INNOVATIVE MULTI-DISCIPLINARY APPROACHES FOR PRECISION STUDIES IN LEUKEMIA

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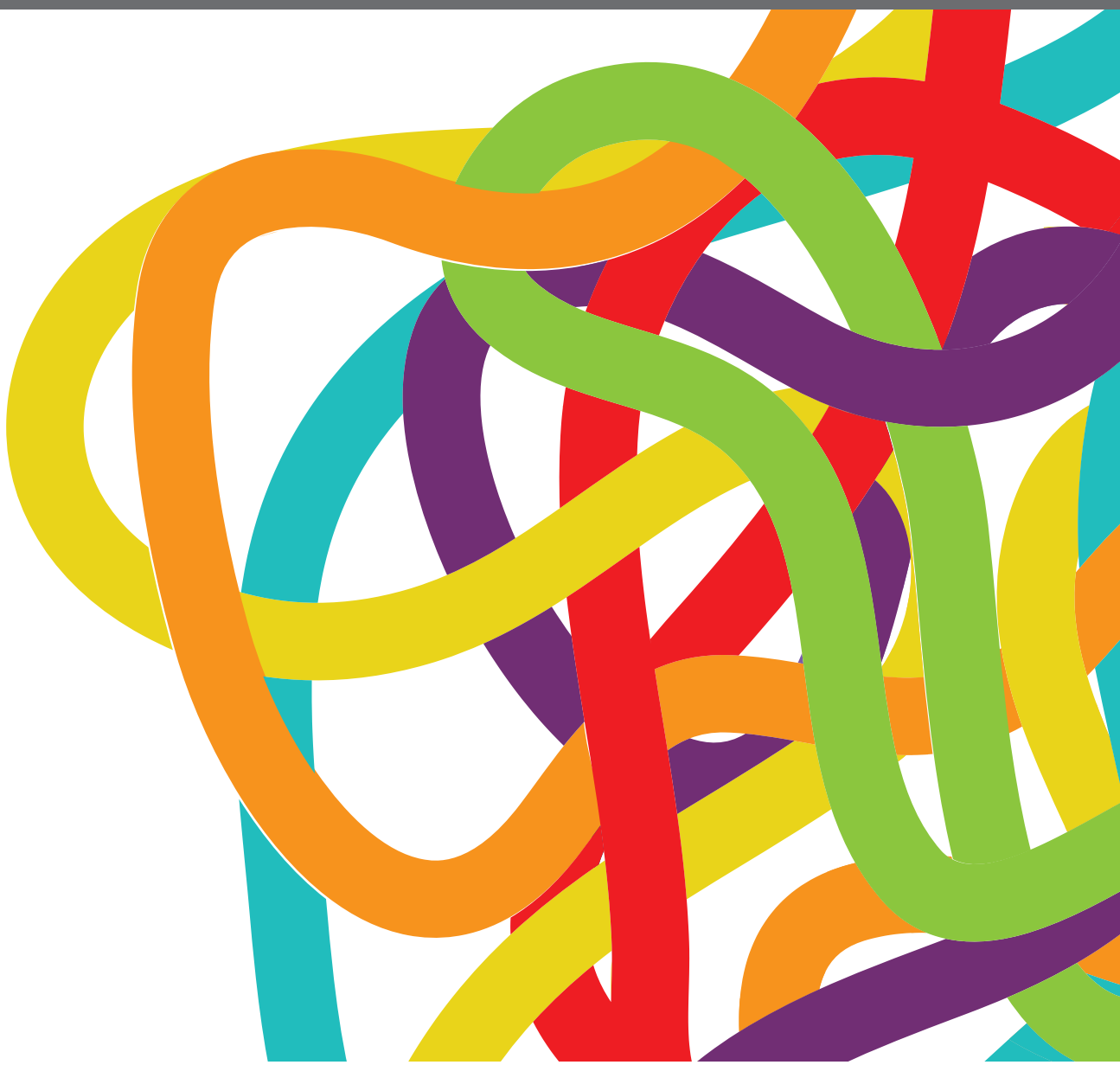

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INNOVATIVE MULTI-DISCIPLINARY APPROACHES FOR PRECISION STUDIES IN LEUKEMIA

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EDITORIAL
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Editorial: Special Issue on Innovative Multi-Disciplinary Approaches for Precision Studies in Leukemia

Sandra Marmiroli[1], *Xu Huang*[2], *Valentina Serafin*[3] *and Alison M. Michie*[2]*

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Keywords: leukemia, bioinfomatic analysis, risk stratication, metabolism, intracellular signaling, technology, AML – acute myeloid leukemia, therapeutic targets

Editorial on the Research Topic

Special Issue on Innovative Multi-Disciplinary Approaches for Precision Studies in Leukemia

By investigating the role of recurrent mutations, epigenetic modifications and aberrant activation of oncogenic signaling (1–4), our understanding of leukemogenesis has advanced to the stage that precision medicine is capable of transforming diagnostic and therapeutic approaches. This Research Topic focuses on the innovative technologies that will aid in the development of a multi-dimensional integrated molecular network, enabling the prediction of therapy responses and creation of novel treatments for those in most clinical need.

Risk stratification scores in AML are important tools for clinicians and scientists alike, due to the relative ease with which biomarker panels can assist with diagnosis, prediction of prognosis, overall survival (OS) and guide appropriate treatment choices. Our work has identified a gene signature associated with the histone demethylase KDM4A, the KDM4A-9 score, which is associated with poor OS and independent of age, cytogenetic risk and mutation status (2). KDM4A-9 is highly correlative with the LSC17 score, while having no overlap, showing the collective power of integrating risk scoring systems (2, 5). In this Research Topic, risk stratification was addressed by Lu et al. through bioinformatic analysis of the publicly-available databases. The authors explored the relationship of CXCR family members in AML and found that over-expression of specific CXCR receptors correlates with FAB subtypes and AML risk stratification. Of note, CXCR2 expression was identified as an independent prognostic factor. Applying machine learning algorithms to gene expression datasets, Mosquera Orgueira et al. described a new model containing 123 variables (ST-123) that was capable of predicting survival of AML patients. Two genes were identified, namely *KDM5B* and *LAPTM4B*, that have an established role in the pathogenesis of myeloid malignancies. Similarly, Sanchez Corrales et al. reviewed machine learning potential to dissect those heterogeneities that render AML hard to tackle with effective therapies, by identifying specific leukemic populations and the biomarkers that define these populations. They propose to design precision immunotherapy strategies, accounting for the heterogeneous sub-populations characteristic of each disease.

Van Gils et al. offered a comprehensive review of the diverse, mainly non-genetic, mechanisms of resistance to chemotherapy in AML, from survival pathways mediated by the bone marrow microenvironment to altered epigenetic profiles and metabolic reprogramming. Unlike most reviews on the same subject, it focused also on AML "persisters" highlighting that they are not

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necessarily pre-existing but arise by distinct mechanisms and may be induced by therapy. Understanding the plasticity of these mechanisms in different patients could lead to effective personalized therapy.

Metabolic reprogramming is a key hallmark of cancer and gaining a fundamental understanding of disease biology in terms of metabolic profiling may enable personalized therapies that exploit the unique weaknesses in leukemic cells. As such, Zhang et al. analyzed publicly-available databases to investigate metabolism-related prognostic factors and identified four genes (*PLA2G4A, HMOX2, AK1, SMPD3*) that when highly expressed represented a poor prognostic predictor for AML patients. Dembitz and Gallipoli described recent evidence demonstrating the validity of developing therapies that target metabolic pathways in AML. The review highlights the differential metabolic pathways that are present in distinct AML subtypes and disease stages, which may enable truly personalized treatments for these patients, supported by FDA approval of the first metabolic inhibitors targeting IDH1/2 in AML. Further promising avenues for targeting metabolic pathways are discussed.

The impact that the microenvironmental niche has on tumor persistence is now becoming appreciated, and as such the stromal component of the leukemic niche may represent a novel therapeutic target, as has been demonstrated in B cell malignancies (6). Two reports tackled different angles of this important topic. Sletta et al. explored the role of colony stimulating factor 1R (CSF1R) within the stromal compartment. High levels of CSF1R are associated with shorter patient survival in AML and RUNX1, a key driver of AML, regulates CSF1R. This review assessed the role of CSFR1 in immunoregulation of macrophage function/polarization and discussed recent pre-clinical studies and clinical trials incorporating distinct CSFR1 inhibitors, providing evidence to support the use of CSF1R-targeted therapies in AML. Simioni et al. reviewed the role of the tumor microenvironment in acute lymphoblastic leukemia. The authors summarized the main aspects concerning experimental, pre-clinical and clinical information in the field, highlighting the potential of therapeutic strategies, which include cytokines and cytokines receptors, such as CXCL12/CXCR4, the MEK/ERK and PI3K/AKT signaling networks. The importance of oncogenic signaling is highlighted also in a review by Ratti et al., that provided an in depth description of the role of phosphoinositide (PI) lipid second messengers in the oncogenesis of AML. The authors emphasize key enzymes in PI metabolism, which are emerging as putative druggable targets in leukemia. They suggest that combining bioinformatic analysis with PI pathsway targeting can lead to new combination therapies to reprogram transcriptional output, control the cell state and attenuate uncontrolled AML cell growth.

Finally, Sun et al. present data from a clinical trial (NCT03412409) in which older patients with acute leukemia or MDS underwent haploidentical-SCT using a novel conditioning regime. Hematopoietic stem cell transplantation (SCT) represents a potential cure for acute leukemia, however as most hematological malignancies occur in older individuals, in which the myeloablative conditioning regimen prior to SCT is linked to a high risk of treatment-related mortality (TRM), this is not always possible. Incorporating reduced intensity conditioning (low dose fludarabine and reduced cyclophosphamide) in patients with acute leukemia/MDS of ≥55 years old revealed that OS, leukemia free survival, TRM, and cumulative incidence of relapse were comparable to a historical, younger cohort of patients that underwent the normal conditioning regimen. This small study suggests that a reduction in the conditioning regimen prior to SCT may be a feasible option for older acute leukemia patients.

Although the treatment of leukemia in all its forms still represents a significant challenge, these articles all highlight novel methods/strategies that can be utilized to identify targets/biomarkers that enable scientists and clinicians the opportunity to develop novel stratified diagnostics or therapeutic interventions for specific patient cohorts. As the development of 'omics approaches is a highly competitive and fast-moving field, an ongoing challenge will be how to integrate individual approaches into a robust multi-omics-based platform that can be used as a routine clinical and diagnostic tool to form the basis of digital healthcare. Indeed, artificial intelligence-lead machine learning technologies, collecting and analyzing molecular-level 'omics information, will represent the forefront of innovation in personalized therapy for prevention and early detection of diseases such as leukemia, coupled with precision prognosis and personalized treatment pathways.

AUTHOR CONTRIBUTIONS

The Editorial was drafted by SM and AM, and further edited by SM, XH, VS and AM. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Comprehensive Analysis of Prognostic Markers for Acute Myeloid Leukemia Based on Four Metabolic Genes

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Background: Metabolic reprogramming is the core characteristic of tumors during the development of tumors, and cancer cells can rely on metabolic changes to support their rapid growth. Nevertheless, an overall analysis of metabolic markers in acute myeloid leukemia (AML) is absent and urgently needed.

Methods: Within this work, genetic expression, mutation data and clinical data of AML were queried from Genotype-Tissue Expression (GTEx) database, The Cancer Genome Atlas (TCGA) database and Gene Expression Omnibus (GEO) database. The tumor samples of TCGA were randomly divided into a training group (64 samples) and an internal validation group (64 samples) at one time, and the tumor samples of GEO served as two external validation groups (99 samples, 374 samples). According to the expression levels of survival-associated metabolic genes, we divided all TCGA tumor samples into high, medium and low metabolism groups, and evaluated the immune cell activity in the tumor microenvironment of the three metabolism groups by single-sample gene set enrichment analysis (ssGSEA) algorithm. Finally, we examined the mutations and prognostic effects of each model gene.

Results: Four metabolism-related genes were screened and applied to construct a prognostic model for AML, giving excellent results. As for the area under the curve (AUC) value of receiver operating characteristic (ROC) curve, the training group was up to 0.902 (1-year), 0.81 (3-year), and 0.877 (5-year); and the internal and external validation groups also met the expected standards, showing high potency in predicting patient outcome. Univariate and multivariate prognostic analyses indicated that the riskScore obtained from our prognostic model was an independent prognostic factor. ssGSEA analysis revealed the high metabolism group had higher immune activity. Single and multiple gene survival analysis validated that each model gene had significant effects on the overall survival of AML patients.

Conclusions: In our study, a high-efficiency prognostic prediction model was built and validated for AML patients. The results showed that metabolism-related genes could become potential prognostic biomarkers for AML.

Keywords: bioinformatic analysis, metabolism, acute myeloid leukemia, survival, prognosis

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INTRODUCTION

AML is a malignant disease of myeloid hematopoietic stem/progenitor cells, which is mainly characterized by primitive and immature myeloid cell dysplasia in the bone marrow and peripheral blood. Its clinical manifestations are anemia, hemorrhage, infection and fever, organ infiltration and metabolic abnormality, etc. In most cases, the condition is urgent and severe, and the prognosis is poor, which may endanger life if not treated in time (1). Chemotherapy, targeted drugs and hematopoietic stem cell transplantation are still the main approaches for AML treatment. Among them, even the most ideal treatment methods such as intensive chemotherapy and allogeneic hematopoietic stem cell (HSC) transplantation, the cure rate is relatively low (2). Therefore, it is urgent to explore new and accurate biomarkers to evaluate the diagnosis and prognosis of AML patients.

More and more evidences show that the metabolic pattern of cell carcinogenesis has changed significantly, which involves many aspects such as glycolysis, TCA cycle, oxidative phosphorylation, amino acid metabolism, fatty acid metabolism and nucleic acid metabolism (3). This phenomenon is known as the metabolic reprogramming of tumor cells (4). Metabolic reprogramming is one of the important hallmarks of tumors. The rapidly proliferating tumor cells take high rate glycolysis as the primary energy supply method to promote the adaptation to the stress environment such as hypoxia and increase the malignant potential of tumors (5). Thus, metabolic reprogramming can be further used to diagnose, monitor and treat cancer. In recent years, new metabolic inhibitors have been developed for the clinical treatment of cancer (6–9).

Metabolic reprogramming also has a significant impact on the progression, treatment and prognosis of AML (10, 11). More precisely, there are abnormalities in the metabolic processes of AML such as glycolysis, amino acid metabolism, fatty acid metabolism, epigenetic modification and autophagy pathway (12). In addition, metabolic abnormalities may promote the immune escape of AML, resulting in immunotherapy limitations (13). A series of novel drugs targeting the metabolic processes of AML have been developed and studied in preclinical and clinical trials (14–16).

The popular idea is that metabolism regulates the strength of the immune system (17). An important study found that arginine metabolism in AML inhibited T cell proliferation, becoming a potential therapeutic target (18). However, a proteomic analysis showed that lipid metabolism increased the melanoma sensitivity to T-cell-mediated killing by promoting antigen presentation (19). Similarly, in melanoma and breast tumors, the treatment targeting pyruvate metabolic reprogramming increased the immune cells infiltration by reducing the lactic acid production and neutralizing the tumor acidity, and finally played a significant role in inhibiting tumor growth (20). Relevant metabolic reprogramming agents, such as leptin, which provides metabolic support for tumor immunity, are also being developed (21). Thus, for tumor microenvironment (TME), different metabolic pathways have different effects on immune cell adaptability and effector function. Overall, the association between the metabolic state of AML and immune response may be the basis for improving the immunotherapy response in the future, with broad exploration space and application prospects.

We used a variety of bioinformatics analysis methods to explore metabolism-related prognostic factors in AML. COX regression analysis helped us screen out significant prognostic markers for further study. The metabolic prognostic model constructed by the training group showed excellent predictive performance after the double validation and the model gene survival analysis. These results provide a basic direction for further exploration of the molecular mechanism and diagnostic markers of AML.

Abbreviations: AML, Acute myeloid leukemia; GTEx, Genotype-Tissue Expression; TCGA, The Cancer Genome Atlas; GEO, Gene Expression Omnibus; GSEA, Gene set enrichment analysis; ssGSEA, single-sample gene set enrichment analysis; AUC, Area under the curve; ROC, Receiver operating characteristic; HSC, Hematopoietic stem cell; TME, Tumor microenvironment; UCSC, University of California Santa Cruz; LASSO, Least absolute shrinkage and selection operator; GO, Gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; GEPIA, Gene Expression Profiling Interactive Analysis.

METHODS

Data Acquisition and Differential Analysis

GTEx database contains healthy human samples of 42 tissue types, covering almost all the transcriptional genes (22). TCGA database stores a large amount of genomic data and clinical

TABLE 1 | Clinical characteristics of AML patients in the TCGA database.

Characteristics		Total	%
All		**171**	**100.00**
Age (y)	≥60	73	42.69
	<60	98	57.31
Gender	Male	92	53.80
	Female	79	46.20
FAB category	M0	14	8.19
	M1	36	21.05
	M2	38	22.22
	M3	18	10.53
	M4	40	23.39
	M5	18	10.53
	M6	3	1.75
	M7	3	1.75
	Not classified	1	0.58
Cytogenetic risk category	Favorable	35	20.47
	Intermediate	100	58.48
	Poor	33	19.30
	Unknow	3	1.75
Immunophenotype	CD33+	124	82.1
	CD34+	99	65.6
	CD117+	134	88.7
Mutation	DNMT3A	18	12.6
	FLT3	45	30.6
	NPM1	33	22.0
	RAS	8	5.3
	IDH1	26	17.2

data, which provides a basis for the exploration of meaningful genomic changes and biological mechanisms affecting tumor initiation, development, differentiation and metastasis (23). In order to unify standards, we downloaded the GTEx gene expression dataset (GTEX_RSEM_gene_fpkm), the GTEx sample information file (GTEX_phenotype), the TCGA-LAML gene expression dataset (TCGA-LAML.htseq_fpkm) and the TCGA-LAML sample information file (TCGA-LAML.survival) from the University of California Santa Cruz (UCSC) genome database (24). We also retrieved AML probe matrix files (GSE71014_series_matrix, GSE37642_series_matrix) and platform files (GPL10558-50081, GPL96-57554) from the GEO database (25). Metabolism-related genes were obtained from the Gene Set Enrichment Analysis (GSEA) website (26). In addition, the AML mutation data (TCGA.LAML.varscan.e595f93d-41ac-435e-8c90-06df7e9d6742.DR-10.0.somatic) was also downloaded from TCGA website. All AML samples from the TCGA and GEO databases were bone marrow samples from patients with initial diagnosis.

All AML gene expression data from TCGA and GEO databases had been processed by log2(x+1) and genes with expression values close to 0 were deleted to exclude the influence of extreme values or outliers. Then, we extracted the gene expression data of normal blood samples (337 samples) from

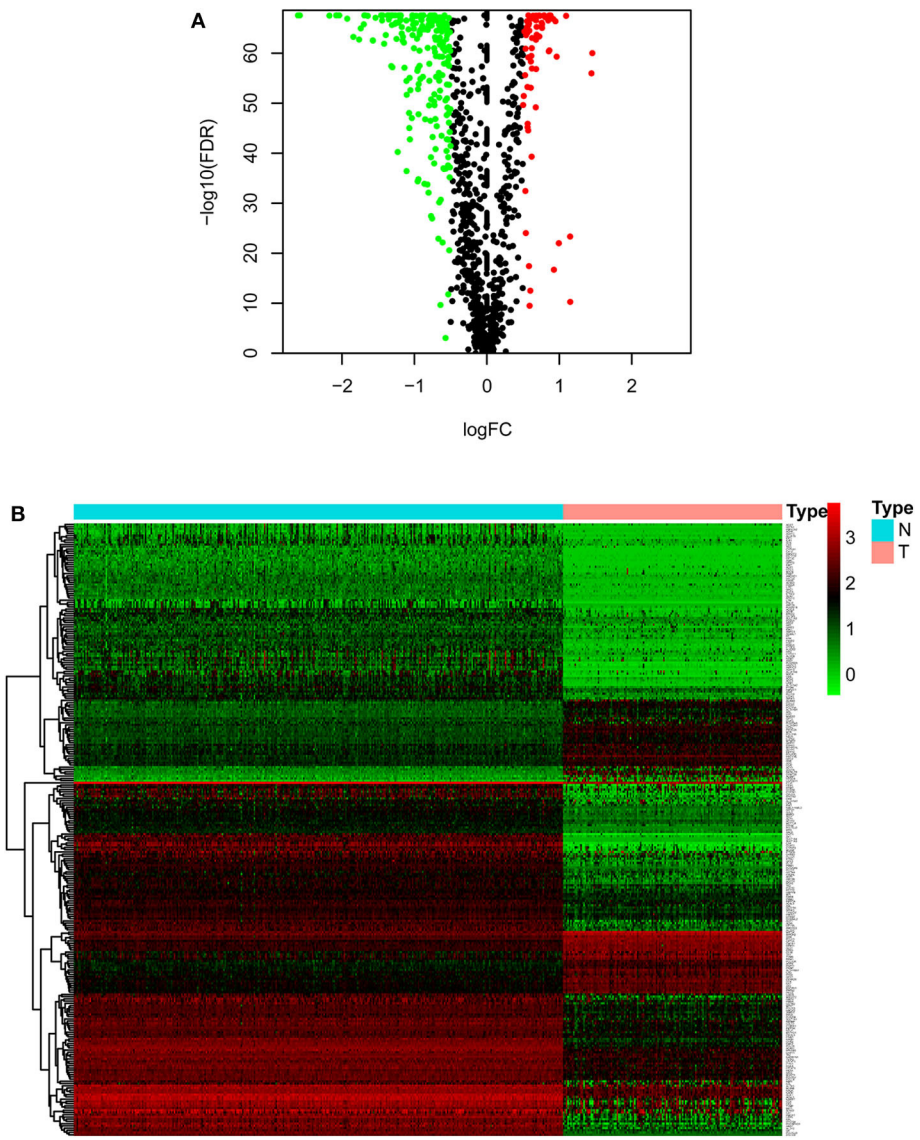

FIGURE 1 | Volcano plot and heatmap of differential metabolic genes. **(A)** Volcano plot. Black dots, green dots and red dots represent undifferentiated genes, down-regulated genes in the tumor group and up-regulated genes in the tumor group, respectively. **(B)** Heatmap. The blue type represents the normal samples and the red type represents the tumor samples. Green color, black color and red color in the main body correspond to low expression, medium expression and high expression.

the GTEx gene expression dataset, and the gene expression data of AML samples (151 samples) from the TCGA-LAML gene expression dataset. These two gene expression data were merged, using the limma package (27) for standardized processing, and the expression of metabolic genes (945 genes) was extracted simultaneously to facilitate subsequent analysis. We intersected the metabolic genes of GEO and TCGA, correcting the batch effect with the sva package (28). We used the Wilcox test to analyze the difference between the normal group and the tumor group, screening differential metabolic genes (275 genes) according to FDR <0.05 and | logFC | >0.5, and drawing differential heatmap (pheatmap R package) and volcano plot finally.

Construction of Prognostic Model and Survival Analysis

After combining the expression of differential metabolic genes with survival time, we divided TCGA samples (128 samples) randomly at one time into a training group (64 samples) and an internal validation group (64 samples), with the GEO samples as two external validation groups (99 samples, 374 samples). For the training group, univariate COX analysis ($P < 0.01$) found survival-associated metabolic genes (12 genes), and the least absolute shrinkage and selection operator (LASSO) (29) removed the high correlation genes to prevent the over-fitting of the model by using the R package glmnet (8 genes). Finally, stepwise multivariate Cox regression analysis was performed to

TABLE 2 | Multivariate COX regression analysis results of model genes.

Id	Coef	HR	HR.95L	HR.95H	P value
PLA2G4A	0.588468436	1.801227607	1.30398577	2.488079983	0.000356501
HMOX2	1.333908151	3.795849187	1.692436314	8.513449476	0.001209189
AK1	3.500312611	33.12580585	4.336098654	253.0659703	0.000740836
SMPD3	1.162351687	3.197443829	1.529613372	6.683811234	0.00200342

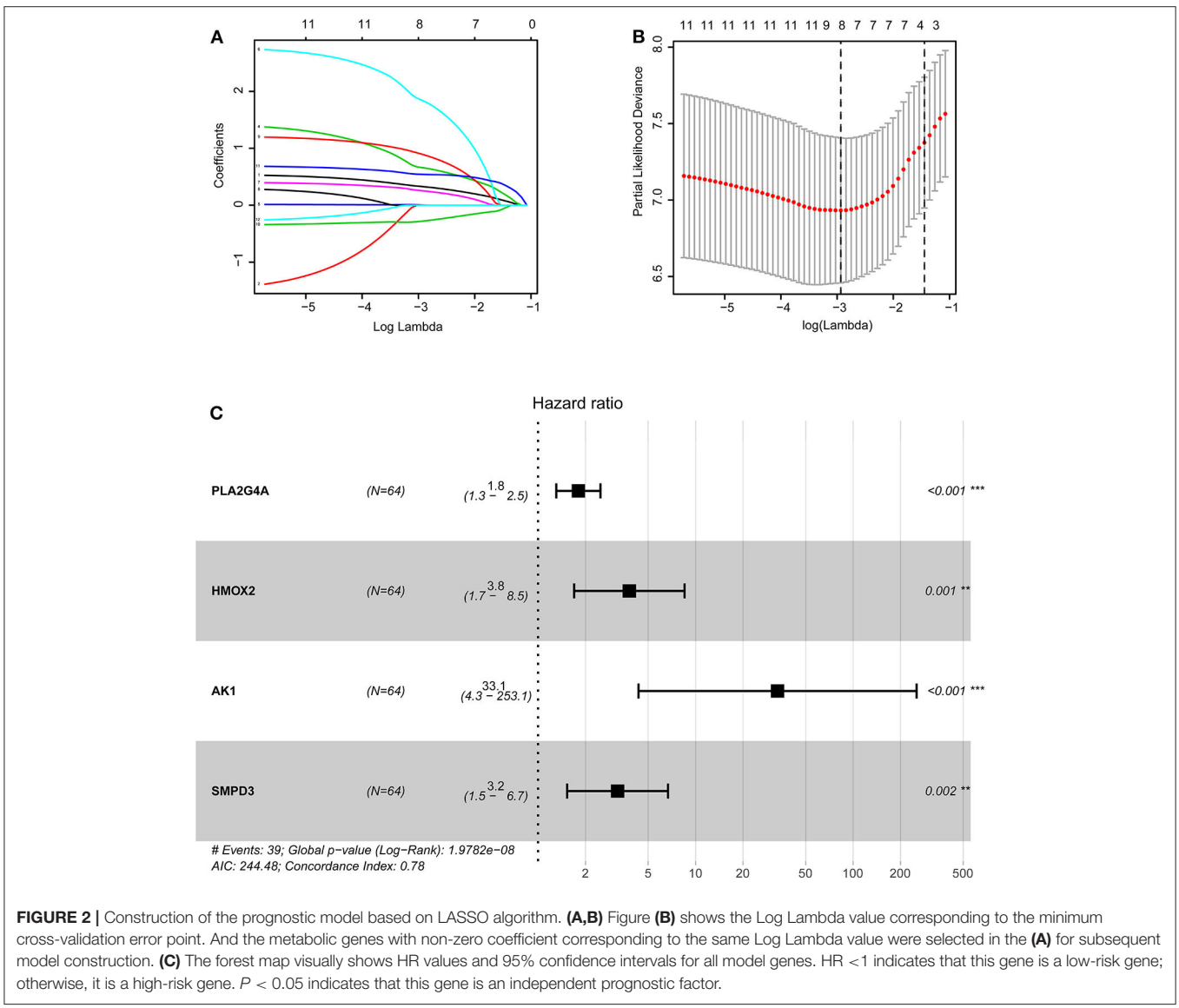

FIGURE 2 | Construction of the prognostic model based on LASSO algorithm. **(A,B)** Figure **(B)** shows the Log Lambda value corresponding to the minimum cross-validation error point. And the metabolic genes with non-zero coefficient corresponding to the same Log Lambda value were selected in the **(A)** for subsequent model construction. **(C)** The forest map visually shows HR values and 95% confidence intervals for all model genes. HR <1 indicates that this gene is a low-risk gene; otherwise, it is a high-risk gene. $P < 0.05$ indicates that this gene is an independent prognostic factor.

construct the optimal prognostic model (4 genes). Meanwhile, in the SPSS 26 version software, we used Schoenfeld residuals to carry out the PH assumption test on model genes, and also drew a plot of the Schoenfeld Residuals against the transformed time for each model gene to ensure the assumptions of proportional hazards is met.

The patient riskScores of the training and validation groups were calculated according to the constructed prognostic model formula. With the median riskScore of the training group as the threshold, we divided the patients in the training and validation groups into a high-risk group and low-risk group, plotting the survival curve (survival R package), ROC curve (survivalROC R package) and risk curve of the training and validation groups, respectively. Finally, univariate and multivariate prognostic analyses were performed for the training group ($P < 0.05$) to judge whether the riskScore obtained from the model could be an independent prognostic factor.

ssGSEA Analysis and Mutation Data Visualization

We conducted ssGSEA analysis using GSVA package (30) to obtain the immune activity of 29 immune-related genesets in TCGA-AML samples, and the correction results were between 0 and 1. According to the expression of survival-associated metabolic genes ($P < 0.05$), hclust function was applied to cluster TCGA-AML samples, generating high, medium and low metabolism groups. Based on the correlation analysis using ESTIMATE algorithm between metabolism groups and tumor microenvironment, the heat map (pheatmap R package) and violin plots (ggpubr R package) of tumor microenvironment were drawn. The survival curves of the three metabolism groups were plotted after survival analysis. We performed the GSEA enrichment analysis using the org.Hs.eg.db R package between the high and low metabolism groups, drawing the top 5 Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, respectively, that were enriched most significantly in the high metabolism group ($P < 0.05$).

The maftools package (31) helped to visualize the TCGA-AML mutation data, drawing waterfall plots of the high, medium and low metabolism groups, respectively.

Mutation Status and Multiple Validations of Model Genes

We entered the cBioportal website (32) and selected the study (Acute Myeloid Leukemia TCGA PanCancer data) to download the mutation status of model genes.

To validate the expression differences of model genes, the differences of model genes were analyzed between the high-risk group (69 samples) and the low-risk group (59 samples) of TCGA-AML, with the boxplots of each model gene plotted using the ggpubr package.

In order to validate the prognostic effect of model genes, Gene Expression Profiling Interactive Analysis (GEPIA) website (33) was used to conduct a single gene survival analysis for each model

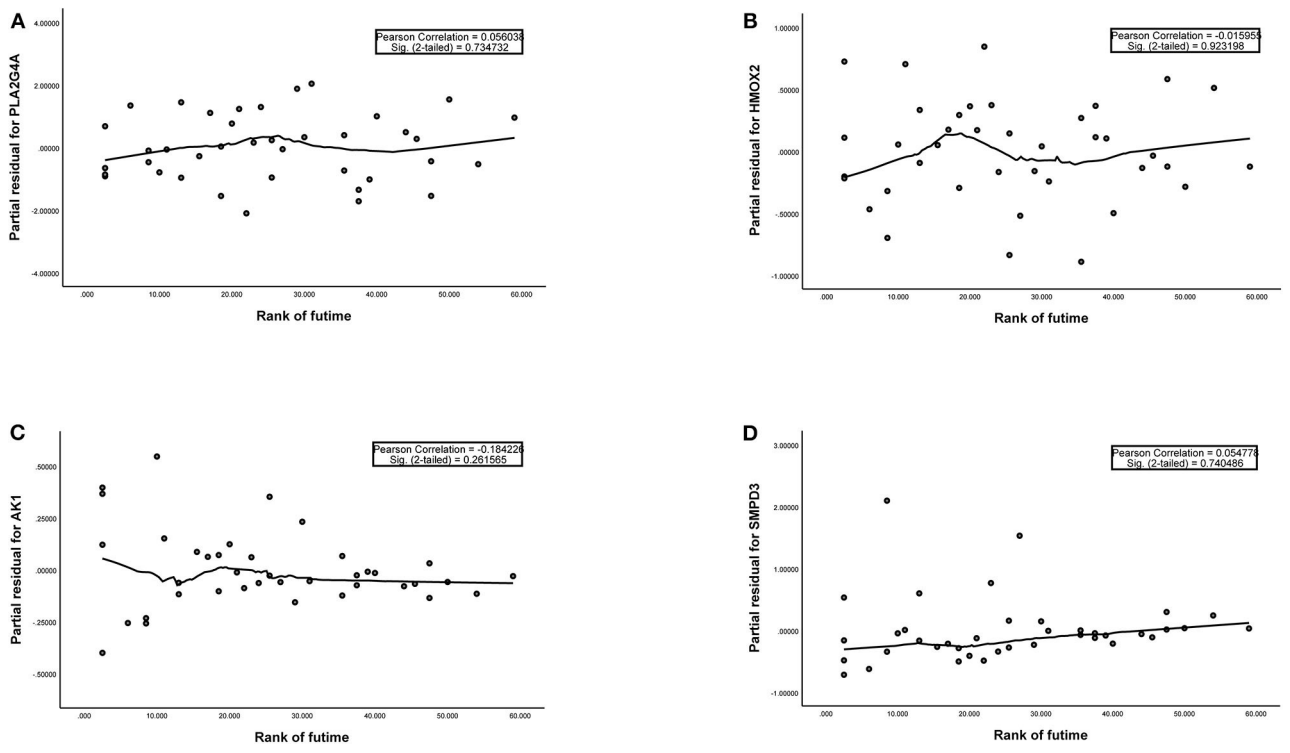

FIGURE 3 | (A–D) The plots of the Schoenfeld Residuals against the transformed time for model genes. According to the variation trend of the smooth curve and statistical analysis, it can be determined that there is no correlation between the partial residuals of four model genes and time rank ($P > 0.05$), suggesting that all model genes meet the assumptions of proportional hazards and suitable for Cox regression analysis.

gene firstly, in which Cutoff-High was 70% and Cutoff-Low was 30%; Next, we applied the PROGgeneV2 online tool (34) and selected the TCGA–AML dataset to conduct multiple gene survival analysis for all model genes.

Statistical Analyses

All statistical analyses were conducted by using R software (version 3.6.3) unless otherwise stated. Univariate and multivariate Cox regression analyses were conducted to investigate the prognostic value of AML-related metabolic signature. All statistical results with a $P < 0.05$ were considered significant.

