBCL	Diffuse large B cell lymphoma
FACS	Fluorescence-activated cell sorting
FFPE	Formalin-fixed paraffin-embedded
FLT3	FMS-like tyrosine kinase 3
GMP	Granulocyte macrophage progenitors
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
IDH1	isocitrate dehydrogenase 1
LepR	Leptin receptor
LSCs	Leukemia stem cells
LT-HSCs	long-term hematopoietic stem cells
MM	multiple myeloma
MPP	Multipotent progenitor cell
MSCs	Mesenchymal stem cells
NGS	Next-generation sequencing
NK cells	Natural killer cells
NPM1	Nucleophosmin 1
OLC	Osteolineage cells
OxPhos	Oxidative-phosphorylation
OS	Overall survival
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PDX	Patient-derived xenografts
PFS	Progression-free survival
QSCs	Quiescent stem-like cells
RNA-seq	RNA sequencing
RP	Ribosomal protein
sc	Single-cell
scRNA-Seq	Single-cell RNA sequencing
SMART-seq	Switching mechanism at the 5'
SORT-seq	FACS sorting and robot-assisted transcriptome sequencing
TCR	T-cell receptor
Tm	Memory T cells
T-regs	Regulatory T cells
WES	Whole exome sequencing
WGS	Whole genome sequencing.

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Nico Gagelmann,
University Medical Center Hamburg-Eppendorf, Germany

REVIEWED BY
Xiaojiang Xu,
Tulane University, United States
Anna M. Eiring,
Texas Tech University Health Sciences Center El Paso, United States

*CORRESPONDENCE
Beizhong Liu
liubeizhong@cqmu.edu.cn

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Lysosome-related genes predict acute myeloid leukemia prognosis and response to immunotherapy

Peng Wan[1], Liang Zhong[2], Lihua Yu[3], Chenlan Shen[1], Xin Shao[1], Shuyu Chen[1], Ziwei Zhou[1], Meng Wang[1], Hongyan Zhang[1] and Beizhong Liu[1,2]*

[1]Central Laboratory of Yongchuan Hospital, Chongqing Medical University, Chongqing, China, [2]Key Laboratory of Laboratory Medical Diagnostics, Ministry of Education, Department of Laboratory Medicine, Chongqing Medical University, Chongqing, China, [3]Clinical Laboratory of Yongchuan Hospital, Chongqing Medical University, Chongqing, China

Background: Acute myeloid leukemia (AML) is a highly aggressive and pathogenic hematologic malignancy with consistently high mortality. Lysosomes are organelles involved in cell growth and metabolism that fuse to form specialized Auer rods in AML, and their role in AML has not been elucidated. This study aimed to identify AML subtypes centered on lysosome-related genes and to construct a prognostic model to guide individualized treatment of AML.

Methods: Gene expression data and clinical data from AML patients were downloaded from two high-throughput sequencing platforms. The 191 lysosomal signature genes were obtained from the database MsigDB. Lysosomal clusters were identified by unsupervised consensus clustering. The differences in molecular expression, biological processes, and the immune microenvironment among lysosomal clusters were subsequently analyzed. Based on the molecular expression differences between lysosomal clusters, lysosomal-related genes affecting AML prognosis were screened by univariate cox regression and multivariate cox regression analyses. Algorithms for LASSO regression analyses were employed to construct prognostic models. The risk factor distribution, KM survival curve, was applied to evaluate the survival distribution of the model. Time-dependent ROC curves, nomograms and calibration curves were used to evaluate the predictive performance of the prognostic models. TIDE scores and drug sensitivity analyses were used to explore the implication of the model for AML treatment.

Results: Our study identified two lysosomal clusters, cluster1 has longer survival time and stronger immune infiltration compared to cluster2. The differences in biological processes between the two lysosomal clusters are mainly manifested in the lysosomes, vesicles, immune cell function, and apoptosis. The prognostic model consisting of six prognosis-related genes was constructed. The prognostic model showed good predictive performance in all three data sets. Patients in the low-risk group survived significantly longer than those in the high-risk group and had higher immune infiltration and stronger response to immunotherapy. Patients in the high-risk group showed greater sensitivity to cytarabine, imatinib, and bortezomib, but lower sensitivity to ATRA compared to low -risk patients.

Conclusion: Our prognostic model based on lysosome-related genes can effectively predict the prognosis of AML patients and provide reference evidence for individualized immunotherapy and pharmacological chemotherapy for AML.

KEYWORDS

acute myeloid leukemia, lysosome, prognostic model, immune infiltration, chemotherapy

1 Introduction

Acute myeloid leukemia (AML) is a highly invasive and destructive hematological malignancy and characterized by abnormal proliferation of hematopoietic cells and early blockage of myeloid differentiation, which impairs normal hematopoiesis with fatal consequences (1). For the past 40 years, the treatment regimen for AML has remained the standard induction chemotherapy regimen based on anthracyclines. Although the majority of patients experience complete remission after initial treatment, the presence of relapses and refractory events results in a 5-year survival rate below 30% (2). Advances in sequencing technology have helped us to gain insights into the pathogenesis of AML and accordingly develop new drug targets and formulate risk stratification, such as Fms-like tyrosine kinase 3 - internal tandem duplication (FLT3-ITD), Isocitrate dehydrogenase(IDH) mutations (3–5). The crosstalk between multiple genetic variants and the lack of clarity on the specific mechanisms of AML development ultimately leads to a mismatch between risk stratification and clinical outcomes, which in turn affects the quality of survival of AML patients (6). Therefore, it is urgent and necessary to further study the pathogenesis of AML, develop appropriate risk assessment methods and improve risk stratification.

Lysosomes are organelles produced by the Golgi apparatus that contain a variety of hydrolytic enzymes and have a unique ph value (7). Previous studies generally regarded lysosomes as organelles that break down substances, but in recent years, studies have pointed out that they not only break down substances and replenish nutrient metabolism, but also influence cell growth, disease generation, tumor progression, and other biological processes by mediating cellular signaling and participating in autophagy (8, 9). During tumor progression, lysosomal function undergoes a significant up-regulation to meet the energy demands necessary for the excessive proliferation and invasion of cancer cells (10). In contrast to normal cells, cancer cells exhibit a greater abundance and larger size of lysosomes, along with elevated lysosomal enzyme activities. Several lysosomal enzymes, such as cathepsin B and cathepsin D, besides their known role in mediating programmed cell death, are strongly implicated in poor patient prognosis (11–15). Additionally, the lysosomal fusion derivative known as Auer rods is predominantly observed in hematologic tumors, with current research focusing on their utility as diagnostic markers (16). However, the functional significance of this lysosomal derivative in acute myeloid leukemia remains poorly understood. Based on the aforementioned evidence, we hypothesize that the expression levels of lysosome-related genes could be utilized to categorize AML patients into distinct molecular subtypes, thereby guiding AML risk stratification and prognosis.

In our research, we collected lysosomal genes, constructed a prognostic model based on lysosome-related genes through systematic analysis, and conducted a preliminary validation of the model's accuracy and usefulness. The aim is to improve the prognosis of AML and provide new reference evidence for individualized treatment of AML.

2 Methods

2.1 Data download and pre-processing

All data used in this study were obtained from two high-throughput sequencing platforms, TCGA(https://portal.gdc.cancer.gov/) and GEO (https://www.ncbi.nlm.nih.gov/geo/), which contained 984 samples from GSE37642 (17), 151 samples from TCGA-LAML, and 304 samples from GSE10358 (18). We then adopted the following criteria to further screen the samples: 1, The tumor primary site of all samples should be bone marrow or peripheral blood. 2, All samples should have complete RNA-seq data and clinical information. 3, All samples shall have complete survival information. After screening, we included 367 samples from GSE37642-GPL96 as our training set, 132 TCGA-LAML samples and 91 GSE10358-GPL570 samples as test set, totaling 590 samples. In addition, the GSE114868 (19) and GSE149237 datasets were downloaded from the GEO database for screening genes that were statistically different ($|logFC>1|$ and $p<0.05$) between healthy donors and AML patients for subsequent screening. Preprocessing of the data is shown in Supplementary Figure S1A.

2.2 Lysosome-related gene sets

A total of 191 lysosome-associated genes from five gene sets were obtained by searching the MsigDB database(https://www.gsea-msigdb.org/gsea/msigdb) with the keyword lysosome, 169 genes were extracted from the expression matrix of the training set

GSE37642-GPL96 for subsequent analysis, and the specific gene sets and genes are provided in Supplementary Table 1.

2.3 Consensus unsupervised clustering

We extracted the expression of 169 lysosome genes from the training set GSE37642-GPL96, and obtained the sample clustering information by repeating the calculation 1000 times using the R package "ConsensusClusterPlus". The differences were initially evaluated by principal component analysis (PCA) and Kaplan-Meier (KM) survival curves, and the expression of genes in different clusters was represented by heatmaps. For secondary clustering, we obtained 87 genes that differed between the two lysosomal clusters and between healthy donors and AML patients by taking the intersection of DEGs from between the two clusters and differential genes from GSE114868 and GSE149237 respectively, and subsequently obtained the results of the secondary clustering of the samples using the same method.

2.4 Differential analysis of gene expression, PPI and enrichment analysis

According to the unsupervised consensus clustering, we divided the test set into different clusters, and analyzed the differential genes between the two clusters using the R package "limma" (|logFC>0| p<0.05) (20), and represented them as volcano plot. We obtained 646 differential genes, exported the network through the string (https://cn.string-db.org/), imported it into Cytoscape_v3.8.0, and selected the top30 nodes to obtain the protein-protein interaction (PPI) network after calculating the degree by cytohubba. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes functional enrichment analysis of differentially expressed genes using the R package "clusterProfiler" (21).

2.5 Immunoinfiltration analysis

The ESTIMATE and CIBERSORT scores were computed using the R package "IOBR" (22). The marker genes of immune cells were sourced from the TISIDB database(http://cis.hku.hk/TISIDB/), and the immune cell enrichment scores were obtained by single-sample gene enrichment score estimation (ssGSEA) analysis with the R package "GSVA" before comparing immune cell infiltration between clusters (23). Immune checkpoint gene set from ref (24).

2.6 Construction and validation of a prognostic model

For the 87 DEGs screened, 26 genes were obtained by univariate cox regression (p<0.05), 6 genes were screened by stepwise multivariable cox regression (p<0.05), lasso regression was performed to prevent overfitting, and finally, lysosome-related gene scoring models were constructed according to the following formulae,

$$Risk\ \ Score = \sum_{x=1}^{n}(Gene_x \times coef_x)$$

$Gene_x$ is the gene expression, $coef_x$ is the coefficient of this gene. In accordance with the median value, the dataset is stratified into High-risk and Low-risk groups. The receiver operating characteristic (ROC) curve for the first, third, and fifth year between the High-risk and Low-risk groups were analyzed using the R package "timeROC". The R packages "regplot" and "rms" were used to produce nomogram and calibration curves. TCGA-LAML, GSE10358-GPL570 were used as test sets and the same calculations were performed.

2.7 Prognostic modeling and immunotherapy response

Tumor Immune Dysfunction and Exclusion(TIDE) score was calculated from the website (http://tide.dfci.harvard.edu/), then group comparisons are made by R. The immune infiltration score and the abundance of immune cells were calculated using the R package "IOBR" before group comparisons were made.

2.8 Drug sensitivity

The drug sensitivity of the expression matrix of the training set was calculated using the R package "pRRophetic" (25), compared in R according to the grouping information, and finally presented in a box plot.

2.9 Cell culture

Cell lines HS-5, KG-1a, HL-60, NB4, U937, and PBMC were obtained from American Type Culture Collection (ATCC), and OCI-AML2 was obtained from the German Collection of Microorganisms and Cell Cultures. KG-1a, HL-60, NB4, and U937 cells were cultured in RPMI-1640 medium (Gibco, USA), while HS-5 was cultured in DMEM (Gibco, USA). OCI-AML2 was cultured in MEM-α medium (Gibco, USA). The media used above contained 10% fetal bovine serum (FBS, USA) and 1% penicillin-streptomycin (Beyotime, Shanghai, China). The PBMC was not cultured after obtaining but was used directly for RNA extraction.

2.10 Real-time quantitative reverse transcription PCR

Total cellular RNA was extracted with TRIzol reagent (Takara, Japan) and then reverse transcribed into cDNA using PrimeScript™ RT Master Mix (Takara, Japan). RT-qPCR was performed in a CFX Connect™ RT-qPCR System (Bio-Rad, USA) using Hieff® qPCR SYBR Green Master Mix (Yeasen, Shanghai, China). Pre-denaturation was conducted for 5 min at 95°C, followed by cycling with denaturation at 95°C for 10 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s,

repeated for a total of 40 cycles. Up to 40 cycles without results were counted as the maximum of 50 cycles. The relative expression values of six genes in different cell lines were calculated using the method of $2^{-\Delta\Delta Ct}$, with GAPDH and PBMC used as reference, respectively. The experiments were repeated three times to obtain the data. All primer sequences, synthesized by Sangon Biotech (Shanghai, China), are shown in Supplementary Table 2.

2.11 Research flowchart

The flow chart for this research is placed in Supplementary Figure S1B.

2.12 Statistical analysis

Statistical analysis of all data was performed through R (R-4.3.1).t test and Kruskal-Wallis test were used for comparison of two and more groups, respectively. log-rank test was used to evaluate the significance of statistical differences. Where $p< 0.05$ was considered statistically significant. * $p< 0.05$; ** $p< 0.01$; *** $p< 0.001$.