RESULTS

Metabolic Genes Differentially Expressed Between Normal and Tumor Samples

As described in the methods section, we downloaded AML clinical information (**Table 1**, **Supplementary File 1**) and

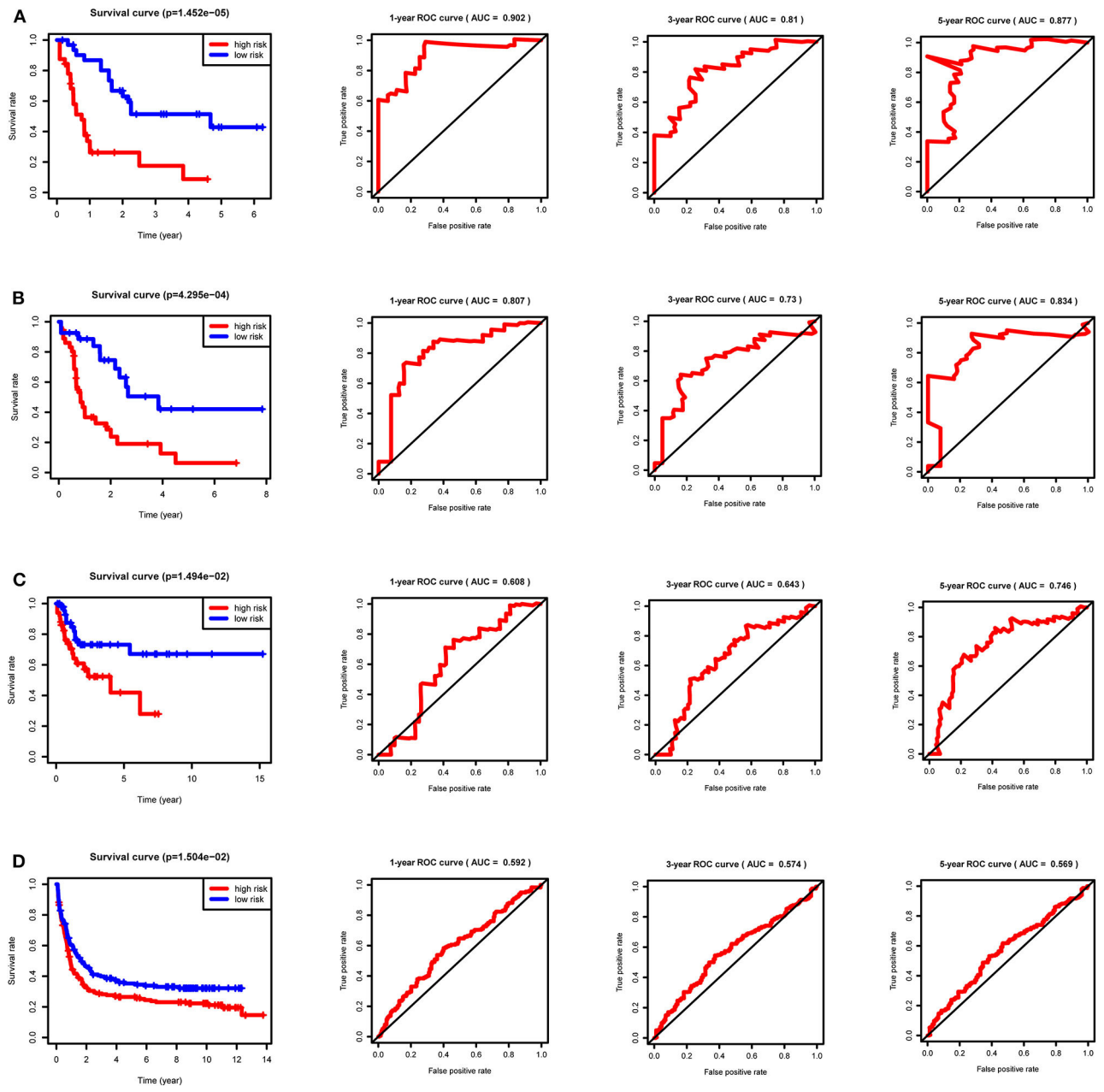

FIGURE 4 | Survival curves and ROC curves for the training, internal validation and external validation groups. For the survival curves, survival rates decrease over time in both the high and low risk groups. $P < 0.05$ indicates survival differences between the high and low risk groups. For ROC curves, AUC > 0.9 suggests that the accuracy of the model is very high; The AUC value is between 0.7 and 0.9, indicating that the model has a certain accuracy. The AUC value is between 0.5 and 0.7, showing the accuracy of the model is general. **(A)** Training group. **(B)** Internal validation group. **(C)** External validation group GSE71014. **(D)** External validation group GSE37642.

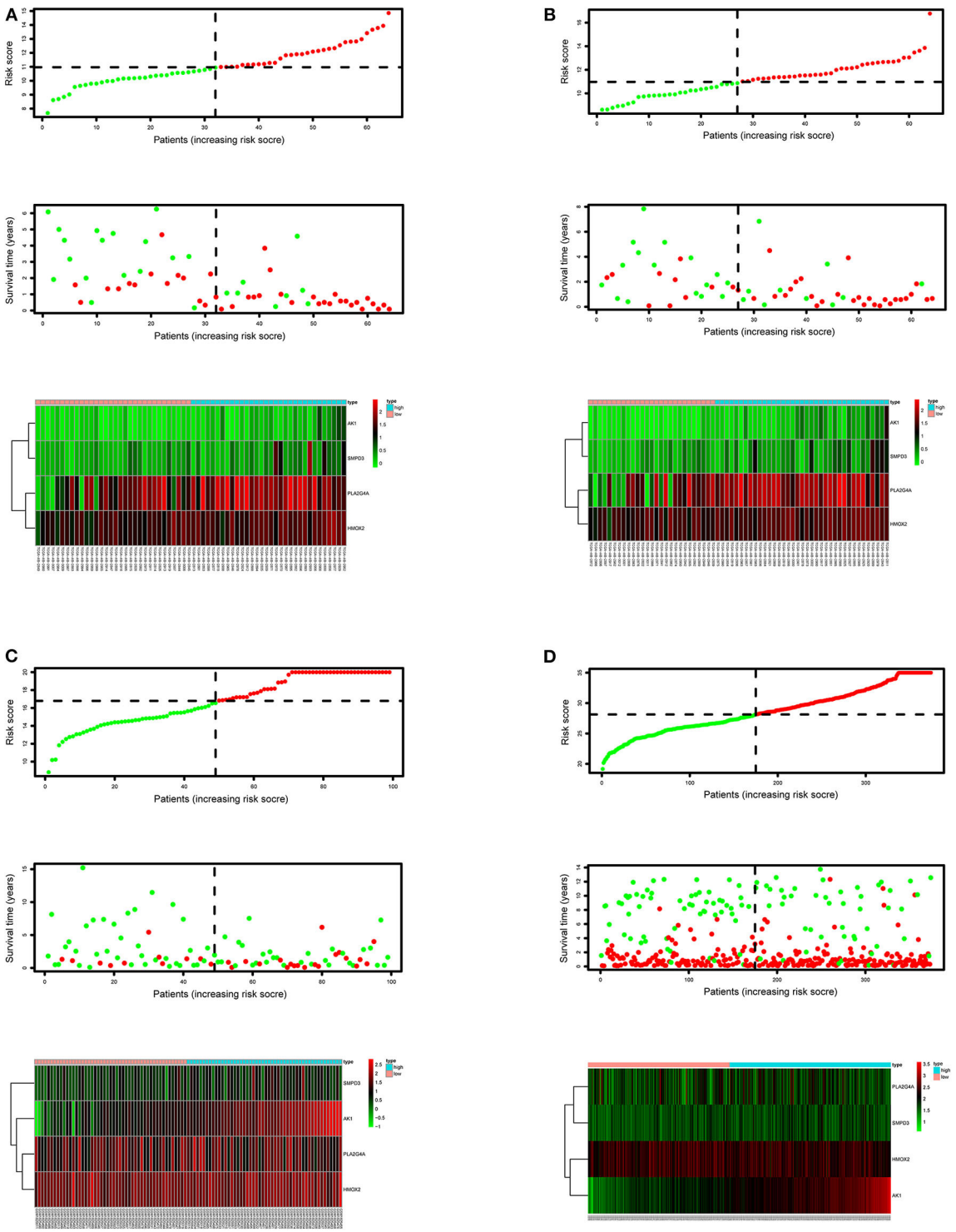

FIGURE 5 | Risk score curves, survival status figures and risk heatmaps for the training, internal validation and external validation groups. The upper part of each subfigure represents the risk score curve (low risk patients are represented by green dots, high risk patients by red dots, and dash lines correspond to the median riskScore of training group). The middle section represents the distribution of survival status and survival time of patients ranked by riskScore (more green dots on the left for low-risk patients, and more red dots on the right for high-risk patients. From left to right, with the increase of the riskScore, more and more patients died, indicating that the riskScore is related to survival). The bottom heatmap displays the expressing pattern of the metabolic genes (the color transition from green to red indicates that the expressing level of the corresponding metabolic gene increases from low to high). **(A)** Training group. **(B)** Internal validation group. **(C)** External validation group GSE71014. **(D)** External validation group GSE37642.

corrected AML gene expression data from the public platforms, and analyzed the differences between normal and tumor samples by Wilcox test, screening 275 differentially expressed metabolic genes by FDR <0.05 and | logFC | >0.5 (**Supplementary File 2**) and drawing differential heatmap and volcano plot finally. The volcano plot shows the differences distribution in the expression levels of metabolic genes between normal and tumor samples on the whole (**Figure 1A**). The heatmap depicts expression changes of each metabolic gene between normal and tumor samples (**Figure 1B**).

The Constructed Prognostic Model and Survival Analysis

The training group (**Supplementary File 3**), the TCGA internal validation group (**Supplementary File 4**) and the GEO external validation groups (**Supplementary Files 5, 6**) were generated from one-time random grouping. After univariate COX analysis ($P < 0.01$) for the training group (**Supplementary File 7**), we put the 12 survival-related metabolic genes obtained into the LASSO algorithm (**Figures 2A,B**). After the stepwise multivariate Cox regression analysis, four metabolic genes realized the prognostic model construction finally. The final model formula is that each patient's riskScore = 0.588*PLA2G4A + 1.334*HMOX2 + 3.500*AK1 + 1.162*SMPD3 (**Figure 2C**, **Table 2**). All model genes are high-risk genes and independent prognostic factors, and also meet the assumptions of proportional hazards (**Figure 3**).

After calculating all patients' riskScores, we divided the patients in the training and validation groups into the high and low risk groups (**Supplementary Files 8–11**) for subsequent survival analysis. For the training group, the internal validation group and the external validation groups, the survival curves between the high and low risk groups have significant differences, and the survival rate of the low risk group is significantly higher than that of the high risk group. According to the AUC values of ROC curves, the accuracy of the prognostic model constructed by us is very high (**Figure 4**). Similarly, the risk curves make a good distinction between the high and low risk groups on the whole. For the upper risk score curves, the patient's riskScore increases from left to right. The middle survival status figures present the survival time decline and the mortality enhancement as the patients' riskScores increase. In the bottom risk heatmaps, each model gene expression increases as the patient's riskScore increases, indicating that all model genes are high-risk (**Figure 5**). Finally, univariate and multivariate prognostic analyses ($P < 0.05$) prove that the riskScore obtained from the model is an independent prognostic factor (**Figure 6**).

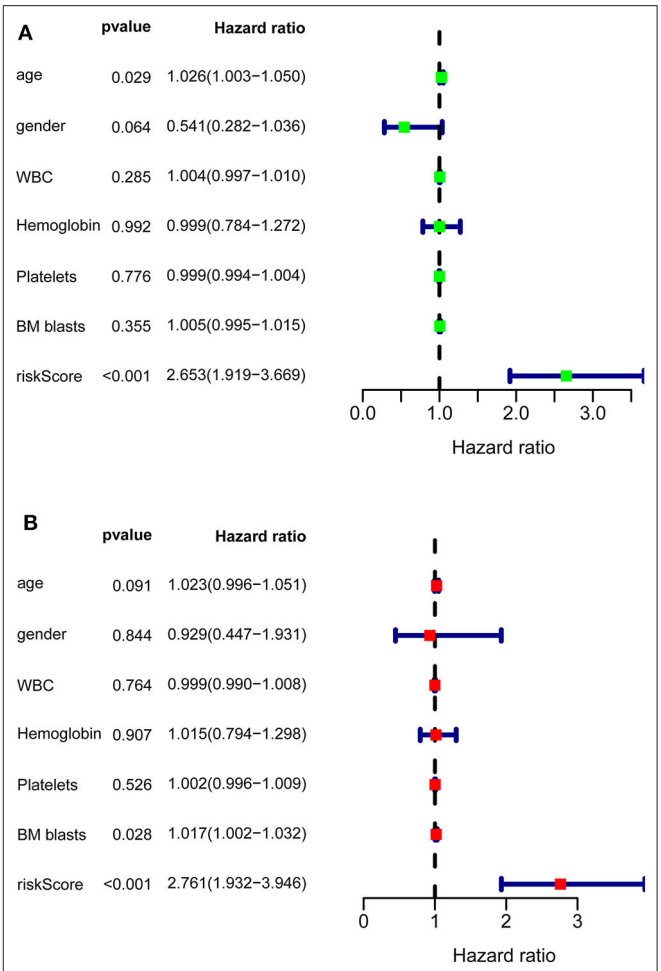

FIGURE 6 | Univariate and multivariate prognostic analyses of clinical parameters and riskScore in TCGA-AML. **(A)** Univariate prognostic analyses. For a clinical parameter or riskScore, if the $P < 0.05$, it is related to survival; if HR >1, it is a high-risk factor. **(B)** Multivariate prognostic analyses. For the results of univariate and multivariate independent prognostic analysis, if the riskScore *P*-value of both is < 0.05, it indicates that riskScore is independent of other clinical parameters and can be used as an independent prognostic factor in clinical practice.

ssGSEA Analysis and Mutation Data Visualization

TCGA-AML samples were clustered to produce high, medium and low metabolism groups (**Figure 7A**, **Supplementary File 12**). The survival analysis reveals that there are significant differences among the three metabolism groups, and the lower the metabolic activity is, the longer the survival time is (**Figure 7B**). By analyzing the correlation between metabolism groups and tumor microenvironment, we plotted the heatmap (**Figure 8A**) and the violin plots (**Figures 8B–E**) of the tumor microenvironment. In summary, metabolic activity is positively correlated with StromalScore, ImmuneScore, and ESTIMATEScore, and negatively correlated with TumorPurity. GSEA enrichment analysis was carried out between high and low metabolism groups, obtaining the top 5 GO terms (**Figure 9A**) and KEGG pathways (**Figure 9B**) with most significant enrichment in high metabolism group ($P < 0.05$). These GO terms and KEGG pathways are primarily associated with immunity and metabolism.

By visualizing the TCGA-AML mutation data, the waterfall plots of the high, medium and low metabolism groups generated (**Figures 10A–C**). It can be seen that there is no significant correlation between metabolic activity and total mutation frequency. The mutation frequency of NPM1 and DNMT3A are the highest among the three metabolism groups. Among

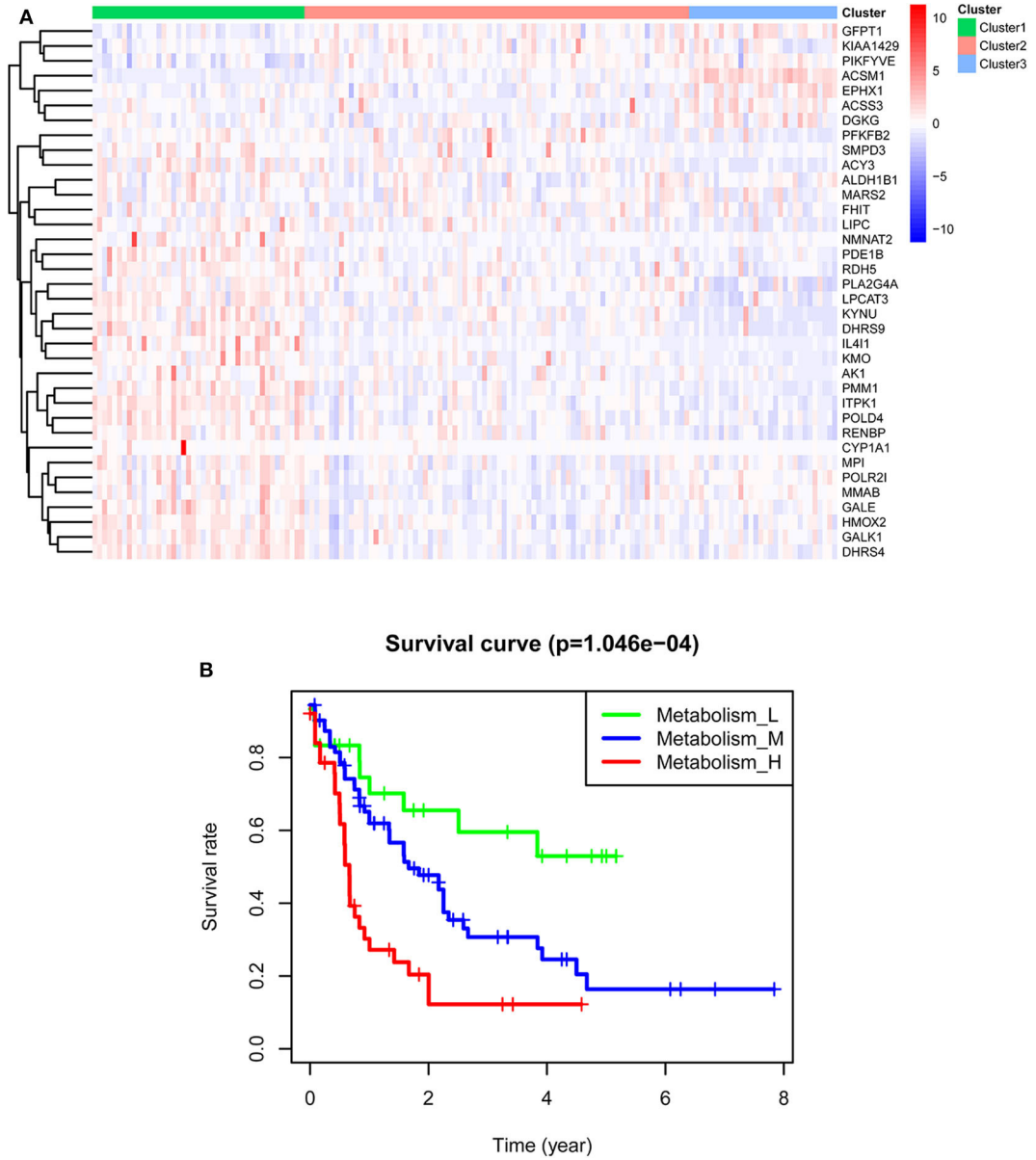

FIGURE 7 | Clustering results of TCGA-AML samples. **(A)** Clustering heatmap. In the main body of the figure, red color represents the high expression of metabolic genes, and blue color represents the low expression of metabolic genes. Cluster1 is mainly red, that is, the metabolic genes are highly expressed, so it is a high metabolism group. Cluster2 has both red and blue colors, so it is a medium metabolism group. Cluster3 is mainly blue, that is, the metabolic genes are low expressed, so it is a low metabolism group. **(B)** Survival curve. $P < 0.05$ indicates that there is a difference in survival among the three groups.

them, NPM1 mutation is mainly frameshift insert mutation, and DNMT3A mutation is mostly missense mutation.

Mutation Status and Multiple Validations of Model Genes

The general picture of genetic alteration (**Figure 11A**) and the domain mutation diagrams (**Figures 11B–E**) of the model genes were downloaded from the cBioportal website. The mutation frequencies of these model genes are very low, and the mutations hardly affect the functional change of the structural domains.

In order to validate the expression differences of model genes between the high-risk group and the low-risk group, boxplots of model genes were drawn (**Figure 12**). All model genes are significantly highly expressed in the high-risk group, which to some extent supports the previous conclusion that all model genes are high-risk genes.

Single-gene survival analysis again validates that each model gene is a high-risk gene and has a significant impact on the prognosis of AML patients (**Figures 13A–D**). Furthermore, the multiple gene survival analysis combining all model genes

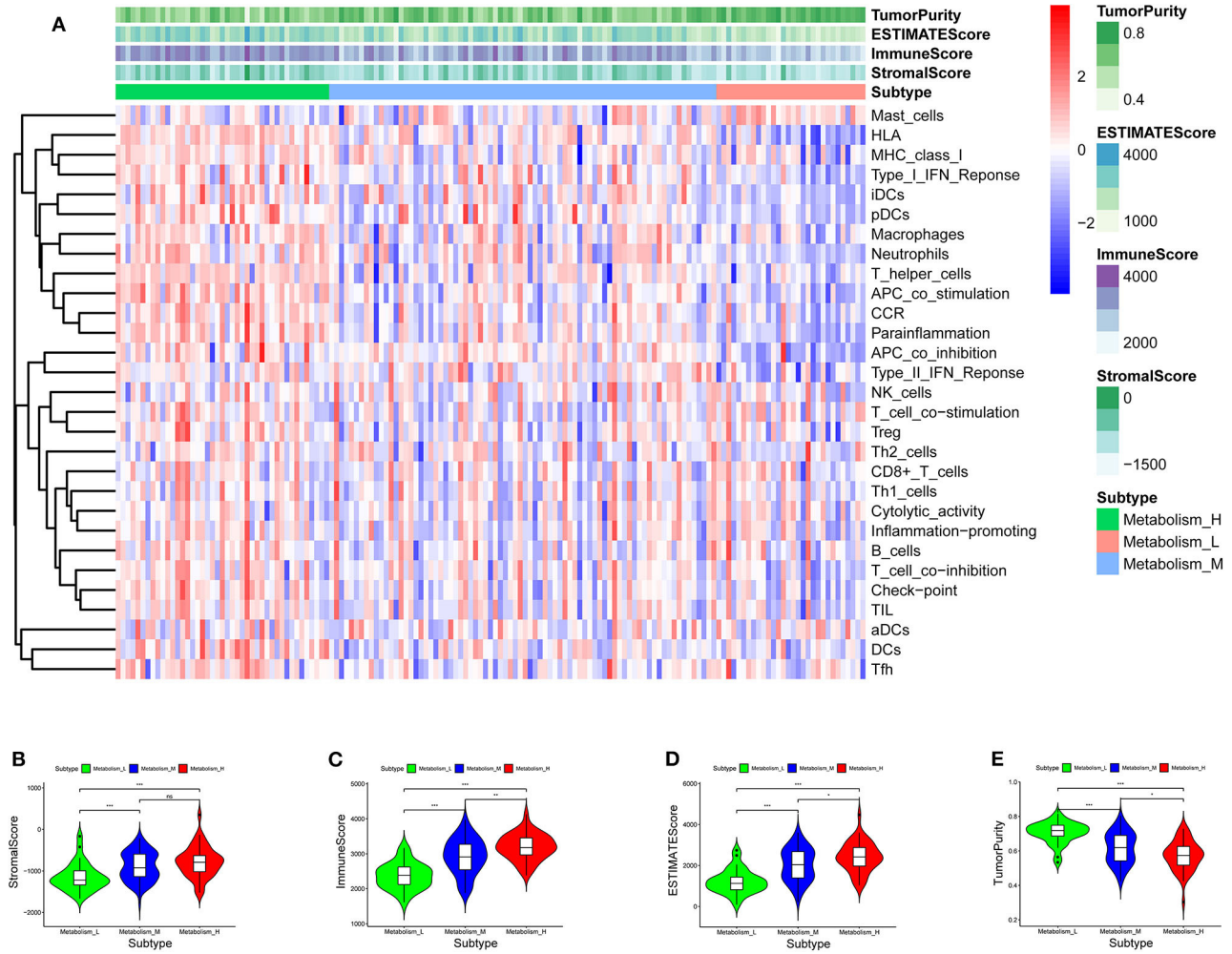

FIGURE 8 | Heatmap and violin plot of tumor microenvironment. **(A)** Heatmap. The x-coordinate represents the sample names, and the y-coordinate represents the immune genesets. The upper part is the tumor microenvironment score, in which StromalScore is the stromal cell score (The color is lighter from left to right, the lower the score, the lower the stromal cell content. That is, with the decrease of metabolic activity, the content of stromal cells decreases), and ImmuneScore is the immune cell score (The color is lighter from left to right, the lower the score, the lower the immune cell content. That is, with the decrease of metabolic activity, the content of immune cells decreases), and ESTIMATEScore is the combined score of stromal cells and immune cells (The color is lighter from left to right, the lower the score, the lower the total content of stromal cells and immune cells. That is, with the decrease of metabolic activity, the total content of stromal cells and immune cells decreases). The higher ESTIMATEScore is, the lower TumorPurity is (The color is darker from left to right, the higher the score, the higher the tumor purity. That is, with the decrease of metabolic activity, the tumor purity increases). **(B–E)** Violin plot. With the increase of metabolic activity, the change trend of various tumor microenvironmental parameters can be seen. $P < 0.001$, *** $P < 0.01$, ** $P < 0.05$, * $P > 0.05$, ns.

successfully validates the accuracy and effectiveness of the prognostic model we constructed (**Figure 13E**).

DISCUSSION

The exploration of metabolic prognostic effects has broadened our horizon and our understanding of traditional transcriptome molecular biomarkers. In this study, we adopted a variety of bioinformatics methods to mine prognosis-related metabolic genes by integrating the metabolic characteristics and clinical information of AML patients, and constructed an accurate and efficient metabolic prognosis model with internal and external validations. Furthermore, the association between metabolism and immunity in AML was also explored, as well as the mutation situation. Finally, multiple validations of the model genes further improved the rigor of our study.

Current diagnosis and treatment of AML rely on histopathological diagnosis and clear classification. With the application of second-generation sequencing and other technologies, AML has more and more molecular targets, which has led to the rapid development of targeted drugs, such as some small molecular targeted drugs, immune-targeted drugs and all kinds of cutting-edge new drugs in clinical research, so the choice of treatment has become more diversified. Therefore, potential biomarkers can be mined and used to predict patient outcomes and develop new treatment strategies.

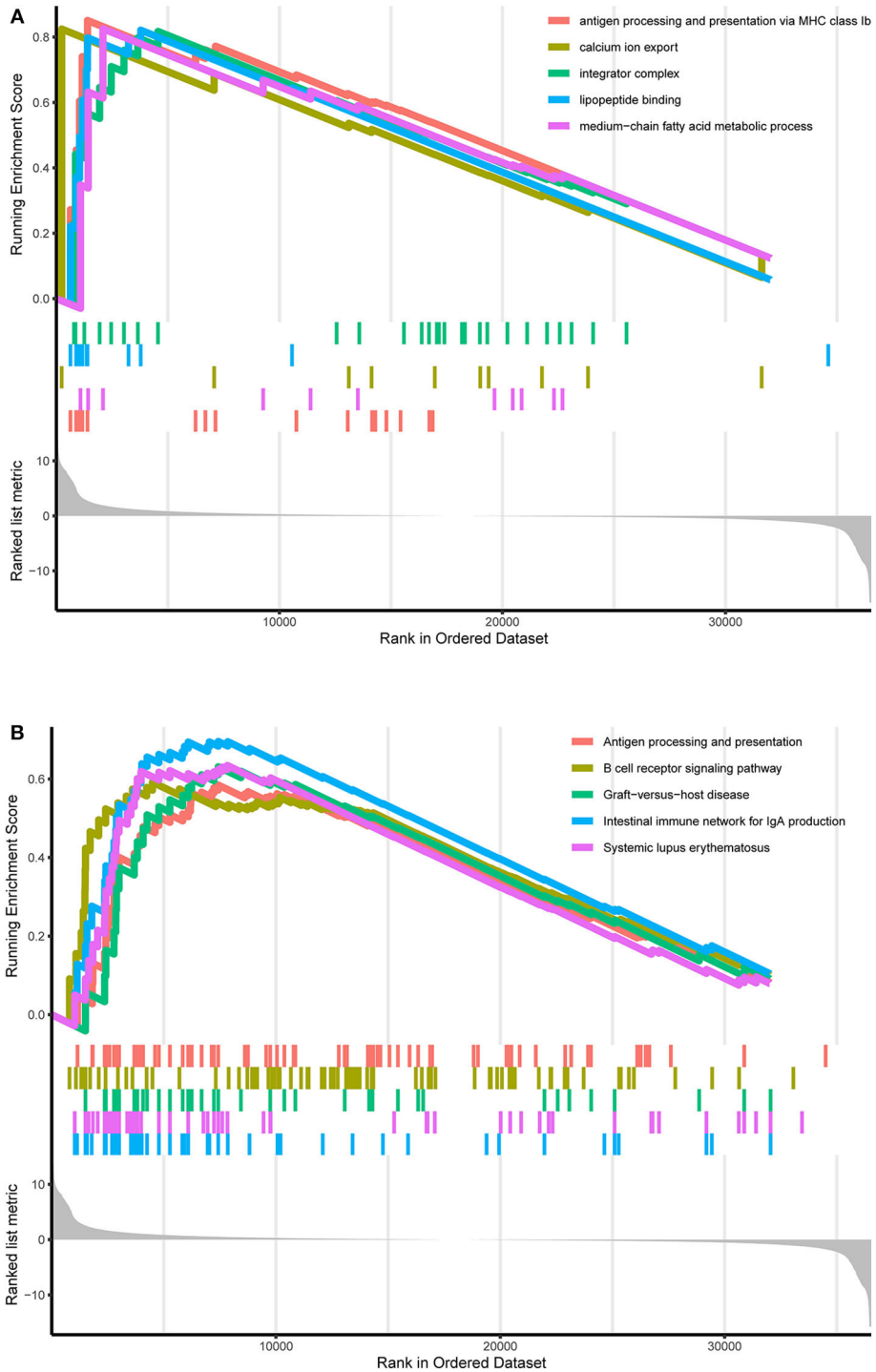

FIGURE 9 | GO and KEGG enrichment results. **(A)** GO enrichment result. At the bottom, the genes are ranked by their logFC values (the closer to the left, the higher the logFC value; the closer to the right, the smaller the logFC value). In the middle section, each color represents the gene on the corresponding GO term. In the upper section, the highest score of each GO term is the GO enrichment score. These GO terms are the top 5 GO terms with the most significant enrichment in the high metabolism group. **(B)** KEGG enrichment result. At the bottom, the genes are ranked by their logFC values (the closer to the left, the higher the logFC value; the closer to the right, the smaller the logFC value). In the middle section, each color represents the gene on the corresponding KEGG pathway. In the upper section, the highest score of each KEGG pathway is the KEGG enrichment score. These KEGG pathways are the top 5 KEGG pathways with the most significant enrichment in the high metabolism group.

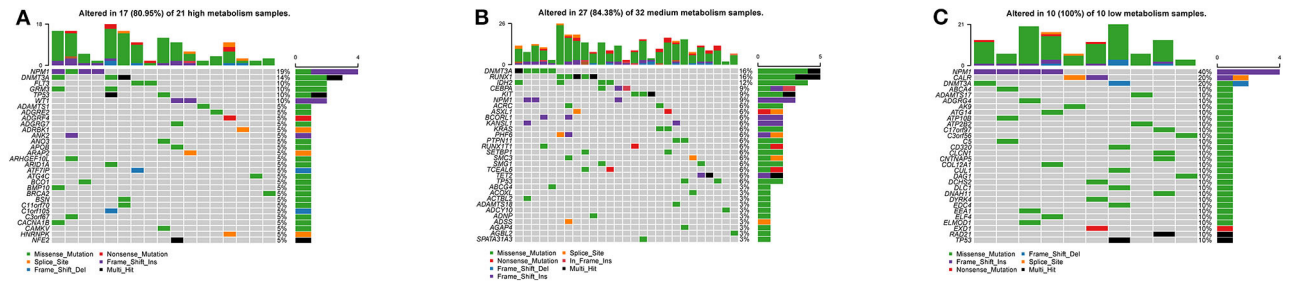

FIGURE 10 | Waterfall plots, visualizing the top 30 mutation genes. The ordinate is the gene name, and the abscissa is the sample name. If the gene is not mutated in the sample, it is shown in gray. If the gene mutates in the sample, it is shown in other colors (different colors represent different mutation types). On the right, the number of samples in which the gene is mutated is counted. At the top, the number of mutations in the sample is counted. **(A)** High metabolism group. **(B)** Medium metabolism group. **(C)** Low metabolism group.

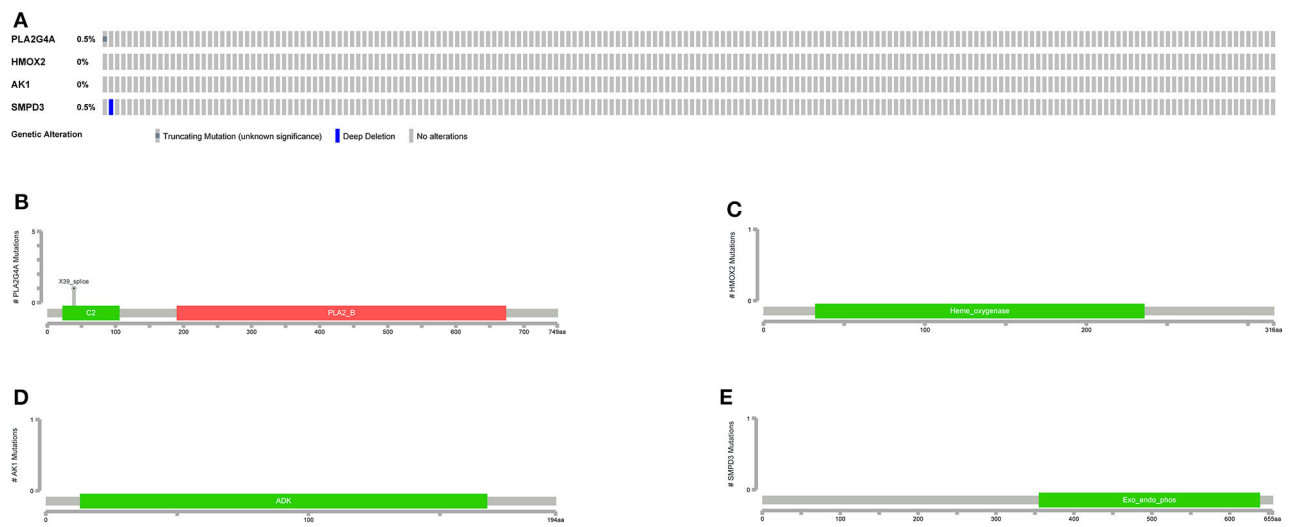

FIGURE 11 | Mutations in model genes. **(A)** The general picture of genetic alteration (mutation and copy number variation). One rectangle represents a sample. The gray rectangle represents no alteration in the sample, and the other colors represent alterations in the sample (different colors represent different alteration types). On the left, the alteration frequency of each gene in all samples is counted. **(B–E)** Mutation patterns in gene domains. The x-coordinate represents the number of amino acid bases, and the y-coordinate represents the number of mutant samples. Different colors represent different domains.

The next-generation sequencing technology developed in recent years employs the whole genome sequencing method, which brings great advantages to multi-group data mining. Prior to this, some studies performed metabolic analysis for some types of cancer and constructed prognostic signatures for cancer prognosis monitoring, including clear cell renal cell carcinoma (35), endometrial adenocarcinoma (36) and glioma (37). This computational bioinformatics analysis can open different perspectives on the clinical application and potential pathological mechanisms of metabolic biomarkers at the macro level. A number of previous studies have proposed transcriptome signatures associated with the prognosis of AML through bioinformatics analysis (38–40). Our study further explored metabolic biomarkers as prognostic predictors and broadened our understanding of the transcriptome clinical significance.

Multiple validations can basically determine that the metabolic model genes we have found are high-risk genes and independent prognostic factors. A recent paper shows that bioinformatic analysis identifies that PLA2G4A has physical interactions with several oncogenic proteins (such as RUVBL2, CAP1, STAT3, and MYCBP) in AML, resulting in multiple effects on the malignant phenotype of AML cells (41). Also, another recent paper reports that AK1 is an independent adverse prognostic factor for AML patients receiving chemotherapy, and patients with high AK1 expression may be recommended for early Allo-HSCT (42). Current studies on the roles of HMOX2 and SMPD3 in AML are largely blank, but they play important roles in other cancers (43, 44), so more preliminary single-gene bioinformatics analyses are needed. Based on previous studies in AML, PLA2G4A and AK1 may be new oncogenes that are more worthy of molecular functional experiments to explore further.

ssGSEA analysis reveals that overall metabolic activity is positively correlated with immune activity in AML. There are two undeniable trends. Firstly, cancer cells tend to have more

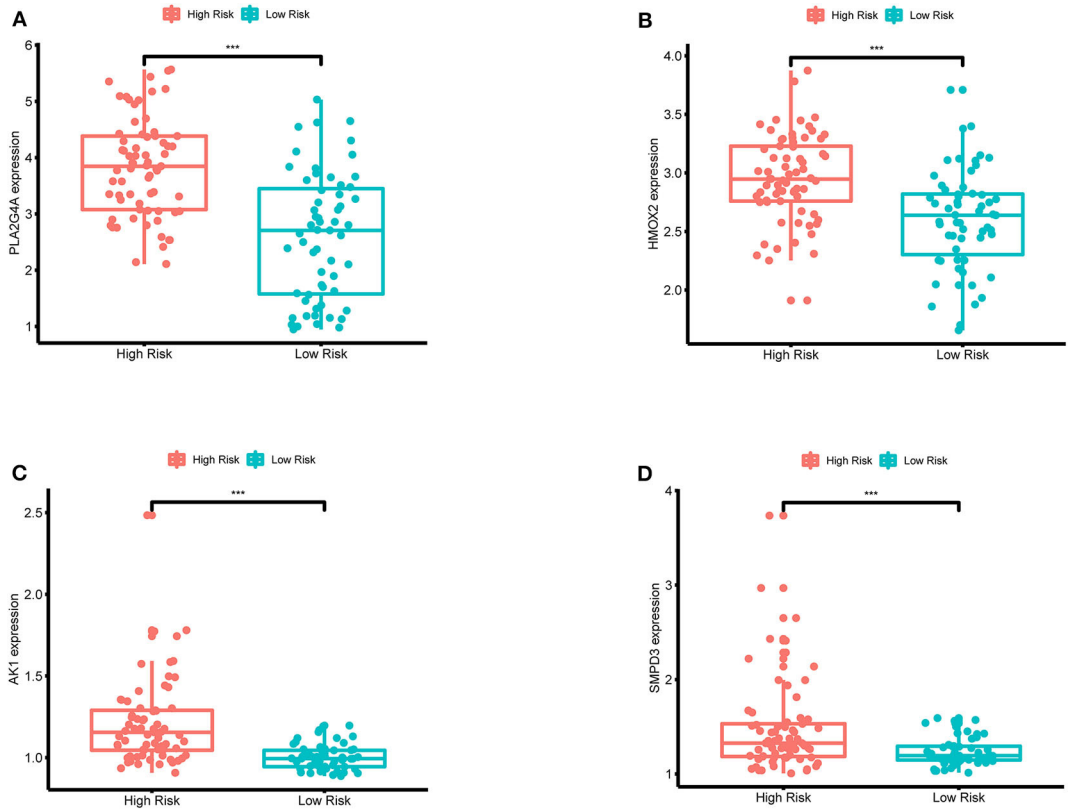

FIGURE 12 | (A–D) Boxplots for the difference validation of model genes. The abscissa is the grouping type, and the ordinate is the expression quantity of the model genes. All model genes are highly expressed in the high-risk group ($P < 0.001$), suggesting that all model genes are high-risk genes. ***, $P < 0.001$.

vigorous metabolism in order to absorb more energy and proliferate malignantly. Secondly, specific antigens of cancer cells can be recognized by the immune system, and the activated immune cells can further play a killing role on the tumor. Based on the survival differences between the three metabolism groups, these two common senses may provide a crude explanation for the high metabolic activity associated with high immune activity in AML. However, the mysteries of the human body are always more complex and wonderful than we think. The crosstalk between different metabolic pathways will seriously affect the tumor microenvironment and eventually impair the immune cells' adaptability and effector function, restricting the success of immunotherapy (17). In tumors, metabolism and immunity do not have a strictly one-way cause-and-effect relationship; More precisely, they always cause and affect each other. In recent years, the most popular studies have always been related to the relationship between metabolic reprogramming and immune escape in tumors (45, 46), including glycolysis with general significance (47). Metabolic reprogramming associated with AML cells, including competition with substrates, large release of bioactive metabolites and overall microenvironmental metabolic remodeling conducive to the survival of immunoregulatory cell subgroups, has become an important mechanism of immune escape in AML and severely hinders the efficacy of immunotherapy (13). Nevertheless, these studies of metabolic changes at the micro level do not contradict our findings, as our conclusion is for the overall metabolic activity of AML at the macro level.

Similarly, GSEA enrichment results in the high metabolism group are mainly related to immunity and metabolism, which is also a supporting basis for the previous ssGSEA analysis results, in which immunity includes antigen processing and presentation, B cell receptor signaling pathway, intestinal immune network for IgA production and lipopeptide binding, presenting pervasive immune response processes; metabolism includes calcium ion export, integrator complex and medium–chain fatty acid metabolic process, also involving a wide range of metabolic processes. As the research object is the overall metabolic activity, GSEA enrichment results are not limited to a single type of biological process. Previous studies have shown that B-cell receptor signal transduction strictly regulates the growth and proliferation of B-cells, and activated B-cells respond to changes in energy and biosynthetic demands and conduct metabolic reprogramming to adapt to metabolic pressure in tumors (48). Another focus of AML research is the intestinal flora. Intestinal flora can keep people healthy by participating in the metabolic process and regulating the immune system, and the reconstruction of healthy intestinal

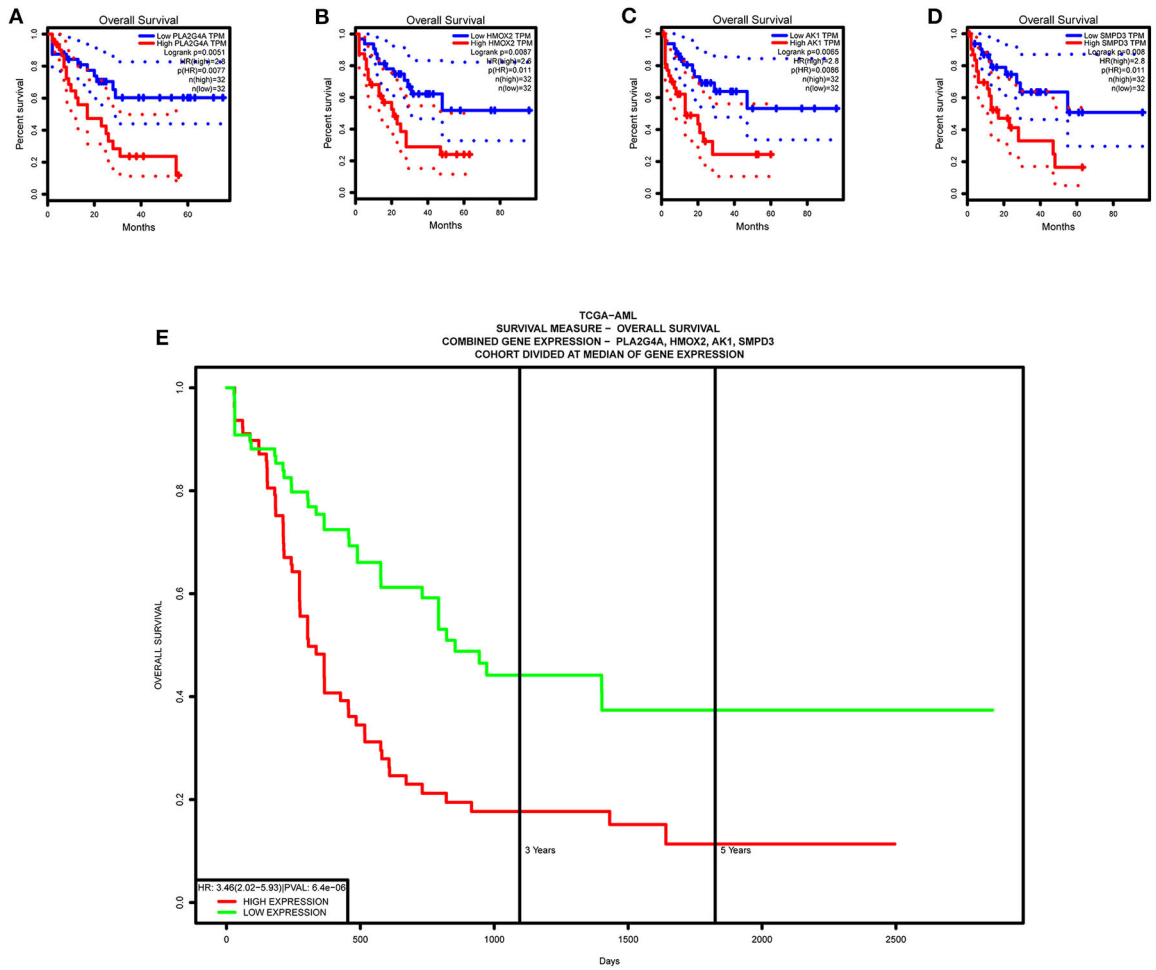

FIGURE 13 | Survival validation of model genes. **(A–D)** Single-gene survival analysis, from the GEPIA website. **(E)** Multiple gene survival analysis, from the PROGgeneV2 online tool.

microflora by adjusting the diet can improve the progression and prognosis of AML (49). Calcium signal transduction plays a key role in several biological processes, such as cell growth, differentiation, metabolism and death, and abnormal calcium signaling and loss of calcium homeostasis can lead to tumor proliferation, angiogenesis and other vital processes of cancer progression (50).

The results of mutation analysis indicate that the mutation frequencies of other genes, especially our model genes, are not high except for the well-known two genes, NPM1 and DNMT3A. Perhaps from the side, our model genes do not depend on structural and functional changes, that is, qualitative changes, to affect the prognosis of AML; they play high-risk roles in the prognosis of AML by the quantitative change. This point can be inferred from our previous discussion on model genes (41–44).

We believe that the data of public platforms we use are appropriately standardized, and multiple validations enhance the rigor of our research. In addition, our conclusion needs to be further validated by wet experiments to be more convincing.

CONCLUSIONS

In conclusion, we adopted a variety of bioinformatics methods to establish an accurate and efficient prognostic model for AML patients, and carried out multiple validations. In addition, the relationship between metabolism and immunity and mutations in AML were explored. These findings provide fundamental insights into the molecular mechanisms and diagnostic markers of AML and contribute to the development of new genomic models for clinical cancer management.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

YZ and SM conceived of and designed the study. YZ, SM, and MW performed the literature search, generated the figures

and tables, and wrote the manuscript. WS and YH supervised the study and reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.578933/full#supplementary-material

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Risk Stratification in Acute Myeloid Leukemia Using CXCR Gene Signatures: A Bioinformatics Analysis

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The role of CXC chemokine receptors in tumors has been an increasingly researched focus in recent years. However, significant prognostic values of CXCR members in acute myeloid leukemia are yet to be explored profoundly. In this study, we firstly made an analysis of the relationship of CXCR family members and AML using samples from TCGA. Our results suggested that transcriptional expressions of CXCRs serve an important role in AML. CXCR transcript expressions, except CXCR1 expression, were significantly increased in AML. It displayed the expression pattern of CXCR members in different AML subtypes according to FAB classification. The correlations of CXCR transcript expression with different genotypes and karyotypes were also present. High CXCR2 expression was found to have a significantly worse prognosis compared with that of low CXCR2 expression, and CXCR2 was also found to be an independent prognostic factor. We also established a CXCR signature to identify high-risk subgroups of patients with AML. It was an independent prognostic factor and could become a powerful method to predict the survival rate of patients.

Keywords: acute myeloid leukemia, CXC chemokine receptor, FAB subtypes, risk stratification, gene signature, prognostic role

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INTRODUCTION

Acute myelocytic leukemia (AML) is a malignant clonal disease of hematopoietic stem cells, which is characterized by a block of leukemic blasts in the bone marrow and other tissues (1). Adult patients could attain complete remission (CR) after standardized chemotherapy treatment; however, the short duration of CR is still an urgent problem for clinician due to a high relapse rate (2). Recent studies suggest that the interaction of the CXC chemokine receptor (CXCR) members and their ligands as well as the complex regulatory network of them take an effect on certain tumor-related processes (3) including activation, proliferation and invasion of leukemic cells (4).

The CXCR family consists of proteins CXCR1–7 (5). CXCR1 and CXCR2 exhibit a high affinity toward a common ligand IL-8. This receptor-ligand interaction induces leukocyte chemotaxis, cell proliferation, and migration and is critical for inflammation and metastasis of tumors (6–8).

The CXC chemokine ligand (CXCL) 9, 10, 11/CXCR3 axis regulates tumor differentiation and activation and the paracrine signal transduction for immune cell development (9, 10). CXCR4, expressed on the surface of hematopoietic stem cells and leukemia blast cells, is activated by CXCL12, and it participates in leukemia cell proliferation and infiltration, as well as in conferring resistance to chemotherapy drugs (11–13). CXCL13 is the ligand for CXCR5, also known as the Burkitt lymphoma receptor 1 (BLR1), and the CXCL13/CXCR5 axis is necessary for B cell homing to lymph node follicles and for the production of immunoglobulin, which coordinates the humoral immunity of the body (14). CXCL16 selectively binds to its sole receptor, CXCR6, and is mainly expressed in natural killer, $CD8^+$ T, and $CD4^+$ T cells. CXCL16/CXCR6 binding plays an essential role in cell adhesion and activation of the immune response (15). CXCR7 is a receptor of CXCL12; however, it is unable to mediate G-protein activation to directly induce cell migration and is considered to be an atypical chemokine receptor (ACKR3). Ligand CXCL-11 or CXCL-12, when bound to CXCR7, can rapidly mediate ligand internalization and degradation (16).

MATERIALS AND METHODS

Original Data

Original expression and clinical data of CXCR family members in AML were down from The Cancer Genome Atlas (TCGA) database (http://Cancegenome.nih.gov/). They were divided into eight subtypes (M0-M7) according to the French-American and British (FAB) classification of AML. The CXCR family mRNA expression level, gender, age, survival status, survival time, risk stratification, chromosome karyotype analysis, gene detection, and other clinical data were extracted from AML patients in TCGA. This study was approved by the Ethics Committee at hospital. Validation of mRNA prognostic power in this study used expression data of TARGET database.

GEPIA

Differences of the expression levels of CXCRs between AML patients and normal tissues were obtained from the GEPIA website (http://gepia.cancer-pku.cn/). the Gene Expression Profiling Interactive Analysis server (GEPIA) is a newly developed interactive web server (17) for analyzing the RNA sequencing expression data of thousands of tumor and normal samples from the TCGA and the GTEx projects using a standard processing pipeline (18). Since AML samples from TCGA database were all tumor samples, GEPIA matches normal samples from the GTEs database.

cBioPortal

cBioPortal (http://www.cbioportal.org) is a multi-functional open network platform, which is a set of tools that propose data mining, data integration, and visualization based on the TCGA database. The intuitive web interface enables the complex cancer genome profiles to be integrated and explored by clinicians (19).

STRING

STRING (https://string-db.org/) is a database of known and predicted protein-protein interactions networks designed for protein functional enrichment analysis. The interactions include direct (physical) and indirect (functional) associations. They stem from computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other (primary) databases. The STRING database currently covers 24,584,628 proteins from 5,090 organisms (20, 21). The Search Tool by choosing Multiple Proteins was used to construct the PPI networks of CXCR member

Statistical Methods

SPSS 19.0 software (SPSS, Chicago, IL) and R language (3.6.3) were used for the statistical analysis. Non-parametric tests were used to compare two or more independent sample sets of data. The patient samples were divided into high- and low- expression groups based on their median expression value. Kaplan-Meier survival analysis and log-rank tests were conducted to analyze the overall survival (OS) using survival R package. Univariate and multivariate analyses were performed on the categorized data to identify independent predictors of outcome. *P* values below 0.05 were considered significant. We chose the candidate genes and they were subjected to multiple proportional risk regression to construct a gene signature as a risk score model. The risk score model included the expression level of mRNA for each optimal prognosis, with weights determined by the estimated regression coefficients of their multivariate Cox regression model, as shown below

$$\text{Risk Score (patient)} = \sum_i \text{Coefficient (mRNAi)} * \text{expression (mRNAi)}$$

RESULTS

Transcript Expression of *CXCRs* in Patients With AML

We compared the transcript expressions of the *CXCRs* in patients with AML with those of normal control subjects, using server GEPIA (22). 173 patients with AML from TCGA and 70 normal subjects from the Genotype-Tissue Expression (GTEx) portal were displayed on the website. Remarkably, all *CXCR* transcripts, except for *CXCR1*, showed a higher expression of mRNA in patients with AML, in comparison to that in normal subjects (**Figure 1A**). Of note, the expression of the *CXCR4* transcript was much higher in the AML group. The decreasing order of expression levels was as follows: *CXCR4*, *CXCR7*, *CXCR2*, *CXCR6*, *CXCR5*, *CXCR3*, and *CXCR1*. Although, *CXCR2* expression is down-regulated in samples from most types of cancers compared to samples from normal subjects, it was found to be upregulated in AML (**Figure 1B**).

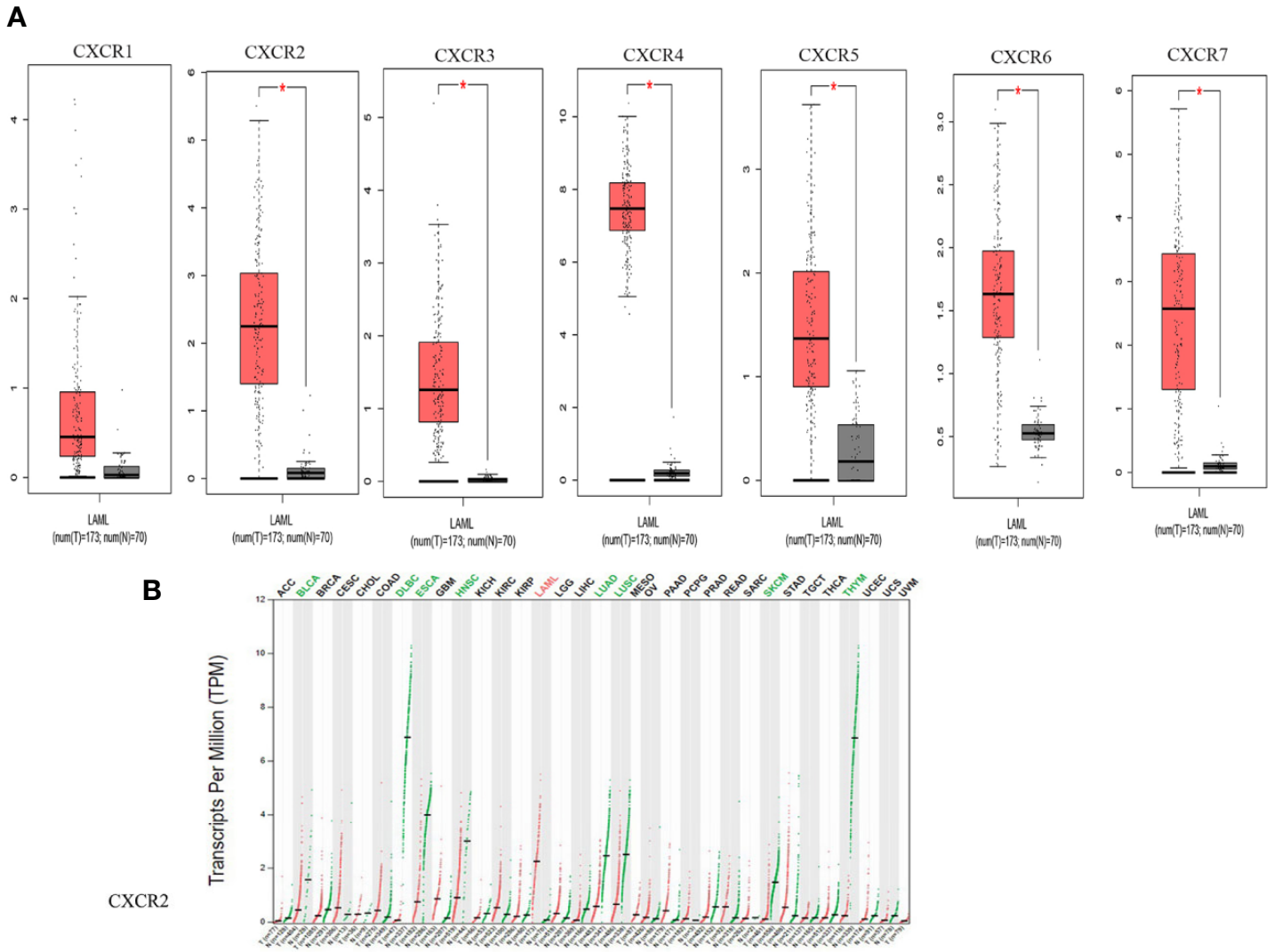

FIGURE 1 | The mRNA expression from GEPIA. **(A)** CXCRs between AML and normal samples. (*P < 0.05). **(B)** CXCR2 expression profile across all tumor samples and paired normal tissues.

Transcript Expression of *CXCRs* in French-American and British (FAB) Subtypes of AML

We analyzed transcript expression of each *CXCR* in the M0 to M5 FAB subtypes of 151 patients with AML; two cases of AML-M6 and one case of AML-M7 were not analyzed. Higher expression of *CXCR1* transcripts was found more frequently in the AML-M5 subtype than in other subtypes ($P = 0.0038$). *CXCR2* showed the highest expression in AML-M5, and the lowest expression was seen in AML-M3 ($P < 0.0001$). *CXCR3* transcripts showed a higher expression in AML-M3, as compared to that in the other subtypes ($P = 0.013$). AML-M4/M5 patients showed an increased expression of *CXCR4* transcripts compared to that in other subtypes, with the highest expression found in AML-M5 ($P = 0.003$). Moreover, no significant difference was found between the expressions of *CXCR5* and *CXCR6* among the different AML subtypes. *CXCR7* showed the highest expression in AML-M0, while the lowest expression was observed in AML-M5 patients ($P = 0.027$) (**Figure 2**).

Correlation of *CXCR* Transcript Expression With Different Genotypes and Karyotypes

To assess the relationship between *CXCR* transcript expression and the mutation status of traditional prognostic genes, we compared transcript expression of *CXCRs* in patients having mutations in *FLT3*, *IDH1*, and *NPM1*, with that in the mutation-negative control group. Patients with a *FLT3* mutation showed a significant decrease in the expression of *CXCR3*, *CXCR5*, and *CXCR6* transcripts compared to that in mutation-negative patients (**Figure 3A**). Similarly, patients with a *NPM1* mutation showed a significant decrease in the expression *CXCR3* and *CXCR6* transcripts compared to that in mutation-negative patients (**Figure 3B**). As for the mutant *IDH1*, the *CXCR4* transcript showed a significant increase in its expression in mutant *IDH1* patients compared to that in the control group (P = 0.032, **Figure 3C**)

We also analyzed the relationship between the *CXCR* transcript expression and cytogenetic karyotype, which is an important parameter for AML prognosis. Patients with the karyocyte t(15;17), t(8;21), and inv(16) were designated the favorable group; patients with normal karyocytes were designated the normal group; patients with the karyocyte 5q-/7q- or complex karyotype were designated the poor group. The results showed that patients of the favorable group had the highest *CXCR7* expression (P = 0.004), those of the normal group had the highest *CXCR2* transcript expression (P = 0.023), and those of the poor group had the highest *CXCR6* transcript expression (P = 0.019, **Figure 3D**).

Correlation of *CXCR* Transcript Expression With AML Risk Stratification

To assess the diagnostic and prognostic significance of *CXCRs* in AML, the correlation between expression of each *CXCR* transcript with AML risk stratification was estimated. The patients were divided into two groups according to their risk stratification. The intermediate-risk and high-risk patients were combined into one medium/high-risk group, and the expression of each *CXCR* transcript in the medium/high-risk group was compared to that of the low-risk group. The expression of *CXCR1*, *CXCR2*, and *CXCR6* transcripts was significantly higher in patients of the medium/high-risk group than that in patients of the low risk group ($P < 0.05$) (**Figure 4**).

Clinical Characteristics of 122 TCGA Samples of Patients With AML

The information of 122 patients with AML who are eligible for survival analysis was retrieved from TCGA database. The screening criteria for these patients as follows: 1) complete survival data present; 2) survival time longer than 30 days; 3) RNA-sequencing expression data present. The relevant clinical characteristics are shown in **Table 1**.

The Role of *CXCRs* in AML Prognosis

The prognostic value of *CXCRs* was estimated by analyzing the survival data and *CXCR* transcript levels in 122 patients from TCGA database. For each *CXCR* transcript, patients were divided into either the high- or low-expression group according to the median value of *CXCR* transcript expression. The median values of each CXCR mRNA expression (FPKM) were shown as follows: CXCR1 0.287, CXCR2 1.772, CXCR3 1.052, CXCR4 92.207, CXCR5 0.065, CXCR6 1.137, and CXCR7 2.400. Using Kaplan-Meier survival analysis, it was found that the survival time of the *CXCR2* high-expression group was significantly shorter than that of the low-expression group ($P = 0.029$). However, no difference in survival times was observed between the high- and low-expression groups for other *CXCR* transcripts (**Figure 5**).

Furthermore, univariate and multivariate COX analyses were used to determine whether *CXCR2* was an independent prognostic factor. Gender, age, white blood cell (WBC) counts, blast cell percentage, risk stratification, and *FLT3* and *IDH1* gene mutation were also analyzed to identify the independent factors affecting patient survival. Age, risk stratification, and *CXCR2* expression were found to be the independent factors affecting patient survival (**Table 2**).

Prognostic Value of *CXCR* Signature in AML

Given the increasing focus on the prognostic value of gene signatures, and the prognostic significance of *CXCR* transcripts in AML, the potential of *CXCR* signatures as a risk score model for AML was explored.

We chose *CXCR1–7* as the candidate genes; these were subjected to multiple proportional risk regression analysis to construct a risk score model based on gene signatures. The risk score model was constructed as follows:

$$Risk\ score = 0.65 \times (CXCR1exp.) + 2.01 \times (CXCR2exp.) + 1.89 \times (CXCR3exp.) + 0.65 \times (CXCR6exp.)$$

where exp. represents expression levels.

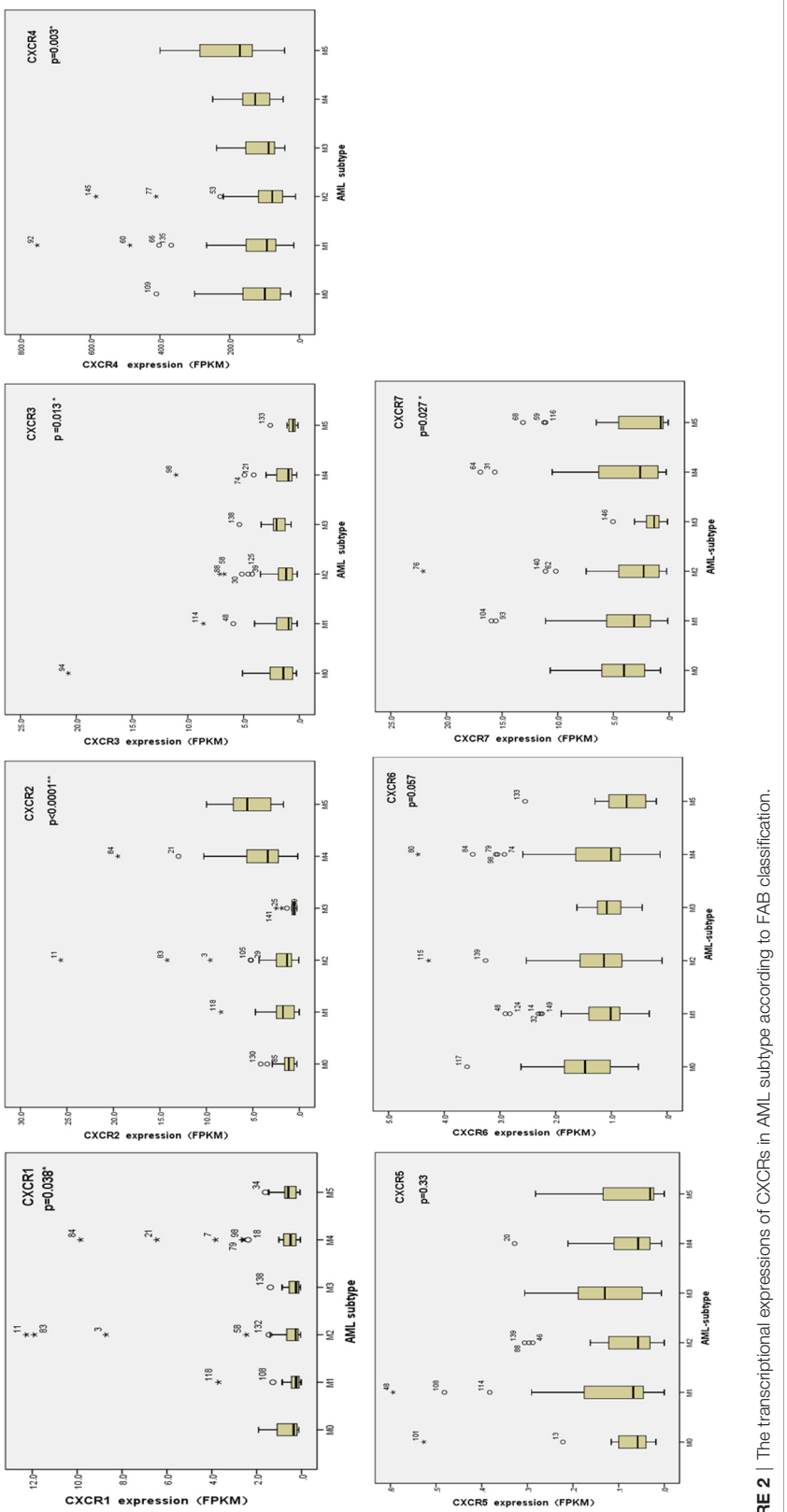

FIGURE 2 | The transcriptional expressions of CXCRs in AML subtype according to FAB classification.

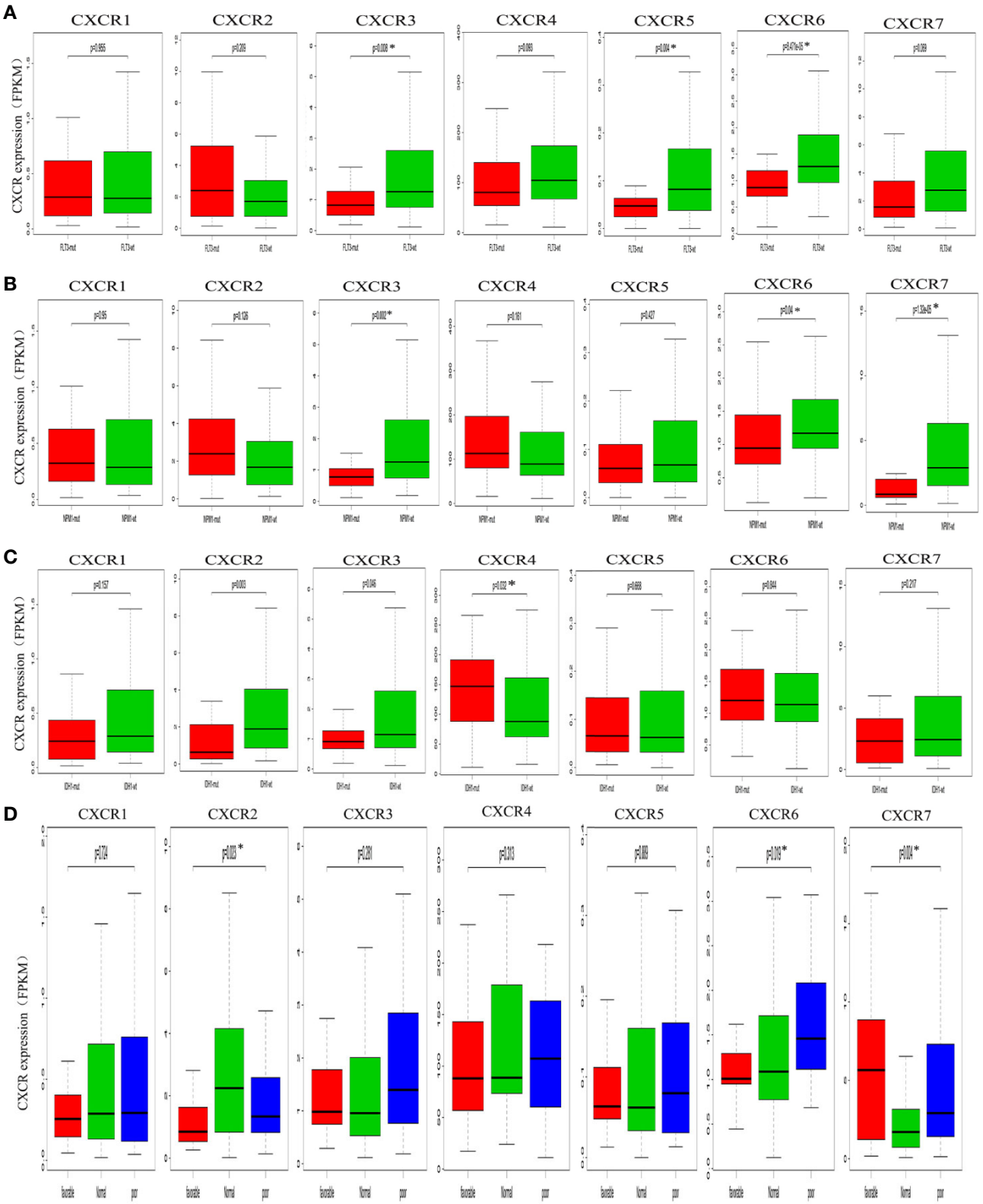

FIGURE 3 | Analysis of prognostic gene expressions between the mutant group and the wild-type group. **(A)** *FLT3* mutation. **(B)** *NPM1* mutation. **(C)** *IDH1* mutation. **(D)** cytogenetic karyotype.

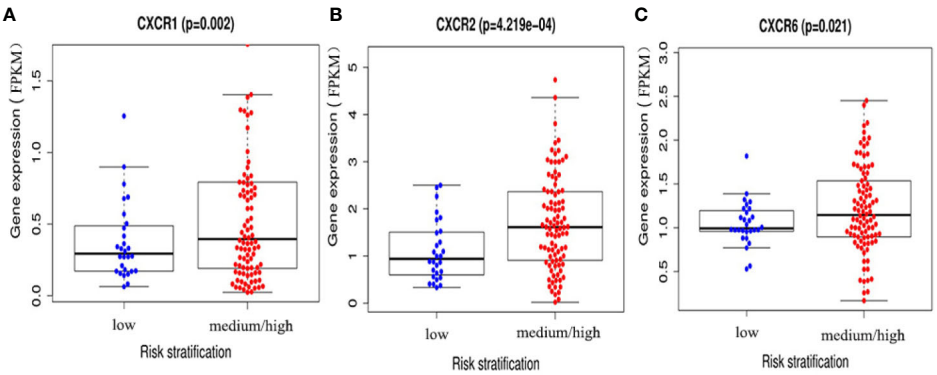

FIGURE 4 | The expressions of **(A)** CXCR1, **(B)** CXCR2, and **(C)** CXCR6 between AML low and medium-high risk group.

TABLE 1 | Clinical characteristics of 122 AML patients enrolled from the TCGA database for survival analysis.

Characteristic	Category	Cases
Age	<60 years	77
	>= 60 years	45
Gender	Male	66
	Female	56
FAB-subtype	M0	12
	M1	28
	M2	29
	M3	12
	M4	26
	M5	12
	M6	2
	M7	1
karyotype	Normal/mediate	65
	Poor	22
	Favorable	24
	NA	11
FLT3 gene	Mutant	34
	WT	84
	NA	4
NPM1 gene	Mutant	29
	WT	89
	NA	4
IDH1 gene	Mutant	22
	WT	100
Survival state	Alive	51
	Dead	71

The forest plot for the gene signature model and its concordance index (C-index = 0.66, P = 4.216e-4) are shown in **Figure 6**. The patients were divided into high- and low-risk groups according to the median risk score, and it was found that the high-risk group displayed a significant reduction in OS compared to that of the low-risk group (P = 2.28e–04, **Figure 7A**). The heatmap of the core genes and risk-score of patients with AML in the two groups are shown in **Figures 7B, C**. Additionally, receiver operating characteristic (ROC) curves were built to evaluate the performance of the *CXCR* signature risk model at three time points (**Figure 8**). The AUC corresponding to 1, 2, and 3 years was 0.719, 0.705, and 0.684, using the Kaplan-Meier method.