3 Results

3.1 Lysosomal subcluster

To investigate whether lysosomal genes exhibit specific expression patterns in AML, we employed unsupervised consensus clustering to categorize 367 AML samples. The most obvious expression variations were detected when k=2, resulting in the split of the training set AML samples into two subclusters. Cluster1 (n=185) and Cluster2 (n=182) (Figures 1A, B, Supplementary Figure S2A). The results of PCA indicated a significant differentiation in gene expression between the two subclusters (Supplementary Figure S2B). Based on this, the KM curve suggested a noteworthy survival difference between the distinct subclusters, with the overall survival (OS) time of patients in Cluster1 significantly prolonged compared to Cluster2 (Figure 1C). Moreover, patients with the runx1-mutation had a

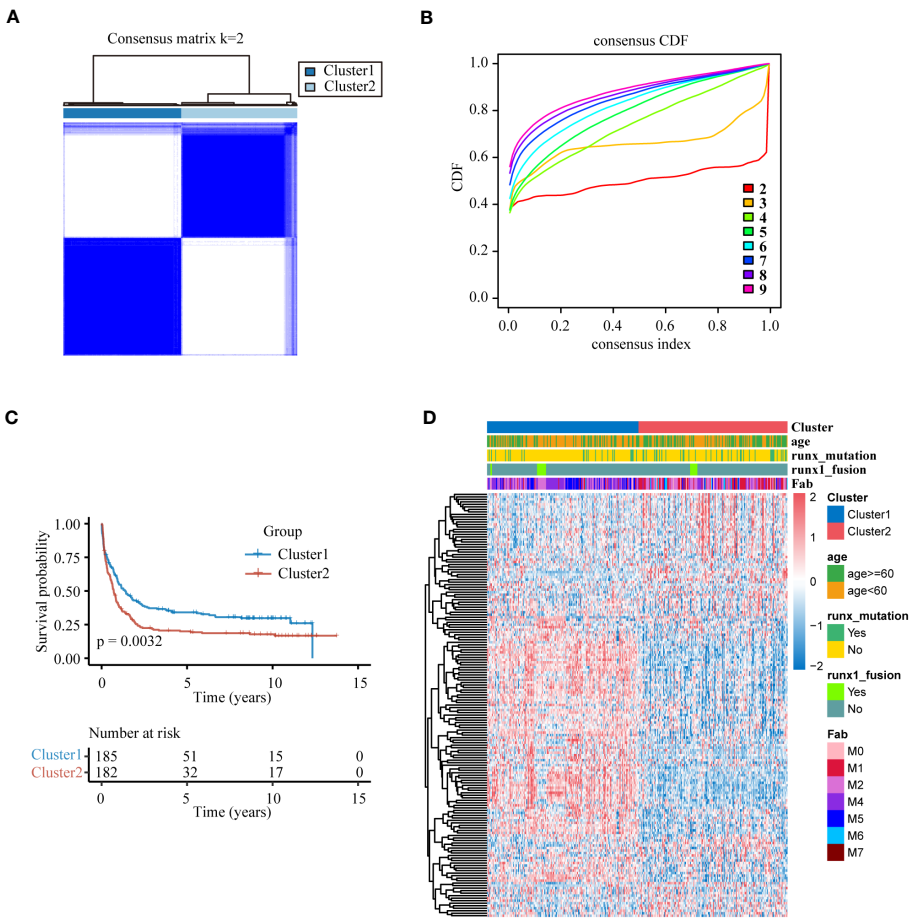

FIGURE 1
Lysosomal genes clustered for AML patients **(A)** Matrix heatmap at k=2. **(B)** Cumulative distribution function (CDF) for different k values. **(C)** KM survival analysis curves between two clusters at K=2. **(D)** Heat map distribution of lysosomal genes in the training set.

significantly higher representation in Cluster 2, and most lysosomal genes exhibited lower expression in Cluster2 (Figure 1D).

3.2 Molecular expression and biological processes among lysosomal subclusters

To delve further into the distinctions between these two subclusters, We utilized the R package "limma" to analyze the genes responsible for these differences, resulting in the identification of 672 differentially expressed genes (DEGs) (|logFC| > 0.5 and p< 0.05), with 166 up-regulated and 480 down-regulated (Figure 2A). To identify the core genes among these DEGs, we computed the top 30-degree core genes by Cytoscape, revealing two modules centered on spleen tyrosine kinase (SYK) and toll-like receptor 4 (TLR4), both tightly linked to the regulation of immune function (Figure 2B). These DEGs were enriched into lysosomes, cellular vesicles, immune cell functions, apoptosis, and some signaling pathways analyzed by KEGG enrichment (Figure 2C). GO enrichment analysis demonstrated the involvement of DEGs in cytoskeletal regulation, vesicle membrane composition, and other aspects (Figure 2D). Similar results were obtained by enrichment analysis of up- and down-regulated genes separately (Supplementary Figures S3A–D). These findings tentatively corroborated the subcluster results of our study.

3.3 Immune infiltration between lysosomal subclusters

The results from the previous PPI core gene and enrichment analyses revealed significant differences in immunomodulatory pathways between the two lysosomal subclusters. To gain a deeper understanding of the immune microenvironmental distinctions between the subclusters, we computed ESTIMATE scores for both subclusters using the R package "IOBR". The ESTIMATE scores indicated that in cluster1, there was greater immune cell infiltration and lower tumor purity compared to cluster2 (Figure 3A). The infiltration of these immune cells may play an anti-tumor role. The relative abundance of selected immune cells was further estimated for all training set samples using CIBERSORT and ssGSEA (Figures 3B, C). The results demonstrated predominant enrichment of monocytes, macrophages, and neutrophils in cluster1, while T cell subsets such as CD8 and CD4+ T cells were enriched in cluster2. The tumor immune response is influenced by the crosstalk between

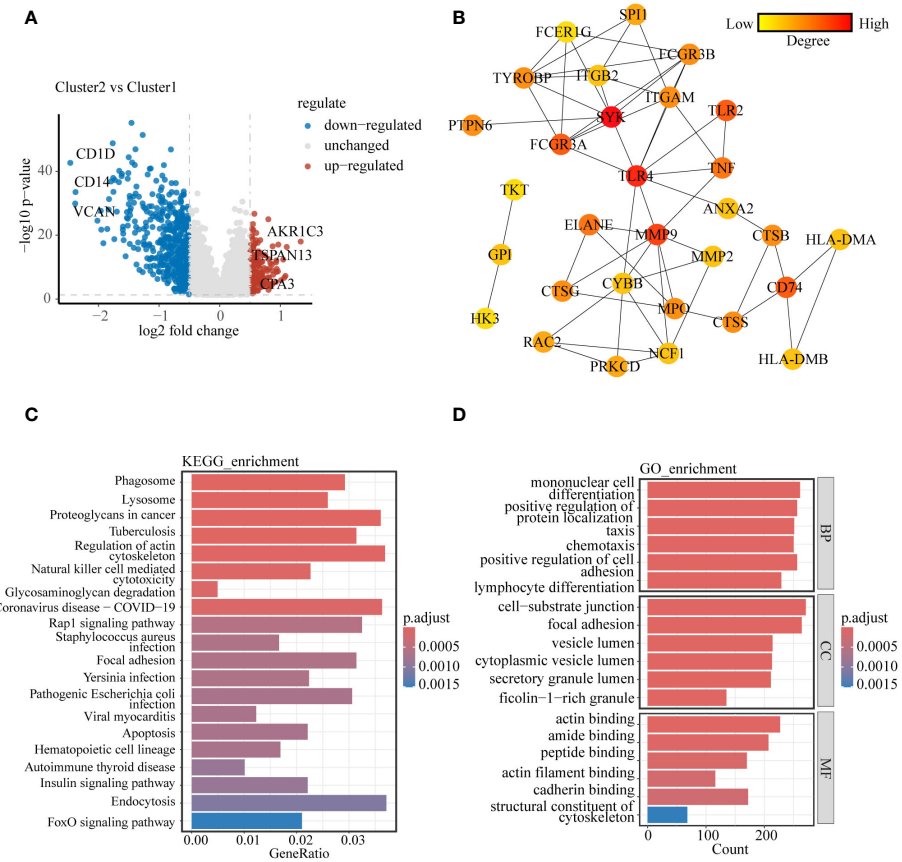

FIGURE 2
Molecular and biological processes differences between lysosomal clusters **(A)** Volcano diagram showing molecular differences between clusters. **(B)** PPI network was constructed to uncover core genes in DEGs. **(C)** KEGG enrichment analysis and **(D)** GO enrichment analysis to explain the biological processes involved in DEGs between clusters.

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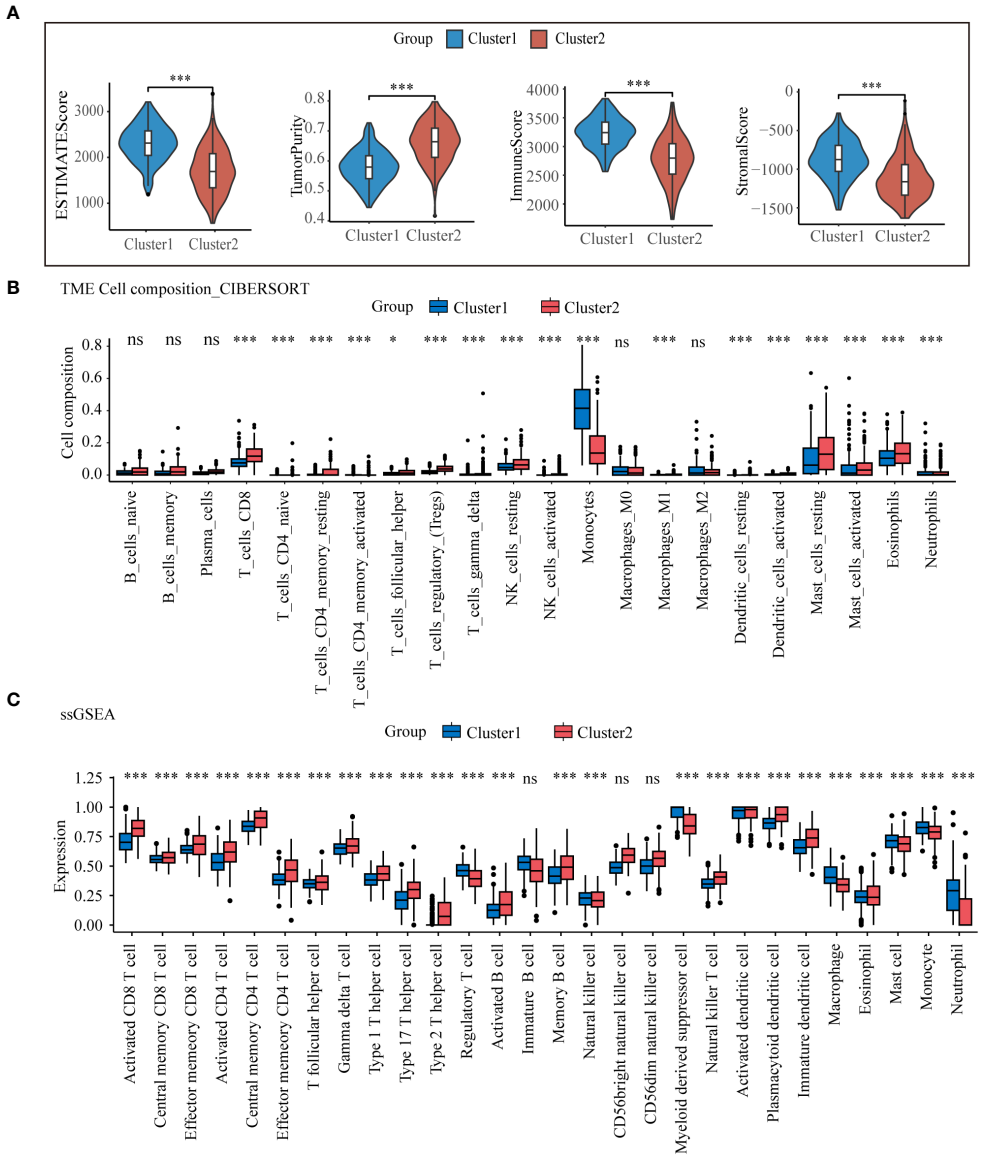

FIGURE 3
Tumor microenvironmental analysis of lysosomal clusters Estimate analysis comparing **(A)** estimate score, **(B)** CIBERSORT calculated the relative compositional abundance of 22 immune cells. **(C)** ssGSEA calculated the relative abundance of immune cells in 28. ns, non-significant; * p< 0.05; *** p< 0.001.

tumor cells, immune cells, and immune molecules. According to the expression of immune checkpoint genes (Supplementary Figure S4), partial immune checkpoint genes were significantly different between the two groups.CD86, whose expression was significantly lower in cluster2 than in cluster1, exerts anti-tumor effects by binding to CD28, inducing T cells to continue proliferating and differentiating into effector T cells (26). The above results indicate significant differences in the immune microenvironment of the two lysosomal subclusters, with cluster1 exhibiting stronger immune cell infiltration and a more robust immune response than cluster2. These differences offer potential therapeutic targets for achieving individualized treatment.

3.4 Secondary clustering

To enhance integration with clinical diagnosis, we initially identified genes exhibiting expression disparities (|logFC| > 1 and p< 0.05) between AML patients and healthy donors from datasets GSE114868 and GSE149237, respectively. We then intersected this selection with genes from the training set GSE37642-gpl96, which had expression differences (|logFC| > 0.5 and p< 0.05) between the two molecular subtypes, to obtain 84 DEGs (Figure 4A). We employed these 87 differentially expressed genes for unsupervised consensus clustering. The clustering results indicated optimal typing at K=2 (Figure 4B, Supplementary Figures S5A–C), and

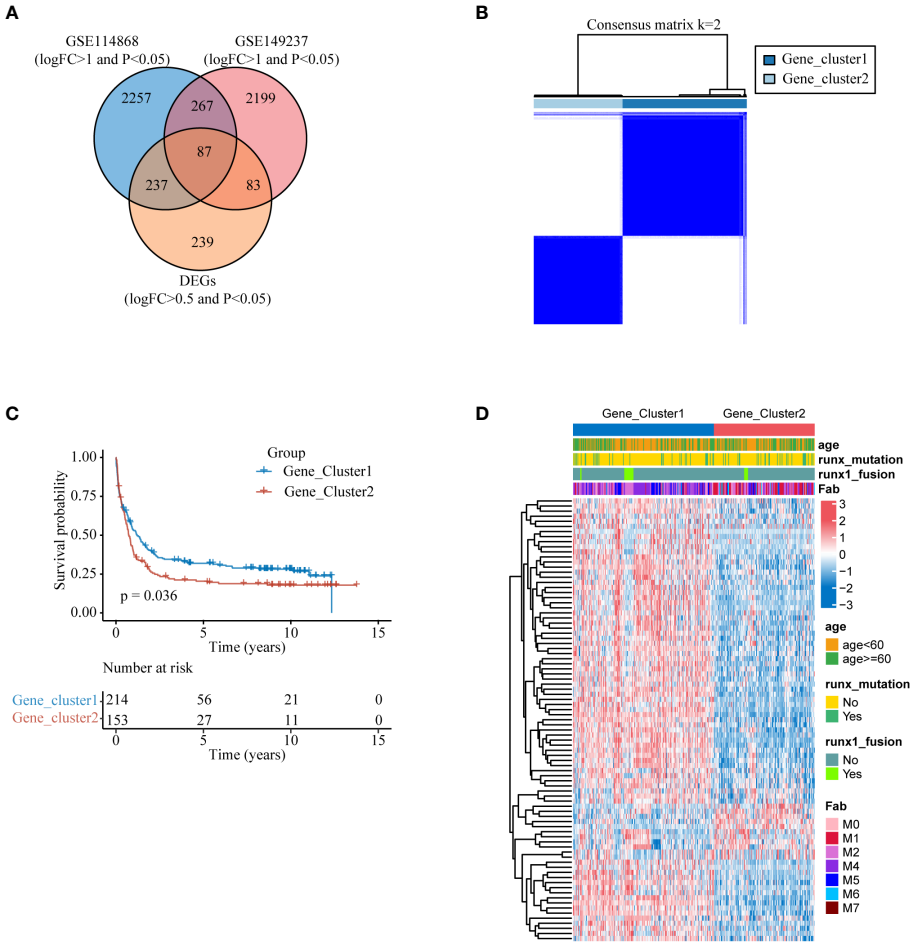

FIGURE 4
Secondary clustering **(A)** DEGs that vary among lysosomal isoforms and also exhibit differences in expression between AML patients and healthy individuals. **(B)** Heat map of the consensus matrix of the sample at k=2. **(C)** KM survival analysis curves between two gene subtypes at K=2. **(D)** Heatmap of the expression of 87 differential genes between the two gene subtypes.