To determine if *CXCR* signature risk score could be used as an independent prognostic predictor for OS, we performed univariate and multivariate Cox regression analysis. The factors included were similar to those used in the previous regression analysis. The results showed that *CXCR* signature risk-score was an independent prognostic predictor of OS. Additionally, we found that age greater than 60 years and high-risk of AML (in the traditional risk stratification) were also independent predictors of a shortened OS (**Table 3**).

Mutation Status of *CXCRs* and Their Correlation With Each Other

We used the c-Bioportal online tool to estimate the correlation between the *CXCRs* (RNA Seq V2 RSEM); the Pearson's correlations are listed in **Table 4**. The data showed that *CXCR1* expression was associated with *CXCR2* (r = 0.523). The expression of *CXCR3* was found to be closely correlated to that of *CXCR5* (r = 0.538) and *CXCR6* (r = 0.412).

Protein-Protein Interaction Network and Functions of *CXCR* and Their Neighboring Genes

The STRING database was used to cluster and construct a network of *CXCRs* and the 47 most frequently altered neighboring genes (**Figure 9A**). Cytoscape software was used to screen the hub genes from the constructed network. *CXCR4* and *IL-10* were found to be important hub genes of the network (**Figure 9B**).

Gene ontology (GO) enrichment analysis was used to predict the functional role of target host genes, and classify them into functional categories, including biological process (BP), cellular component (CC), and molecular function (MF). GO:0070098 (chemokine-mediated signaling pathway), GO:0030595 (leukocyte chemotaxis), GO:0060326 (cell chemotaxis), GO:0019221 (cytokine-mediated signaling pathway), and GO:0007166 (cell surface receptor signal pathway) were found to be significantly regulated by *CXCRs* and were classified under BP (**Figure 10A**) (23). These are well-known signal pathways involving cytokines and their receptors. Several important GO

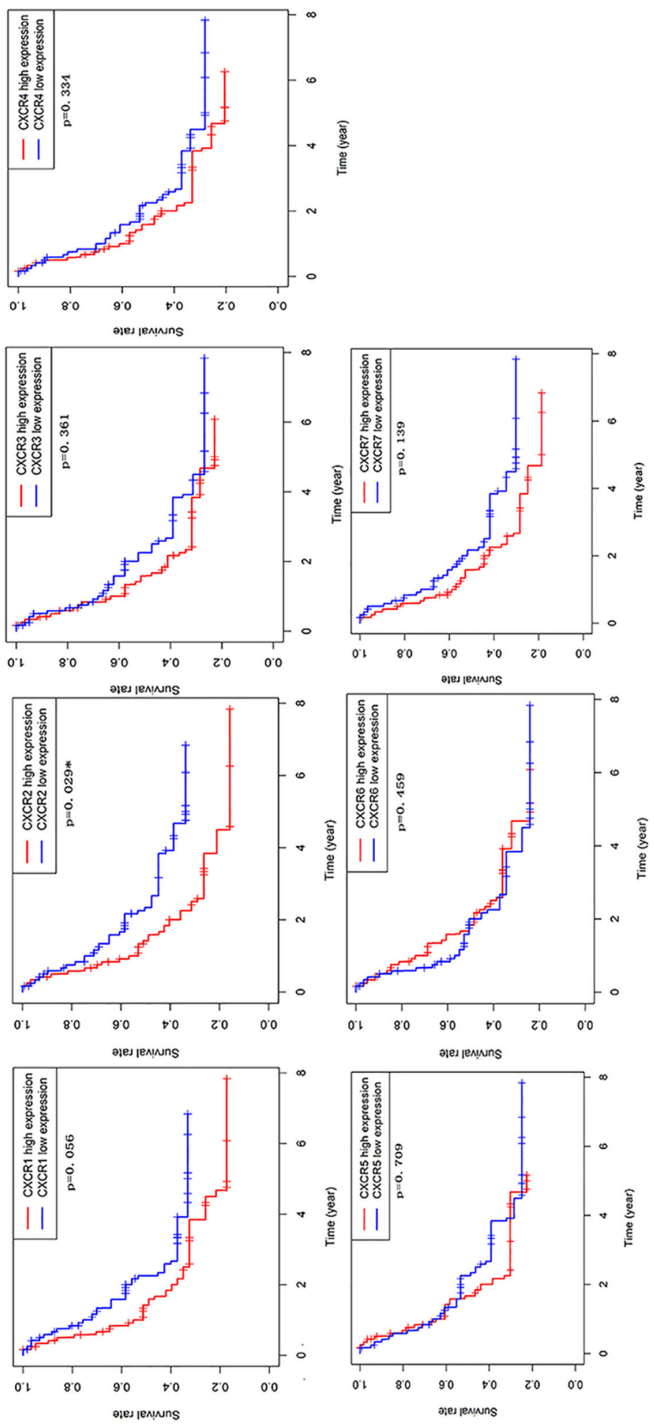

FIGURE 5 | The prognostic values of CXCR family members in AML patients.

TABLE 2 | Univariate and multivariate overall survival (OS) analysis of CXCR2 in AML patients.

parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	P value
Gender(male/female)	0.937	0.586–1.497	0.785	1.183	0.705–1.987	0.522
Age(<60 y/>=60y)	2.268	1.415–3.636	0.00066**	2.153	1.264–3.665	0.0047*
WBC(<2G/L/>=2G/L)	1.245	0.779–1.991	0.359	1.028	0.579–1.824	0.924
Blast (%)	1.005	0.996–1.015	0.261	1.004	0.994–1.015	0.390
Risk stratification (low/median/high)	1.822	1.274–2.606	0.0010*	1.540	1.029–2.305	0.036*
FLT3 (mut/wt)	1.392	0.840–2.305	0.199	1.286	0.719–2.297	0.396
IDH1(mut/wt)	0.832	0.446–1.549	0.561	0.771	0.392–1.517	0.451
CXCR2 (high/low)	1.226	1.067–1.408	0.0039*	1.185	1.005–1.398	0.043*

*$p > 0.001$, **$P < 0.001$ ($P < 0.05$)

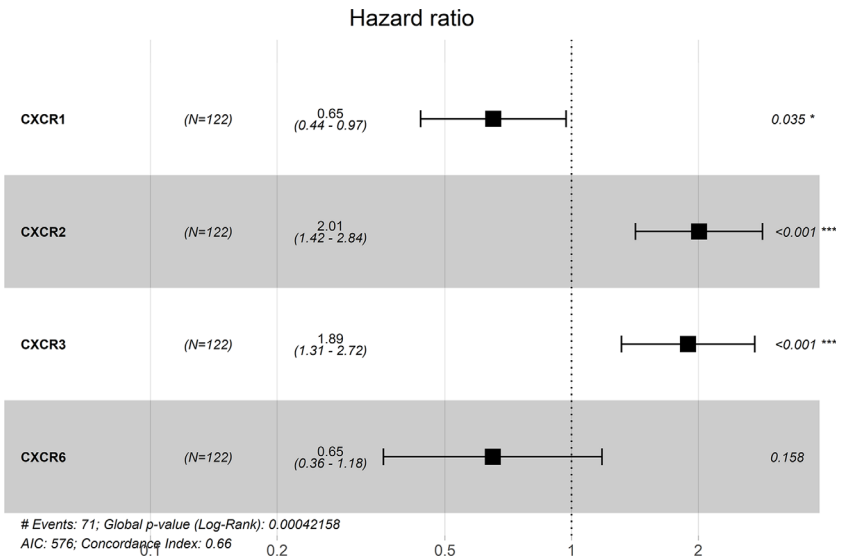

FIGURE 6 | The forest plot for the gene signature model and Concordance Index.

enriched BP pathways are associated with anti-inflammatory and immune responses. The top 20 GO terms for CCs and MF are shown in **Figures 10B, C**.

We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) to functionally analyze the *CXCRs*, as well as frequently altered neighboring genes. The 20 most significantly *CXCR*-enriched pathways, using KEGG analysis in patients with AML, are shown in **Figure 10D** ($P < 0.0001$). KEGG pathway analysis showed that these *CXCR* genes are most significantly enriched in cytokine-cytokine receptor interaction, chemokine signaling pathway, IL-17 signaling pathway, TNF signaling pathway and NF-kappa B signaling, and Toll-like receptor signaling pathway, which is related to the process of tumor invasion and metastasis.

Validation in the TARGET Database

The prognostic value of *CXCR2* and the *CXCR* signature risk score model were verified using the TARGET database. We selected 295 patients with AML from the TARGET database to analyze the prognostic value of *CXCR* genes and evaluate the risk score model. The median value of CXCR2 expression (counts) in this cohort was 65. It was observed that *CXCR2* could act as an independent prognostic factor ($P = 0.030$, **Figure 11A**). We used the risk score model [Risk score = 0.65×(*CXCR1*exp.) + 2.01 × (*CXCR2*exp.) + 1.89 × (*CXCR3*exp.) + 0.65 × (*CXCR6*exp.)] established to calculate the risk score of each patient. The median value of CXCR signature risk score was 940.872. The risk score model could accurately categorize the patients in the TARGET database into high- and low-risk groups according to the median value ($P = 4.079e\text{-}02$, **Figure 11B**).

DISCUSSION

The *CXCR* family members have been reported to play important roles in different types of cancers (24, 25). Although *CXCRs* are known to be involved in tumorigenesis and prognosis of numerous cancers, there is a lack of detailed bioinformatic

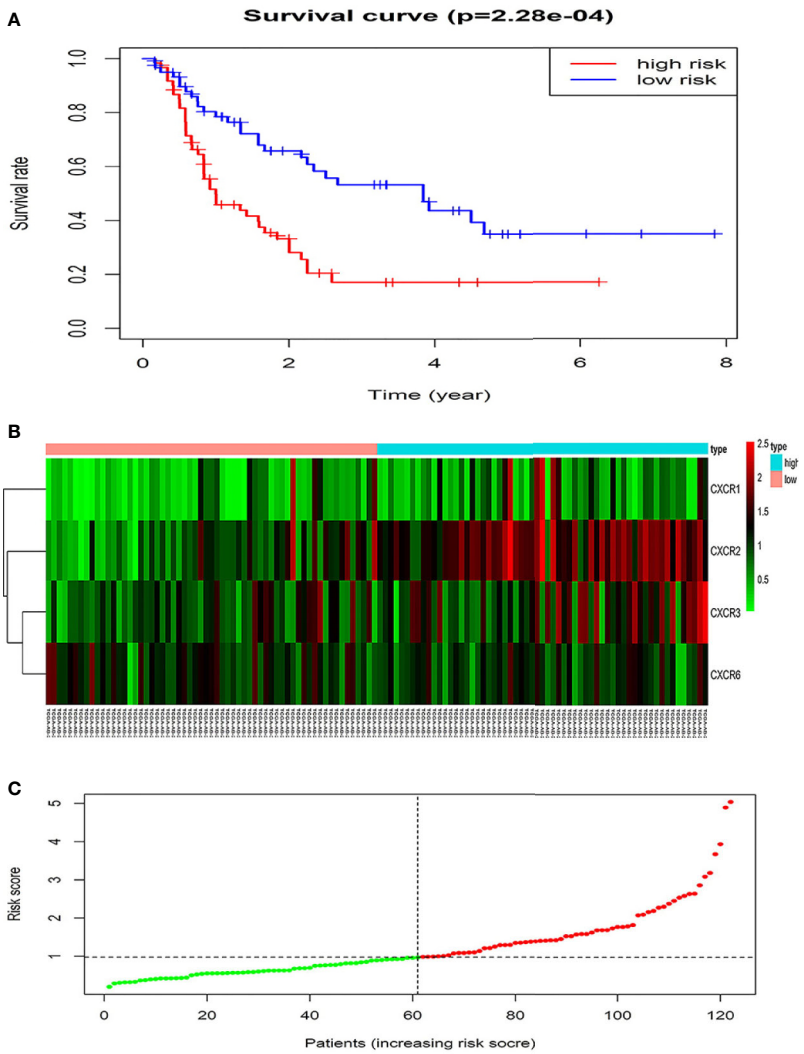

FIGURE 7 | The prognostic values of CXCR signature. **(A)** The survival curves of low- and high- risk group. **(B)** The heatmap of gene signature. **(C)** The risk score of AML patients in two groups.

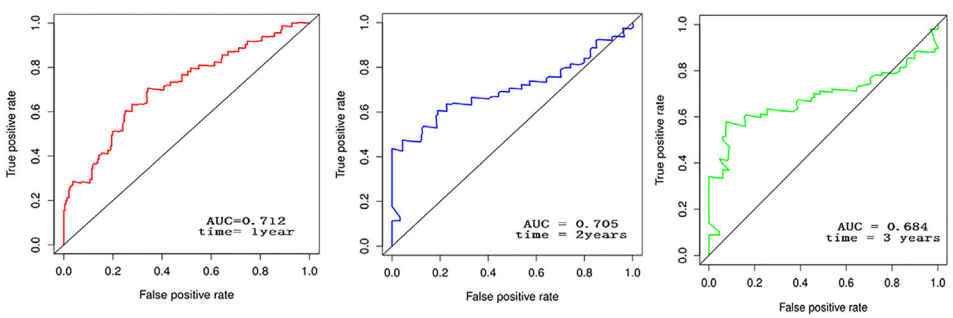

FIGURE 8 | The AUC corresponding to 1, 2, and 3 years of CXCR signature using K-M plot.

TABLE 3 | Univariate and multivariate overall survival (OS) analysis of CXCR signature in AML patients.

parameter	univariate analysis			multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
Gender(male/female)	0.950	0.594–1.518	0.830	1.148	0.683–1.930	0.602
Age(<60y/>=60y)	2.240	1.397–3.592	0.00081**	1.882	1.086–2.261	0.024*
WBC(<2G/L/>=2G/L)	1.262	0.789–2.017	0.331	1.065	0.622–1.825	0.817
Blast (%)	1.005	0.996–1.015	0.245	1.005	0.994–1.015	0.382
Risk stratification (low/median/high)	1.856	1.296–2.659	0.00073**	1.551	1.012–2.374	0.043*
FLT3 (mut/wt)	1.416	0.855–2.346	0.176	1.434	0.797–2.576	0.228
IDH1(mut/wt)	0.861	0.462–1.604	0.637	0.949	0.485–1.858	0.879
Riskscore (high/low)	2.041	1.626–2.564	8.23E-10**	1.944	1.516–2.493	1.62E-07**

*$p > 0.001$, **$P < 0.001$ ($P<0.05$).

TABLE 4 | The correlations of CXCRs with each other in AML (RNA Seq V2 RSEM).

	CXCR1	CXCR2	CXCR3	CXCR4	CXCR5	CXCR6	CXCR7
CXCR1	1	**0.523***	**0.258**	0.0974	**0.274**	0.0902	–0.0495
CXCR2	**0.523***	1	–0.0566	**0.142**	3.61E-03	–0.0548	–0.0678
CXCR3	**0.258**	–0.0566	1	–0.025	**0.538***	**0.412***	**0.199**
CXCR4	0.0974	**0.142**	–0.052	1	0.0838	–0.0397	–0.159
CXCR5	**0.274**	3.61E-03	**0.538***	0.0838	**1**	**0.367**	–0.0532
CXCR6	0.0902	–0.0548	**0.412***	–0.0397	**0.367**	1	0.155
CXCR7	–0.0495	–0.0678	**0.199**	–0.159	–0.0532	0.155	1

Red indicates positive correlation, blue indicates negative correlation, and the depth of color indicates the degree of correlation.

analyses of *CXCRs* in AML. This is the first study to investigate the transcript levels and prognostic value of *CXCRs* in AML. Additionally, we evaluated *CXCR* signatures as predictors of the risk of developing AML.

Our analysis revealed that all *CXCRs*, except *CXCR1*, were up-regulated in patients with AML compared to normal subjects. Notably, *CXCR2* expression was found to significantly increase in AML but is known to be no apparent increase or even decreased in most of other tumors, indicating a critical role of *CXCR2* in AML.

We also showed that *CXCR4* expression was increased AML-M4 and AML-M5. This is consistent with the findings of our previous study, wherein we demonstrated that *CXCR4* expression is higher in AML-M4 and AML-M5 than in subtypes M2 and M3 (26). Further, we found that the transcript expression of other *CXCRs* was significantly different in different AML subtypes. Very few studies have reported the correlation between the FAB subtypes and transcript levels of *CXCRs*; however, this correlation needs to be investigated further.

We assessed the correlation between *CXCR* transcript expression and the cytogenetic and molecular profile of patients with AML. Previous studies have reported a correlation between *CXCR4* and *FLT3* in AML. Rombouts et al. showed that *CXCR4* showed a higher expression in *FLT3*-ITD mutant group than in the *FLT3*-wild-type group (27). This finding is consistent with our previous study as well (26). These results showed that patients with a *FLT3* mutation had lower expression of *CXCR3*, *CXCR5*, and *CXCR6* transcripts than that in mutation-negative patients, suggesting a possible anti-cancer effect of *CXCR3/5/6* in AML. However, we have not been able to demonstrate this.

Konoplev et al. evaluated the relationship between *CXCR4* and *NPM1* in a group of 117 untreated adults with AML and found that a mutation in NPM1 is not correlated with CXCR4 or pCXCR4 protein levels, suggesting that *CXCR4* and *NPM* pathways play independent roles in adult AML (28). In contrast, Mannelli et al. demonstrated that the *NPM1*-mutated cases of AML displayed a significantly higher expression of *CXCR4* compared to *NPM1*-wild-type cases (29). With respect to studies investigating the relationship between *CXCR4* and other prognostic genes, Kuo et al. found that *CXCR4* expression was significantly higher in *CEBPA* wild-type patients than in *CEBPA* mutant patients; thus, *CEBPA* has been speculated to affect the *CXCR4* expression (30). *In vitro* studies first detected the increased expression of endogenous *CXCR4* in AML cell lines and demonstrated that *CEBPA* mutants modulated CXCR4 activation. Currently, the correlation between *CXCR4/CXCR7* expression and *IDH1* has been reported in human glioma (31, 32) but has not been reported in AML yet. In this study, we found that *CXCR4* transcript expression in mutant *IDH1* patients was significantly higher than that in *IDH1* wild-type patients. This helps to better understand the relationship between *CXCR4* and *IDH1* in AML. Despite these results, there are very few reports on the correlation between other *CXCRs* and AML prognostic genes; hence, this further investigation is required.

Risk stratification for AML the molecular and genetic characteristics takes into account, as well as age, WBC count, and several other factors. Correlation analysis of *CXCRs* with AML risk stratification revealed that *CXCR1*, *CXCR2*, and *CXCR6* transcript expressions were higher in the medium/high-risk group than those in the low-risk group. To determine

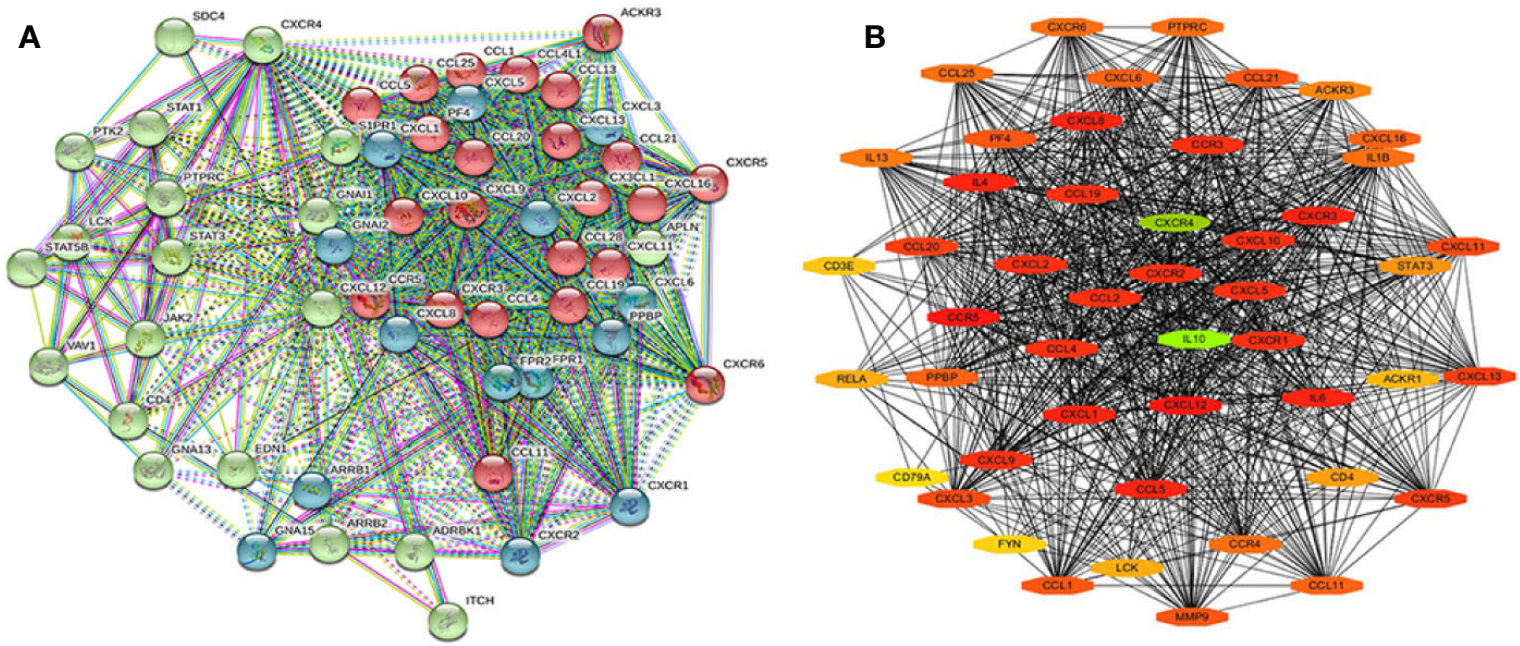

FIGURE 9 | The PPI of CXCR family members and their neighbor genes **(A)** the PPI network analyzed by String. **(B)** The hub genes analyzed by Cytoscape.

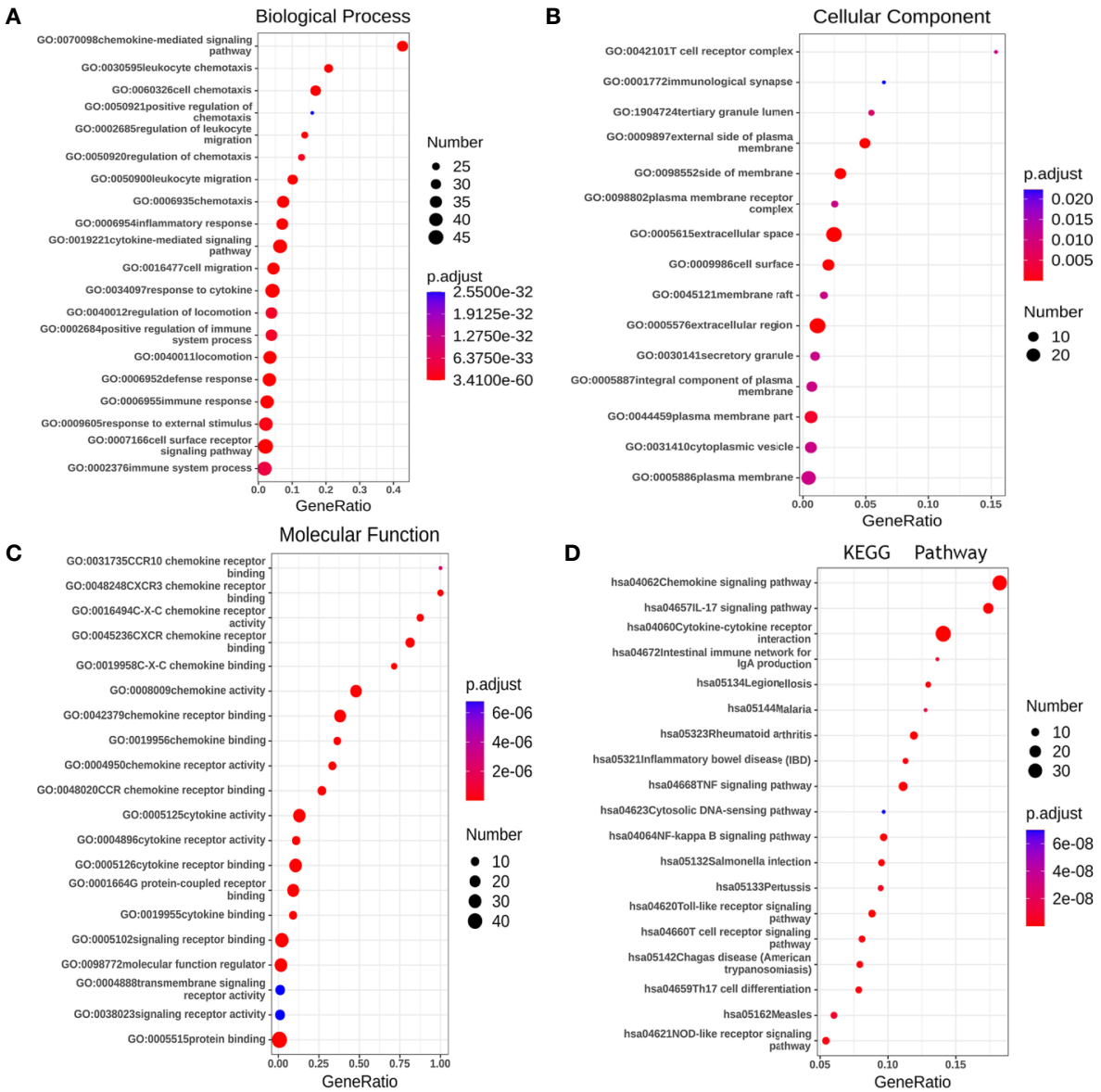

FIGURE 10 | The functions of CXCRs and neighborhood genes predicted by GO and KEGG analysis by String. **(A)** Biological Process. **(B)** Cellular Component. **(C)** Molecular Function. **(D)** KEGG enrichment.

the prognostic value of *CXCRs*, we analyzed the OS of patients with AML. OS analysis showed that *CXCR2* was a predictor of shorter OS and was independent of other classical factors such as age, gender, WBC counts, blast cell percentage, risk stratification, and *FLT3*/*IDH1* gene mutation.

The CXCR1/CXCR2 pathway is the most widely studied pathway in tumors. CXCL1, 2, 3, and 8 are angiogenic chemokines that bind to receptor CXCR2, with the highest affinity exhibited by CXCL1 (33). While studying the effects and ligands of CXCR1/CXCR2, Cheng et al. found that CXCL8 is up-regulated in co-cultures of bone marrow mesenchymal cells and leukemia cell lines compared with CXCL8 expression in single cultures (34). Inhibition of CXCL8/CXCR2 binding can lead to cell cycle arrest in G0/G1 phase, inhibition of AML cell proliferation, inhibition of AKT phosphorylation, and cell apoptosis. Elevated expression of *IL-8* and *CXCR2* was found in stem and progenitor cells isolated from AML and myelodysplastic syndrome (MDS) patients. Schinke et al. demonstrated that an increased *CXCR2* expression was a poor prognostic factor for AML and MDS, further reinforcing the prominent role of the IL-8/CXCR2 axis in AML and MDS (35). Hao et al. investigated the relationship between CXCL1/CXCL2, clinical characteristics, and prognosis in patients with AML (36). Expression of *CXCL1* and *CXCL2* was detected using quantitative-PCR in bone marrow samples from 160 patients with *de novo* AML. Furthermore, CR was assessed and event-free

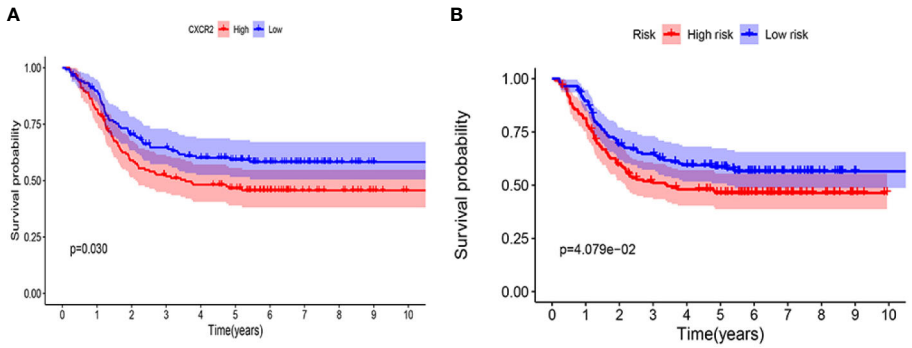

FIGURE 11 | Kaplan-Meier survival curves in validation cohort using TARGET database. **(A)** CXCR2. **(B)** CXCR gene signature.

survival (EFS) and overall survival were calculated. An increased expression of *CXCL2* was found to correlate with the monochromosomal karyotype (P = 0.001). However, *CXCL2* was negatively correlated with EFS (P = 0.069) and overall survival (P = 0.055), although this was not statistically significant.

The role of CXCR4 in the development of AML has become an attractive subject of investigation in the recent years. CXCL12 binds and activates its homologous receptor CXCR4 in the microenvironment of the bone marrow to mediate the transport of leukemia cells, while keeping in close contact with stromal cells and the extracellular matrix to generate growth-promoting and anti-apoptotic signals. Increased *CXCR4* expression in AML cells is associated with poor prognosis (37). Rombouts et al. found that patients with increased *CXCR4* expression in the $CD34^+$ subset of cells had significantly reduced chances of survival and a higher probability of relapse, suggesting that the Stromal Cell-Derived Factor-1(SDF-1)/CXCR4 axis may influence responsiveness to therapy and contribute to an unfavorable prognosis of AML (27). Additionally, we have previously suggested that *CXCR4* is an independent prognostic factor for AML (26). The results of a previous report suggested that *CXCR4* is expressed in a subset of patients with AML and is associated with poor prognosis, and *CXCR4* expression appears to be an independent prognostic factor for reduced survival in a heterogeneous group of patients with AML (38). However, we did not find any obvious prognostic value of *CXCR4*. This may be due to insufficient samples in the dataset or erroneous methods of detection for *CXCR4* expression.

CXCR7 is a newly discovered receptor of CXCL12 that co-exists with CXCR4, and CXCR4/CXCR7 has been declared to play a role in AML (39). Kim et al. investigated the expression levels and function of *CXCR7* in AML cells *in vitro*, and showed that *CXCR7* was involved in the regulation of autocrine CXCL12 in AML cells (40). Faaij et al. analyzed the expression of chemokine receptors in children with skin involvement of AML and showed that skin-residing AML cells intracellularly expressed CXCR4 and CXCR7 in 90.9% of evaluated cases (41). These results suggested that chemokine receptor interactions are involved in the homing and retention of AML blast cells in the skin.

The role of the CXCR3, CXCR5, and CXCR6 axes in AML is not well known. The therapeutic potential of CXCR3-CXCL9/10/11 and CXCR5/CXCL13 signaling pathways in tumors has been investigated previously (42, 43). Abnormally high levels of CXCR5 and CXCL13 in the serum of lymphoma patients are significantly associated with poor prognosis (44). Similarly, abnormally high expression levels of *CXCR6* and *CXCL16* are found to be closely related to tumor proliferation and metastasis, and have been reported to be associated with human ovarian cancer (45) and the metastasis of liver cancer cells (46).

The traditional approach of predicting the prognosis of a disease using one single gene cannot compete with the predictive value of several different potential biomarkers; thus, there is a growing concern over the prognostic value of gene signatures. Yu et al. had reported a *CXCR* signature for gastric cancer, and a Kaplan-Meier survival analysis revealed that the OS significantly reduced in the high-risk *CXCR* signature group compared with that of the low-risk *CXCR* signature group (47). In this study, we established a *CXCR* signature for AML, which comprehensively determined the patient's prognosis based on the expression of each *CXCR* transcript and its prognosis coefficient. Using the Kaplan-Meier survival analysis for the *CXCR* signature, median OS in the low risk-group was shown to be significantly higher than that of the high-risk group. Additionally, it is known AML prognosis increasingly relies on detection of multigene Panel Testing. We did a bioinformatics analysis and verified it in different database. Experimental validation need to be further studied.

In conclusion, we analyzed the expression, clinical features, and prognostic value of *CXCRs* in AML. Our results suggested that transcriptional expressions of *CXCRs* serve an important role in AML. *CXCR* transcript expressions, except *CXCR1* expression, were significantly increased in AML. High *CXCR2* expression was found to have a significantly worse prognosis compared with that of low *CXCR2* expression, and *CXCR2* was also found to be an independent prognostic factor. We established a *CXCR* signature to identify high-risk subgroups of patients with AML. These results might help improve the treatment and prognosis of patients with AML.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: TCGA database (http://cancergenome.nih.gov/).

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Academic Committee of Wuhan Union Hospital, Huazhong University of Science and technology. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

The concept of the study was designed by CL, SH, and JY. WX, YH, and CL made the literature research. JZ provided data acquisition. XC and CL made the statistical analysis. CL and JY wrote the manuscript with inputs from all co-authors. The first version was prepared by CL. WX, YH, and JZ made a revision. JY and SH conceived and coordinated the project. SH was the guarantor of integrity of entire study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.584766/full#supplementary-material

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Haploidentical Stem Cell Transplantation With a Novel Conditioning Regimen in Older Patients: A Prospective Single-Arm Phase 2 Study

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Objective: Haploidentical stem cell transplantation (haplo-SCT) has demonstrated encouraging results in younger patients. There is also an increasing need for haplo-SCT in older patients. However, the high risk of treatment-related mortality (TRM) in older patients is still a major concern. We aimed to investigate a novel conditioning regimen (Bu/Flu/Cy/ATG) followed by haplo-SCT in older patients.

Method: This prospective, single-arm clinical trial was performed at Peking University Institute of Hematology, China. Patients were enrolled if they were (1) diagnosed with acute leukemia or MDS; (2) without MSD and MUD, and with HID available; and (3) age ≥55 years. The Bu/Flu/Cy/ATG regimen consisted of the following agents: Ara-C (2 g/m^2/day, injected i.v.) on days-10 and−9; BU (9.6 mg/kg, injected i.v. in 12 doses) on days-8,−7, and−6; Flu (30 mg/m^2/day, injected i.v.) from day−6 to day−2; Cy (1 g/m^2/day, injected i.v.) on days−5 and−4; semustine (250 mg/m^2, orally) on day-3 and antithymocyte globulin (ATG) [2.5 mg/kg/day, rabbit, SangStat (Lyon, France)] on days−5,−4,−3, and−2. The primary endpoint was 1-year TRM.

Results: From April 1, 2018 to April 10, 2020, a total of 50 patients were enrolled. All patients achieved neutrophil engraftment with complete donor chimerism. The cumulative incidence of grade 2-4 aGVHD at day-100 was 22.0%. The cumulative incidences of CMV viremia and EBV viremia on day 100 were 68.0 and 20.0%, respectively. The cumulative incidence of TRM at 1-year was 23.3%. and the cumulative incidence of relapse (CIR) at 1 year after transplantation was 16.5%. The overall survival (OS) and leukemia-free survival (LFS) at 1 year were 63.5 and 60.2%, respectively. The outcomes were also comparable with patients who received Bu/Cy/ATG regimen using a propensity score matching method.

Conclusions: In conclusion, this study suggested that a novel conditioning regimen followed by haploidentical HSCT might be a promising option for older patients. The study was registered as a clinical trial.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier: NCT03412409.

Keywords: haploidentical transplant, elderly, anti-thymocyte globulin, cyclophosphamide, fludarabine

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-SCT) remains the main and often only curative method for most of the hematological malignancies (1, 2). Historically, allo-SCT was performed from a matched sibling donor (MSD) with a myeloablative conditioning regimen. Allo-SCT was mainly performed in relatively younger patients since the risk of treatment related mortality (TRM) in older patients who received myeloablative conditioning regimen transplant procedures was relatively higher. However, most adult hematological malignancies develop in older individuals, with a median age of ∼60–70 years. The prognosis for older patients treated with conventional chemotherapy is very poor, and allo-SCT still provides superior long-term survival compared with conventional chemotherapy in older patients (3). Therefore, there is an increasing need for allo-SCT in older patients and an unmet need to decrease the TRM of allo-SCT to further improve long-term survival.