KM curve revealed that Gene_cluster1 had significantly higher overall survival time than Gene_cluster2 (Figure 4C).The heatmap illustrates the expression patterns of the 87 DEGs between the two gene subtypes and their correlation with clinical features (Figure 4D).

3.5 Construction of a prognostic model for lysosome-related genes

To identify genes influencing prognosis between the two lysosomal subclusters, we conducted univariate cox regression ($p< 0.05$) on the 87 DEGs obtained from the intersection (Supplementary Figure S6). We identified 26 DEGs significantly impacting prognosis. Further screening was performed using multivariate cox regression ($p< 0.05$) (Figure 5A). To prevent overfitting, we employed lasso regression and constructed a prognostic model comprising 6 genes (Figures 5B, C). The sample's risk score was computed based on the formula:

$$\begin{aligned} Risk\ \ Score = {} & expression(PILRA) \times coef(-0.370) + expression(LILRA2) \\ & \times coef(0.221) \\ & + expression(MYO1F) \times coef(0.214) + expression(NCF1) \\ & \times coef(0.110) \\ & + expression(HPGDS) \times coef(-0.137) + expression(MPO) \\ & \times coef(-0.095) \end{aligned}$$

The samples from the dataset GSE37642-GPL96 were divided into two groups based on the median values of the risk scores. The sankey diagram illustrates the association between several subtypes and patient survival outcomes(Figure 5D). Cluster2 and Gene_cluster2, associated with worse prognosis, exhibited significantly higher risk scores than Cluster1 and Gene_cluster1(Figures 5E, F).

3.6 Validation of the lysosome related-genes prognostic model

To test the predictive effect of lysosomal related-genes prognostic model on the prognosis of AML patients, we first

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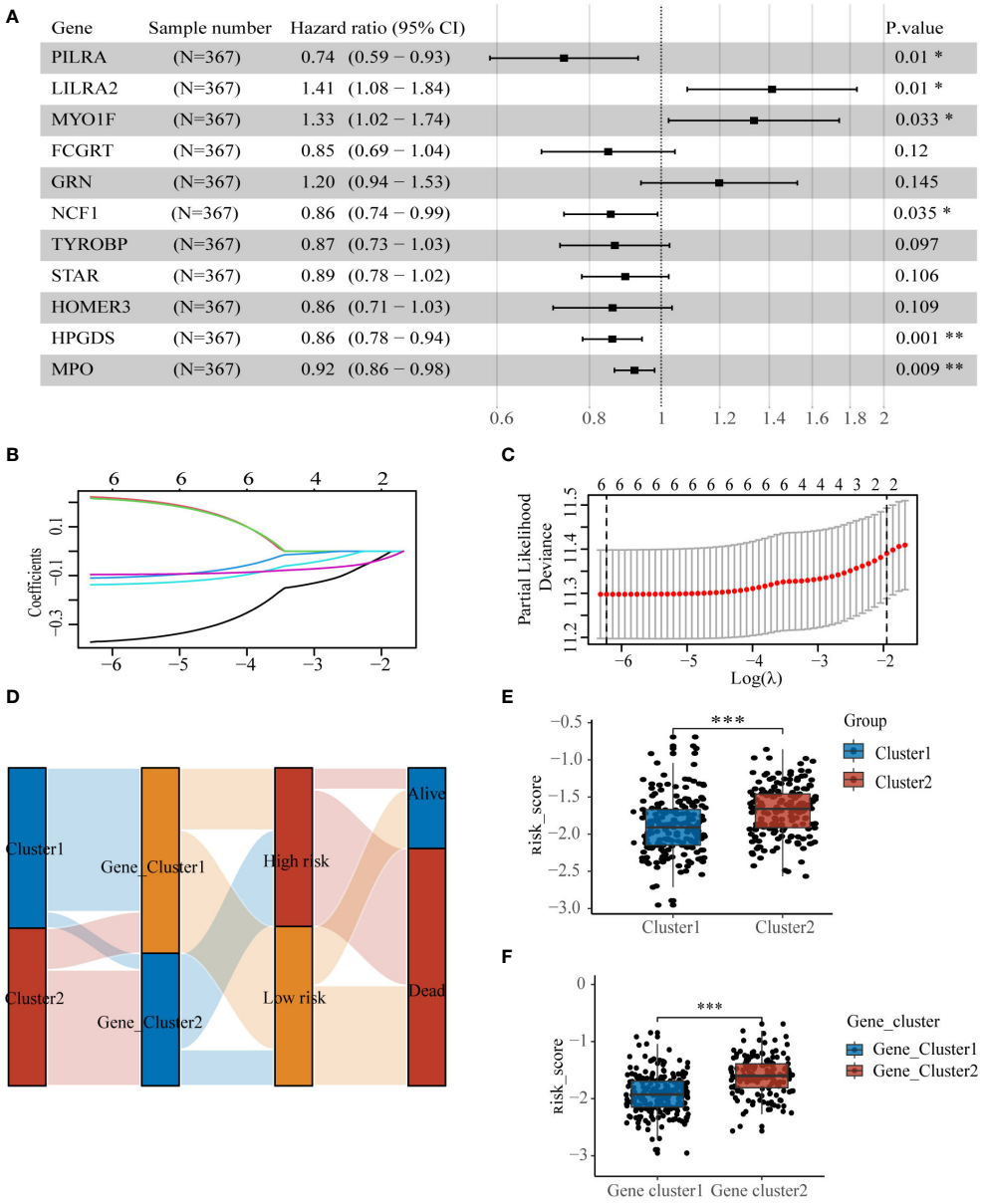

FIGURE 5
Construction of lysosome-related gene prognostic model **(A)** Multivariate regression results screened 6 DEGs, **(B, C)** lasso regression screened 6 DEGs for construction of prognostic model. **(D)** Sankey diagrams clearly show the distribution of patients among different subgroups and the outcome of **(E)** molecular subtypes and **(F)** risk scores for genetic subtypes. * p< 0.05; ** p< 0.01; *** p< 0.001.

examined the distribution of risk scores of patients in the training set by ggrisk (Figure 6A), and the patients with greater risk scores had higher risk of death. The results of the KM curves hinted to the fact that patients in the high-risk group had a much lower OS than those in the low-risk group (Figure 6B), and the 1-, 3-,and 5-year AUC of ROC were 0.659,0.706,0.709 respectively (Figure 6C). These results demonstrated the good performance of the lysosomal risk score model in predicting the survival of AML patients. Further, we observed similar results in the test set data TCGA-LAML (Figures 6D–F) and GSE10358-GPL570 (Figures 6G–I). As the risk score increases, the risk of patient death increases, which provides an important basis for identifying high-risk patients. These results suggest that our lysosomal prognostic model can be used as a reliable survival predictor, which can help to more accurately stratify patients and assess prognosis.

3.7 Nomogram

We plotted the nomogram in conjunction with other clinical characteristics such as age, FAB typing for the purpose of further evaluating the model, patients with lower risk scores and younger age had better prognosis (Supplementary Figure S7A). The calibration curve showed the agreement between our prognostic model and real events (Supplementary Figure S7B). Similar results were observed in the test set data TCGA-LAML (Supplementary

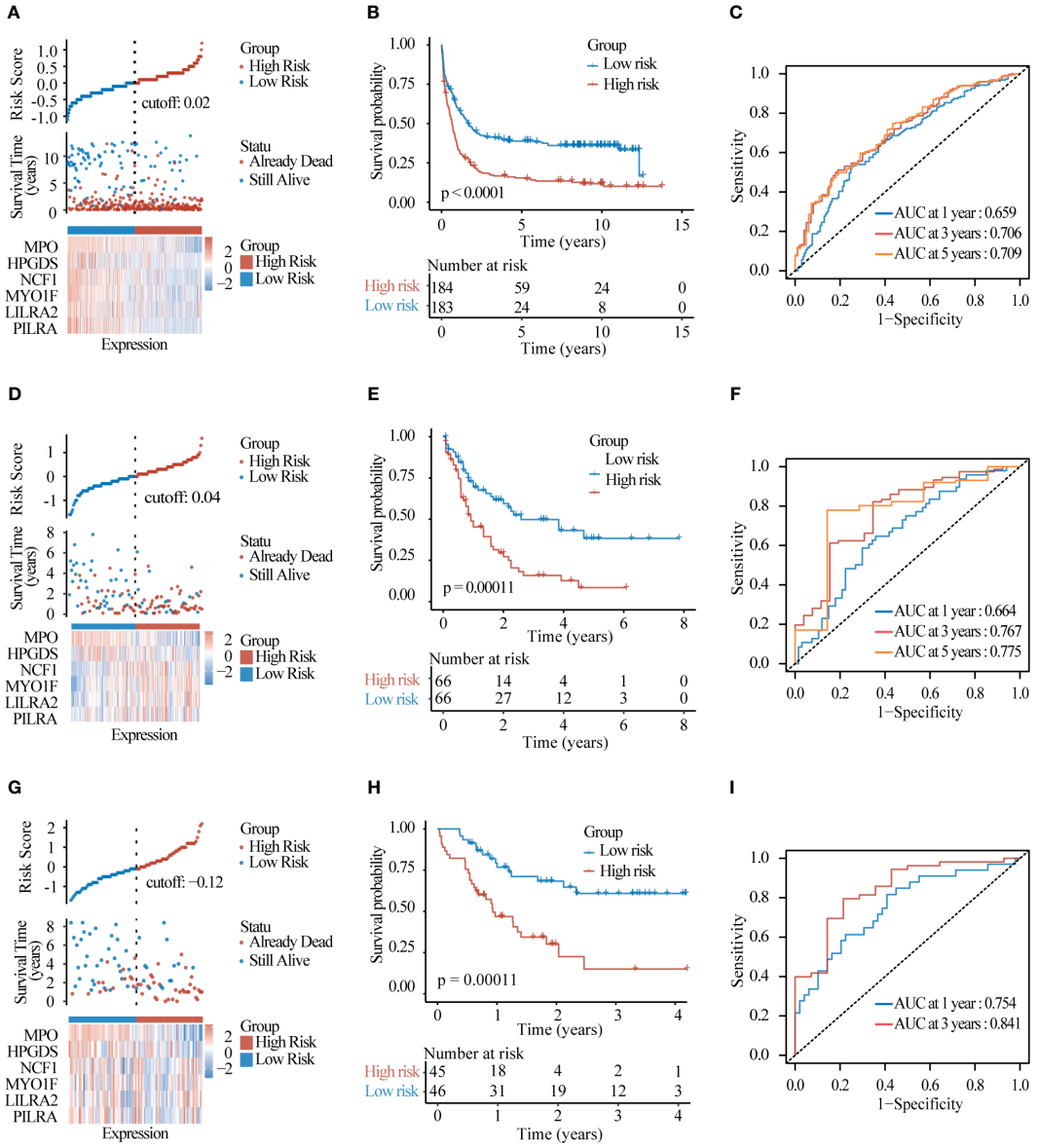

FIGURE 6
Model evaluation and validation **(A)** Risk factor distribution plots and **(B)** KM survival curves and **(C)** timeROC curves assessing the predictive accuracy of the risk model in the test set GSE37642-GPL96. **(D–F)** The same was performed in test set TCGA-LAML and **(G–I)** test set GSE10358-GPL570.

Figures S7C, D) and GSE10358-GPL570 (Supplementary Figures S7E, F). In addition, expanding the sample size and wider data validation are more helpful to strengthen the predictive power and clinical application value of risk lysosomal risk score in different populations.

3.8 Lysosomal scores predict immunotherapy effects

TIDE scores were calculated for the purpose of evaluating the role of risk scores in immunotherapy, and the TIDE scores of the high-risk group were significantly higher than those of the low-risk group (Figures 7A–D), suggesting that the high-risk group may be more susceptible to immune escape. Some immune checkpoints associated with MHC-II molecules were significantly less expressed in the high-risk group compared to the low-risk group(Figure 7E). The results of the ESTIMATE scores showed that immune infiltration was significantly stronger in the low-risk group than in the high-risk group (Supplementary Figures S8A–D), and the infiltration abundance of most immune cells was significantly with the high-risk group (Supplementary Figure S8E). These results suggest that there is a significant difference between the high-risk and low-risk groups in terms of response to immunotherapy in the training set data, and that the scoring model can effectively guide immunotherapy.