Among the strategies to reduce TRM, using a regimen with less toxicity is an important option. Since much of the regimen-related toxicity(RRT) (such as the cardiac toxicity, veno-occlusive disease and hemorrhagic cystitis) in myeloablative BuCy regimens is believed to be due to Cy (4), fludarabine has been widely used to reduce the RRT in the setting of MSD and unrelated donor(URD) (5–8). Besides, the development of a reduced intensity conditioning (RIC) regimen has led to improved outcomes of allo-SCT in older patients. These advances have led to the increased use of allo-SCT in older patients (9) in the setting of MSD or URD.

Unfortunately, MSDs are only available for less than one-quarter of patients. Haploidentical donors are a good alternative for those without MSDs (10) with several advantages. First, haploidentical donors (HID) can be available in ∼100% of cases and can be used very quickly. Second, the sibling donor for the older patients is usually older and therefore at higher risk of comorbidities and complications during donation. Furthermore, there is evidence that younger haploidentical donors may be superior to older matched sibling donors for older patients. Therefore, haploidentical stem cell transplantation for older patients is a promising option when MSD or URD is unavailable.

Currently, haplo-SCT is mainly performed in younger patients due to the concerns about increased TRM in older patients. Therefore, there is an unmet need to decrease the TRM and further improve the overall survival (OS) of haplo-SCT in older patients. We previously attempted a fludarabine-based regimen (replacing of Cy 3.6 g/m^2 with fludarabine 150 mg/m^2) in patients with older age or comorbidities. The OS, leukemia-free survival (LFS), TRM and cumulative incidence of relapse (CIR) were quite encouraging and seemed to be comparable to those receiving the Bu/Cy/ATG regimen. However, the incidence of primary graft failure (PGF) was higher in the Bu/Flu/ATG group than in the Bu/Cy/ATG group (17.6 vs. 3.0%, $P = 0.04$) (11). Several other studies also reported a possible association of Flu with poor engraftment. Therefore, in the current study, we used a novel conditioning regimen with a combination of reduced doses of Cy and fludarabine to ensure engraftment and tolerance. In this prospective study, we demonstrated encouraging results using the Bu/Flu/Cy/ATG regimen for older patients receiving haplo-SCT.

PATIENTS AND METHODS

Study Design

This prospective, single-arm clinical trial was performed at the Peking University Institute of Hematology, China. This study was approved by the ethics committee of Peking University People's Hospital. All patients provided written informed consent before enrollment. The study was registered as a clinical trial (ClinicalTrials.gov: NCT03412409). The inclusion criteria were as follows: patients aged ≥ 55 years and diagnosed with acute leukemia or myelodysplastic syndrome (MDS) with a HID available. The exclusion criteria were (1) with pregnancy; (2) active infection without control; (3) enrollment in other clinical trials within 1 month (4) other contradiction to HSCT; and (5) no informed consent. The exit criteria were (1) refusal to comply with the clinical trial protocol; (2) treatment termination due to the patients' decision; and (3) investigator determination that continuing the clinical trial was inappropriate.

The estimation of sample size was performed using PASS software (version 11.0.7; PASS, NCSS, LLC). The incidence of 1-year TRM was 40% according to our previous study (11). This study proposed reducing the incidence of 1-year TRM from 40 to 20%. The settings included $\alpha = 0.05$, two sided, $\beta = 0.20$, and a sample size of 43 was determined to achieve the statistical power (power = 80%). Meanwhile, considering sample loss due to follow-up, death due to early treatment and other unexpected factors, an additional 10% sample size was required; thus, the final sample size was ∼49 patients. No interim analysis was planned.

Patients who received the myeloablative Bu/Cy/ATG conditioning regimen during the current study period were compared with patients who received Bu/ Flu/Cy/ATG in this study. Finally, 100 patients were selected as control group using propensity score matching method with a 1:2 ratio.

Conditioning Regimens and Other Transplant Procedures

The Bu/Flu/Cy/ATG regimen consisted of the following agents: Ara-C (2 g/m^2/day, injected i.v.) on days-10 and−9; BU (9.6 mg/kg, injected i.v. in 12 doses) on days-8,−7, and−6; Flu (30 mg/m^2/day, injected i.v.) from day−6 to day−2; Cy (1,000 mg/m^2/day, injected i.v.) on days−5 and−4; semustine (250 mg/m^2, orally) on day-3 and antithymocyte globulin (ATG) [10 mg/kg, rabbit, SangStat (Lyon, France)] on days−5,−4,−3, and -2.

Donor Selection, Mobilization, and Stem Cell Collection

The donor selection rule was based on previous literature. Patients were eligible for haploidentical HSCT if a MSD or URD was unavailable. All recipients received G-CSF-mobilized bone marrow and peripheral blood-derived stem cells.

Supportive Therapy

All patients received cyclosporine (CsA), mycophenolate mofetil (MMF) and short-term methotrexate (MTX) for graft-vs.-host disease (GVHD) prophylaxis as previously described (1, 12). The dosage of CsA was 2.5 mg/kg per day, i.v., from day 9 before transplantation until bowel function returned to normal. Then, the patient was switched to oral CsA. MMF was administered orally, at 0.5 g every 12 h, from day 9 before transplantation until hematopoietic recovery after transplantation. The dosage of MTX was 15 mg/m^2, administered i.v. on day 1, and 10 mg/m^2 on days 3, 6, and 11 after transplantation. Prophylaxis and treatment of CMV infection after alloHSCT were performed as described previously (13, 14). Ganciclovir was administered during conditioning (through day−2) and acyclovir (400 mg twice a day) was given until the discontinuation of all immunosuppressive agents. Patients also received prophylactic drugs to prevent infection by fungi.

Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were monitored twice per week via real-time PCR. Hematopoietic chimerism was evaluated by fluorescence *in situ* hybridization (FISH) (for sex-mismatched pairs), or the short tandem repeat technique. The HCT-comorbidity index (HCT-CI) score was evaluated according to the literature (15).

Study Endpoints

The primary endpoint was 1-year TRM, which was defined as any cause of death other than relapse. The secondary endpoints were engraftment, acute graft-vs.-host disease (aGVHD), chronic GVHD (cGVHD), CMV reactivation, EBV reactivation, relapse, OS, and LFS.

Neutrophil engraftment was defined as the first of 3 consecutive days with an absolute neutrophil count $\geq 0.5 \times 10^9$/l. Platelet engraftment was defined as the first of 7 consecutive days with a platelet count $\geq 20 \times 10^9$/l without transfusion dependence. Complete donor chimerism was defined as the presence of at least 95% donor hematopoietic cells. Primary graft failure (PGF) was defined as the failure to surpass a threshold absolute neutrophil count of 0.5×10^9/L by day 28 after transplantation. Acute graft-vs.-host disease (aGVHD) and chronic GVHD (cGVHD) were graded according to previous criteria (16, 17). OS was defined as the time from the first day of transplantation to death as a result of any cause. Follow-up data for survival were censored when the patient was last verified to be alive. LFS was defined as the time from transplantation to relapse, disease progression, or death, whichever occurred first. Relapse was defined as the reappearance of blasts in the blood, BM (>5%) or any extramedullary site after complete remission (CR).

Statistical Analysis

Continuous variables are represented as the median, and categorical variables are represented as the percentages. OS, and LFS were estimated using the Kaplan-Meier method. The cumulative incidences of engraftment, GVHD, TRM and relapse were estimated using a competing risks model. Death and relapse without developing GVHD were treated as competing events for

TABLE 1 | Patient characteristics.

Variables	Numbers
Age, median (range)	59 (55–64)
Male (%)	29 (58%)
Disease	
AML	19
CR1/CR2/>=CR3/NR	12/1/2/0
ALL	10
CR1/CR2/>=CR3/NR	7/1/2/0
MDS	21
Low/int-1/int-2/high	0/4/12/5
Disease risk index (low/high)	26/24
HCT-CI	
HCT-CI 0	24
HCT-CI 1-2	21
HCT-CI>=3	5
Donor sex, male (%)	28 (56%)
Donor age, median (range)	32 (23–60)
Donor-recipient ABO blood type	
Match/	27
major /minor/major+minor mismatch	6/14/3
Donor-recipient CMV sero status	
+/+	49
+/–	0
–/+	1
–/–	0
Missing	0
Donor-recipient EBV sero status	
+/+	49
+/–	0
–/+	1
–/–	0
Missing	0
Graft	0
BM+PB	42
PB	8
MNC	8.63 (5.81–18.19)
CD34	2.30 (0.50–7.39)

AML, Acute myeloid leukemia; ALL, Acute lymphocyte leukemia; MDS, Myelodysplastic syndromes; CR, Complete remission; HCT-CI, Hematopoietic stem cell transplantation Comorbidity Index; CMV, Cytomegalovirus; EBV, Epstein-Barr virus; BM, bone marrow; PB, peripheral blood; MNC, mononuclear cells.

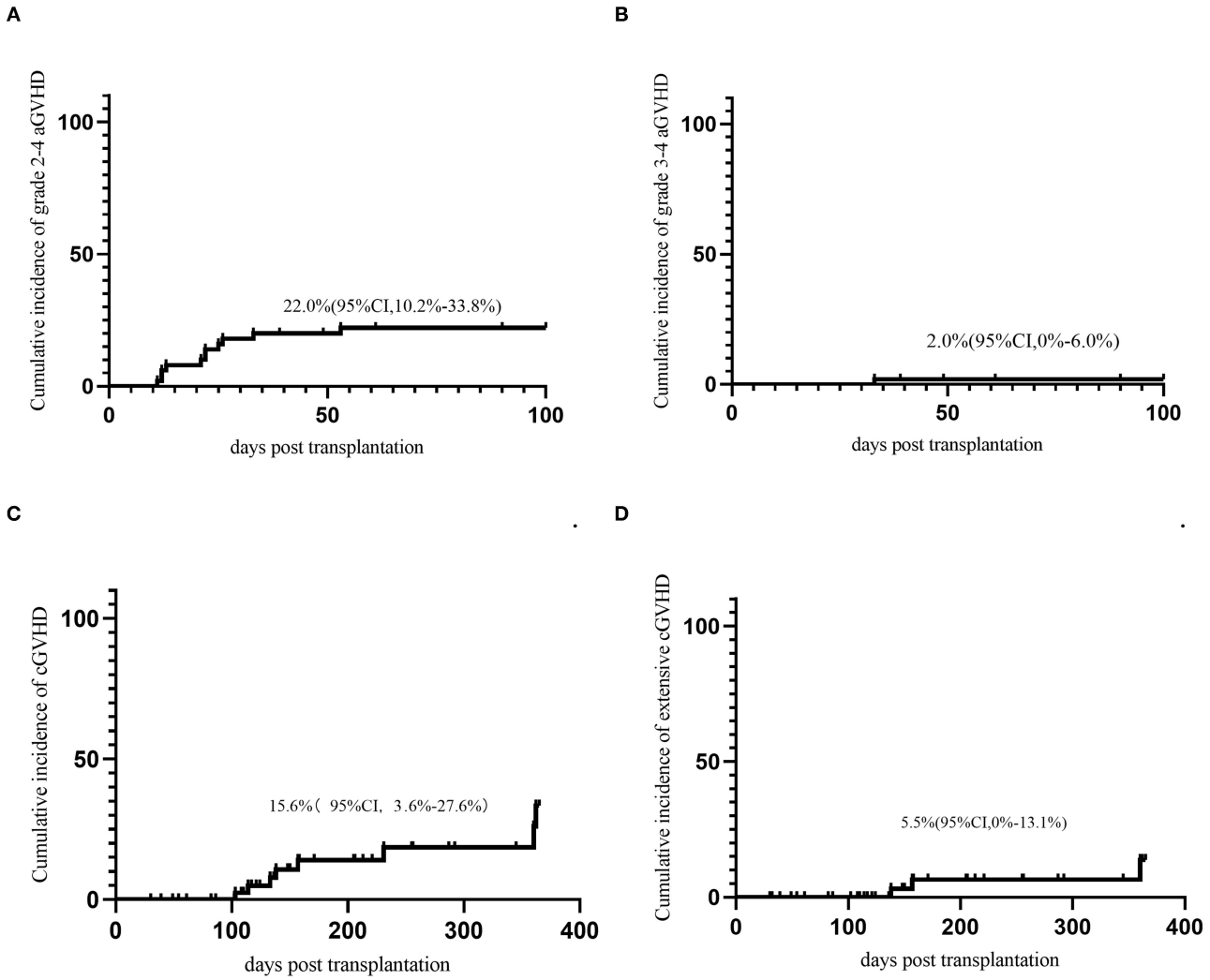

FIGURE 1 | GVHD. **(A)** Acute GVHD grade 2-4; **(B)** Acute GVHD grade 3-4; **(C)** Chronic GVHD; **(D)** extensive chronic GVHD.

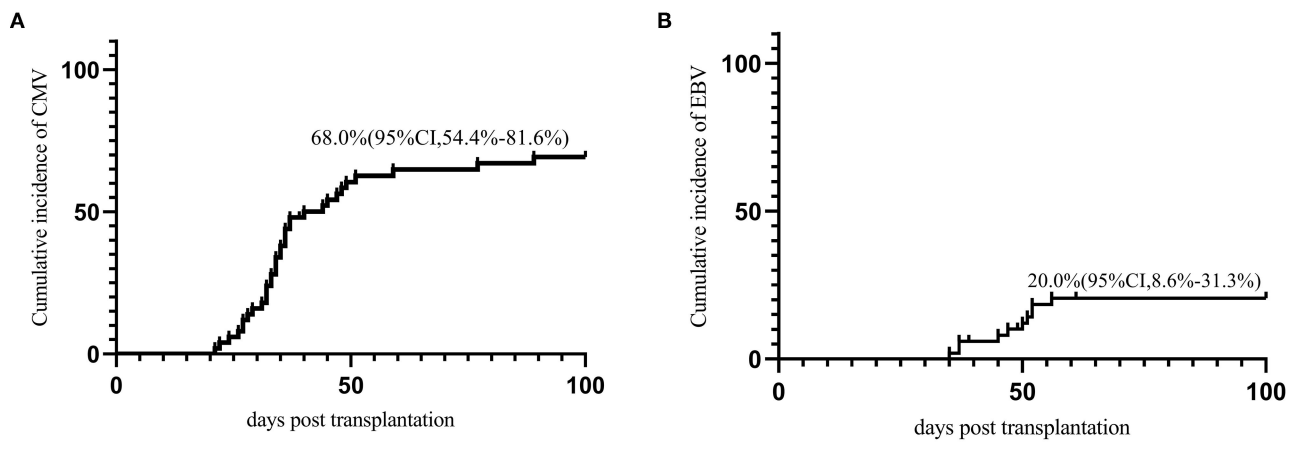

FIGURE 2 | Virus infection **(A)** CMV; **(B)** EBV.

GVHD, whereas relapse and TRM were treated as competing events. A $p < 0.05$ for a two-sided test was considered significant. The multivariate Cox proportional model and survival analysis were calculated with SPSS software (SPSS 22.0, Chicago, IL, USA). The cumulative incidence was calculated with R statistical software, version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

Logistic regression was used for the propensity score calculation from the following variables: age, sex, and disease. A 1:2 matching by propensity score was performed by using the nearest neighbor matching method with a caliper width fixed at 0.2. Propensity score matching was carried out using SPSS software (SPSS 22.0, Chicago, IL, USA).

RESULTS

Patients and Donor Characteristics

From April 1, 2018 to April 10, 2020, a total of 50 patients were enrolled. In addition, the last follow-up date was May 10, 2020. The median age was 59 (55–64) years old. Among the 50 patients, 5 (10%) had HCT-CI $\geq$3. Most patients (94%) received a combination of bone marrow and peripheral blood as grafts. Details of the patients, disease, transplant characteristics and donor characteristics are shown in **Table 1**.

Transplant Outcomes

TRM

The cumulative incidence of TRM on day 30, day 100, and 1-year was 0%, 6.4% (95% CI 0–37.5%,) and 23.3% (95% CI, 9.1–37.5%), respectively. There were 14 deaths on study, five primarily due to relapse, two due organ failure and seven due to infection.

Engraftment

All the 50 patients achieved neutrophil engraftment with a median time of 13.5 (9–23) days. All patients achieved complete donor chimerism. Forty-two patients (42/50, 84.0%) achieved platelet engraftment with a median time of 13 (8–47) days after HSCT. The cumulative incidence of platelet engraftment at 100 days was 84.2% (95% CI, 73.4–95.0%). Five patients did not achieve platelet engraftment due to a TRM and other 3 patients did not achieve platelet engraftment till the last follow-up.

GVHD

Seventeen (30.0%) patients developed aGVHD within 100 days. The median onset time of grade 2-4 aGVHD was 18.4(11–94) days after transplantation. The cumulative incidence of grade 2-4 aGVHD and grade 3-4 aGVD at day-100 was 22.0% (95% CI, 10.2–33.8%) and 2.0% (95% CI 0–6%), respectively (**Figures 1A,B**). Nine patients developed cGVHD and 4 patients developed extensive cGVHD. The cumulative incidence of cGVHD and extensive cGVHD at 1 year were 15.6% (95% CI, 3.6–27.6%) and 5.5% (95% CI 0–13.1%) (**Figures 1C,D**).

Infection

Thirty-four (68.0%) patients developed CMV viremia within 100 days post HSCT. The median onset time of CMV viremia was 34.5 (21–89) days post-HSCT. The cumulative incidence of CMV viremia on day-100 post-HSCT was 68.0% (95% CI, 54.4–81.6%) (**Figure 2A**). There are two patients developed CMV associated pneumonia. Twelve (24.0%) patients developed EBV reactivation with a median time of 50 (35–101) days post-HSCT. The cumulative incidence of EBV reactivation at day-100 was 20.0% (95% CI, 8.6–31.3%) (**Figure 2B**). No patients developed PTLD.

Relapse and Survival

The last follow-up date was May 10, 2020. The median follow-up time for survivors was 231.5 (30–754) days after transplantation. Thirty-six patients were still alive at the last follow-up. The cumulative incidence of relapse (CIR) at 1 year after transplantation was 16.5% (95% CI, 1.9–31.1%). The OS and LFS at 1 year were 63.5% (95% CI, 46.5–80.5%) and 60.2% (95% CI, 42.4–78.0%), respectively (**Figure 3**). No factors were identified to be associated with TRM, LFS, OS, or CIR in multivariate analysis.

Comparison With the Bu/Cy/ATG Regimen

The patient characteristics of the two groups of patients are summarized in **Supplementary Table 1**. The baseline variables were balanced in the two groups except for age, which was younger in the Bu/Cy/ATG group. There were 2 cases of graft failure in the Bu/Cy/ATG group, while there was no graft failure in the Bu/Flu/Cy/ATG group. Compared with the Bu/Cy/ATG group, there were no differences in CMV, EBV, aGVHD, CIR, TRM, LFS, or OS (**Supplementary Table 2**).

DISCUSSION

In the present study, we demonstrated that the novel conditioning regimen of Bu/Flu/Cy/ATG followed by haplo-SCT was a feasible option in older patients. This novel haplo-SCT protocol had acceptable TRM with a good profile of engraftment, GVHD, relapse and survival. This encouraging result suggests that haplo-SCT is a potentially promising method for older patients.

Older age historically has been considered a relative contraindication for haplo-SCT, as physicians are often concerned about high TRM in the setting of MAC (18, 19). To address this problem, various reduced-intensity conditioning regimens have emerged. Fludarabine has been widely used to reduce RRT in the setting of MSD and URD (5–8). A fludarabine-based regimen was also used in most reports using haplo-SCT in older patients. Retrospective reports of haplo-SCT using a non-myeloablative regimen (NMA) with either T cell depletion or post-cyclophosphamide (PTCY) have demonstrated that the 2-year TRM is ∼27–35%. However, none of the previous retrospective studies reporting haplo-SCT in older patient aimed to evaluate the safety and efficacy of a uniform regimen, and the regimens in the previous retrospective studies were heterogeneous. The incidence of 1-year TRM was 40% according to our previous study (11). With the objective of reducing the TRM, we used a novel MAC regimen consisting of low doses of Cy and fludarabine to reduce the incidence of 1-year TRM from 40 to 20% in our current study. Actuarially, the 1-y TRM was 23% in the present study which seemed to be

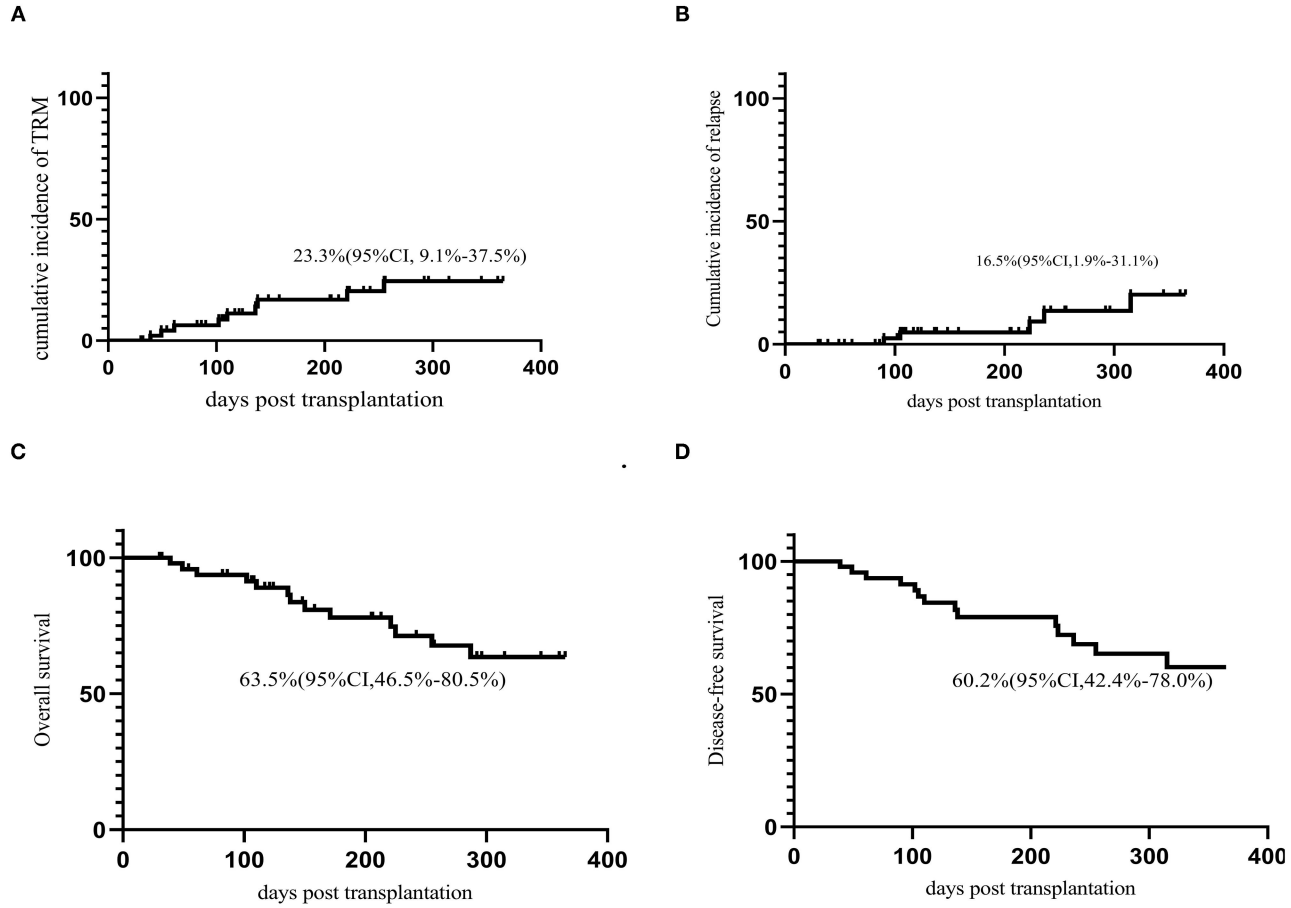

FIGURE 3 | Outcomes **(A)** TRM; **(B)** CIR; **(C)** OS; **(D)** DFS.

lower than that in our previous study (Bu/Cy/ATG 29.8–40%) (11). Therefore, it shows that age alone seems to be not a contraindication with current novel method. However, the actual result is discordant with the expected results. For this limitation, we need to expand the samples for further conformation in the future.

Graft failure may be a major concern in situation with reduced intensity of the conditioning regimen. In our previous attempt to complete replacement of Cy 3.6 g/m^2 by fludarabine 150 mg/m^2, the incidence of PGF was higher in the Bu/Flu/ATG group than in the Bu/Cy/ATG group (17.6 vs. 3.0%, $P = 0.04$) (11) despite the OS, LFS, TRM and CIR being comparable. Several other studies also reported a possible association of Flu with poor engraftment. In a prospective randomized study comparing BuCy with BuFlu (20), the five graft failures all occurred in the Flu group and the median percentage of recipient hematopoietic chimerism at 4 weeks after transplantation was significantly greater in the BuFlu group (BuCy, 0% [range, 0–7%]; BuFlu, 5.5% [range, 0–94%]; $P < 0.001$). In addition, a group from Nagoya reported that the Flu regimen was a risk factor for donor-type aplasia in children with bone marrow failure diseases (ASH 2012, Abstract 3474; Abstract 959). Another study found that the flu-based conditioning regimen was associated with an increased need for retransplantation compared with the Cy-based regimen (12). Rizzieri et al. reported patients with hematologic malignancies or marrow failure with a 6% incidence of GF and a 10.2% rate of TRM after the Cy (2 g/m^2) + fludarabine protocol with additional alemtuzumab (16). While transplants in a fludarabine-based conditioning regimen with a high dose of Cy (150 mg/kg) have reportedly been well-engrafted, but with a high of TRM (17). Therefore, it seems that the dose of Cy should be optimized to balance the TRM and engraftment (21). However, the optimal dose of Cy to balance the toxicity and engraftment is still unknown (22). In the current study, we added a reduced dose of Cy (2 g/m^2) combined with fludarabine to promote engraftment. As expected, our study demonstrated that the addition of Cy to previous Bu/Flu/ATG could promote engraftment compared with a previous report with Bu/Flu/ATG regimen. In accordance with these findings and our experience, this study showed that Cy 2 g/m^2 may be a balanced dose in combination with Flu + ATG + BU for haplo-HSCT for hematological malignancies, with good engraftment and safety profiles. Further prospective controlled study is warranted to validate the result of this novelconditioning regimen.

Graft-vs.-host disease (GVHD) is the most common complication that affects patients undergone HSCT and it can significantly decrease the quality of life (23–25) and leads to increased late mortality. Acute and chronic GVHD rates appeared to be increased with advancing age (a-GVHD 38 vs. 64% and c-GVHD 45 vs. 54%) in the report from the MD Anderson group (26). Numerous studies have demonstrated that the risk of GVHD is increased with age (27–30). Haploidentical HSCT has comparable or lower GVHD rates than matched-related donor HSCT in patients with PTCY as GVD prophylaxis (31–33). Compared to RIC or NMAC, MAC have a higher incidence of acute and chronic GVHD, which is an important factor contributing to its high TRM (34, 35). However, Scott et al. retrospectively analyzed 1,325 patients aged 18–70 years with AML, ALL, and MDS and received T-cell replete haploidentical HSCT (36). The results showed that there was no difference in grade II-IV aGVHD and c-GVHD according to regimen intensity for older patients. This present study showed a comparable incidence of aGVHD and cGVHD with a novel RTC regimen compared with patients received conventional MAC regimens.

The impact of RIC on TRM in the haplo-SCT setting from retrospective studies is conflicting (18, 36, 37). Although some studies have demonstrated the decreased TRM and reduced toxicity of RIC in older patients or patients with comorbidities, it is also controversial that whether decreased TRM will further translate into improved survival when comparing RIC and MAC regimens. In a propensity score adjusted analysis in haplo-SCT (38), RIC was associated with less TRM but higher relapse and no significant differences in OS, LFS, or GVHD. As for this RTC regimen for older patients, a decreased TRM has been achieved but if the survival was prolonged?

The propensity score matching (PSM) analysis was used to balance the variables affecting the choices of a treatment among different treatment options. So, we conducted a PSM analysis in patients with acute leukemia and MDS to evaluate the effect of the novel conditioning regimen vs. our conventional MAC regimen in our center. The PSM analysis showed that older patients could achieved similar TRM, OS, DFS, and CIR with younger patients. However, whether decreased TRM of this novel regimen could be further translated into improved DFS should be further confirmed in the further prospective RCTs.

In conclusion, our study with a novel conditioning regimen in a homogenous population demonstrated very encouraging results compared with our historical data. It suggested that a novel conditioning regimen of Bu/Flu/Cy/ATG might be a feasible option for older patients receiving unmanipulated haploidentical HSCT. And this novel regimen is worthy of further RCT studies.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethics committee of Peking University People's Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

X-JH conceived and designed the study, reviewed, and edited the manuscript. T-TH collected and analyzed the data. Y-QS assisted in the analysis of the data and wrote the paper. All authors contributed to the revision of the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.639502/full#supplementary-material

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REVIEW
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Colony Stimulating Factor 1 Receptor in Acute Myeloid Leukemia

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Acute myeloid leukemia (AML) is an aggressive heterogeneous blood cancer derived from hematopoietic stem cells. Tumor-stromal interactions in AML are of importance for disease development and therapy resistance, and bone marrow stroma seem like an attractive therapeutic target. Of particular interest is colony stimulating factor 1 receptor (CSF1R, M-CSFR, c-FMS, CD115) and its role in regulating plasticity of tumor-associated macrophages. We discuss first the potential of CSF1R-targeted therapy as an attractive concept with regards to the tumor microenvironment in the bone marrow niche. A second therapy approach, supported by preclinical research, also suggests that CSF1R-targeted therapy may increase the beneficial effect of conventional and novel therapeutics. Experimental evidence positioning inhibitors of CSF1R as treatment should, together with data from preclinical and early phase clinical trials, facilitate translation and clinical development of CSF1R-targeted therapy for AML.

Keywords: colony stimulating factor 1 receptor, tumor-stroma, signal transduction, biomarkers, acute myeloid leukemia, targeted therapy, therapy development

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INTRODUCTION

Acute myeloid leukemia (AML) is the most common aggressive blood cancer in adults with a median age at diagnosis of 71 years and with an overall incidence of approximately 4/100.000. In patients older than 70 the incidence is 17/100 000 (1). The 5-year survival rate for AML was less than 20% (2) before a wave of FDA-approved novel agents (introduced from 2017) were incorporated into standard treatment regimens and entered late-stage development in therapy combinations (3, 4). Even with these recent improvements, there have been few therapy developments that address tumor-host interactions. It is foreseen that AML will continue to represent a therapeutic challenge requiring novel treatment modalities (5).

AML is characterized by disruptive hematopoiesis through block in myeloid differentiation and enhanced proliferation leading to accumulation of non-differentiated myeloid cells/myeloblasts in bone marrow and peripheral blood (6). AML is diagnosed when myeloblast count comprise at least 20% of the bone marrow (7). Normal blood production is interrupted, and a typical AML patient may present low numbers of functionally intact granulocytes, platelets, and erythrocytes. Symptoms often include fatigue, shortness of breath, easy bruising, and frequent infections.

AML is a heterogeneous disease comprising both recurrent and rare chromosomal translocations and mutations. Next generation sequencing (NGS) analyses of leukemic samples have contributed to reveal the genetic landscape of AML, showing an enormous mutational diversity and identifying over

30 recurrent mutations (7–10). In some subsets of AML, molecular diagnostics can suggest the therapies most likely to give a favorable outcome such as expression of cell surface marker CD33 or mutations in specific genes such as FLT3/IDH1/IDH2 (8, 11, 12). Among the most frequent mutations in AML are those affecting nucleophosmin 1 (NPM1) which is possible to target experimentally, as recently demonstrated (13–16). Similarly, mutations in TP53 are associated with chemo-resistance and therapies aimed at restoring P53 function are in development (17).

It has been postulated that the process of leukemogenesis is initiated by relatively few steps, and a "two hit" model of AML development has been proposed, in which class 1 and class 2 mutations can suffice for AML initiation (18). This theory has mostly been supported by the analyses of large AML sample cohorts (10, 19). Observations indicate that AML is a disease that starts with a single clone that acquires novel mutations over time, thereby further contributing to tumor heterogeneity and early relapse (20–22).

The acquisition of somatic mutations is a relatively common event in most cell types and increases with age (23, 24). Certain mutations occur in hematopoietic stem cells (HSC) and gain a competitive advantage, resulting in "clonal hematopoiesis" that could lead to expansion of a clonal population of blood cells (25). Clonal hematopoiesis is predisposing individuals to hematological disease (26, 27) but studies have discovered that clonal hematopoiesis-harboring mutations in AML-associated genes like DNMT3A and TET2 are ubiquitous in the elderly population between 50 and 70 (28). Although prevalence of clonal hematopoietic mutations is very common, progression to hematological malignancy is extremely rare. A recent investigation found no significant association between clonal hematopoiesis and long-term risk of developing AML between cases and controls (29). Future research should rely on methods to distinguish between high-risk and low-risk clonal mutations for development of aggressive disease. This imperative should gain interest in sequencing-based non-invasive screening and in future tailored therapy guided by cytogenetics and mutational profile.

The AML therapies implemented from 2017 filled a nearly 40-year paucity in drug development (12). These new agents include lipid formulated chemotherapy, antibodies directed against AML cells, Bcl-2 family inhibitors, metabolic enzyme inhibitors of IDH1/2, and tyrosine kinase inhibitors. Although developing targeted therapy presents challenges, the accumulated knowledge about AML will continue to translate into novel treatment approaches that improve patient outcomes (30). In fact, treatment based on molecular diagnostics should increase overall survival of AML above the glass ceiling of 50% long-term survival currently observed in younger and fit patients (7, 31). The recent therapeutic landscape of AML does not include stroma-targeting therapy, although intensive induction chemotherapy and consolidating allogeneic stem cell transplantation eradicate most of the host stromal environment. At the same time, it appears clear that broad acting kinase inhibitors like midostaurine and gilteritinib (predominantly inhibitor of FLT3, AXL) may affect stromal function (32).

Colony Stimulating Factor 1 receptor (CSF1R) is a particularly interesting target since its expression and signaling is prominent in the supportive stromal compartment. The use of enzymatic inhibitors and blocking antibodies of CSF1R represents two novel approaches that addresses tumor-stroma interactions in an attractive way.

CSF1R BIOLOGY

CSF1R (M-CSFR, c-FMS, CD115, c-fms proto-oncogene, McDonough feline sarcoma oncogene) is a cell surface glycoprotein encoded by the CSF1R gene located on the distal end of the long arm chromosome 5 (5q32) (**Figure 1**) (33). CSF1R is a class III receptor tyrosine kinase and member of the platelet-derived growth factor (PDGF) receptor family along with FLT3, c-KIT, and PDGF-α and -β receptors (34). In comparison, CSF2R (GM-CSFR, CD116) belongs to class 1 hematopoietic receptor systems and CSF3R (G-CSFR, CD-114) is related to the cytokine (hematopoietin) receptor family (35). CSF1R is expressed primarily on mononuclear phagocytes, namely monocytes, macrophages, and dendritic cells where its activation is crucial for their growth and differentiation during immune responses. CSF1R is involved in promoting the physiological properties of monocytes and macrophages which entail cytotoxicity, phagocytosis, and chemotaxis through the release of cytokines and chemokines. CSF1R is also found on a diversity of cells of the body such as Langerhans cells of the skin, Paneth cells in the small intestine, osteoclasts, brain microglia, cells in the female reproductive tract, and at low levels on hematopoietic stem cells (36).