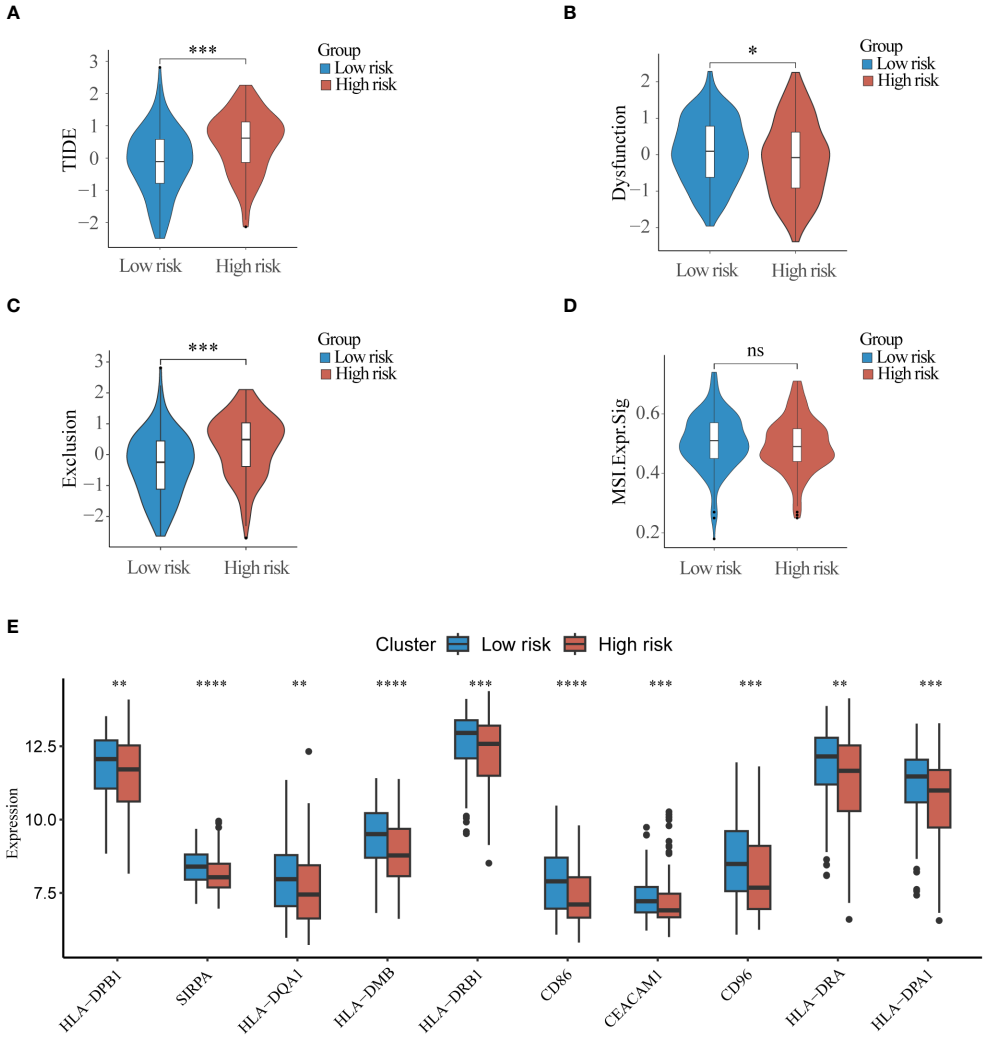

FIGURE 7
Immunotherapy response differences between high and low-risk groups **(A)** TIDE score, **(B)** Dysfunction score **(C)** Exclusion score and **(D)** Microsatellite instability score was used to compare differences in response to immunotherapy between high and low risk groups. **(E)** Top10 immune checkpoint molecules differentially expressed in high and low risk groups. ns, non-significant; * p< 0.05; ** p< 0.01; *** p< 0.001.

3.9 Drug sensitivity

We analyzed the sensitivity of the training set samples to several drugs by using the R package "pRRophetic". As compared with the low-risk group, the high-risk group was more sensitive to cytarabine (Figure 8A), ATRA (Figure 8C), imatinib (Figure 8E), and bortezomib (Figure 8F). There was no significant difference between the two groups in sensitivity to doxorubicin (Figure 8B) and midostaurin (Figure 8D). The above results provide important reference evidence for clinical treatment.

3.10 Validation of gene expression

To validate the expression of the six genes utilized in model construction, we initially selected dataset GSE114868 to compare gene expression between healthy donors and AML patients. The results revealed significant downregulation of PILRA, LILRA2, MYO1F, and NCF1 in AML, while HPGDS and MPO exhibited heightened expression levels (Supplementary Figure S9). Subsequently, we corroborated these findings using cell lines. Consistent with dataset GSE114868, we observed notable reductions in PILRA, LILRA2, MYO1F, and NCF1 expression, alongside significant elevations in HPGDS and MPO expression in AML cell lines compared to normal cells (Figures 9A–D). Notably, HPGDS was predominantly overexpressed in KG-1a cells, with relatively low expression in other AML cell lines (Figure 9E), while MPO expression in NB4 and U937 cells surpassed that of normal cells by more than 50-fold (Figure 9F).

4 Discussion

The current risk assessment for AML relies predominantly on the identification of genetic traits through gene sequencing and other methods for risk classification. However, this approach is

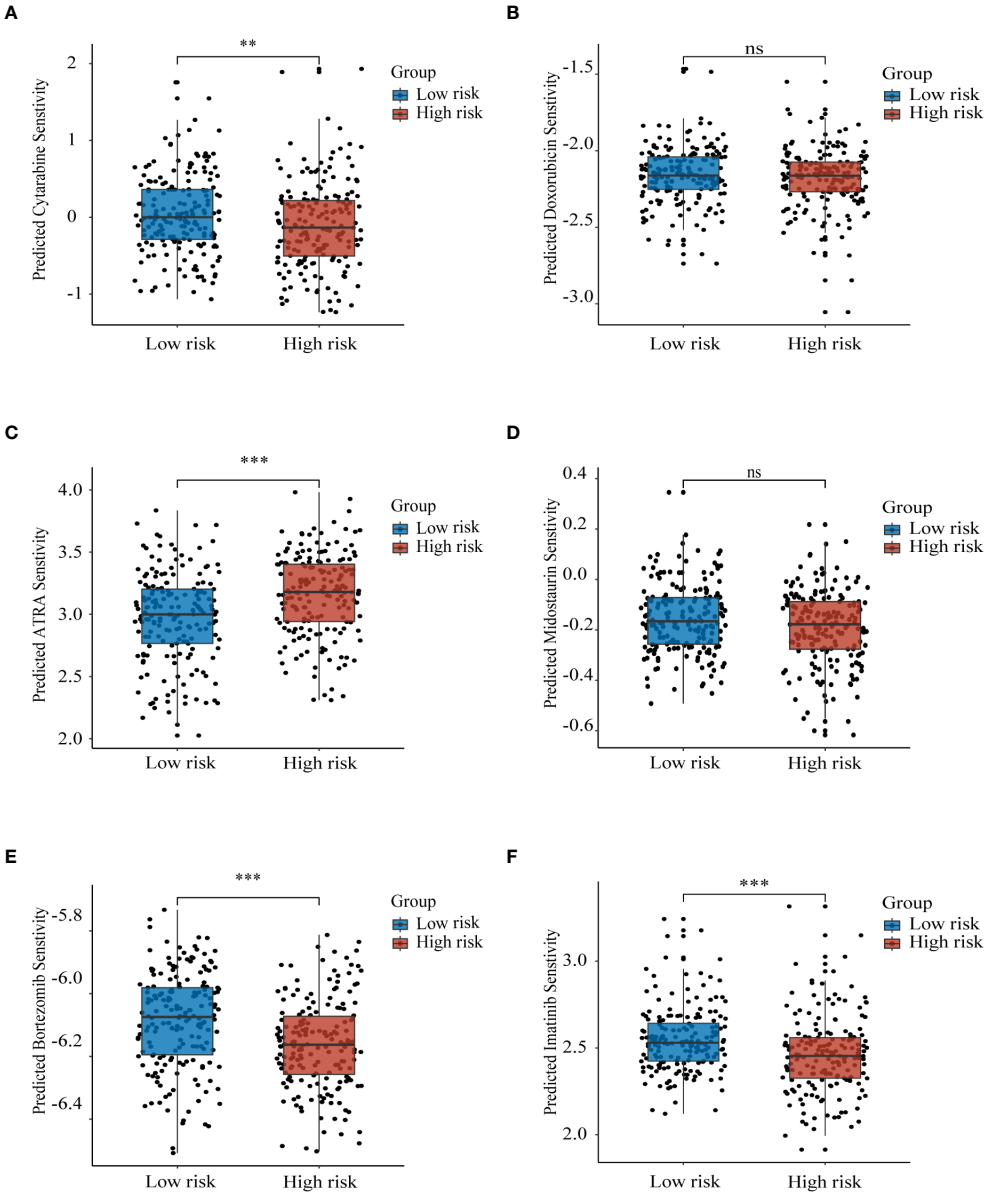

FIGURE 8
Prognostic model to guide drug therapy Sensitivity of high and low risk groups to different drugs in the training set GSE37642-GPL96 samples with **(A)** cytarabine, **(B)** doxorubicin, **(C)** ATRA, **(D)** midostaurin, **(E)** bortezomib, and **(F)** imatinib. ns, non-significant; ** p< 0.01; *** p< 0.001.

hindered by issues such as prolonged duration, reliance on a single method, and low accuracy (27, 28). In contemporary cancer research, molecular classification and prognostic modeling are increasingly turning their focus toward the intricate roles played by subcellular entities, or organelles. While existing risk models have predominantly centered on mitochondria, particularly in the context of cancer cells undergoing metabolic reprogramming, the lysosome, despite its equally pivotal role in cancer cell metabolism, has been relatively neglected (29–32).

Our study has developed molecular subtype and prognosis-related risk models in AML centered on lysosomal-related genes. This is the first model constructed based on lysosomal genes in AML. In this study, disparities in lysosomal gene expression were instrumental in classifying AML patients into distinct molecular subtypes, which differed significantly in terms of patient prognosis, molecular expression, and immune infiltration. However, unlike other similar studies (33), we refrained from conducting prognostic screening of the gene set prior to molecular subtyping. While this approach may diminish the model's prognostic predictive capacity, it facilitates the identification of other essential biological features beyond prognosis.

Our PPI results reveal that the pivotal differential genes distinguishing between the two lysosomal isoforms are SYK and TLR4. SYK, a non-receptor tyrosine kinase, has garnered significant attention in numerous studies as a promising target for hematologic malignancies and inflammation-related diseases (34). In prior research, SYK's pro-carcinogenic mechanism has been proposed to regulate the activation of associated pathways through signal transduction, thereby promoting AML cell survival and drug

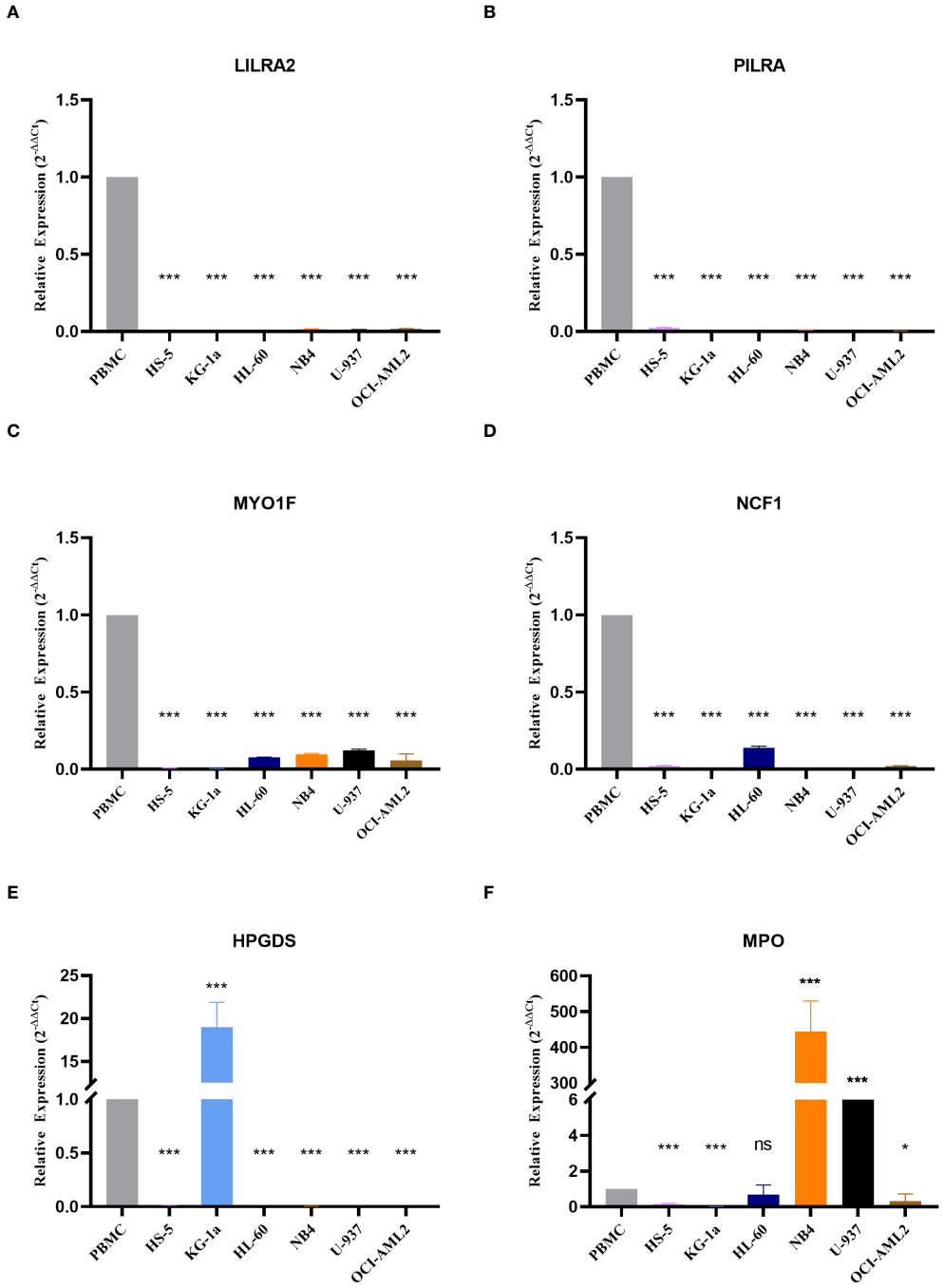

FIGURE 9
Expression of the six genes modeled Expression values of the six genes used to construct the model in normal and AML cell lines are depicted, including **(A)** LILRA2, **(B)** PILRA, **(C)** MYO1F, **(D)** NCF1, **(E)** HPGDS, and **(F)** MPO. ns, non-significant; * p< 0.05; *** p< 0.001.

resistance (34, 35). Our study suggests, for the first time, a connection between SYK and lysosomes in AML, a proposition supported by several studies in non-tumor cells (36, 37). Exploring this connection may bring new insights into SYK inhibitor resistance. Toll-like receptor 4 (TLR4) belongs to the Toll-like receptor (TLR) family, plays a pivotal role in pathogen recognition and innate immune activation (38). TLR4 responds to stimulation to activate signaling pathways, such as AMPK, and also regulates the tumor microenvironment, thereby influencing tumor progression (39, 40). A connection between lysosomes and TLR4 has been unveiled, with lysosomes serving as a site for TLR4 degradation (41). However, whether TLR4 modulates lysosomal function remains elusive, and our findings offer additional reference evidence for this avenue of investigation, more extensive studies are warranted to delve into the TLR4-lysosomal connection and its precise mechanism.

We constructed prognostic model comprising 6 genes (PILRA, LILRA2, MYO1F, NCF1, HPGDS, MPO) and categorized patients

into high- and low-risk groups. The distribution of risk scores between clusters and Gene_clusters provided a preliminary indication of the accuracy of the scoring model. The results from risk factor distribution plots, KM curves, and TimeROC demonstrated that the high-risk group had worse prognostic outcomes. Nomograms and calibration curves further illustrated the reliability of our model, and these results were validated in two other distinct datasets. Among these genes, MPO, HPGDS, and PILRA were considered favorable prognostic factors. Myeloperoxidase (MPO) regulates inflammatory responses and participates in the regulation of oxidative stress homeostasis (42). It is a common diagnostic marker in hematological neoplasms and aids in differentiating between myeloid and lymphoid lineages in acute leukemias (43). High expression of MPO is correlated with a favorable prognosis in AML patients (44). Hematopoietic prostaglandin d synthase (HPGDS) is an enzyme that catalyzes the isomerization of prostaglandin h2 (PGH2) to prostaglandin d2 (PGD2) (45). It exerts antitumor effects by catalyzing the production of PGD2 (46). Paired immunoglobulin-like type 2 receptor alpha (PILRA) is predominantly expressed on monocytes and macrophages (47) and is involved in the regulation of neutrophil infiltration (48). High expression of PILRA enhances the effect of antitumor immunotherapy (49), but its effects vary in different cancers (50).On the other hand, MYOIF, ILRA2, and NCF1 are considered prognostically unfavorable factors. Studies have demonstrated that MYOIF enhances the adhesion and migration of immune cells (51), promotes M1 polarization of macrophages (52), and in some tumor patients, MYO1F is mutated to form fusion proteins (53, 54), promoting tumorigenesis and progression (55). Activation of LILRA2 inhibits monocyte function and antigen presentation by dendritic cells (56, 57), and high expression of LILRA2 has been associated with a poor tumor prognosis (58). NCF1 encodes a protein that is one of the subunits of NADPH oxidase, and inhibition of NCF1 induces differentiation of APL cells as well as inhibits melanoma cell growth (59, 60). These pieces of evidence strongly support the reliability of our model. However, with the exception of MPO, the above genes have been rarely reported in AML, and follow-up studies are needed to delve deeper into their functions and mechanisms in AML.

Immunotherapy serves as a pivotal therapeutic approach in which lysosomes assume a significant role. Lysosomes facilitate immune evasion by cancer cells through the degradation of crucial proteins, including PD-L1 and MHC- I (61, 62). Only a minute fraction of current AML studies have delved into the influence of lysosomes on immunotherapy (63). Our findings reveal distinct immune responses and variations in the expression of immune checkpoint molecules between high and low-risk groups. Remarkably, multiple immune checkpoint molecules exhibited significant downregulation in the high-risk group, potentially contributing to the observed differences in immunotherapeutic responses (64). Notably, heightened expression of MHC-II class molecules has been consistently linked to favorable prognoses across various tumor types. This link has been confirmed by some studies in AML (65–68). While considerable attention has been devoted to exploring the impact of MHC-II molecules on antitumor immunotherapy, there appears to be a dearth of research investigating the relationship between MHC-II molecules and lysosomes in AML, despite such associations being reported in other disease models (69, 70). Our study might shed light on subsequent lysosome-mediated immunotherapy for AML. Furthermore, our study uncovered lysosome-associated differences in drug sensitivity between high- and low-risk groups, Consistent with this finding, lysosomes have been implicated in conferring drug resistance in cancer cells through mechanisms involving the segregation of drugs within the lysosomal compartment (71).

In conclusion, we have constructed a prognostic model centered on lysosome-related genes for the first time in AML. Our model can effectively assess the prognosis of patients and guide their clinical treatment, which provides new reference evidence for individualized treatment of AML. However, our study also has many limitations. One limitation is that, the study only focused on the association between lysosome-associated mRNAs and AML prognosis, lacking research on non-coding RNAs such as lncRNAs, circRNAs, and tRNAs. Second, external validation of clinical samples is required to ensure the accuracy of the scoring model.

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/Supplementary Material.

Author contributions

PW: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization. LZ: Project administration, Supervision, Writing – review & editing. LY: Funding acquisition, Writing – review & editing, Resources, Supervision. CS: Conceptualization, Methodology, Writing – review & editing. XS: Data curation, Investigation, Software, Writing – review & editing. SC: Data curation, Investigation, Software, Writing – review & editing. ZZ: Data curation, Investigation, Software, Writing – review & editing. MW: Data curation, Investigation, Software, Writing – review & editing. HZ: Data curation, Investigation, Software, Writing – review & editing. BL: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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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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Luca Vincenzo Cappelli,
Sapienza University of Rome, Italy

REVIEWED BY
Francesca Kaiser,
Sapienza University of Rome, Italy
Francesco Malfona,
Sapienza University of Rome, Italy

*CORRESPONDENCE
Magdalena Karasek
karasek.magdalena@gmail.com

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A hybrid protocol CLAG-M, a possible player for the first-line therapy of patients with mixed phenotype acute leukemia. A Polish Adult Leukemia Group experience

Magdalena Karasek[1*], Anna Armatys[2], Marek Skarupski[3,4], Łukasz Bołkun[5], Katarzyna Budziszewska[6], Joanna Drozd-Sokołowska[7], Ewa Zarzycka[8], Patrycja Mensah-Glanowska[9], Małgorzata Gajewska[10], Janusz Hałka[11,12], Agnieszka Kopacz[13], Witold Prejzer[8], Olga Chyrko[1], Tomasz Wróbel[1], Agnieszka Wierzbowska[14,15] and Marta Sobas[1]

[1]Department of Hematology, Blood Neoplasms and Bone Marrow Transplantation, Wroclaw Medical University, Wroclaw, Poland, [2]Department of Hematology and Bone Marrow Transplantation, University of Silesia, Katowice, Poland, [3]Department of Applied Mathematics, Faculty of Pure and Applied Mathematics, Wroclaw University of Science and Technology, Wroclaw, Poland, [4]Department of Mathematics and Computer Science, Eindhoven University of Technology, Eindhoven, Netherlands, [5]Department of Hematology, Internal Diseases and Angiology with a Subdivision of Blood Cell Transplantation, University Teaching Hospital in Białystok, Białystok, Poland, [6]Department of Hematology, Institute of Hematology and Transfusion Medicine, Warsaw, Poland, [7]Warsaw Medical University, Department of Hematology, Oncology and Internal Medicine, Warsaw, Poland, [8]Department of Hematology and Transplantology, Medical University of Gdansk, Gdańsk, Poland, [9]Department of Hematology, Jagiellonian University, Collegium Medicum in Kraków, Kraków, Poland, [10]Department of Internal Medicine and Hematology, Military Institute of Medicine, Warsaw, Poland, [11]Department of Hematology and Bone Marrow Transplantology, Clinical Hospital of the Ministry of Internal Affairs and Administration with the Warmia-Mazury Oncology Centre in Olsztyn, Olsztyn, Poland, [12]Department of Oncology, University of Warmia and Mazury, Olsztyn, Poland, [13]Department of Hematology, University Teaching Hospital them. Fryderyk Chopin in Rzeszów, Rzeszów, Poland, [14]Department of Hematology, Medical University of Łódź, Łódź, Poland, [15]Department of Hematology, Provincial Multi-specialized Oncology and Trauma Center, Łódź, Poland

Introduction: Mixed-phenotype acute leukemia (MPAL) is a rare disease with poor prognosis. So far, no standard approach has been established as the "know-how" of MPAL is based only on retrospective analyses performed on small groups of patients.

Materials and methods: In this study, a retrospective analysis of the outcomes of adult MPAL patients included in the PALG registry between 2005 and 2024 who received the CLAG-M hybrid protocol as induction or salvage therapy was performed.

Results: Sixteen of 98 MPAL patients received CLAG-M: eight as first-line and eight as salvage therapy. In the first line, two patients achieved partial response (PR), and six achieved complete remission (CR), of whom four successfully

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underwent allogeneic hematopoietic stem cell transplantation (alloHSCT). Two patients who did not undergo alloHSCT promptly relapsed. Within the whole group, the overall response rate (ORR) was 75% (n = 12/16). With the median follow-up of 13 months, six out of eight patients remain in CR, however, two of them died due to acute graft versus host disease. Out of eight patients who received CLAG-M in the second line, four patients (50%) obtained CR. AlloHSCT was conducted in seven cases, six of which were in CR. Only two patients remained in CR at the time of the last follow-up. Tolerance to treatment was good. The median times for severe neutropenia and thrombocytopenia were 22 days (range, 16–24) and 17 days (range, 12–24), respectively. Overall, grade 3-4 infections were observed in 12 cases, and all infections presented successful outcomes.

Conclusions: CLAG-M is an effective first-line salvage regimen for MPAL with an acceptable safety profile. Early achievement of CR with prompt alloHSCT allows for satisfactory disease control.

KEYWORDS

mixed phenotype acute leukemia, ambiguous leukemia, induction treatment, MPAL, hybrid regimen, methylome targeted therapy

Introduction

Mixed phenotype acute leukemia (MPAL) is a rare disease, representing 2%–5% of acute leukemias (1–3). Contrary to the established lineage-specific antigen expression in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), MPAL is characterized by blast cells co-expressing lymphoid and myeloid lineage antigens. Due to the heterogeneity of this rare disease and the fact that patients with MPAL are usually excluded from acute leukemia clinical trials, no unified treatment protocol has been established. According to retrospective studies and case reports, the outcomes of therapy based on an ALL-like regimen seem to prevail over those of therapy with an AML-like or hybrid regimen combining both approaches (1, 2, 4–8). Furthermore, the united front is maintained for allogeneic stem cell transplantation (alloHSCT), as outcomes in patients who underwent the procedure are superior to those who received only chemotherapy (3, 4, 9–11).

However, in light of reports implying the possibility of lineage switch (12) and favorable response if treatment matching the DNA methylation patterns of blast cells (13) is applied, the discussion about the most appropriate induction regimen remains doubtful. Uncertainty about introducing therapy based on immunophenotype, cytogenetic, or molecular biology has led to the consideration of a hybrid protocol combining both AML-like and ALL-like regimens. Notwithstanding the studies strongly contradicting that approach (2, 7), some other reports present the benefits of the hybrid regimen (3, 14–16). In accordance with these findings, we retrospectively analyzed MPAL patients who received the hybrid protocol based on cladribine, cytarabine, granulocyte colony-stimulating factor (G-CSF), and mitoxantrone (CLAG-M). Based on the significant research of the Polish Adult Leukemia Group (PALG), which revealed that CLAG-M is a well-tolerated and effective salvage regimen in refractory or relapsed AML (17), we assessed the advantages and safety profile of this treatment protocol in poor-risk MPAL. Furthermore, the prominent effect of cladribine included in treatment protocols for AML, especially in patients with unfavorable cytogenetics and FLT3-ITD mutations, has been proven in previous PALG studies (18–20), which enhanced the advantage of CLAG-M.

Finally, we present a comprehensive analysis of the treatment course and its outcome in MPAL patients treated with the CLAG-M protocol as the first line of induction and salvage therapy.

Materials and methods

The present study is the outcome of the close cooperation of hematological centers associated with the Polish Adult Leukemia Group, which reported MPAL patients diagnosed and treated with regimens according to the centers' individual experiences between 2005 and 2024. Of all reported cases, we selected and retrospectively analyzed the group of patients treated with the CLAG-M regimen in the first-line (Group A) of induction and as the salvage protocol (Group B) in case of refractoriness or relapse after the first-line of therapy.

The comprehensive database included information about the patients' state characterized by age, sex, Eastern Cooperative Oncology Group (ECOG) Performance Status Scale, and presence

of hepatosplenomegaly, lymphadenopathy, or central nervous system (CNS) infiltration by leukemia cells. In addition, precise data regarding diagnosis and treatment were collected. The diagnosis was based on blast immunophenotyping according to The European Group for the Immunological Characterization of Leukemias (EGIL) (21)and criteria included in the World Health Organization (WHO) classification and verified according to the WHO 2022 classification (22).

All patients were administered the standard CLAG-M regimen in both induction and salvage protocols, including cladribine at a dose of 5 mg/m^2 intravenously (iv.) on days 1–5; cytarabine (Ara-C) at a dose of 2,000 mg/m^2 iv.; on days 1, 2, 3, and 5; granulocyte-colony stimulating factor (G-CSF) 30 MU subcutaneously (sc.) on days 0, 1, 2, 3, 4, and 5, and mitoxantrone at a dose of 10 mg/m^2 on days 1, 2, and 3 (17). Furthermore, consolidation treatment with a high dose of cytarabine (3,000 mg/m^2 iv. Every 12 h on days 1, 3, and 5) and intrathecal prophylaxis or treatment with methotrexate (15 mg), cytarabine (40 mg), and dexamethasone(4 mg) were considered. For consolidation treatment, Ara-C was administered at a dosage adjusted for patient age. Patients under 60 years old received high-dose Ara-C (2 g/m^2–3 g/m^2 every 12 h i.v. on days 1, 3, and 5), whereas patients above 60 years old received intermediate-dose Ara-C (1 g/m^2–1.5 g/m^2 every 12 h i.v. on days 1, 3, and 5). Eventually, all eligible patients were scheduled to undergo alloHSCT. Furthermore, according to the results of cytogenetic analysis, imatinib was included in the treatment of two cases with BCR::ABL rearrangement.

Regarding the high risk of infectious complications associated with intensive treatment, prophylaxis was implemented on the last day of the protocol. The patients were administered levofloxacin (500 mg orally every 24 h), acyclovir (800 orally mg every 12 h), and posaconazole (200 mg orally every 8 h).

Treatment response was assessed according to the ELN criteria (23). To evaluate minimal residual disease (MRD) multiparameter flow cytometry was performed, and quantitative RT-PCR was employed for the t (9, 22) MPAL type.

Furthermore, we analyzed the safety profile of the CLAG-M regimen in first-line of treatment by examining the time to neutrophil and platelet recovery and infectious and non-infectious complications at the time of therapy. Considering the reported prolonged hematological recovery following CLAG-M administration and the consequent heightened risk of infectious complications, two distinct time frames were evaluated for hematological recovery (24). First, the durations of severe neutropenia and thrombocytopenia were evaluated. It was defined as the time from the date of induction implementation to the date of the first stable absolute neutrophil count (ANC) above 500/μL and platelet count above 50,000/μL. Second, the criteria for complete hematological recovery time to ANC >1,000 μL and platelet count >1,000,000 μL were calculated.

Overall survival (OS) was assessed from the date of diagnosis in group A and from CLAG-M administration in group B to the patient's death or the last follow-up. Eventually, we incorporated the overall response ratio (ORR), defined as the percentage of patients who responded to a CLAG-M regimen with a partial or better response.