CSF1R is a cell surface protein with an extracellular glycosylated domain comprising five immunoglobulin (Ig)-like domains (D1-D5), a transmembrane domain and a cytoplasmic kinase domain (**Figure 2**). The intracellular portion of the receptor is composed of eight tyrosine phosphorylation sites situated on the juxtamembrane section, the kinase insert, the major kinase domain, and distal kinase domain (37). In the inactive state, CSF1R presents an autoinhibitory conformation (38). The two activating ligands; CSF1/M-CSF and the more recently identified interleukin 34 (IL34) differ slightly in structure but show undistinguishable downstream signaling pathways according to Boulakirba et al. (39). However, they discovered differences in cytokine/chemokine production when CSF1- or IL34-differentiated monocytes are polarized into different phenotypes. This suggest that macrophages derived from either ligand may behave differently and thus exert different polarization potential. Another study found that CSF1 and IL34 have different spatiotemporal expression but serve complementary roles in regulating the development and maintenance of macrophages (40). Binding of CSF1 or IL34 to CSF1R induces non-covalent dimerization of the receptor chains and transphosphorylation of tyrosine residues (41). The first tyrosine to be phosphorylated is Tyr561 which is necessary for full receptor activation (42). The phosphorylated residues function as docking sites for several different proteins that

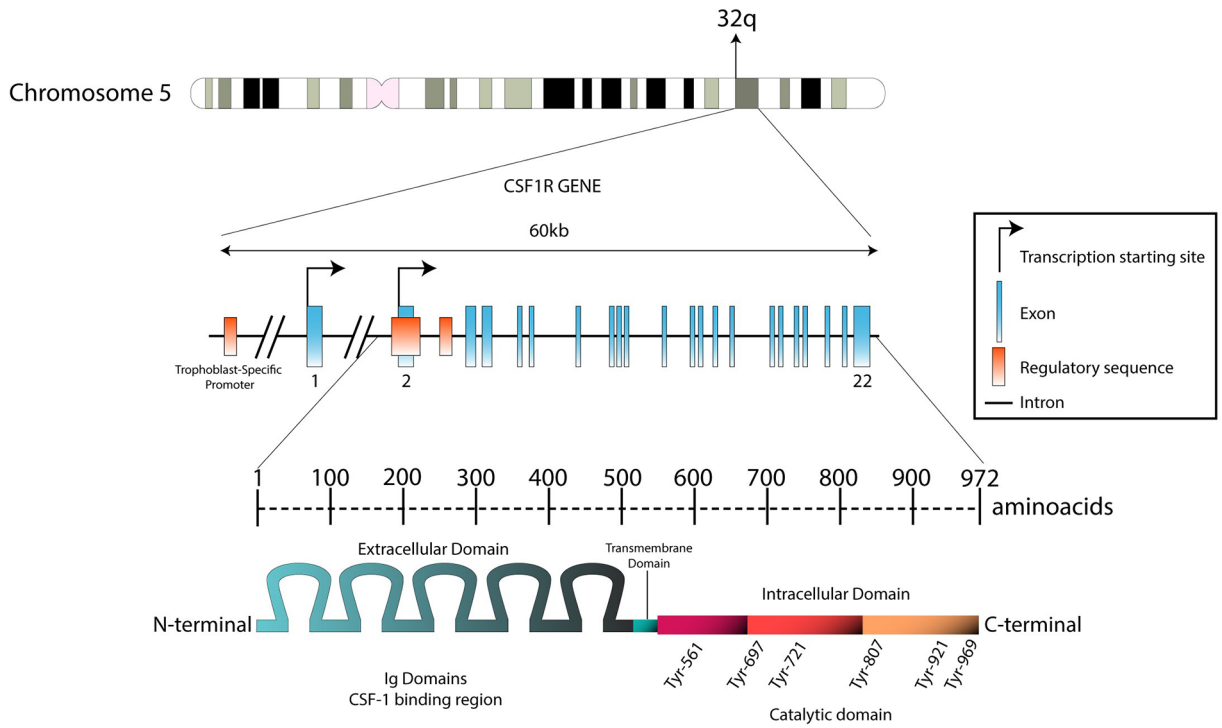

FIGURE 1 | Genomic structure of the CSF1R locus in human and protein structure. The CSF1R locus is located at the distal end of the q arm in chromosome 5 (5q32). With 60 kb length, CSF1R gene is composed by 22 exons. Transcription between the first exon and exon 22 is exclusively regulated by a trophoblast-specific promoter. In other tissues (such as macrophages), transcription takes place only between exon 2 and exon 22. The transcript produced is predicted to be 3.9 kb long. This product will translate in a 972-aminoacid protein with a molecular weight of 108 kDa. The N-terminal extracellular domain is composed by five immunoglobulin domains (512 aa), which contain the ligand binding region. The hydrophobic transmembrane domain is 25 amino acids long. The intracellular domain contains the tyrosine residues that will be phosphorylated upon receptor stimulation (435 aa).

subsequently activate signaling molecules. Among them are members of the Src family kinases, phospholipase Cγ2, phosphatidylinositol 3-kinase (PI3K), and suppressor of cytokine signalling-1 (SOCS1) (43). Following the different downstream signal transduction pathways, the resulting gene expression mechanisms promote proliferation, differentiation, and survival of the cell (44). Studies analyzing the effects of CSF1R-mutations in macrophages suggest that the PI3K/Akt pathway has a pivotal role in ensuring CSF1-mediated survival of macrophages (40). Macrophage proliferation is primarily associated with the two pathways through PI3K and MEK, but multiple ERK tyrosine kinases may also be involved. Studies have shown that macrophage differentiation is mediated through the PLC-ϒ2 pathway activated by phosphorylation of Tyr-721 and Tyr-807 in CSF1R (40). Knowledge about CSF1R biology and its role in cancer is evolving rapidly, especially regarding the supportive tumor microenvironment.

CSF1R AND THE MICROENVIRONMENT

Bone marrow stromal cells facilitate growth of normal hematopoietic and leukemic cells through the continuous production of growth factors. In AML, malignant cells are thought to polarize the surrounding stroma through a cytokine-regulated mechanism facilitating a stroma-mediated protection of AML. This complex process involves several cytokines, chemokines, growth factors, receptors, and adhesion molecules (45). This can be seen *in vitro* by increased AML cell proliferation and protection from drug-induced apoptosis when these cells are in direct or indirect contact with the human stromal cell line HS-5 (46–48). Thus, targeting the niche and the interaction between leukemic cells and their environment appear promising for AML treatment.

The bone marrow microenvironment (BME) in AML plays an important role by contributing to both leukemic development and therapy resistance. Situated in the BME we find the bone marrow niche; a controlled perivascular and endosteal space where hematopoietic stem cells (HSC) are regulated and maintained by the surrounding stroma (**Figure 3**) (49, 50). The surrounding stroma that affects HSC consists of mesenchymal stromal cells, osteoblasts, endothelial cells, macrophages, and CXCL12-abundant reticular cells in addition to neurons and glial cells (51–56). CSF1R signaling is present in several cellular subpopulations that regulate hematopoiesis and homeostasis. Detectable expression of CSF1R is found mainly on

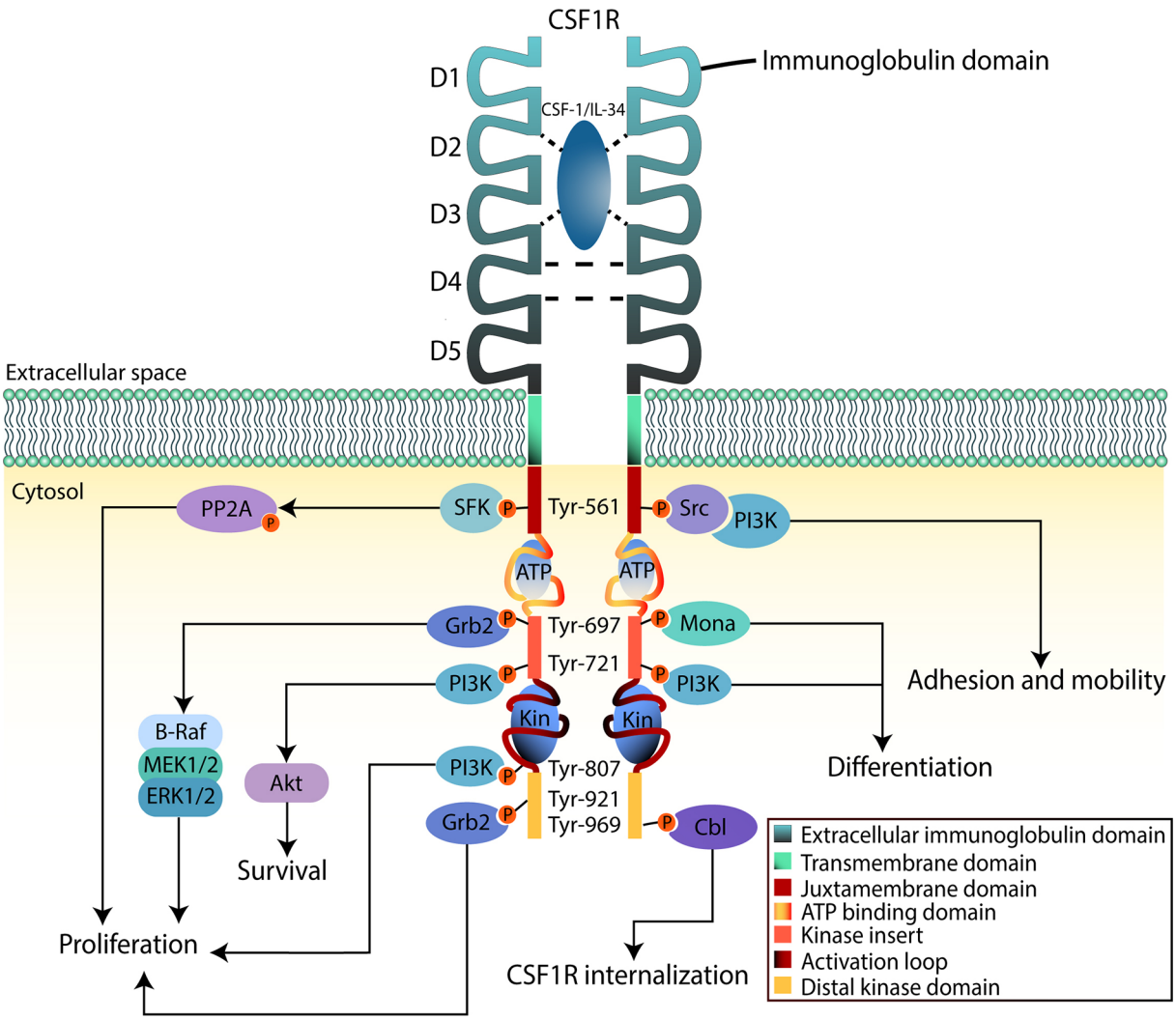

FIGURE 2 | CSF1R downstream signaling in myeloid cells. The binding region of CSF-1 or IL-34 to CSF1R is contained in the second and third domain (D2 and D3) of the extracellular region, D4 mediates homotypic interactions. Upon binding to the ligand, CSF1R dimerizes inducing tyrosine phosphorylation that will lead to activation of downstream signaling pathways. This will promote proliferation, survival, and differentiation of the cell. The intracellular region, apart from the tyrosine residues, contains an ATP-binding domain as well as catalytic domains where substrates bind (Kin). When activation takes place, these domains will fold to phosphorylate signaling mediators.

macrophages, osteoclasts, and at low levels on HSC (44). Interestingly, recent studies have revealed CSF1R-expression on leukemic stem cells (LSC) (10), which share phenotypical and functional similarities with HSC. LSC have the capacity to produce a cellular hierarchy of leukemic progenitors as well as remodeling the bone marrow niche, reshaping it into an environment conducive to support leukemic expansion. Thus, the leukemia niche is created where cells are not subject to the same signals as normal HSC and consequently contributes towards malignant progression. More specifically, angiogenesis increases and stromal cells acquire supportive features for the leukemic cell population (57). Failure to eradicate LSC following chemotherapy often contributes to early relapse (58–60).

Another important component that characterizes the AML bone marrow niche includes the interaction between leukemic and endothelial cells. For example, AML cells can intravasate into the vasculature and fuse with endothelial cells thereby creating a favorable vasculature for expansive cell proliferation (61, 62). The level of proangiogenic vascular endothelial growth factor (VEGF) is high in AML patients, and high levels of VEGF and can also lead to increased secretion of granulocyte-macrophage colony stimulating factor (GM-CSF) which is known to stimulate cell growth in AML (63). Fibroblasts also participate in AML development, and studies have found several functional cancer-associated fibroblasts (CAF) in AML patient samples (64). Evidently, all components of the AML bone

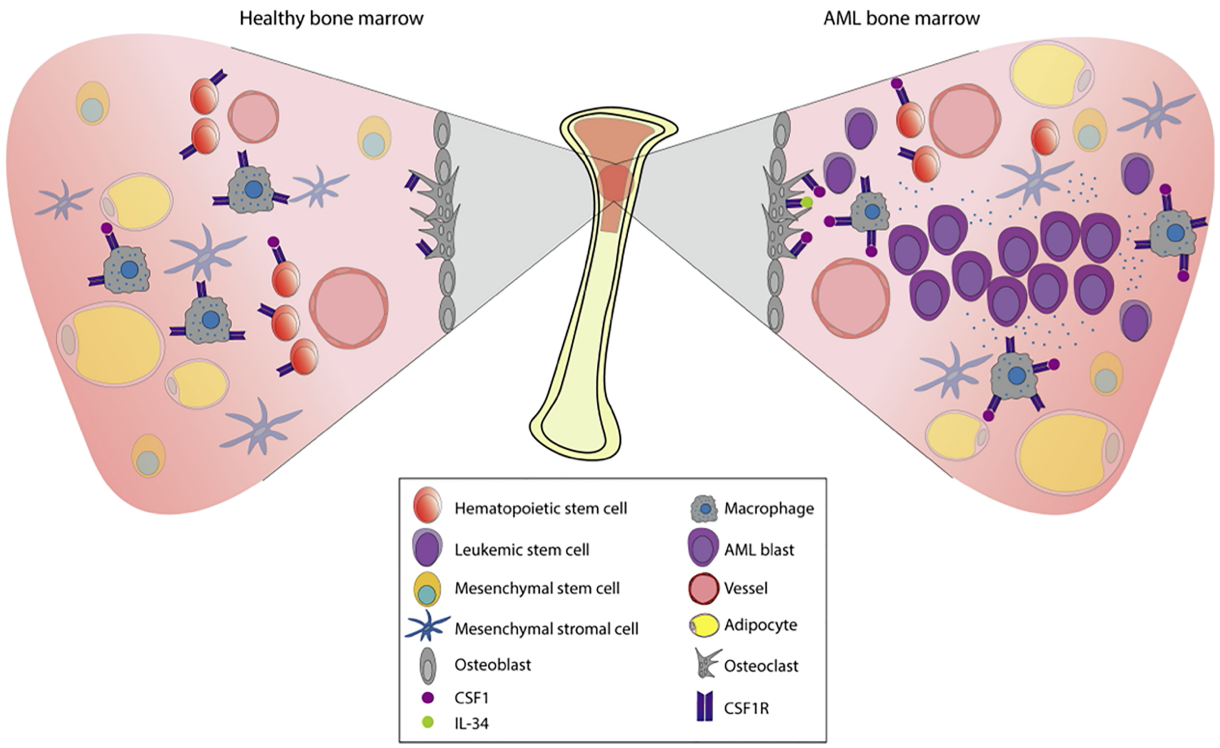

FIGURE 3 | Pathophysiology of AML in the bone marrow. The bone marrow is a tissue organized to protect cell proliferation. Under normal conditions (left), the multipotent hematopoietic stem cells (HSCs) will give rise to the myeloid and the lymphoid lineages. Myeloid lineage transcription factors regulate the expression of CSF1R, already present in macrophage precursors. The stromal environment is composed by osteoblasts, osteoclasts, adipocytes, macrophages, mesenchymal cells, and vessels that will allow this differentiation process to take place. When there is a dysregulation of proliferation and apoptosis in myeloid precursors, hematologic malignancies such as AML may appear. In AML (right), proliferation and survival rely on the bone marrow stromal cells signals. These signals are strongly mediated by the CSF1R which is expressed in certain cell types (shown in the figure), contributing to paracrine communication.

marrow niche interact with leukemic cells and affect their proliferation, differentiation, adhesion, quiescence, migration, and clonal expansion (45). These interactions between leukemic cells and the BME can determine the fate of leukemic cells following chemotherapy, which and ultimately has implications for residual disease and early relapse (65).

For most intermediate and high-risk AML patients, allogeneic stem cell transplantation is the only curative therapy available (7). Depending on intensity of the transplant conditioning therapy, the bone marrow stroma is more or less damaged by the stem cell transplant process, but donor stem cell engraftment is difficult if the radiation-resistant recipient resident macrophages are eradicated. In an attempt to elucidate this process *in vivo*, mice were inserted with a CSF1R-eGFP construct used as a myeloid reporter gene, where GFP is under the control of the CSF1R promoter. After the stem cell niche was exposed to lethal radiation, subsequent analysis of the niche was performed following lethal irradiation and autologous hematopoietic stem cell transplantation (66). Recipient CD169+ CSF1R-eGFP resident macrophage number in bone marrow aligned with the persistent engraftment of long-term reconstituting HSC within bone marrow, illustrating the complex properties of macrophages in stem cell niches of the bone marrow.

CSF1R AND TUMOR-ASSOCIATED MACROPHAGES

Macrophages are myeloid cells derived from monocytes (67) present in the tumor micromilieu of the bone marrow (68). The major regulators of macrophage proliferation and survival are the growth factors M-CSF/CSF1 and IL34. These play an autocrine/paracrine role in various solid tumors, attracting and differentiating incoming monocytes into tissue resident macrophages in the tumor microenvironment (69–71). Monocytes and macrophages have come to the attention of cancer researchers because of their plasticity and influence on malignant progression as well as their role in cancer-related inflammation (72–76). Increasing evidence demonstrates a correlation between macrophage density within the tumor micromilieu and malignant progression carrying a poor prognosis (77–80).

Macrophages are thought to be polarized by various cytokines towards pro-inflammatory or anti-inflammatory behavior, which means that they exert either influence on tumor development (81). In endometrial and breast cancer, macrophages undergo cancer-specific reprogramming which

significantly alters their distribution and function (71). In general, it is thought that a complex bidirectional communication between the macrophages and the tumor ultimately forge the anti-inflammatory, immune regulatory myeloid cells into tumor-associated macrophages (TAM).

To describe macrophage activity, a functional classification of macrophages into M1 and M2 has been proposed, where M1 are pro-inflammatory and tumoricidal, and M2 are anti-inflammatory and immune regulatory. M1 macrophages are differentiated by another growth factor; granulocyte-macrophage colony stimulating factor (GM-CSF, or CSF2) and other pro-inflammatory agents (82). This simple dichotomy excludes the spectra of diverse phenotypes within the tumor microenvironment (82, 83). In response to local microenvironmental cues, TAM display an impressive adaptability that elicit functions supporting tumor growth and resistance to therapy (84). Convincing experimental and clinical evidence has shown that macrophages promote cancer initiation, angiogenesis, migration, and invasion suggesting that specialized subpopulations of macrophages may be important therapeutic targets (85).

Our knowledge of the roles of macrophages and their functions in the development of AML is still limited, although a recent *in vivo* study showed correspondence between macrophage infiltration and overall survival (53). Further, AML cells have also been shown to polarize macrophages and orchestrate the invasion of monocytes into bone marrow of mice, suggesting that AML and surrounding stroma affect monocytic infiltration and transformation into a tumor supportive phenotype. Altogether, the complex interplay between TAM and malignant cells further provides rationale for targeting CSF1R in AML as the receptor is essential for macrophage differentiation and survival.

CSF1R MUTATION AND EXPRESSION

Recent analyses employing whole-exome and whole-genome sequencing [from the collaborative Beat AML research program and the Cancer Genome Atlas Program's (TCGA) study on AML] did not detect significant mutational events in CSF1R (10, 19). When we searched for genetic alterations in CSF1R in the accessible databases TCGA (86, 87) and COMSIC (88) we found that 0.9% of all patients (n = 2,034) had CSF1R deletions in one allele. Yet, CSF1R mutations found at codon 301 (L301S) and 969 (Y969F) have earlier been identified in some patients with AML (89). Interestingly, mutations at codon 301 are believed to contribute to constitutive activation of the receptor, while the tyrosine residue at codon 969 have shown to be involved in negative regulatory activity (90). However, the total incidence of mutations in codon 969 was 12.7% and only 1.8% in codon 301. These studies date back to 1990 and included a samples size of 110 patients with myelodysplastic syndromes and AML. Furthermore, studies have revealed that a carboxy-terminal truncation and the two point-mutations (L301S and A374X) in the extracellular D4 domain are crucial for activation of the oncogene (91, 92). In addition, another oncogenic derivative with two translocations and a constitutively active CSF1R fusion protein joined to the carboxy-terminal 399 amino acids is reported in megakaryoblastic AML (FAB classification M7) (93). Conclusively, as recent sequencing analyses did not reveal any significant mutational events in a large sample of AML patients, CSF1R mutations do not appear to be relevant as a target in AML.

Nevertheless, inappropriate expression of CSF1R has been associated with several malignancies, including breast cancer, prostate cancer, ovarian cancer, leukemias, and Hodgkin's lymphoma (67, 84). More importantly, analysis of CSF1R expression levels in AML patient samples found a correlation between high levels of CSF1R expression and shorter overall survival (94).

Moreover, it has been demonstrated that the runt-related transcription factor 1 (RUNX1), which plays a critical role in the development of AML, is involved in CSF1R transcriptional regulation. RUNX1 plays a key role in the regulation of growth and survival of macrophages by controlling CSF1R gene expression and, in turn, RUNX1 expression is repressed in CSF1-stimulated cells (95). Specifically, RUNX1 regulates expression of RUNX3, CSF1R, and CEBPA genes (96). RUNX1 has also been shown to be a key mediator (directly or indirectly) of tumorigenesis. In BRAF inhibition resistant melanomas, for example, RUNX1 has been shown to autocrinally upregulate expression of CSF1R which possibly contribute to growth and invasion (97). In contrast, RUNX1 loss-of-function mutations in hematopoietic stem progenitor cells (HSPC) reduce rates of apoptosis and increase stress resistance with a consequent selective advantage over normal HSPC (98). Also; a recent investigation showed correlation between inversion of chromosome 16 and CSF1R overexpression in AMl blasts (99).

Further research is needed to determine if specific genetic subsets of AML influence CSF1R expression.

We hypothesize that dysregulation of CSF1R expression through other mutations may benefit from CSF1R inhibitors.

PRECLINICAL ACTIVITY OF CSF1R INHIBITORS

The complex interaction between leukemic blasts and the surrounding stroma could be exploited therapeutically, as novel treatment in combination with standard treatment regiments. Inhibition of CSF1R has been proposed to be an effective target for blocking monocytes and TAM that infiltrate the tumor stroma and support tumor growth (100). Several companies have produced small molecule inhibitors of CSF1R kinase activity; and most of these have been claimed to be highly specific (44, 100). However, given the high level of conservation of the tyrosine kinase domains of the type III protein tyrosine kinases (CSF1R, Fms-like tyrosine kinase-3, KIT, platelet-derived growth factor receptor), it would be difficult to predict off-target impacts *in vivo*, based on the *in vitro* data.

Edwards and co-workers have recently performed an *ex vivo* functional screen of patient-derived leukemic cells from the "Beat AML consortium," with the goal of identifying new therapeutic targets (94, 101). Interestingly, small-interfering RNA (siRNA) tyrosine kinome screen identified CSF1R to significantly reduce cell viability in primary AML patient samples. Sensitivity towards CSF1R inhibition (reduced cell viability *ex vivo*) was found in 23% of patient samples. The CSF1R-inhibitor GW-2580 showed high degree of specificity compared with other class III receptor tyrosine kinases and was selected to be CSF1R inhibitor activity in all subsequent experiments. Screening of 315 AML patient samples for sensitivity towards GW-2580 revealed a wide range of responses ranging from highly sensitive to non-sensitive. Nevertheless, GW-2580 significantly induced apoptosis in patient samples but not in samples from healthy donors. Analysis of the patient samples that had undergone inhibitor screening from the "Beat AML" patient cohort revealed a significant association between resistant samples and poor prognostic markers. Cytogenetic abnormalities included complex karyotypes, inversion 3, monosomy 5/deletion 5q, the gene mutations TP53, NRAS, KRAS, and genetic adverse prognostic risk group correlated with increased CSF1R inhibitor sensitivity. The authors concluded that the samples resistant towards CSF1R inhibitors were potentially non-sensitive towards all forms of treatment, which could explain its ineffectiveness.

Examinations of CSF1R expression patterns by flow cytometry and mass cytometry (CyTOF) of samples from AML patients and healthy donors revealed overall expression of CSF1R in AML samples is found on a subpopulation of CD14-expressing monocytes which seem to diminish after exposure to CSF1R inhibitors, while negligible expression of CSF1R was found on a small portion of leukemic blasts (94). In solid tumors, cells expressing CSF1R almost exclusively defines a population of tumor infiltrating macrophages. However, because AML can arise from macrophage-lineage precursor cells, it can be challenging to determine the origin of supportive CSF1R-expressing cells, and to know whether they are infiltrating monocytes/macrophages or tumor derived.

Analyses of CSF1R ligand stimulation suggest that receptor signaling occurs through a ligand-dependent mechanism, and CSF1R inhibitors eliminate CSF1R-expressing supportive cells in AML (94). Furthermore, CSF1R-expressing cells protect AML cells through paracrine cytokine secretion of hepatocyte growth factor (HGF) and CSF1 and that utilizing CSF1R inhibitors may be an effective treatment in a subpopulation of AML patients (94, 101).

It has been hypothesized that using CSF1R inhibitors is most effective in the early stages of the disease (101), which presents an ongoing issue in clinical development as Phase 1 clinical trials usually enroll relapsed/refractory (R/R) AML patients. Increased clinical response in *de novo* disease compared to late-stage cancer is common for many malignancies but studying these patterns requires comprehensive clinical trials. For instance, it took nearly a decade to complete the Phase III registration trial for the first FLT3 targeted treatment in otherwise healthy patients with *de novo* AML (102). Therefore, we expect it will take time to develop CSF1R inhibitors for early stage and first line AML.

CLINICAL DEVELOPMENT OF CSF1R INHIBITORS

A wide variety of clinical trials have used different treatment strategies to target CSF1R (**Table 1**). Ongoing studies aim to decipher the safety profile and the clinical activity of CSF1R inhibitors alone and in combination in various malignant diseases. Some of these studies have been previously reviewed by Cannarile et al. (100), describing different approaches for targeting CSF1R in different cancer types. The FDA has recently approved the oral small-molecule CSF1R inhibitor pexidartinib (PLX3397/PLX10801) as monotherapy for the CSF1-driven non-malignant diffuse-type Tenosynovial Giant Cell Tumor (dt-GCT) (103). Pexidartinib also has inhibitory activity against FLT3 and cKIT, two commonly mutated genes in AML. Results of a phase I/II open-label clinical study of pexidartinib in relapsed/refractory (R/R) FLT3-ITD-mutated AML have recently been published (NCT01349049) (104). In this study, 90 patients were treated either in dose escalation or in dose expansion with the aim of assessing safety and tolerability of pexidarnitib, and the maximum tolerated dose (MTD) was not reached. The overall response rate (ORR) was 21%, probably due to patients having received multiple lines of therapy. Although this study focused on treating R/R FLT3-ITD$^+$ AML, it also considered the possibility of targeting CSF1R with pexidartinib

TABLE 1 | Summary of CSF1R targeted therapies for leukemias in clinical trials (2020).

Compound	Class	Target	Clinical phase	Status	ClinicalTrials.gov identifier	Sponsor
Pexidartinib (PLX3397/ PLX10801)	Small molecule	CSF1R, FLT3, cKIT	I/II	Completed [44]	NCT01349049	Daiichi Sankyo, Inc.
			I/II	Ongoing	NCT02390752	National Cancer Institute (NCI)
JNJ-40346527	Small molecule	CSF1R	II	Terminated	NCT03557970	OHSU Knight Cancer Institute
NMS-03592088	Small molecule	CSF1R, FLT3, cKIT	I/II	Ongoing	NCT03922100	Nerviano Medical Sciences
Emactuzumab (RG7155)	mAb	CSF1R	I	Ongoing	NCT02323191	Hoffmann-La Roche

in patients with wild type FLT3. This study reported tolerability and an antileukemic effect of pexidartinib in highly pretreated R/R AML. Another ongoing study with similar objectives is a Phase I/II trial of pexidartinib in children and young adults with R/R leukemias (AML or ALL) or solid tumors is currently under investigation (105). The results of this trial will help to decipher safety profiles of CSF1R inhibition in the younger population.

Other CSF1R inhibitors have also been considered for treatment of R/R AML. One example is the selective small molecule CSF1R inhibitor JNJ-40346527, for which preclinical studies have shown interesting immunomodulatory effects in murine models of Crohn's Disease (106). Although this component's safety and efficacy have been tested previously in patients with rheumatoid arthritis and advanced Hodgkin's Lymphoma (107, 108), it has yet to be studied in patients with R/R AML. A phase II open-label clinical trial with the aim of studying efficacy of JNJ-40346527 in patients with R/R AML had to be terminated due to insufficient patient enrolment, unfortunately. The reasons for poor enrolment are unknown, however, it might be increasingly difficult to secure accrual in trials with monotherapy in an aggressive disease like AML (109).

A different approach for treating R/R AML is the ongoing open-label phase I/II, first-in-human clinical trial assessing the clinical activity of the combined FLT3, KIT, and CSF1R inhibitor NMS-03592088 (110). This multi-center non-randomized study aims to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of NMS-03592088 in patients with R/R AML or chronic myelomonocytic leukemia (CMML) (111). Like pexidartinib, the three targets FLT3, KIT, and CSF1R of NMS-03592088 are connected to the molecular physiopathology of AML, being relevant mediators to target for treatment strategies.

In addition to small-molecule therapeutics, monoclonal antibodies (mAbs) are also being considered as valid options for targeting CSF1R. The most promising example of anti-CSF1R therapy is emactuzumab (RG7155) given as monotherapy for dt-GCT (112). A Phase I clinical trial with emactuzumab in combination with the chemotherapeutic agent paclitaxel in solid tumors reduced TAM at the optimal biological dose (OBD) (113). The efficacy of this monoclonal antibody is yet to be tested in AML, but more clinical data from studies investigating the behavior of emactuzumab (NCT02323191) will give us further knowledge about this agent.

DISCUSSION

Clinical development of CSF1R inhibitors for AML treatment is in its early development. The activity of some CSF1R inhibitors is modest and more studies are needed to understand the therapeutic potential of these inhibitors. It is important to understand the possible negative side-effects of on-target toxicities like macrophage depletion outside the tumor (114). Similarly, central nervous side effects like fatigue should be considered when investigating CSF1R-targeted therapies for AML (115, 116). A recent study discovered that pexidartinib affects CNS microglia but also has long-term effects in the myeloid and lymphoid compartments of the bone marrow, spleen, and blood (117). The long-term effects on circulating and tissue macrophages have implications for future development of CSF1R inhibition as treatment, because peripheral monocytes repopulate the central nervous system. AML is a highly heterogenic disease with specific therapies, and CSF1R inhibition may represent a more universal approach that targets the stroma. Although single agent sensitivity to CSF1R inhibitor is observed in AML, monotherapy will most likely not be sufficient for efficient AML treatment (94, 101). We suggest a combined approach for targeting the leukemia cells directly as well as the surrounding stroma. For other tumor types, various combinations with CSF1R-mediated TAM depletion are currently under clinical investigation (114). A strategy that may be attractive uses CSF1R inhibitors in combination with a CXCR2 antagonist (118). Kumar and coworkers employed CSF1R inhibition to disrupt chemokine secretion by cancer associated fibroblasts (CAF) abolishing recruitment of pro-tumor granulocytic myeloid-derived suppressor cells (MDSCs). In addition, combining CSF1R inhibitors with a CXCR2 antagonist blocked the infiltration of these cells and showed strong anti-tumor effect (118). CSF1R-targeting agents in combination with checkpoint blockade inhibitors, other targeted therapies, anti-angiogenic therapies, chemotherapy, and adoptive T-cell transfer approaches are all currently undergoing clinical investigations (114). Though several inhibitors are targeting leukemic cells directly by inhibiting FLT3 and TAM by inhibiting CSF1R, we speculate that these inhibitors may be beneficial to use in a carefully designed sequences with immunotherapeutics like immune checkpoint inhibitors.

CONCLUDING REMARKS

The supportive microenvironment in the bone marrow of AML patients significantly contributes to early relapse and death and is a major challenge for successful treatment. The contribution of tumor-associated macrophages (TAM) to malignant progression in AML is substantial, involving bidirectional communication between leukemic cells and TAM. Targeting CSF1R-expressing TAM may be an effective treatment for depleting supportive cells and kill leukemic cells (112, 119). Limited evidence has demonstrated that CSF1R-inhibition has been a beneficial approach for a subset of AML patients (94). At present, the most effective therapy for AML is combinations and sequences of anti-leukemic therapeutics, for example, the recent combination of venetoclax plus hypometylating agents or the sequence of intensive chemotherapy followed by allogeneic stem cell transplantation (120, 121). We suggest a novel approach for eliminating leukemic cells directly and attacking the leukemia-supporting surrounding stroma by inhibiting CSF1R signaling. Future work needs to address the optimal CSF1R targeting combinations and sequences that secure the most clinical benefit for AML patients.

AUTHOR CONTRIBUTIONS

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

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BRIEF RESEARCH REPORT
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Personalized Survival Prediction of Patients With Acute Myeloblastic Leukemia Using Gene Expression Profiling

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Acute Myeloid Leukemia (AML) is a heterogeneous neoplasm characterized by cytogenetic and molecular alterations that drive patient prognosis. Currently established risk stratification guidelines show a moderate predictive accuracy, and newer tools that integrate multiple molecular variables have proven to provide better results. In this report, we aimed to create a new machine learning model of AML survival using gene expression data. We used gene expression data from two publicly available cohorts in order to create and validate a random forest predictor of survival, which we named ST-123. The most important variables in the model were age and the expression of *KDM5B* and *LAPTM4B*, two genes previously associated with the biology and prognostication of myeloid neoplasms. This classifier achieved high concordance indexes in the training and validation sets (0.7228 and 0.6988, respectively), and predictions were particularly accurate in patients at the highest risk of death. Additionally, ST-123 provided significant prognostic improvements in patients with high-risk mutations. Our results indicate that survival of patients with AML can be predicted to a great extent by applying machine learning tools to transcriptomic data, and that such predictions are particularly precise among patients with high-risk mutations.

Keywords: acute myeloid leukemia, cancer, survival, machine learning, gene expression, prognosis

INTRODUCTION

Acute Myeloid Leukemia (AML) is a heterogeneous neoplasm characterized by cytogenetic and molecular alterations that drive patient prognosis. Currently established AML risk stratification guidelines, like the *European Leukemia Net* (ELN) risk classification (1), are based primarily on a limited number of cytogenetic and molecular variables. However, these guidelines don't take into

account the whole mutational profile of AML, the different layers of biological complexity in the tumor and the complex intertwining between patient outcomes and the complexity of molecular interactions. Therefore, there is substantial room for improving predictions of AML survival. In this report we present a new machine learning model of AML patient survival based on gene expression data, which achieves high predictability independently of high risk mutations.

In the last years, the emergence of artificial intelligence has brought new expectations to the field of medicine, particularly for disease diagnosis and prognostication. Machine learning (ML) is a field of artificial intelligence that performs outcome prediction based on complex interactions between multiple variables by making little assumptions about the relationship between the dependent and independent variables (2). In ML, a model is trained with examples and not programmed with human-made rules (3). The implementation of ML-based survival models is becoming increasingly popular in order to provide patient-centered risk information that can assist both the clinician and the patient.