Event rate analysis

First, to evaluate survival, we incorporated Kaplan–Meier analysis. However, due to the small group size and short follow-up time, a specific parametric Weibull model was used to determine whether the risk ratio for each endpoint was constant over time. In this model, the survival function is given as $S(t) = e^{-(t/\eta)^{\beta}}$, $t \geq 0$; η, $\beta > 0$; therefore, the mortality rate is thepower function $h(t) = \eta^{-\beta}\beta t^{\beta-1}$. These parameters were estimated using the Maximum Likelihood Method (MLE). The Weibull plot was used to determine whether the dataset followed the Weibull distribution. If $0 < \beta < 1$, we are in the infant mortality phase; i.e., the mortality rate is decreasing. For $\beta = 1$, we deal with random causes of mortality (constant rate), while for $\beta > 1$, we deal with aging and increasing rates. The Weibull shape parameter was estimated using 90% confidence intervals (CIs). Due to the small sample size in both groups, we developed a model based on Bayesian methods combined with profile likelihood to increase the statistical power (see Appendix). Using Monte Carlo Markov Chain methods, the estimator values and 90% credibility intervals (CrIs) of the shape parameter were determined. To check the similarity between two posterior distributions of beta, we calculated the overlapping index (OI) (25). All calculations were made using R version 4.2.1 in RStudio 2023.06.0. To estimate the parameters, we used the packages WeibullR, surv, survival, flexsurv, and overlapping.

Results

The characteristics of patients

Within the presented period, 16 of 98 MPAL patients were treated with the CLAG-M protocol. In eight cases, the CLAG-M regimen was used as the first induction (group A) and also in eight cases as salvage treatment (group B). In group B, CLAG-M was administered after ALL-like or AML-like induction therapy, in three and five cases, respectively. In all cases, there was no response to first-line treatment and all patients were diagnosed with refractory disease.

Half of the patients (n = 8/16, 50%) were diagnosed with B/myeloid type MPAL, six patients in group A and two patients in group B. T/myeloid type MPAL was more frequent in group B, as diagnosed in four patients. One case of MPAL t (9, 22) was reported in groups A and B. Additionally, in group B, B/T/myeloid type MPAL was reported in one case. The median age at diagnosis was 44 years (range, 21–64 years). Nevertheless, the patients in group B were younger, with a median age of 34. The entire research group was characterized by equal numbers of women and men and good performance status in all patients, with a median ECOG score of 1 (range, 0–2). Lymphadenopathy occurred in 43,75% (n = 7/16) of patients and was the most common extramedullary involvement. At the time of diagnosis, each patient underwent lumbar puncture and optional magnetic resonance imaging (MRI) for signs of a cerebral mass. Eventually, one patient was diagnosed with blast cells in the cerebrospinal fluid (CSF) as the only case of central nervous system

involvement. Intrathecal chemotherapy was administered twice a week until no blast cells were detected in the CSF, and then twice per consolidation cycle. As for the diagnosis of recurrence, each patient also underwent lumbar puncture and optional MRI, but no CNS involvement was diagnosed.

Half of the patients (n = 8/16, 50%) presented with cytogenetic aberrations; however, karyotype data were missing in three cases in Group B. Complex karyotypes were detected in four cases (n = 4/16, 25%), whereas the other four had single chromosomal aberrations. One case of *BCR-ABL* rearrangement was detected in each group. As far as gene mutations are considered, *RUNX1* and *FLT3-ITD* mutations were most frequent. However, *FLT3-ITD* mutations were only found in Group B. A detailed characterization of the patients is presented in Table 1.

The safety of treatment

The patients presented with severe neutropenia for a median of 22 days (range, 16–24 days) in Group A and 24 days (range, 18–27 days) in Group B, and severe thrombocytopenia for a median of 17 days (range, 12–24 days) in Group A and 20 days (range, 18–25 days) in Group. B. Regarding complete hematological recovery, half of the patients in Group A met the criteria. Of the eight patients in this group, seven (87.5%) had platelet counts > 100,000/μL, with a median thrombocytopenia duration of 25 days (range, 19–44). Additionally, four patients (50%) had ANC levels >1,000/μL, with a median time to neutrophil recovery of 25.5 days (range, 23–28 days).

Regarding infectious complications, the incidence and classification according to the Common Terminology Criteria for Adverse Events (CTCAE version 5.0) were similar in both groups: six (75%) cases of grade 3–4 infection in each group. In all cases, the epidemiological factor was the bacterium, and all had successful outcomes. All infections were reported within an ANC >500/μL. No noninfectious complications were observed. Finally, neither admission to the intensive care unit nor death during induction treatment was observed in either group.

The results of treatment with CLAG-M

Six of eight patients (75%) treated with CLAG-M as the first line of induction achieved complete remission (CR); however, three of them had partial hematologic recovery (CR_h). The remaining two patients were evaluated for partial response (PR). Additionally, 67% (n = 4/6) of CR patients had negative MRD. The outcome of CLAG-M as salvage

TABLE 1 The characteristics of patients.

Number	Sex	Age	MPAL type (WHO 2022)	Blasts in BM smear (%)	Karyotype	Genetic mutations
Patients who received CLAG-M in the first line of induction						
1.	Female	51	MPAL t(9;22)	92.5	46 XX, t(9;22) [85%]	None
2.	Male	64	B/Myeloid	83	46, XY, t(8;21) del(9)/ 45, XY-, t(8;21), del(9)	*RUNX1*
3.	Male	37	B/Myeloid	73	46, XY	None
4.	Male	56	B/Myeloid	56	47, XY +8, t(8;21), del 11	*RUNX1*
5.	Male	59	T/Myeloid	48	42~48, XY,del(9)(p21)[5],+13[4],+mar1[6],+mar1x2[2],+mar2[8],+mar3[5] [cp13]/46,XY[7]	*CEBPA*
6.	Female	43	B/Myeloid	50.7	46, XY	None
7.	Female	35	B/Myeloid	78	46,XX,del(1)(p22p11),del(2)(q33)[10],add(3)(q23)[10],der(9)del(9)(p22)add (9)(q22),add(16)(p13.3)[5],-17,der(18)t(17;18)(q11.1;q12.2),+22[4],+mar [5][20]	None
8.	Female	63	B/Myeloid	76	46, XX, t (3;8)/46, XX	None
Patients who received CLAG-M as salvage treatment						
1.	Female	28	B/T/Myeloid	81	46, XY, del13(q12.31-33)	*RUNX1*
2.	Female	33	T/Myeloid	90	No data	No data
3.	Male	63	T/Myeloid	86.5	No data	No data
4.	Male	21	B/Myeloid	68	46, XX	None
5.	Female	36	T/Myeloid	76	No data	*FLT3-ITD*
6.	Male	45	MPAL t(9;22)	89	No metaphases	None
7.	Female	31	T/Myeloid	90	47, XY+5, t(6;14)(p21,q32)[9]	*FLT3-ITD*
8.	Male	63	B/Myeloid	56.4	46, XX [20]	*FLT3-ITD*

BM, bone marrow.

treatment was less prominent, as only 50% (n = 4/8) of the patients achieved CR. Unfortunately, data on MRD in Group B are lacking. Finally, regarding CLAG-M administration, the ORR were 75% (n = 12/16), 100% in Group A and 50% in Group B (n = 4/8).

Regarding further treatment, in Group A, patients in CR and CR_h were prioritized for alloHSCT after a consolidation cycle with a high or intermediate dose of cytarabine, depending on whether the patient was under or over 60 years old, respectively. Two patients in PR received the therapy based on the best physician experience; in one case, another cycle of CLAG-M and hyperCVAD in another case. At the observation endpoint, both were evaluated for CR2 with complete hematological recovery, MRD negative and positive, respectively.

In Group A all patients were considered eligible for alloHSCT, and successful qualification was performed in seven cases (87.5%) (five in CR1 and two in CR2), as one patient did not consent to the procedure. When the study endpoint was reached, the procedure was performed in four patients who remained in CR1 before allotransplantation. In all cases, the conditioning regimen before alloHSCT was preferably based on a combination of chemotherapy and radiotherapy. One patient in CR1 eventually did not undergo alloHSCT due to the lack of a matching donor. Two patients in CR2 awaited allo-HSCT, as the matching donor was confirmed. Two patients, who did not receive allografts promptly after consolidation, relapsed. They qualified for salvage treatment with azacytidine and venetoclax, with no response. Eventually, both patients died due to relapse and refractory disease. Two more deaths were reported in Group A, both due to complications of acute graft versus host disease (aGvHD).

In Group B, allo-HSCT was performed in seven cases (87.5%), including five with CR and two with refractory disease. The conditioning regimen prior to alloHSCT was similar to that Group A and was based on a combination of chemotherapy and radiotherapy. In Group B, six patients (75%) died at the time of follow-up. In four of them, the cause was the underlying disease: primary resistance and relapse after alloHSCT, each in two cases. The other two patients died of infections.

After a median follow-up time of 13 months (3–131), 10 of 16 (63%) patients died, six due to relapse or refractory disease, four from infection or complications after alloHSCT. In Group A, regardless of deaths due to GvHD complications, six out of eight (75%) patients remained in CR. The median OS was 9 months (range, 3–131 months). In Group B, two patients remained in CR after alloHSCT, with an OS of 11 and 23 months. The median OS was 21.5 months (range, 3–28 months). Details about the treatment outcomes in Groups A and B are presented in Tables 2, 3.

Survival analysis

Survival assessment using the Kaplan–Meier plot is presented in Figure 1. Regarding the outcomes of the individually created statistical model, we compared the shape factor, β. Probability plots with 90% Cis are presented in Figures 2, 3 for Groups A and B. Note that all the points are within the confidence bounds. The numerical results are listed in Table 4.

In both groups, the null hypothesis cannot be rejected. The following results were obtained.

In Group A: $\beta = 0.7078$, CrI = (0.3182; 1.1756), while in Group B: $\beta = 1.2103$, CI = (0.5703; 1.9913).

In Group B, β indicates that we deal with an increasing death rate (since $\beta > 1$). However, in Group A estimation of β indicates that the death rate is decreasing (since $\beta < 1$). The overlapping index (OI) is also calculated. Overlapping can be used to assess the posterior distribution of a Bayesian model. In our case OI = 0.55. This results in a 45% difference between the posterior distributions of the shape parameters.

Discussion

The presented overview analyses of a homogenous group of adults, whose age is predominantly over their 40s. Regardless of age, sex, and MPAL type (B/mielo, T/mielo, or others), 75% of CR was accomplished with CLAG-M in the first-line of induction accompanied by low non-hematological toxicity and no prolongation of severe neutropenia or severe thrombocytopenia. As far as meta-analysis and most treatment guidelines preferably recommend ALL regimens for MPAL treatment (1, 2, 4–8), selective treatment may lead to clonal expansion of blasts resistant to initial lineage-based chemotherapy. The substantiation of these doubts could be found in the recommendations for administering an AML-based regimen if no response to ALL-based treatment was observed (4, 26). Furthermore, MPAL-like phenomena of lineage switches cannot be neglected. The hypothesis presented by Hu et al. implies that leukemic clones involved in lineage switching may be derived from multipotent hematopoietic cells (27). Moreover, the pressure of ALL-like treatment has been reported to be a potential cause of lineage switching. According to published data, CD19 targeted therapy of B-ALL with blinatumomab or αCD19 CAR-T cells may lead to myeloid switch (28–30), and B-cell precursor ALL is prone to myeloid switch under standard intensive ALL-like treatment (31, 32). These phenomena were additionally associated with alternations in transcription factors, such as Pu1 and Pax5, which have also been described as potential causes of lineage switch (33). Considering these findings, hybrid protocols may be a solution to prevent lineage switching.

Nevertheless, previously reported hybrid protocols combining regimens from both ALL and AML protocols are too toxic (2, 7). Therefore, we opted for a CLAG-M regimen characterized by less toxicity and a more multidirectional profile of effectiveness that may overcome the challenges arising from the complex pathogenesis of MPAL without the potential selection of any subclones. To date, the CLAG-M protocol has been widely reported to be beneficial in AML with poor prognosis, but has also proven to be well-tolerated (17, 34–36).

In terms of multidirectional activity, agents in the CLAG-M regimen have significant cytotoxic effects on both myeloid and lymphoid lineages. Their administration is an effective approach in high-risk AML (37) and relapsed or refractory ALL (38), whereas cladribine is commonly administered for the treatment of

TABLE 2 The treatment outcome in patients treated with CLAG-M in the first line of induction.

Patient number	Induction	Treatment response	MRD status	Consolidation	CNS pro-phylaxis with i.th.ch.	AlloHSCT	Relapse	Relapse or refractoriness therapy	Death	Response state at the time of FU	OS (months)
1.	CLAG-M + imatinib	CR	Negative	HD-AraC	Yes	Yes	No	No	No	CR	131
2.	CLAG-M	CR_h	Positive	ID-AraC	Yes	Yes	No	No	No	CR	15
3.	CLAG-M	CR_h	Negative	HD-AraC	Yes	Yes	No	No	Yes, due to bleeding in course of GvHD	CR	8
4.	CLAG-M	CR_h	Negative	HD-AraC	Yes	Yes	No	No	Yes, due to sepsis in course of GvHD	CR	9
5.	CLAG-M	CR	Negative	HD-AraC	No	Not done due to the lack of patient's consent	Yes	AZA+VEN	Yes, due to relapse	Relapsed disease	10
6.	CLAG-M	CR	Positive	HD-AraC	No	Not done due to donor-matching failure	Yes	AZA+VEN	Yes, due to relapse	Relapsed disease	6
7.	CLAG-M	PR (from 78% to 6% of blasts)	Not applicable	Not applicable	Yes	Will be performed after reinduction with CLAG-M	No	CLAG-M	No	CR, MRD (-)	3
8.	CLAG-M	PR (from 76% to 23% of blasts)	Not applicable	Not applicable	Yes	Will be performed after reinduction with hyperCVAD	No	HyperCVAD	No	CR, MRD (+)	5

CLAG-M, cladribine, cytarabine, granulocyte colony-stimulating factor; CR, complete response; MRD, minimal residual disease; CNS, central nervous system; i.th.ch, intrathecal chemotherapy; alloHSCT, allogeneic hematopoietic stem cell transplantation; OS, overall survival; CR, complete response; HD-AraC, high dose cytarabine; AZA + VEN, azacytidine + venetoclax; GvHD, graft versus host disease.

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TABLE 3 The treatment outcome of patients treated with CLAG-M in refractory/relapsed MPAL.

Patient number	1st line regimen	Response to 1st line treatment	2nd line regimen	Response to 2nd line treatment	3rd line regimen	Response to 3rd line treatment	alloHSCT	Relapse after alloHSCT	Death	Response state at the time of FU	OS (months)
1.	PALG ALL7 Ph (−)<55 y.o.	Refractoriness	CLAG-M	CR	No	–	Yes	Yes	Yes, due to infection	CR after second alloHSC	28
2.	DAC	Refractoriness	CLAG-M	CR	No	–	Yes	Yes	Yes, due to relapse	Relapsed disease	23
3.	mini FLAM	Refractoriness	CLAG-M	CR	No	–	Yes	Yes	Yes, due to infection	PR after FLAG-IDA therapy	23
4.	DA (2 + 5)	Refractoriness	CLAG-M	Refractoriness	No	–	No	Not applied	Yes, due to refractory disease	Refractory disease	2
5.	DA + midostaurin	Refractoriness	CLAG-M	Refractoriness	HyperCVAD	CR	Yes	No	No	CR	11
6.	PALG ALL7 Ph (-)< 55 y.o. + imatinib	Relapse	CLAG-M + dasatinib	CR	No	–	Yes	No	No	CR	23
7.	DA	Refractoriness	CLAG-M	Refractoriness	No	–	Yes in active disease	Progression	Yes, due to refractory disease	Refractory disease	9
8.	DA + midostaurin	Refractoriness	CLAG-M	Refractoriness	VEN+AZA	CR	Yes	Yes	Yes, due to relapse	Relapsed disease	20

PALG ALL7 Ph (−)<55 y.o. – treatment protocol by Polish Adult Leukemia Group in acute lymphoblastic leukemia chromosome Philadelphia negative: dexamethasone, vincristine, daunorubicin, pegaspargase; DAC, treatment protocol by Polish Adult Leukemia Group: daunorubicin, cytarabine, cladribine; miniFLAM, fludarabine, cytarabine, mitoxantrone (reduced intensity); DA, daunorubicin, cytarabine; CLAG-M, cladribine, cytarabine, granulocyte colony-stimulating factor, mitoxantrone; hyperCVAD, cyclophosphamide, vincristine, doxorubicin, dexamethasone; VEN + AZA, venetoclax, azacytidine; alloHSCT, allogeneic hematopoietic stem cell transplantation; CR, complete response; OS, overall survival.

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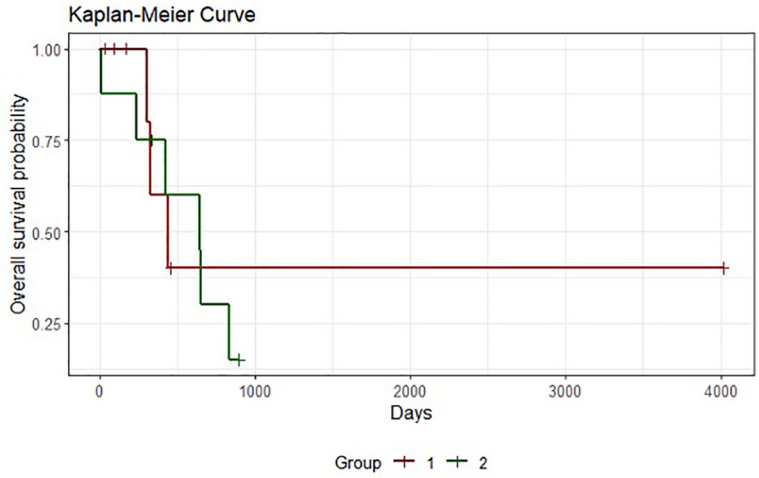

FIGURE 1
Comparison of survival in Groups (A, B). Kaplan–Meier plot.

lymphoma. Another compound in the CLAG-M regimen, mitoxantrone, may be used in both AML and ALL treatment protocols. Mitoxantrone is a DNA-damaging agent whose association with Ara-C and cladribine results in the synergistic inhibition of DNA repair mechanisms (17, 39). The addition of G-CSF potentiates Ara-C sensitivity, especially in cells with low proliferative activity, thereby enhancing treatment response (40). Cladribine increases the cellular uptake of Ara-C and potentiates its intracellular metabolism, thereby intensifying the cytostatic effect (41–43). Furthermore, cladribine actively inhibits DNA synthesis by incorporating it into DNA strands and directly damaging the mitochondrial membrane, leading to cell apoptosis (44).

Cladribine has also been reported to have hypomethylating activity (45), which was proven in previous studies by Libura et al. (46). Patients diagnosed with AML and coexisting *IDH1/2* mutations leading to DNA hypermethylation and epigenetic dysregulation had more successful outcomes when cladribine was applied in the induction protocol. Based on the favorable results of the aforementioned study, cladribine may be characterized as a crucial agent of the regimen, especially since the investigation by Alexander et al. (47) reported a significant contribution of the methylation profile in MPAL pathogenesis. Furthermore, according to Takahashi et al., achieving a complete response in MPAL is more likely if AML-like or ALL-like regimens are administrated according to the methylation profile presented by blasts (13). Since methylome examination is not a standard diagnostic procedure, it could be challenging to use it as an eligibility criterion for treatment. Thus, applying the general hypomethylating agent, cladribine, may restrict methylation changes in lineage-defining transcription factor genes responsible for mixed immunophenotype presentation (13). Eventually, its general effects may limit lineage-specific clone selection and resistance to therapy.

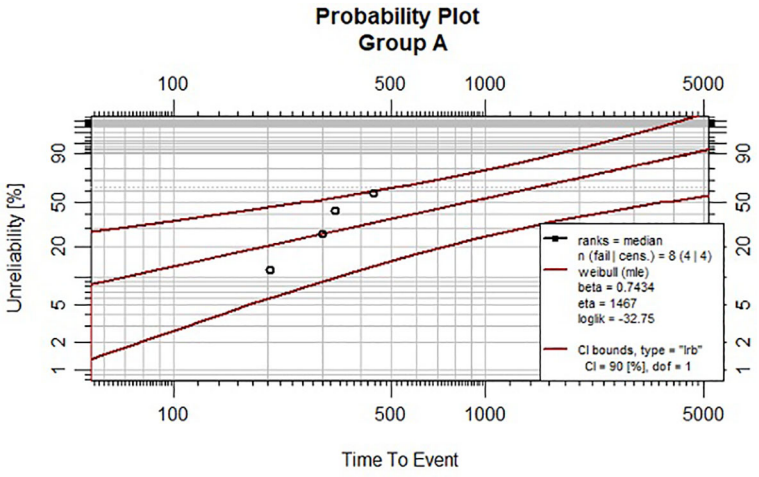

FIGURE 2
Weibull plot with 90% CIs for Group (A).

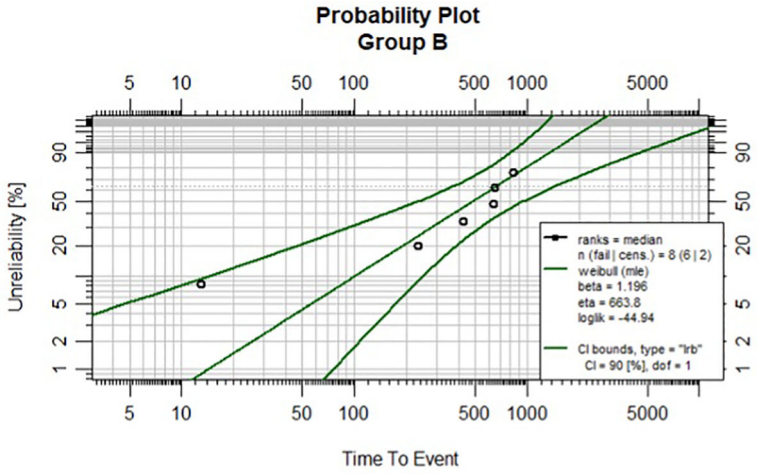

FIGURE 3
Weibull plot with 90% CIs for Group (B).

Nevertheless, decisions regarding further treatment in patients who achieve PR remain a challenging clinical problem. So far, the literature and physicians' experiences suggest that the next line of treatment should be switched to a regimen specified for another lineage than the previous one (48–50). Such a procedure can be justified if lineage-specific treatment is administered; however, in the first-line treatment, we recommend a hybrid regimen with a broader cytotoxic effect involving both lineages. Thus, in light of insufficient literature data on the management of PR patients who have received hybrid protocols, we advocate CLAG-M as a reinduction.

Notwithstanding the broad cytostatic effect of CLAG-M on leukemia cells, the impact of some mutations on the course of the disease, and thus treatment, cannot be neglected. Interestingly, cladribine was reported to overcome the negative effect of *FLT3-ITD* mutation and improve treatment response in patients diagnosed with AML *FLT3-ITD* positive (18). A similar effect is believed to be observed in patients with MPAL *FLT3-ITD* positivity, which implies an additional advantage of the CLAG-M protocol. Nevertheless, the administration of tyrosine kinase inhibitors (TKI) such as imatinib and dasatinib in cases of MPAL with BCR-ABL

TABLE 4 The numerical results of Weibull model for group A and B.

Estimates:	Group A			
	Est	L90%	U90%	se
shape	0.743	0.416	1.326	0.262
scale	1467.49	482.02	4472.71	994.33
N = 8	Events: 4		Censored: 4	
Total time at risk: 5877 (days)			AIC = 69.49478	
Log-likelihood = -32.7474			df = 2	
Estimates:	Group B			
	Est	L90%	U90%	se
shape	1.196	0.653	2.194	0.629
scale	663.84	378.69	1164.32	226.70
N = 8	Events: 6		Censored: 2	
Total time at risk: 4028 (days)			AIC = 93.88921	
Log-likelihood = -44.9446			df = 2	

rearrangement, has a significant impact on improving the prognosis of patients' lifespans (4, 51).

In this analysis, we proved that no prolongation of severe cytopenia was observed in either research group. Although partial hematological recovery was reported in half of the CR patients in Group A, all infectious complications occurred during the period of severe cytopenia and were successfully treated with empirical and targeted antibiotic therapy; thus, no admission to the intensive care unit was needed. All patients were able to continue further treatment safely without significant interruptions due to significant hematological toxicity of the protocol or prolonged serious infections. The tolerance of CLAG-M in the second-line treatment in patients who already received intensive regimens was similar to that in the first-line treatment. However, infectious prophylaxis and strict clinical supervision are necessary because most patients suffer from infectious complications. Targeted infectious therapy is likely to result in a successful outcome, even in elderly patients, as proven in our study.

Discussions about MPAL treatment, including alloHSCT, must be addressed. The outcomes of retrospective studies have demonstrated the beneficial role of alloHSCT. In a report by Heesch et al., the 5-year survival rate of patients with MPAL who underwent the transplant procedure was 70% compared to 19% for those who received only chemotherapy (11). Favorable results of alloHSCT in MPAL were also demonstrated by Munker et al. (3-years OS 56.3%) (9), Shimizu et al. (5-years OS 48%) (51), and Liu et al. (3-years OS 45%) (10). It is worth mentioning that MRD-negative CR achieved by CLAG-M treatment significantly increased the chances of success of the procedure and preserved reasonable disease control. CR obtained with CLAG-M in the first line of induction in most patients allowed four of them to receive allograft quickly and consolidate the treatment response. Nevertheless, deferring alloHSCT for a disease with such a high risk and unfavorable prognosis as MPAL can lead to rapid relapse, as revealed by the case of two patients in Group A. Nowadays, improvements in alloHSCT methodologies and post-transplant care broads patients eligible for allotransplantation, and age and comorbidities no longer present strict limitations (52). Taking this advantage, MPAL patients should be widely qualified for alloHSCT as the greatest change for good disease control and prolonged survival.

On the other hand, regarding alloHSCT outcomes in patients who received CLAG-M as salvage therapy, complete response was maintained in two cases. It is clear that, although statistically irrelevant in clinical practice, patients who undergo alloHSCT in CR1 are more likely to remain in remission. These data contradict the report of Munker et al., who demonstrated no difference in outcomes of alloHSCT between MPAL patients who underwent the procedure in CR1 or CR2. However, these results were not statistically relevant (9). However, further studies are required. Nevertheless, our study is illustrated comparatively to the general analysis of OS after alloHSCT in CR1, CR2, and no response by Bolo et al. (53).

The limitation of this small research group required the development of an individual mathematical model to assess the significance of the impact of CLAG-M on MAPL patient survival. In Group B, the mortality increasing tendency remained unaffected, whereas the death rate was reduced in Group A, which received CLAG-M as the first line, thereby achieving mostly CR. Thus, the differential factor between the two groups can be indicated as an effective intensive regimen with a broad cytotoxic action in induction therapy, which allows for a good response and prompt alloHSCT. Ultimately, the CLAG-M regimen as the first-line treatment may offer a significant opportunity for consolidation treatment and lifespan prolongation. Considering the limitations of this research, we hope for international multicenter cooperation to revise the prepared mathematical model and establish an opportune therapeutic protocol for patients with this rare disease with an adverse prognosis.

Conclusions

Following our findings, we opted for intensive yet acceptable safety profile induction with the hybrid protocol of CLAG-M, which, with prompt alloHSCT, allows for reasonable disease control. All patients included in the study were able to receive CR in the first line of induction and proceeded to alloHSCT with no deaths or serious complications. The protocol is intensive but still well-tolerated, and in the case of a disease with such complex pathogenesis as MPAL, treatment with a broad profile of cytostatic action such as the CLAG-M regimen can improve the chances of achieving CR. Undoubtedly, our study is limited by the group size, comparability, well-documented nature, and short follow-up time. Nevertheless, this study sheds light on possible approaches to MAPL treatment. Further studies on the mechanisms underlying MPAL transformation and ambiguous phenotypes are crucial for a better understanding of the course of the disease and possible targeted therapy. However, until then, CLAG-M protocols will be a promising treatment scheme for MPAL patients to improve their therapy results.

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

The studies involving humans were approved by the local Bioethics Committee of Wroclaw Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

MK: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review & editing. AA:

Investigation, Writing – review & editing. MSk: Data curation, Formal analysis, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. ŁB: Investigation, Writing – review & editing. KB: Investigation, Writing – review & editing. JD-S: Investigation, Validation, Writing – review & editing. EZ: Investigation, Writing – review & editing. PM-G: Investigation, Writing – review & editing. MG: Investigation, Writing – review & editing. JH: Investigation, Writing – review & editing. AK: Investigation, Writing – review & editing. WP: Investigation, Writing – review & editing. OC: Investigation, Writing – review & editing. TW: Supervision, Validation, Writing – review & editing. AW: Methodology, Supervision, Validation, Writing – review & editing. MSo: Methodology, Project administration, Supervision, Validation, Writing – review & editing.