Survival prediction of AML patients has been extensively improved in the last decades. Several biomarker panels based on next-generation sequencing of multiple recurrently mutated or aberrantly expressed genes have been proposed to facilitate improved prognostic stratification. Although multiple somatic alterations have been associated with patient outcome, such as those in *NPM1*, *CEBPA*, *FLT3*, *IDH1*, *IDH2*, *KIT*, *WT1* and *RUNX1* (4), only mutations a few mutations are broadly employed in current clinical routine (5). Multiple studies have proposed novel biomarker panels and personalized survival prediction models aimed to improve AML prognostication. Sherve et al. (6) developed a novel prognostic model that incorporates clinical, cytogenetic and mutational data to determine personalized outcomes for each particular patient (6). In the same line, Patkar et al. (7) created a scoring model that provides a mechanism to risk stratify AML patients with mutated *NPM1* (7); whereas Gerstung et al. (8) reported statistical models that can generate personally tailored clinical decision support from all of the available prognostic information arising from a knowledge bank of AML cases (8). These studies evidence that the application of ML to clinical and molecular data has the potential to predict patient outcomes since the moment of diagnosis, and therefore it may help to improve therapeutic strategies in the field of AML.

In the present study, we applied ML algorithms to gene expression data from AML cases in order to create new individualized models of survival based on retrospective data, and to understand their relationship with high-risk mutations.

METHODS

Two databases available in the *Gene Expression Omnibus* were used for model training and validation. The GSE37642 database was used for training, as it contains gene expression data from 562 adult patients diagnosed with AML who were treated in the multicenter phase III AMLCG-1999 trial. Median age was 45 years (range 18-85 years, 32% aged ≥ 65). The GSE68833 database was used for validation, and it contains gene expression data from 137 adult patients with AML included in *The Cancer Genome Atlas* (TCGA) cohort, with a median age of 59 years (range 18-88 years, 33% aged ≥ 65). Both databases used partially overlapping microarray chips, so that we rank-normalized the log2-transformed expression estimates and selected a set of 44,366 common gene expression probes between both cohorts.

Unsupervised Gene Expression Clusterization

Briefly, the *Mclust* algorithm (9) was used in order to detect the 2 most likely clusters of patients according to the expression of each probe (*Mclust* function, parameter G = 2). Briefly, the *Mclust* algorithm determines the most likely set of clusters according to geometric properties (distribution, volume, and shape). An expectation-maximization algorithm is used for maximum likelihood estimation, and the best model is selected according to Bayes information criteria. The association of each of these probe-level clusters with overall survival was calculated using cox regression. Thereafter, those probes whose clusterization was significantly associated with survival (Bonferroni adjusted p-value < 0.05) were selected for multivariate clusterization using the same *Mclust* algorithm. Cluster prediction was performed on the test set using parameters estimated in the training cohort, and cox regression was used to verify the association of this clusterization with overall survival. The Shoenfeld's test was used to assess the proportional hazards assumption.

Survival Analysis

We analyzed gene expression association with overall survival using cox regression implemented in R. Assumption of proportional hazards was checked with Schoenfeld's method.

Random Forest Survival Analysis

We started our analysis by testing the association of each probe with overall survival in the training set using multivariate cox regression. Schoenfeld's method was used to assess the proportional hazards assumption.

Random forest survival models were created with the *rfsrc* function implemented in the *randomForestSRC* package in R (10). We decided to use this type of model because, in contrast with deep networks, random forest can quantify the relative importance of each variable, and thus enable the filtering of low-importance variables for model reduction and performance improvement. Parameter tuning was performed using the *tune.rfscr* function, which optimizes the *mtry* and nnodes variables. Random forests were implemented on survival data of the training cohort. Bootstrapping without replacement was performed with the default *by.node* protocol. Continuous rank

probability score (CRPS) was calculated as the integrated Brier score divided by time, and represents the average squared distances between the observed survival status and the predicted survival probability at each time point. CRPS is always a number between 0 and 1, being 0 the best possible result. Survival prediction on the test cohort was performed using the *predict.rfsrc* function with default parameters. Harrel's concordance index (c-index) was used to assess model discriminative power on the bootstrapped training set and on the test set. C-index reflects to what extent a model predicts the order of events (e.g., deaths) in a cohort. C-indexes below 0.5 indicate poor prediction accuracy, c-indexes near 0.5 indicate random guessing and c-indexes of 1 represent perfect prediction.

Variable selection was selected by fitting age-adjusted cox regression models of overall survival in the training set. We initially analyzed different sets of genes in order to select the best input sets. Afterwards, we selected different sets of genes according to their multiple testing adjusted p-values; either False Discovery Rate (FDR) or Bonferroni-adjusted p-values below 0.01, 0.05, 0.1. Variable reduction was performed by iteratively removing those variables with low importance. Variable importance was calculated with the *vimp* function, and we iteratively removed those samples with negative or low weight (importance $< 1 \times 10^{-4}$). We replicated the models in the independent set.

RESULTS

Model Selection

We created six different survival models by taking into consideration different sets of transcripts at various thresholds of statistical significance. Importantly, all models achieved c-indexes > 0.67 in both the training and the validation sets. The best model contained 123 variables (absolute age, age ≥ 65, and the expression of 121 probes) (**Supplementary Table 1**). We name this model *Stellae-123* (ST-123). ST-123 achieved c-indexes of 0.7228 in the training set and 0.6988 in the test set, indicating a high reproducibility of the personalized risk prediction (**Figures 1A, B**). Apart from age, the most important variables in ST-123 were the expression of *KDM5B* and *LAPTM4B* (**Supplementary Table 2**).

Performance of ST-123 Over Time

The predictive accuracy of ST-123 was sustained over time, as reflected by the similar c-indexes obtained at 5 years of follow-up. Furthermore, mortality predictions were more accurate among those patients at high-risk of death according to the model. Additionally, CRPS plots indicate a rapid loss of predictability in the first days after diagnosis followed by an stabilization of survival predictions over time (**Figure 1C**). Indeed, c-indexes were less predictive for patients who died in the first days since diagnosis (**Table 1**). (ST-123 could clearly stratify patients in 4 different quartiles with different and reproducible mortality rates (**Figures 2A, B**).

ST-123 Performance in Patients With High-Risk Mutations

We tested the performance of the classifier in patients with high risk mutations in the TCGA cohort (validation set). We selected cases harboring *TP53* mutation and/or deletion (16 patients), *RUNX1* mutation (14 patients) and *ASXL1* mutation (6 patients) (1). We didn't include FLT3-ITD cases because this mutation type was not reported in the TCGA cohort. Using two-year survival predictions, we could observe a statistically significant association with overall survival within these high-risk patients (p-value 4.04×10^{-4}, age-adjusted p-value 2.16×10^{-3}) (**Figure 2C**). Indeed, both cox models achieved high concordance measures: c-indexes of 0.774 (standard error 0.052) and 0.767 (standard error 0.042), respectively.

DISCUSSION

The concordance measure of ST-123 is substantially superior to that described for the ELN risk classification (0.59) (6), which suggests an increased performance. Furthermore, ST-123 seems particularly useful to better stratify high-risk patients. These results are in line with other ML-based models of survival based on mutational data described so far (8), which emphasizes the possibility of improving risk stratification in AML by implementing artificial intelligence. Although other prognostic models, such as that published by *Sherve et al.* (6), report a higher predictive accuracy, these also incorporate several baseline clinical variables. Indeed, CRPS plots indicate that the highest loss of prediction accuracy in our model occurs early after disease diagnosis, which reflects the fact that clinical variables are the main predictors of early mortality in AML (8). On the contrary, we can see how the CRPS curve of our model rapidly reaches a plateau, maintaining values ∿0.15 for long periods of time, reflecting the stability of the predictions over time. It seems reasonable that the incorporation of clinical variables (such as performance status and leukocytosis) will improve the overall predictive accuracy of ST-123 by identifying those patients at risk of early death. On the contrary, little contribution of mutations to gene expression patterns has been observed in myelodysplastic syndromes, and this issue needs to be addressed in AML (11).

It has been hypothesized that a better risk stratification based on the integration of different layers of clinical and biological complexity could, for example, reduce the number of allogeneic stem cell transplants performed in patients with AML by 20-25% while maintaining the same overall survival rate as that with the current treatment recommendations (8). In the same line, high-risk older patients might benefit from innovative drugs and drug combinations (12). This could help to restrict the most aggressive or innovative treatments to those AML patients who really are at the highest risk of death. Therefore, it becomes increasingly important to incorporate these new personalized models of survival in the risk stratification of AML, rather than keep relying on imperfect

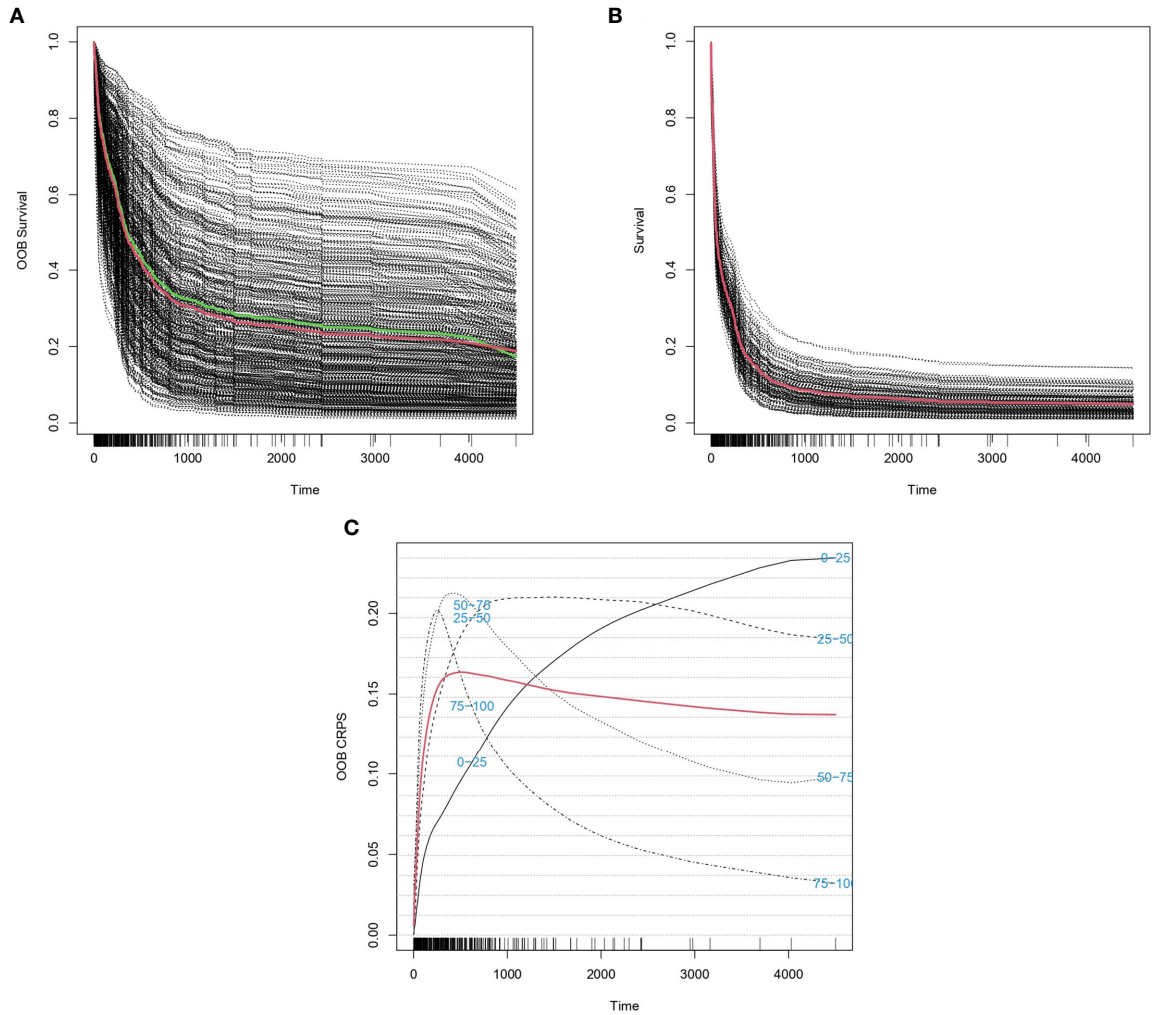

FIGURE 1 | Predicted individual survival curves according to the best random forests model. **(A)** Out-of-bag survival curves predicted for patients within the training cohort. The thick red line represents overall ensemble survival and the thick green line indicates the Nelson-Aalen estimator. **(B)** Individual survival curves predicted for patients within the test cohort. The thick red line represents overall ensemble survival. **(C)** Representation of out-of-bag CRPS over time. Red line is the overall CRPS. Additionally, stratified CRPS by quarters of predicted ensemble mortality are provided. Vertical lines above the x axis represent death events.

predefined risk groups. There is a need to analyze all these predictors in systematic and independent cohorts in order to provide further evidence about their effectiveness, and also to potentially integrate their individual predictive accuracies in more precise estimations of patient outcomes (13). Importantly, accumulated experience indicates that dichotomized molecular classifiers in AML can be reproduced in an unbiased and independent approach (14), but further efforts need to be made to compare the performance of personalized predictors such as ours.

From a biological point of view, the most important genes in ST-123 were *KDM5B* and *LAPTM4B*. Not surprisingly, both genes are deeply vinculated with carcinogenesis. *KDM5B* (Lysine Demethylase 5B) encodes a master epigenetic regulator of H3K4 methylation that regulates the expression of several oncogenes and tumor suppressors during carcinogenesis (15). *KDM5B* downregulates the oncogenic potential of leukemic stem cells by inducing H3K4-specific demethylation in murine and human MLL-rearranged AML cells, thereby promoting cell differentiation (16). Indeed, the KDM5B protein is the target of various inhibitors that are under study for the treatment of cancer (17). In the same line, the oncogene *LAPTM4B* (Lysosomal Protein Transmembrane 4 Beta) plays several roles in carcinogenesis, such as promoting tumor growth and metastasis, inhibiting apoptosis, initiating autophagy and driving multidrug resistance mechanisms (18). Interestingly, the expression of *LTPM4B* has been shown to be prognostic in myelodysplastic syndromes and in different types of solid tumors (19, 20).

In conclusion, our results indicate that survival of patients with AML can be predicted by applying ML tools to

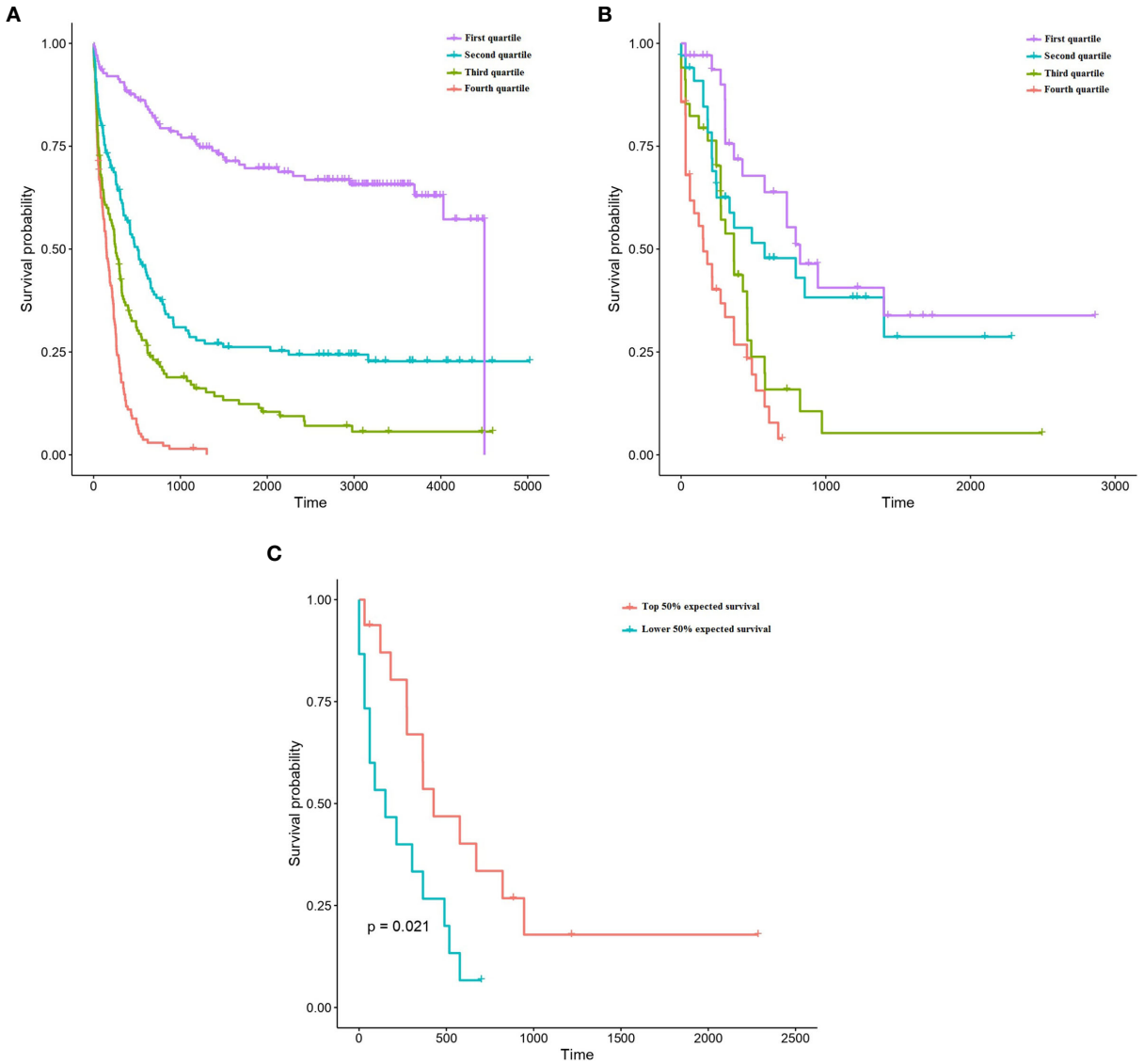

FIGURE 2 | Kaplan-Meyer plots representing the survival of patients depending on their classification to each quartile of predicted survival by ST-123 in the training **(A)** and validation **(B)** cohorts. **(C)** Kaplan-Meyer plots representing the outcomes of patients affected by high-risk mutations (*TP53* mutation/deletion, *ASXL1* mutation or *RUNX1* mutation) depending on their classification to the upper or lower median of predicted mortality by ST-123 in the validation set.

TABLE 1 | C-indexes of ST-123 after restricting the analysis to different time points since diagnosis.

Days since diagnosis	C-index (Training set)	C-index (Test set)
<10	68.65	59.66
<20	70.13	59.54
<30	71.88	60.19
<60	73.86	62.13
<100	74.06	63.11
>100	71.94	69.17

transcriptomic data, and that such predictions are particularly precise among patients with high-risk mutations. The possibility to enrich transcriptomic models such as ours with clinical and mutational data will lead to a more precise and holistic prediction of AML survival, paving the way for the development of new personalized treatment strategies.

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

AM had the idea and performed the research. AM, AP, MC, JD, MG, and BA analyzed the data and wrote the paper. NA, LB, RF, AA, MA, and EF analyzed the manuscript and made suggestions. CC, GM, PM, MM, and JL gave final consent for publication. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.657191/full#supplementary-material

Supplementary Table 1 | Characteristics of the different random forest models created at the different significance thresholds. Error rates (1-concordance) are provided for predictions of survival at 1, 2, 3, 4, 5 and full follow-up periods.

Supplementary Table 2 | Variables included in the final model along with their relative importance. For gene expression probes, we also report the type of input used (either continuous gene expression counts or *Mclust*-based binary clusterization).

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MINI REVIEW
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Taming Cell-to-Cell Heterogeneity in Acute Myeloid Leukaemia With Machine Learning

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Acute Myeloid Leukaemia (AML) is a phenotypically and genetically heterogenous blood cancer characterised by very poor prognosis, with disease relapse being the primary cause of treatment failure. AML heterogeneity arise from different genetic and non-genetic sources, including its proposed hierarchical structure, with leukemic stem cells (LSCs) and progenitors giving origin to a variety of more mature leukemic subsets. Recent advances in single-cell molecular and phenotypic profiling have highlighted the intra and inter-patient heterogeneous nature of AML, which has so far limited the success of cell-based immunotherapy approaches against single targets. Machine Learning (ML) can be uniquely used to find non-trivial patterns from high-dimensional datasets and identify rare sub-populations. Here we review some recent ML tools that applied to single-cell data could help disentangle cell heterogeneity in AML by identifying distinct core molecular signatures of leukemic cell subsets. We discuss the advantages and limitations of unsupervised and supervised ML approaches to cluster and classify cell populations in AML, for the identification of biomarkers and the design of personalised therapies.

Keywords: AML, machine learning, classification, clustering, leukaemia

INTRODUCTION

AML is an aggressive and fast-progressing leukaemia characterised by the accumulation of myeloid progenitors (1). Although most patients achieve remission after first line chemotherapy and haematopoietic stem cell transplantation, about 40% later relapse (2). Long-term survival following relapse is below 20% with a median survival of 4-6 months, an outcome that has not improved over the last two decades with conventional approaches (2–4) and novel therapies are therefore urgently needed (4).

AML is a molecularly heterogeneous group of diseases with a complex mutational landscape, characterised by intra- and inter-patient variation (**Figure 1A**). Advances in next-generation sequencing and single-cell technologies have revealed that AML cells display genetic and epigenetic heterogeneity in different patients and even within the same patient multiple sub-

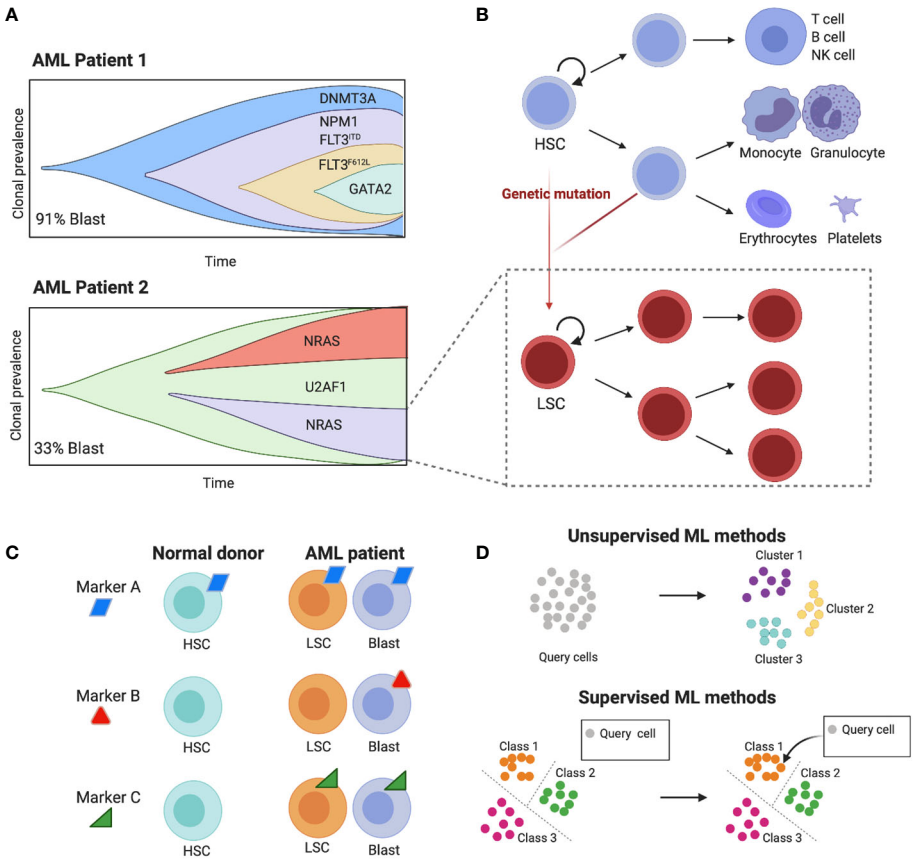

FIGURE 1 | The high cell-to-cell heterogeneity in AML tumours can be dissected using machine learning methods. **(A)** The schematic representing clonal diversity in two putative AML patients highlights the complex intra and inter-patient variation of cell diversity (schematics adapted from Petti et al., 2019). Importantly, each clone carries its own hierarchical structure (here shown for one clone as an example). **(B)** Leukemic populations share the hierarchical organization of normal hematopoietic development, where hematopoietic stem cells (HSCs) differentiate into multiple cell lineages, giving rise to all mature blood cells (blue lineages). Genetic mutations induce malignant transformation and give rise to leukemic stem cells (LSCs) that share some characteristics of their normal counterparts such as unlimited ability to self-renew and the potential to give origin to a variety of more mature leukemic subsets (red lineages). **(C)** Ideal targets for immunotherapy with engineered T cells are those present in both leukemic blast and LSC cells and absent in healthy cell types. Targets that are ubiquitously expressed will fail to target specific leukemic populations and will be toxic for normal cells (on target off, tumour toxicity). Targets that are absent from LSC will render the treatment prone to relapse. Due to the high cell heterogeneity in AML more than one molecule is likely to fulfil these requirements. **(D)** Machine learning methods to identify cell populations can be unsupervised and supervised. The former uses the intrinsic structure of the data to cluster cells in an automatic fashion. The second uses a predefined set of groups to classify unknown cells, leveraging previous knowledge. Figure created with BioRender.com.

clones co-exist, each carrying its own hierarchical structure and possessing distinct immunophenotypes (5).

A non-genetic source of heterogeneity in AML is its proposed hierarchical structure, mimicking the cellular hierarchy in normal hematopoietic development (**Figure 1B**). In healthy individuals, this involves a stepwise differentiation process, with hematopoietic stem cells (HSCs) giving rise to progressively more mature blood cells (6–8). LSCs lie at the top of AML cellular hierarchies, and carry an unlimited ability to self-renew as well as giving origin to a variety of more mature leukemic subsets (1), each expressing characteristic patterns of cell surface markers. LSCs can persist in a dormant state, making them selectively unresponsive to conventional chemotherapies and allowing them to eventually fuel disease relapse. For these reasons, the effective targeting of LSCs underpins any successful treatment for AML.

A promising approach is to target LSCs using immunotherapy with autologous T cells genetically redirected to express Chimeric Antigen Receptors (CARs). In fact, CAR-T cells can effectively target tumour cells irrespectively of their quiescent status. However, the lack of surface markers preferentially expressed on LSCs as opposed to healthy HSCs has hindered the development of cell-based immunotherapy strategies for AML, given the high risk of on-target off-tumour toxicity (9, 10). In addition, some of the targets tested so far (e.g. CD33 or CD123) have heterogenous expression in the LSC compartment, with the risk of relapse due to their incomplete targeting (11). Upon relapse, genetic and immunophenotypic heterogeneity in AML LSCs further increases, complicating the discovery of 'one fits all' drug target (12).

As a result of AML's heterogenous nature, CAR-T cell approaches against a single target are unlikely to be effective,

thus the design of combinations of CAR-T cells against multiple targets requires a systematic characterization of the expression levels of surface antigens in AML cell populations at single-cell resolution (**Figure 1C**) (9).

The unprecedented resolution achieved with single-cell technologies has enabled the dissection of cell populations, including tumour and rare cell types that could not be identified using conventional bulk sequencing (13, 14). In AML, the quantitative phenotyping of leukemic cell profiles has allowed the identification of leukemic subsets without prior knowledge of phenotypic markers for their prospective isolation, opening up new analytical challenges for their clinical interpretation (5, 15–19).

Despite Machine Learning (ML) techniques having shown prognostic utility in classifying patients at high risk of relapse and having been applied to risk-adapted treatments [review by (20)], they have only been recently applied to resolve heterogeneity in single-cell datasets from AML patients (15, 18). Fortunately, there has been an explosion of new algorithms based on ML for the characterization of cell populations in single-cell datasets (**Table 1**) that could be applied to identify molecular markers specific to AML subpopulations.

Here, we review some recent state-of-the-art ML methods with the potential to shed light into cell heterogeneity in AML and identify biomarkers for specific cell populations in single-cell datasets. Benchmarking of some recent methods has been done by (37) and (38). Rather than an extensive discussion of algorithms, we provide a general overview of tools available to identify cell populations in single-cell studies, highlighting ones that have the potential to reveal new and rare cell types in AML and aid the design of personalised treatments.

MACHINE LEARNING FOR CELL TYPE IDENTIFICATION IN SINGLE-CELL DATASETS AND BIOMARKER DISCOVERY FOR PERSONALISED IMMUNOTHERAPY

Single-cell high-throughput techniques, such as scRNA-seq, quantitatively characterise **cell types** within a tissue (39). Typical workflows in single-cell transcriptional profiling include dimensionality reduction and clustering of cells based on their gene expression patterns followed by manual annotation of cell clusters from known cell type **markers** (40). In the context of AML and other cancers, transcriptionally similar malignant cells are expected to group together, and can be unambiguously identified by the expression of certain feature genes that can be used as biomarkers for designing personalised treatments.

The identification of cell types using typical workflows has several drawbacks: first, rare cell types are easily missed and grouped together with some more prevalent ones; second, cell identity is often not discrete but lies in a continuum (for instance, cells with mixed identities or in transition); and third, the clustering can reflect other sources of variability unrelated to cell types (41). To address these issues, ML tools have recently been developed allowing quantitative identification and probabilistic assignment of cell types, thus aiding the identification of rare and heterogeneous cell populations.

In general, ML approaches are either **unsupervised** or **supervised** (**Figure 1D**). The main difference being the use of prior knowledge. Supervised methods are **trained** on an **annotated reference** with known **classes** of cell types, whereas unsupervised models identify patterns in the data without prior knowledge. A summary of recent methods is shown in **Table 1**.

Recent ML Unsupervised Methods

A common task for unsupervised methods is to use the intrinsic structure of the data to find clusters of cells. The advantage of these approaches is that cells can be grouped in an automatic and unbiased manner and thus, have the potential to discover unknown cell populations.

The popular single-cell processing packages Seurat (42) and Scanpy (43) use a graph-based clustering approach combined with modularity optimization to group transcriptionally-similar cells together. Markers differentially expressed in each cluster can be found using different methods, including logistic regression. The cell identity of each cluster is assigned manually according to previous knowledge of cell-type specific markers. The main disadvantage of this approach is that the number of clusters depends on a resolution parameter assigned by the user (higher values will lead to a greater number of clusters) and thus, they may not faithfully reflect cell types.

The recently developed Single-Cell Clustering Assessment Framework (SCCAF) (24) generates an optimal number of clusters automatically. After the data has been clustered, SCCAF builds an ML classifier (logistic regression) using part of the data (training). By applying this model to the rest of the dataset (test), it iteratively merges clusters that appear indistinguishable to the ML classifier to produce the final optimum clustering. The output of the model is a weighted list of feature genes characteristic of every cluster that often include known markers for a given cell type and could potentially be used to detect common biomarkers of leukemic cell subsets from AML patients.

Another unsupervised method, single-cell consensus clustering (SC3) uses the first 4-7% * N (number of cells) **eigenvectors** to build multiple **k-means clustering** solutions (21). After hierarchical grouping, the final clustering is driven by the combination of multiple clustering solutions. The output is a list of marker genes that define each consensus cluster. While SC3 may not be the most sensitive method to find rare populations (such as LSCs), SC3 was successful in identifying clusters of prevalent genetic subclones with different mutations in myeloproliferative neoplasms (21). A disadvantage of this method is that it does not scale well for datasets with more than 5,000 cells (44).

A recent unsupervised method, weighted-nearest neighbour (WNN), was used to cluster cells using multiple data modalities (e.g. surface proteins and transcriptomes) measured in the same cell (25). This method uses **k-nearest neighbours** (kNN) to learn cell-specific modality "weights". When applied to a multiomics dataset generated from human bone marrow samples (45), it showed that the combination of surface proteins and gene

TABLE 1 | Summary of recent ML-based methods to identify cell types.

Algorithm name	Classification type	Method	Input data	Important contribution	Reference
SC3	Unsupervised	Consensus clustering and hierarchical clustering	Normalised expression matrix	Transcriptome-based identification of genetic subclones in myeloproliferative neoplasms	(21)
cNMF	Unsupervised	Non-negative matrix factorization	Expression matrix and several parameters	Identification of previously misclassified immature skeletal muscle cells in a published dataset from brain organoids	(22)
scCOGAPS	Unsupervised	Non-negative matrix factorization	Normalised and log-scaled expression matrix	Identification of gene expression signatures characteristic of discrete cell types in the developing retina	(23)
SCCAF	Unsupervised	Logistic Regression and self-projection	Expression matrix and several parameters	Identification of cell states associated with different stages of erythroid maturation in mouse	(24)
WNN	Unsupervised	K-nearest neighbours and Jaccard distance	Expression matrix and protein matrix (or any other single-cell measurement)	Single-cell multimodal analysis improves resolution of cell states in the immune system and identify previously unreported subpopulations	(25)
CellAssign	Supervised	Expectation-Maximization hierarchical model	List of cell markers, subset of expression matrix containing the marker genes and some parameters	Resolution of malignant and non-malignant cells and their molecular dynamics during disease progression in follicular lymphoma	(26)
Garnett	Supervised	Multinomial elastic-net regression	Hierarchical list of cell markers (positive and negative) and expression matrix	The model trained on a mouse lung dataset is successfully applied to detect both healthy cell types and tumor cells in a human lung cancer dataset	(27)
scmap	Supervised	k-means (scmap-cluster) and k-nearest-neighbour (scmap-cell)	Annotated reference dataset and query expression matrix	Cell types in a test datasets are annotated with high accuracy irrespectively of batch effect	(28)
CHETAH	Supervised	Hierarchical Spearman correlation	Annotated reference dataset and query expression matrix (both normalised and log –scaled)	The cell type identification algorithm correctly identifies cancer cells absent in the reference dataset as “unassigned” or “intermediate”	(29)
scClassify	Supervised	Hierarchical ordered partitioning, ensemble learning and weighted k-nearest-neighbour	Annotated reference dataset and query expression matrix (both log –transformed)	Identification of cell types from the Tabula Muris single cell dataset that were unidentified in the original publication, including very rare populations	(30)
SingleR	Supervised	Correlation to training set	Annotated reference dataset and query expression matrix (both normalised and log-transformed)	Identification of a subgroup of macrophages whose molecular markers are upregulated in samples from patients with idiopathic pulmonary fibrosis.	(31)
SingleCellNet	Supervised	Random Forest	Annotated reference dataset and expression matrix (both raw)	Cells from pancreatic tissue that were “unclassified” in the original study are identified as Schwann cells and gamma cells	(32)
SuperCT	Supervised	Artificial Neural Network	Pre-trained ANN model and a query expression matrix	The model predicts cell types with high accuracy in multiple single cell test datasets including cord blood mononuclear cells and mouse pancreatic cancer.	(33)
ACTINN	Supervised	Artificial Neural Network	Annotated reference dataset and query expression matrix	Model trained on a T cell subtype reference accurately predicts T cell subtypes from an independent peripheral blood mononuclear cells dataset	(34)

(Continued)

TABLE 1 | Continued

Algorithm name	Classification type	Method	Input data	Important contribution	Reference
Moana	Supervised	Support Vector Machine	Pre-trained model and raw query expression matrix	Identification of common and cell type-specific gene expression responses to IFN-β treatment in peripheral blood cells	(35)
scPred	Supervised	Support Vector Machine	Annotated reference dataset and query expression matrix (both normalised)	Prediction of pathological cell states in gastric and colorectal cancer	(36)

expression was superior for identifying cell populations than using one data modality alone. Multiomic single-cell technologies quantifying both surface proteins and transcriptomes of individual cells (e.g. CITE-seq), could be ideally applied to the identification of surface targets for the design of cell based immunotherapies (46).