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EDITED BY
Luca Vincenzo Cappelli,
Sapienza University of Rome, Italy

REVIEWED BY
Anna M. Eiring,
Texas Tech University Health Sciences Center El Paso, United States
Ramzi Abboud,
Washington University in St. Louis, United States

*CORRESPONDENCE
Xueya Zhang
jakey3456@sina.com

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Case report: Combination therapy with selinexor, decitabine and half-dose CAG regimen for relapsed elderly acute myeloid leukemia

Xueya Zhang*, Yuqi Sun and Jinfa Zhong

Department of Hematology, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian, China

The treatment of elderly patients diagnosed with acute myeloid leukemia (AML) poses significant challenges. Currently, one promising strategy in therapeutic interventions for geriatric individuals revolves around the utilization of small molecule targeted drugs either independently or in conjunction with demethylating agents. In this report, we present the successful attainment of complete remission in an elderly female patient with relapsed AML, the patient underwent treatment with a combination of Selinexor, decitabine, and a half-dose CAG regimen for two cycles. Subsequently, the patient has sustained this remission through consolidation therapy involving a medium dose of Ara-c. This therapeutic regimen has demonstrated favorable outcomes in the management of relapsed AML in elderly individuals. Furthermore, the adverse reactions were manageable. In order to devise an efficacious treatment regimen for elderly patients suffering from relapsed and refractory acute myeloid leukemia, it is imperative to incorporate a larger cohort of cases for clinical investigation.

KEYWORDS

elderly acute myeloid leukemia, relapsed, selinexor, decitabine, half-dose CAG regimen

Introduction

The treatment of elderly patients diagnosed with acute myeloid leukemia (AML) poses significant challenges for hematologists due to the presence of strong heterogeneity, poor performance status, multiple comorbidities, and adverse prognostic factors (1). However, in recent years, the introduction and utilization of diverse novel drugs have led to substantial advancements in the management of AML in the elderly population. According to data analysis conducted by the MD Anderson Cancer Center (2), the response rate to intensive chemotherapy in elderly patients with AML is estimated to be between 40% and 50%. However, these patients face a high early mortality rate of 26% to

36% within 1 to 2 months, and their median survival is limited to only 4 to 6 months. Furthermore, the overall one-year survival rate for elderly AML patients is less than 30%, which is significantly lower compared to the response rate of 70% to 80% and the long-term survival rate of 40% to 50% observed in younger patients.

Currently, one promising strategy in therapeutic interventions for geriatric individuals revolves around the utilization of small molecule targeted drugs either independently or in conjunction with demethylating agents. In the case of elderly patients experiencing relapsed and refractory AML, it is advisable to consider participation in clinical trials, explore novel drug therapies, and implement optimal supportive treatment approaches (1, 2). In this report, we present the successful attainment of complete remission in an elderly female patient with relapsed AML, achieved through the administration of a half-dose CAG regimen in combination with selinexor and decitabine.

Case presentation

On December 16, 2022, a 71-year-old female patient was admitted to our hospital presenting with abdominal distension lasting for a duration of one week. The patient's medical history did not reveal any other concurrent illnesses. The complete blood count analysis revealed a white blood cell count (WBC) of 1.7×10^9/L, a hemoglobin level of 94 g/L, and a platelet count of 43×10^9/L. Subsequent bone marrow aspiration revealed AML with 58.5% myeloblasts. Flow cytometry analysis demonstrated that blast cells accounted for 48.1% of the sample, exhibiting positive expression of CD13, CD33, CD34, CD38dim, CD105, CD117, HLA-DR, CD45, and MPO. Additionally, the next-generation sequencing results indicated the presence of IDH2 (c.419G>A p.R140Q), SH2B3 (c.527_528del p.V176Afs*6), ABL1 (c.1826_1828del p.K609del), DDX41 (c.1574G>A p.R525H), KMT2C (c.1173C>A p.C391*), and GNAS (c.608T>C p.L203P) gene mutations. The conventional cytogenetic analysis revealed a karyotype of 46, XX (20). A treatment regimen consisting of azacitidine (AZA: 119mg on days 1-5) and venetoclax (VEN: 20mg on day 1, 40mg on day 2, 80mg on day 3, and 100mg on days 4-28) and voriconazole prophylaxis was given with one cycle. Subsequent bone marrow analysis indicated a 5% of residual blast cells, suggesting complete remission with incomplete hematological recovery (CRi). On April 14, 2023, bone marrow aspiration revealed that approximately 4% of the cells were blasts, while immature monocytes accounted for approximately 1%. Additionally, flow cytometry immunophenotyping identified 3.6% of myeloid progenitor cells with abnormal phenotypes. Following this, on April 14, 2023, AZA+VEN targeted therapy was administered, consisting of AZA 120mg subcutaneous injection on days 1-5 and VEN 100mg oral administration on days 1-28, in combination with antifungal prophylaxis with voriconazole 200mg oral administration every 12 hours. A subsequent review of bone marrow aspiration on May 25, 2023, indicated that blast cells accounted for 8.5% of the total cell population. Flow cytometry analysis revealed the presence of 5.1% abnormal myeloid progenitor cells, indicating an early recurrence after achieving CRi. Consequently, the patient was initiated on the CAG regimen chemotherapy on June 8, 2023. This regimen included the administration of 20mg intravenous bolus injection daily for four days of aclarubicin, 16mg subcutaneous injection every 12 hours for 14 days of cytarabine, and 300ug subcutaneous injection for 14 days of granulocyte colony-stimulating factor (G-CSF). A subsequent review of the bone marrow on July 7, 2023, revealed the presence of 8% blast cells. Considering the patient's unsatisfactory response to first line chemotherapy, we decided to adopt the XPO1 inhibitor selinexor given its promising activity (see Discussion) in combination with decitabine and half dose CAG regimen on July 11, 2023. This consisted of decitabine 25mg administered on days 1 to 5, cytarabine 16mg every 12 hours on days 3 to 10, aclarubicin 20mg on days 3 and 4, G-CSF 300ug on days 3 to 10, and selinexor 60mg once a week for four weeks. On August 25, 2023, a re-examination of the bone marrow revealed a presence of 2% of the original cells and measurable residual disease (MRD) 2.9% by flow cytometry. Commencing from August 27, 2023, the patient was administered selinexor at a dosage of 60mg once a week for four weeks, along with decitabine (25mg d1-5) and a half dose of CAG (aclarubicin 20mg d3-4, cytarabine 16mg q12h d3-9, G-CSF 300ug d3-9). On September 19, 2023, another bone marrow re-examination was conducted, indicating a persistence of 2% cells and MRD of 1%. A subsequent review of the bone marrow on November 9, 2023, confirmed complete remission (CR) with MRD 1%. The patient then underwent a regimen of consolidation chemotherapy consisting of a intermediate dose of cytarabine (Ara-c) (1.5g/d q12h) for three days in one cycle, starting on November 11, 2023. Up to date, the patient was treated with maintenance selinexor + AZA after Ara-c and her bone marrow examination reflects a state of CR (Figures 1, 2). Last date of follow up was July 20, 2024 and overall survival of the patient so far reached 20 months.

Discussion

The efficacy of intensive chemotherapy in elderly patients with acute myeloid leukemia (AML) is frequently suboptimal due to factors such as drug resistance, compromised performance status, multiple organ dysfunction, and significant treatment-related toxicities, resulting in elevated early mortality rates (1). Consequently, there is a growing need to explore alternative therapeutic approaches for this patient population. One such approach involves the utilization of novel agents and treatment regimens. Notably, the combination of venetoclax (VEN) and azacitidine (AZA) has emerged as a standard regimen for AML patients aged ≥ 75 years or those deemed unsuitable for intensive chemotherapy (3, 4). Despite showing initial promise, the issue of resistance to combination therapy poses a significant challenge, leading to disappointing outcomes for patients with relapsed/refractory acute myeloid leukemia (R/R AML).

A patient's Eastern Cooperative Oncology Group performance status greater than 2 rendered our patient unsuitable for intensive chemotherapy. Consequently, she was administered induction chemotherapy utilizing the AZA and VEN regimen. Due to the intricate biological nature of this disease and the variations in initial treatment approaches, it is imperative to prioritize approved therapies

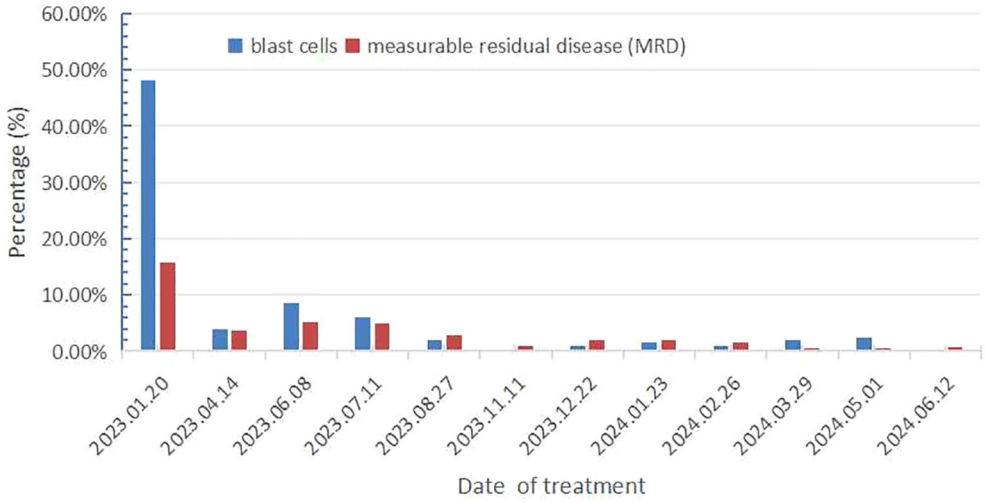

FIGURE 1
A schematic figure illustrating the treatment course from diagnosis to remission.

that specifically target the R/R AML subgroup. Consequently, conducting clinical trials to treat this subgroup of patients is one of the effective strategies For the majority of patients, allogeneic hematopoietic cell transplantation (HCT) stands as the sole potential curative treatment strategy. Prior to HCT, a common practice involves administering a combination of chemotherapy and targeted therapy as a rescue treatment to alleviate the burden of leukemia (1, 2).

The CAG regimen, which consists of cytarabine, aclarubicin, and granulocyte colony-stimulating factor, has been extensively utilized in China and Japan for treating both newly diagnosed and R/R AML. It has shown good tolerance and minimal cardiotoxicity (5). A meta-analysis of CAG revealed a complete remission rate of 60.1% in R/R AML patients, indicating its potential to overcome AML resistance (6). A previous study reported a complete remission rate of 46.5% in R/R AML patients treated with the CAG regimen. Furthermore, the efficacy and safety of CAG in the treatment of AML patients who did not respond to initial induction chemotherapy was demonstrated (7). These findings suggest that the CAG regimen holds promise as a potential therapeutic option for both newly diagnosed and R/R AML cases.

Selinexor, an inhibitor of exportin-1 (XPO-1), effectively promotes nuclear retention and functional activation of tumor suppressor proteins, thereby inducing apoptosis in cancer cells (8). The prevalent overexpression of XPO-1 in various tumors, including AML, underscores the significance of developing novel therapeutic approaches, especially for relapsed AML cases (9). Given the poor prognosis associated with relapse in 10-60% of AML patients, the demand for new treatment strategies is particularly pressing. After a promising phase I trial (10), A phase II study was conducted using a combination of selinexor, cytarabine, and idarubicin in patients diagnosed with R/R AML (11). A total of forty-two patients, with a median age of 59.5 years, were enrolled in the study. However, due to the occurrence of prolonged aplasia and a high incidence of febrile neutropenia (85%)

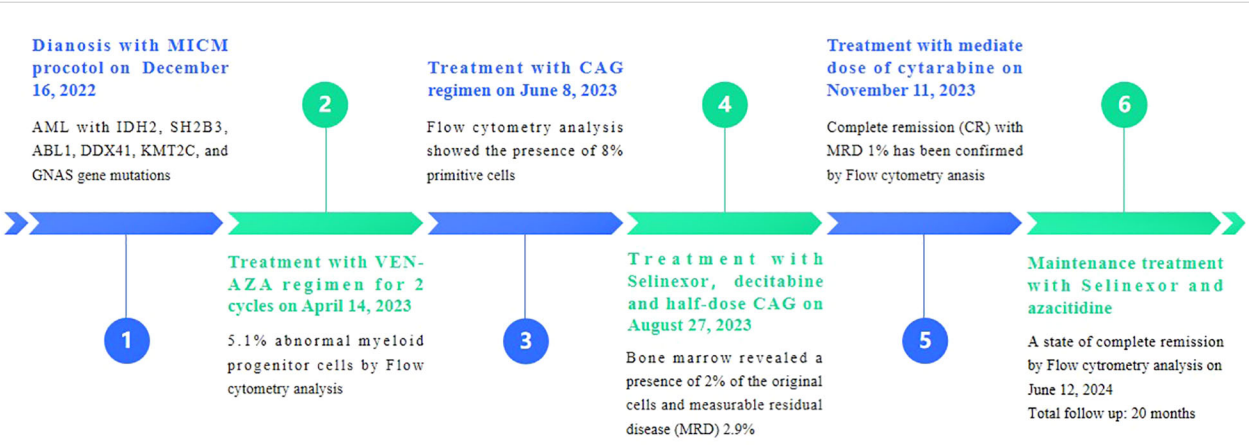

FIGURE 2
Results of bone marrow primitive cell count and flow cytometry immunophenotyping for measurable residual disease.

and grade 3/4 diarrhea (56%), the initial selinexor dosage of 40 mg/m^2 administered twice weekly for a duration of 4 weeks was subsequently reduced to 60 mg twice weekly for a period of 3 weeks. This adjustment resulted in a notable decrease in the occurrence of febrile neutropenia (33%) and severe diarrhea (40%). The overall response rate observed in this study was 50% (8).

Based on above studies and given her frailty, the patient underwent treatment with a combination of Selinexor, decitabine, and a half-dose CAG regimen for two cycles, resulting in complete remission. Subsequently, the patient has sustained this remission through consolidation therapy involving a medium dose of Ara-c. This therapeutic regimen has demonstrated favorable outcomes in the management of relapsed AML in elderly individuals. As side effect, the patient exhibited bone marrow suppression and pancytopenia, which subsequently resolved through symptomatic intervention, leading to a restoration of normal hematopoiesis within two weeks. Furthermore, the adverse reactions were manageable. In order to devise an efficacious treatment regimen for elderly patients suffering from R/R AML, it is imperative to incorporate a larger cohort of cases for clinical investigation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, 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

XZ: Writing – original draft, Writing – review & editing. JZ: Writing – original draft, Writing – review & editing. YS: Writing – original draft, Writing – review & editing.

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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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1391329/full#supplementary-material

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