Other unsupervised methods rely on Non-negative matrix factorization (NMF) methods (22, 23). These methods allow for the identification of cell types and, simultaneously, **cell states.** Given the great transcriptional heterogeneity seen in AML even within clonal populations carrying the same mutational patterns (16), it may be helpful to consider cell identities and activities separately when clustering leukemic populations. Moreover, NMF is potentially useful to identify LSC populations in AML, where the classical surface proteins defining primitive cell types are present in highly similar patterns to healthy HSCs, but a 'malignant stem-like' profile can still be identified (47).

Recent ML Supervised Methods

Supervised methods to classify cell types exploit previously identified cell types and use either known marker genes or annotated reference datasets as an input to probabilistically assign new cells to a given category.

Some methods take a list of markers for each cell type as input (48). For example, CellAssign (26) uses predefined cell types input as a marker gene list to build a hierarchical model that produces a statistical classification of cells. This approach was used to delineate the composition of the tumour microenvironment in serial samples (treatment and relapse) from follicular lymphoma. Garnett (27) also takes as input a list of markers. The format of the input list permits accounting for cellular hierarchy (i.e, cell subtypes) and can include positive and negative markers to define cell types (27).

Other supervised methods use an annotated reference dataset to classify cell types but differ in the features and the ML methods used to train models (see **Table 1**). For instance, SingleCellNet (32) uses the most discriminative **gene pairs** (top pair transformation) to build a **random forest** classifier while methods such as scPred (36) and Moana (35) use principal components as features to fit a **support vector machine** (SVM). Some methods rely on one or several similarity metrics (such as SingleR (31)) and **k-nearest neighbours** (kNN) to map query datasets into a known reference [e.g. scmap (28) and scClassify (30)]. Other methods use the training dataset to build an **Artificial Neural Network** (ANN) model such as SuperCT (33) and ACTINN (34) with an input layer containing as many nodes as the number of genes in the training set and an output layer with nodes equal to the number of cell types. Interestingly, both ANN methods provide pre-trained models that could be used to classify new AML datasets.

An advantage of supervised ML approaches is that cell types are assigned probabilistically and some approaches allow for the possibility of an "unassigned" category (26–28, 32, 34). The unassigned label for cells that are absent or are very different in the reference dataset is key to limit misclassification and to allow the discovery of new cell types.

Algorithms such as CHETAH (29) and scClassify (30) allow for intermediate categories that can highlight populations with a mixture of identities as previously reported in AML (49). These methods are based on hierarchal correlation trees to classify test datasets (29, 30).

As more annotated single-cell datasets become available, the primary advantage of supervised methods is leveraging previous knowledge. Reference datasets of human bone marrow cells from healthy individuals are available from resources such as the Human Cell Atlas (50). Distinct cell populations or patient-specific tumour clones could be identified as unknown (because they are very different or absent in the reference data sets). As AML single-cell datasets become more abundant, they can be integrated with healthy single or multimodal references using ML methods (25).

A disadvantage of supervised methods is that they rely on known markers or accurate cell type annotations to build classification models. Often, markers for rare cell populations, such as LSCs, are unknown, not robust (51) or can be expressed by more than one cell type (15). Further, in many cases, annotation of single-cell datasets requires additional standardisation (29).

DISCUSSION

ML techniques are able to find non-trivial patterns in high-dimensional data (52). In fact, ML has already proven useful in identifying markers in bulk studies in prospectively isolated leukemic sub-populations (53, 54). However, ML has not reached its full potential for the characterisation of AML cell populations at single-cell resolution, partly due to the recent development of large datasets (5, 15–18).

Here we have reviewed tools to aid biomarker discovery using ML at single-cell level resolution. Many ML models explicitly quantify the contribution of individual features (genes) for a given classification. Importantly, genes identified in microarray data as important for classifying samples into "AML" or "no-AML" were not always differentially expressed (55). This means that traditional differential expression analysis could fail to identify biomarkers that are good predictors for assigning a class to a given group of cells (36). Thus, ML algorithms can find biomarkers that otherwise will be missed, expediting the design of suitable target combinations for immunotherapy.

Recently, it was shown that single-cell transcriptomics is capable of dissecting genetic subclones in AML, such as $GATA2^{R361C}$, which cluster separately from normal hematopoietic cell types (16). This observation suggests that subclonal diversity in AML could be associated with distinct gene expression profiles which ML techniques can leverage to identify mutated populations. Some AML mutations create subtle differences in expression profiles (15–17) and isolating these populations represents an analytical challenge contemporary ML methods could address.

Moreover, recent experimental innovations allowing for the simultaneous quantitative assessment of cellular and molecular information at single-cell resolution promise to better dissect cell heterogeneity in AML. Particularly important is the ability to detect mutations in single cells combined with their transcriptional profiling, offering an unprecedent opportunity to identify specific leukemic cell populations (13, 15–17, 56, 57). For instance, the combination of single-cell transcriptomics and mutational profiles allowed the distinction of pre-leukemic clones, LSC and healthy HSC (17). ML such as SVM could be used next to identify molecules that maximise this classification as done before for bulk RNA-seq and microarray data (53).

In addition, the identification of mutant and non-mutant cells allows for applying ML methods to both all and only mutated cells to further characterise subpopulations (16), and can be used to fine-tune ML classification algorithms. For instance, a two-step ML classification strategy was applied to bone marrow samples of AML patients (15). First, a fraction of mutant cells was identified by genotyping and these were classified into one of six normal haematopoietic cell types (monocyte-like, progenitor-like, etc.). Subsequently, these malignant cell types were incorporated as additional classes in a second classifier that successfully identified mutant and normal cells from their transcriptome profiles.

The simultaneous characterization of surface proteins at single-cell resolution (46) is especially important for isolation of heterogeneous cell populations. There are some analytical challenges with the integration of multiple data modalities (58), but combining different data types from the same cell has already shown to improve the identification of cell populations in AML datasets (16, 18) and healthy bone marrow samples (25), thus we anticipate that multimodal datasets will improve the performance of ML models in isolating specific cell populations and may facilitate the identification of relevant surface targets for precision immunotherapy.

All the methods reviewed here will incur a certain degree of **underfitting** and **overfitting**. Thus, it is wise to compare algorithms in the initial cell composition assessment. Some, such as hierarchical methods, are potentially more suitable for AML samples, where there is an intrinsic hierarchy shared with normal hematopoietic development (**Figure 1B**). Also, methods that enable the recognition of intermediate cell types, mixed identities or different cell states would be more suitable for the identification of abnormally differentiated leukemic cells, known to be characteristic of AML (49).

Finally, we anticipate that single-cell resolution phenotyping will be important for the design of cell-based immunotherapy combinatorial strategies accounting for clonality and differentiation states of AML populations, with ML likely playing a pivotal role in the selection of optimal therapeutic targets for the design of personalised workflows tailored to each patient.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version. YC conducted literature review and wrote the manuscript in consultation with RP. SG and AG critically revised the work.

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GLOSSARY

Artificial Neural Network (ANN): A type of supervised learning model where multiple simple functions (artificial neurons) are connected in layers, which sequentially process information. ANNs contain an input layer which passes the information to several "hidden" layers, these are activated depending on the input and feed this information to the output layer, which reflects the assigned class. Deep ANNs are those with many hidden layers.

Annotated Reference Dataset: A (single cell) expression dataset, where the cell types of all cells are known, e.g. through experimental validation. Reference datasets are useful to assign likely labels (classify) to new cells (query) that are similar to cells in the reference.

Cell state: The cellular activities a cell is carrying out at a given moment. These can be general (e.g. hypoxia response) or specialised (e.g. cycling).

Cell type: The kind of cell, e.g. a Red Blood Cell. Cell types are commonly associated with specialised functions, markers and histology. However, it is important to note that cell types are often fluid or non-constant and distinguishing two similar cell types can be difficult.

Classes and Clusters: Both describe grouping data points by measurements made during experiments. The key difference is that clusters refer to groupings obtained through unsupervised learning, whereas classes refer to groups from supervised learning. Importantly, classification is able to assign class-names (based on the training dataset), whereas clusters are "nameless".

Eigenvector and Eigenvalue: Eigenvectors are the vectors which do not change in direction if a matrix is linearly transformed; the eigenvalue is the scalar denoting by how much the eigenvector has changed in magnitude after transformation. In this way eigenvectors and eigenvalues can represent a matrix (eigen decomposition), encoding the fundamental structure of the matrix. An example use of eigen decomposition is Principal Component Analysis.

Gene pairs (top pair transformation): Transformation based on comparing the expression of pairs of genes within each cell, limited to genes that are preferentially expressed in each cell type defined in the training data, as well as those genes that are specifically under-expressed in each type.

K-nearest neighbour algorithm: Training datapoints of known classes are mapped into a (usually dimensionally reduced) space. New datapoints are then mapped into the same space and a class is assigned to each as the most frequent class of their k (e.g. 7) nearest neighbours.

K-means clustering algorithm: Method that aims to partition n observations into k clusters such that each observation belongs to the cluster with the nearest mean.

Marker: A characteristic protein, often expressed on the surface of a cell, or gene, e.g. a transcription factor, that can be used to mark a specific cell type experimentally.

Overfitting: Occurs when a model fits a particular dataset too closely, it will then fail to generalise to unseen data.

Random Forests and Decision Trees: Decision Trees learn a "yes-no flow chart" to sequentially partition data until a classification is reached; individual decision trees are prone to overfitting. Random Forests are multiple independent decision trees trained together. Classification output is the average output of all trees, overcoming overfitting seen in an individual tree.

Single cell gene expression matrix: The processed data obtained from single cell expression experiments is usually represented by a gene expression matrix. This is a large table where every row represents a gene, and every column the reads measured in a single cell.

Supervised Learning: A collection of machine learning approaches where characteristic labels (classifications) are learned from data annotated with known classes.

Support Vector Machine (SVM): Is a supervised learning algorithm that aims to find the hyperplane that best separates two classes, i.e. goes "right through the middle". SVM can be extended to non-linearly-separable data using a kernel function that maps the data to a higher dimensional space in which it is linearly separable.

Training Data and Test Data: When training a machine learning algorithm datasets should be split into training and test data. The model is learned using the training data. Test data are a subsection of the original dataset, that the model has not encountered in training, and can be used to approximate the model's expected performance on unseen data.

Underfitting: Occurs when a model does not adequately learn the underlying structure of the data.

Unsupervised Learning: A collection of machine learning approaches that learn a pattern in the unlabelled data.

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REVIEW
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Escape From Treatment; the Different Faces of Leukemic Stem Cells and Therapy Resistance in Acute Myeloid Leukemia

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Standard induction chemotherapy, consisting of an anthracycline and cytarabine, has been the first-line therapy for many years to treat acute myeloid leukemia (AML). Although this treatment induces complete remissions in the majority of patients, many face a relapse (adaptive resistance) or have refractory disease (primary resistance). Moreover, older patients are often unfit for cytotoxic-based treatment. AML relapse is due to the survival of therapy-resistant leukemia cells (minimal residual disease, MRD). Leukemia cells with stem cell features, named leukemic stem cells (LSCs), residing within MRD are thought to be at the origin of relapse initiation. It is increasingly recognized that leukemia "persisters" are caused by intra-leukemic heterogeneity and non-genetic factors leading to plasticity in therapy response. The BCL2 inhibitor venetoclax, combined with hypomethylating agents or low dose cytarabine, represents an important new therapy especially for older AML patients. However, often there is also a small population of AML cells refractory to venetoclax treatment. As AML MRD reflects the sum of therapy resistance mechanisms, the different faces of treatment "persisters" and LSCs might be exploited to reach an optimal therapy response and prevent the initiation of relapse. Here, we describe the different epigenetic, transcriptional, and metabolic states of therapy sensitive and resistant AML (stem) cell populations and LSCs, how these cell states are influenced by the microenvironment and affect treatment outcome of AML. Moreover, we discuss potential strategies to target dynamic treatment resistance and LSCs.

Keywords: therapy resistance, acute myeloid leukemia, leukemic stem cells, minimal residual disease, plasticity

INTRODUCTION

The major problem with cancer treatment is that many patients obtain impressive remissions after a wide variety of treatments yet retain residual tumor cells after the initial therapy, which can develop into recurrence or metastasis. Intratumor heterogeneity in relation to therapy response is the key factor contributing to this treatment failure. For several decades, initial therapy for AML remained unchanged and typically consisted of repetitive courses of intensive combination chemotherapy

with anthracyclines and cytarabine, the so-called "7 + 3" standard regimen, aiming at achieving complete remission (CR, <5% of leukemic cells). In general, and dependent on several risk factors, for patients under 60 years the 5-year overall survival (OS) rate after this treatment is 40–50%, while for patients older than 60 years the OS rate is only 15–20%. This poor OS rate in the elderly is partly explained by a higher proportion of patients with an unfavorable disease biology and an inability to tolerate intensive chemotherapy (1). The poor treatment outcome of AML is in part of the patients due to refractoriness to chemotherapy at diagnosis but in the major part caused by relapse originating from a small subpopulation of therapy-resistant leukemia cells (minimal residual disease, MRD) (2, 3) (**Figure 1**). Relapses can occur after months or even years. One of the causes of resistance to anthracyclines is the altered function of the efflux pumps (4), while the efficacy of cytarabine is significantly reduced by enzymatic degradation (5). MRD is the sum of resistance mechanisms to initial therapy,

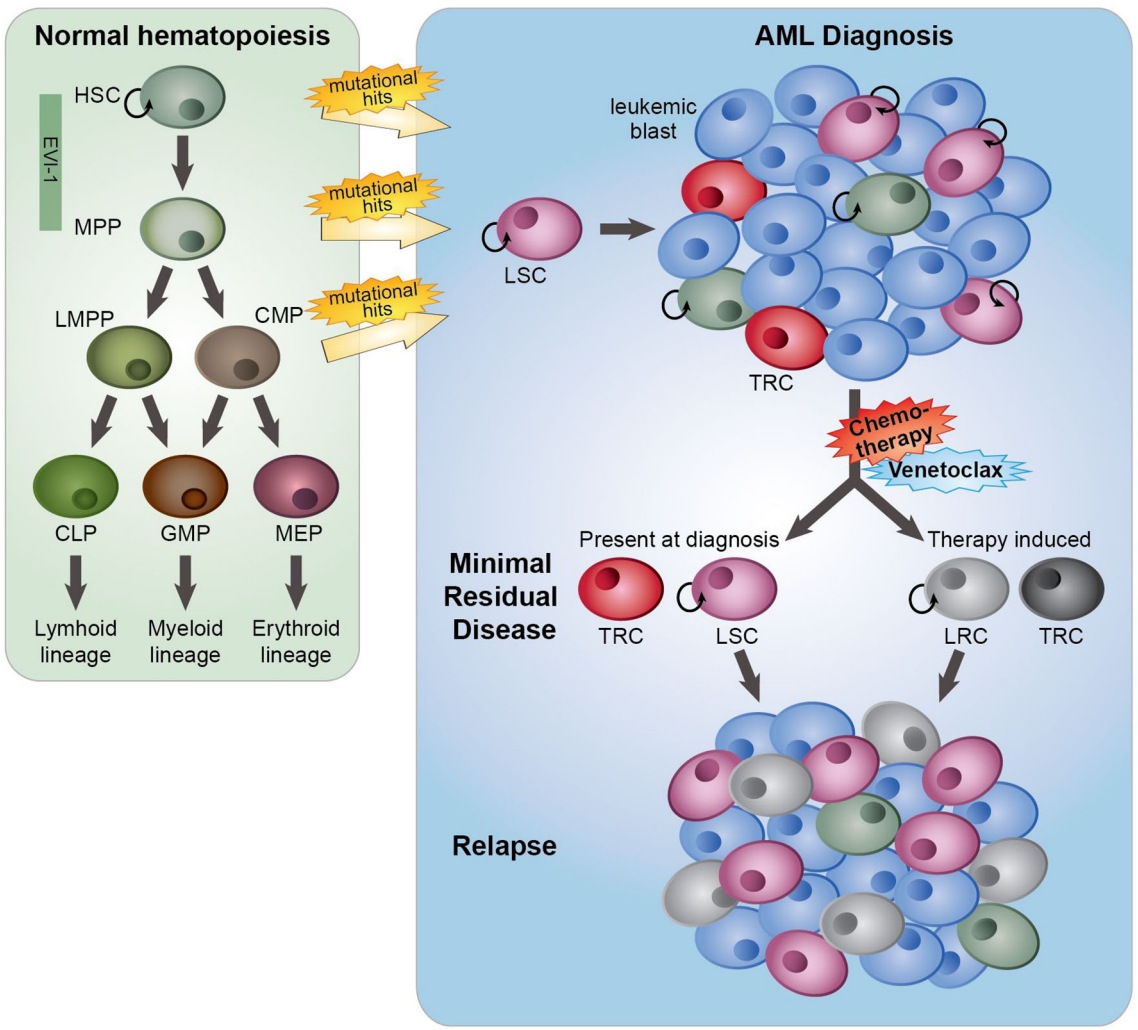

FIGURE 1 | The role of minimal residual disease, therapy resistance and LSCs in AML relapse development. In normal hematopoiesis (green box), quiescent hematopoietic stem cells (HSCs) with self-renewal capacity give rise to multipotent progenitors (MPPs), which can differentiate towards lymphoid primed multipotent progenitors (LMPPs), common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), granulocyte-macrophage progenitors (GMPs) and megakaryocyte erythroid progenitors (MEP). These lineage-committed progenitors can produce terminally differentiated lymphoid, myeloid and erythroid blood cells. AML originates from the transformation of normal HSCs, MPPs or more committed progenitors, developing in leukemic stem cells (LSCs) that subsequently can give rise to full-blown leukemia. AML initiated from HSC and MPP highly express the transcription factor EVI-1. At AML diagnosis (blue box), a heterogeneous leukemia cell population with a variety of sensitivities to therapy exists. Moreover, LSCs and normal hematopoietic (stem) cells, responsible for reconstituting the normal healthy blood cells after therapy, co-reside in the patient's bone marrow. While treatment with standard induction chemotherapy results in complete remission in the majority of AML patients, a population of (chemo)therapy-resistant cells (TRCs) (minimal residual disease) constituting AML cells with leukemia-initiating potential survive the treatment. LSCs with leukemia-initiating potential within MRD could initiate sooner or later a relapse. Instead of (chemo)therapeutic selection of pre-existing subpopulations of LSCs, AML cells might adaptively obtain a leukemia re-initiating cell (LRC)-phenotype upon exposure to treatment.

and MRD load is prognostic for OS and relapse-free survival of AML patients (2, 3), indicating that therapeutic targeting of MRD may delay or prevent a relapse, but may also improve the chance of a more successful stem cell transplantation. Leukemia cells with stem cell features ("leukemic stem cells", LSCs) residing within MRD are thought to be responsible for re-initiation of the tumor (6, 7) (**Figure 1**). Changes in the chromatin and epigenetic landscape, facilitating transcriptional changes, are part of non-genetic therapy resistance, and the presence of a LSC gene expression signature in AML predicts the risk of developing a relapse (8, 9). Also, a LSC expression signature mainly consisting of genes that are epigenetically regulated showed an association with response to chemotherapy (10). Notably, during chemotherapy treatment the frequency as well as the phenotype of LSCs changed, indicating that the treatment itself affects the appearance of relapse-initiating cells (11, 12) (**Figure 1**).

Venetoclax-based therapy could induce responses in approximately 70% of untreated older AML patients (13, 14). However, upfront resistance to venetoclax as well as relapse following CR was appearing (14–17). Recently, it was shown that monocytic AML is less sensitive to venetoclax than immature AML due to the metabolic properties of the monocytic AML cells (18).

The biggest challenge in the treatment of AML is relapse and refractoriness caused by persistent AML cells that survive the initial treatment. The term "drug tolerant persisters" is frequently used to describe cancer cells with non-mutational mechanisms of drug resistance. Persistent leukemia cells might exist prior to drug treatment; however, they might also become resistant upon exposure to a treatment (**Figure 1**). As shown decades before with bacteria, persistent tumor cells can resume their drug sensitivity upon drug removal (19). Leukemia "persisters" are characterized by their quiescence state, different energy consumption, adaptation to the bone marrow (BM) microenvironment, changing identity, and phenotypic plasticity. Mechanisms that cause their persistence include a variety of epigenetic, transcriptional, and metabolic processes that often co-exist (**Figure 2**). Clinical targeting of persistent AML cells (MRD) will increase efficacy of treatment and finally the survival of patients. For the development of successful therapeutic strategies targeting AML MRD, it will be crucial to understand the mechanisms that drive this persistence.

THE HETEROGENEITY AND PLASTICITY OF LSCs AND MINIMAL RESIDUAL DISEASE

AML has a hierarchical cellular organization, with a small fraction of self-renewing LSCs at the apex of the hierarchy. LSCs are defined as cells that can self-renew, which was experimentally shown by the capacity of re-initiation of leukemia when (serial) transplanted into immunodeficient mice. Moreover, LSCs can differentiate into non-LSC blasts (20, 21). The identity of LSCs is influenced by clonal genetic evolution, epigenetic alterations, their metabolic state, and their microenvironment, finally resulting in intra- and interpatient heterogeneity in their response to therapy (8, 22–24) (**Figure 2**). In AML, LSCs have been described as a heterogeneous and relatively rare cell population that could be isolated from the leukemic bulk population by flow cytometry based on expression of a set of specific cell surface markers, including CD34+CD38− (20, 21). In general, CD34+CD38− AML cell populations display a higher leukemia-initiating cell frequency than CD34+CD38+ AML cell populations (8, 22). Interestingly, in 80% of CD34-positive AML cases, at least two distinct LSC populations were identified, a CD34+CD38− fraction resembling normal lymphoid primed multipotent progenitors (LMPP-like LSCs) and a CD34+CD38+ fraction resembling granulocyte-macrophage progenitors (GMP-like LSCs) which have been derived from the LMPP-like LSC population. In almost 15% of CD34-positive AML cases, there is a dominant population of LSCs that resembles multipotent progenitors (MPP-like LSCs) (25) (**Figure 1**). Understanding the biological properties of AML LSCs, particularly their similarities and differences from normal CD34+CD38− hematopoietic stem cells (HSCs) and their heterogeneity and plasticity in individual patients is important for the development of therapies that can specifically eradicate these cells during the course of disease.

In the past, the identification of cell surface markers differentially expressed between LSCs and HSCs has been intensively studied, but thus far no unique marker universally expressed on CD34+CD38− LSCs across AML patients but not on normal HSCs has been discovered. This is mainly due to the intra- and interpatient heterogeneity of AML. Many lymphoid and myeloid antigens are aberrantly expressed in AML, which give rise to complex leukemia-associated (immune)phenotypes (LAIPs) that are highly heterogeneous and differ between the individual AML patient (26). Expression of these LAIPs can also change during the course of the disease (27). Despite this heterogeneity, it was shown that specific cell surface markers are increased on LSCs compared to HSCs or progenitors in part of the AML patients, including CD123 (28), C-type lectin-like molecule-1 (29), CD25 (30), CD32 (30), CD44 (31), IL1RAP (32), and GPR56 (33) (**Figure 2**).

During the development of AML, genetic aberrancies induce epigenetic changes, leading to increased epigenetic plasticity in leukemic cells. While it was shown that genetic mutation-driven, unique epigenetic profiles in leukemic cells could be developed (34), LSCs demonstrated to share an epigenetic signature that is mostly independent of genetic mutations (10). Accordingly, transcriptional profiling of AML LSCs revealed a molecular signature that is associated with leukemia "stemness" and leukemia-initiating capacity and that is highly correlative with AML prognosis (8, 9), indicating that the presence of a transcriptional "stemness" profile affects response to treatment. Moreover, reactivation of a self-renewal-associated transcriptional signature was shown to be an important characteristic of the transformation of normal progenitors into LSCs (35). Together, these results implicated that there is high

degree of plasticity in imposing "stemness" on leukemic cells, and showed the importance of stem cell features for the response to chemotherapy of AML cells.

Recently, several studies challenged the idea that LSCs are less sensitive to chemotherapy than non-LSCs. These studies showed that LSCs are not selectively resistant to chemotherapy (12, 36, 37), and that the identity of the LSC is transient and dynamic during the AML disease course (11, 12). Enhanced LSC frequencies and phenotype diversity were observed at relapse as compared to diagnosis, suggesting that current AML

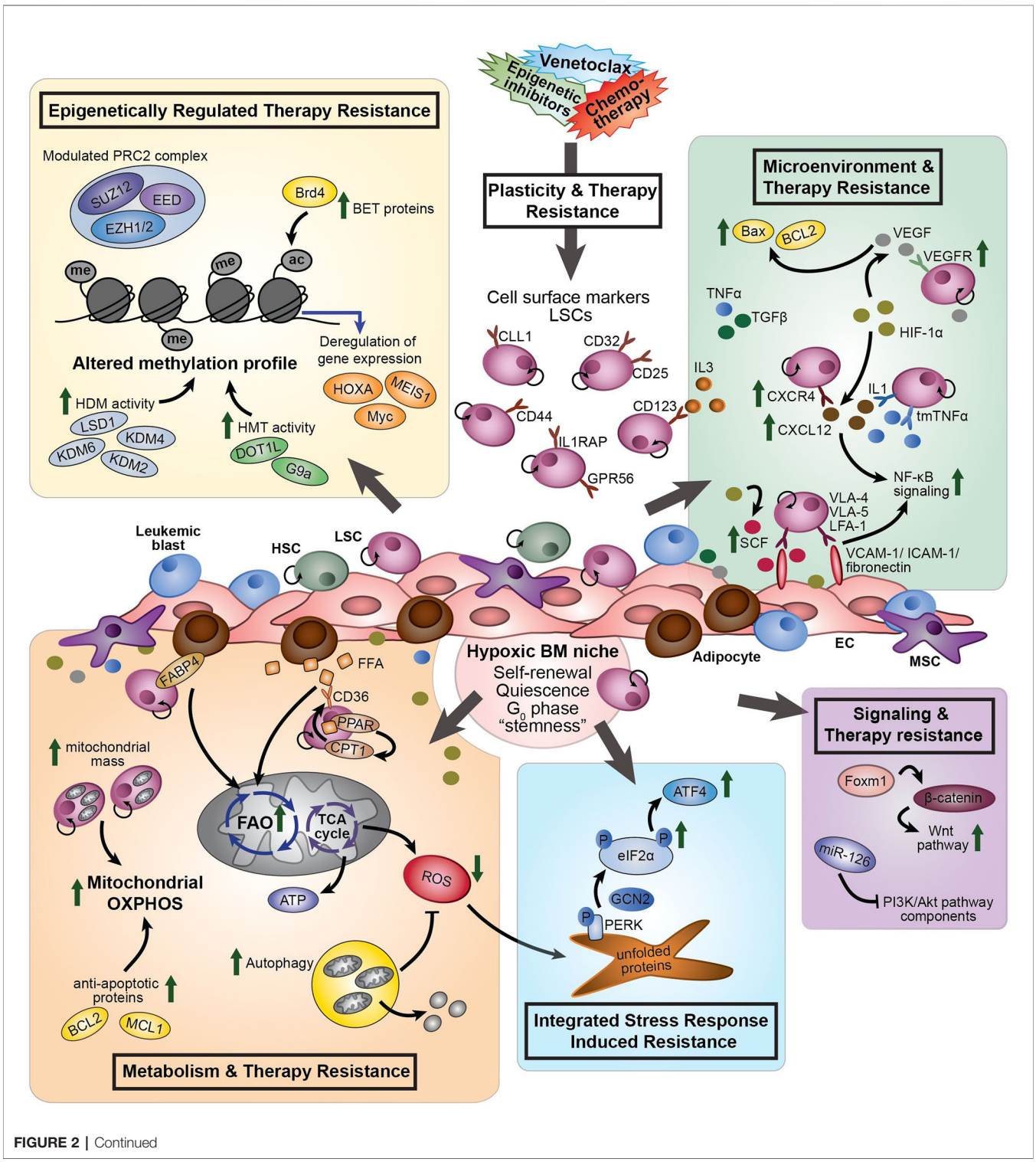

FIGURE 2 | Continued

FIGURE 2 | The different processes involved in therapy resistance in AML (stem) cells. *Plasticity and therapy resistance*: Within the hypoxic BM niche, persistent AML (stem) cells might exist prior to drug treatment or might become resistant and obtain leukemia re-initiating potential upon exposure to a treatment, such as chemotherapy or venetoclax. The transcriptional signature of AML LSCs is associated with "stemness" and leukemia-initiating capacity. LSCs aberrantly express lymphoid and myeloid antigens, including CD123, CCL1, CD25, CD32, CD44, GPR56 and IL1RAP. These cell surface markers differ within and between individual AML patients and can change during the course of the disease. Different processes are involved to induce therapy resistance: *Epigenetically regulated therapy resistance* (yellow box): LSCs and therapy-resistant AML cells show modulated expression of components of the PRC2 complex (i.e. EZH1/2), upregulation of BET proteins (*i.e.* Brd4), and altered methylation profile caused by enhanced HDM activity (i.e. LSD1, KDM2, KDM4, and KDM6) and HMT activity (*i.e.* DOT1L and G9a). These differential epigenetic processes induce transcriptional deregulation of genes, like MEIS1, Myc and HOXA. *Microenvironment and therapy resistance* (green box): In response to hypoxia, HIF-1α signaling promotes expression of VEGF, CXCR4, and SCF. CXCR4 on AML cells interact with CXCL12, increasing stromal protective effects. VEGF expressing ECs protect VEGFR3-expressing AML cells from chemotherapy-induced apoptosis, due to increased BCL2/Bax ratios. LSCs express VLA-4, VLA-5 and LFA-1 on their cell surface, which interact with the stromal ligands VCAM-1, ICAM-1 and fibronectin to support attachment to stromal cells, promoting NF-κB signaling. SCF enhances anti-apoptotic and proliferative effects of fibronectin expressed on AML cells. Pro-inflammatory cytokines, including TNFα, influence cell adhesion, promoting LSC survival and chemotherapy resistance through modulation of NF-κB signaling. Several members of the TGF*β* family suppress proliferation of AML cells and enhance chemotherapy resistance. *Metabolism and therapy resistance* (orange box): AML LSCs often lack the ability to enhance glycolysis and therefore switch from anaerobic glycolysis to mitochondria-mediated OXPHOS to generate ATP. Therapy-resistant AML cells show increased mitochondrial mass and high OXPHOS. In addition to glucose, FFA can by metabolized to drive the TCA cycle and OXPHOS. Adipocytes, the major MSC present in the BM, support survival of AML cells by stimulating FAO and OXPHOS through fatty acid transfer. Part of the LSC population expresses the fatty acid transporter CD36 to control uptake of FFA. CPT1, regulated by PPAR, controls FAO translocation by conjugating FFA with carnitine for translocation into the mitochondrial matrix. FABP4 is involved in the interaction of adipocytes with LSCs. Furthermore, LSCs are able to reduce ROS production generated by mitochondria in response to hypoxia, by activation of autophagy. Inhibition of BCL2 by venetoclax efficiently reduces LSC survival by impairing homeostasis and inhibiting OXPHOS. *Integrated Stress Response induced resistance* (blue box): In response to stress stimuli, such as ROS, the PERK-eIF2*α* ISR pathway is activated in LSCs. eIF2*α* is phosphorylated by GCN2 or PERK, reducing global protein synthesis while allowing translation of specific genes, including ATF4. Increased activity of the ISR pathway protects LSCs to enable restoration of homeostasis favoring survival. *Signaling and therapy resistance* (purple box): Upregulation of FOXM1 activates the Wnt/*β*-catenin signaling pathway by direct binding to *β*-catenin, inhibiting its degradation, preserving LSC quiescence and promoting LSC self-renewal. Overexpression of miR-126 repress multiple components of the PI3K/Akt pathway, resulting in the proliferation of LSCs, delayed G_0 exit of LSCs and enhances resistance to combination chemotherapy. AML, acute myeloid leukemia; ac, acetyl group; ATF4, activating transcription factor 4; ATP, adenosine tri-phosphate; BET, bromodomain and extra-terminal motif; Brd4, bromodomain-containing 4; BM, bone marrow; CCL1, C-type lectin-like molecule 1; CPT1, carnitine O-palmitoyltransferase 1; CXCR4, CXC chemokine receptor-type 4; CXCL12, CXC motif chemokine ligand 12; eIF2*α*, eukaryotic initiation factor 2*α*; EC, endothelial cells; EED, embryonic ectoderm development; EZH1/2, zeste homolog 1 or 2; FAO, fatty acid oxidation; FABP4, fatty acid binding protein 4; FFA, free fatty acids; GCN2, general control non-derepressible 2; HDM, histone demethylase; HIF-1*α*, hypoxia-inducing factor 1*α*; HMT, histone methyl transferase; HSC, hematopoietic stem cell; ICAM-1, intracellular adhesion molecule 1; IL3, interleukin 3; ISR, integrated stress response; KDM, histone lysine demethylase; LFA-1, lymphocyte function-associated antigen 1; LSD1, lysine-specific histone demethylase 1; LSCs, leukemic stem cells; me, methyl group; MSC, mesenchymal stromal cells; NF-κB, nuclear factor κB; P, phosphorylation; PPAR, peroxisome proliferator-activated receptor; PERK, protein kinase RNA-like ER kinase; PI3K, phosphatidylinositol 3-kinase; PRC2, polycomb complex 2; ROS, reactive oxygen species; SCF, stem cell factor; SUZ12, suppressor of zeste 12; TCA, tricarboxylic acid cycle; TGF*β*, transforming growth factor *β*; tmTNFα, transmembrane tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VLA-4, very late antigen-4; VLA-5, very late antigen-5.

therapeutic regimens promote dramatic changes in the LSC compartment. Instead of chemotherapeutic selection of pre-existing subpopulations of drug-resistant AML LSCs, AML cells adaptively acquired a leukemia re-initiating cell (LRC)-specific signature, implying that LRCs are developed in response to chemotherapy (12). Interestingly, as these LRCs express a LRC-specific gene signature, it might be very useful to measure LRC markers after initial therapy for the early detection of relapse initiation. Thus, LRCs were described to emerge after chemotherapy treatment and differed from LSCs at diagnosis (12) (**Figure 1**), indicating that it is most relevant to study the relapse-initiating cells after the initial treatment.

THE QUIESCENT AND THERAPY RESISTANT STATE OF LSCs

Cell-intrinsic, epigenetic, and transcriptional reprogramming leading to reduced cell proliferation is associated with reduced sensitivity to treatment and an increased tumor-initiating potential (**Figure 2**). This reduced proliferative cell state might exist before therapy but can also be acquired as a result of treatment. For example, persistent cancer cells derived from human glioblastoma patients entered a slow-proliferation state following treatment with the tyrosine kinase inhibitor dasatinib (38). In patient-derived xenografts (PDX) of primary acute lymphoblastic leukemia (ALL), a rare dormant subpopulation of ALL cells resembling relapse-inducing cells were treatment resistant and contained "stemness" properties (39). While most of the clonogenic AML cells in AML patient samples were actively cycling, a small number of AML progenitors were quiescent. Furthermore, leukemia cells capable of engrafting in NOD/SCID mice showed to be quiescent and in the G_0 of the cell cycle prior to transplantation. After serial transplantation a rare quiescent long-term human SCID leukemia-initiating cell population with extensive self-renewal capacity and extremely low proliferation rate was identified, suggesting that only a small proportion of the LSC pool has extensive self-renewal potential and drives progression to AML (40).

CD34+CD38− LSCs in AML reside in the endosteal region of mouse bone marrow, wherein they are primarily quiescent and protected from cytarabine-induced apoptosis (7). These AML LSCs could be activated to enter the cell cycle and become sensitized to cytarabine by administration of exogenous granulocyte colony-stimulating factor (41). Also, interleukin (IL)-3, a critical cytokine involved in myeloid differentiation and the ligand of CD123, could enhance the proliferation rate of AML blasts. CD123 is involved in the potential of LSCs to engraft in NOD/SCID mice, as blocking CD123 with the monoclonal

antibody 7G3, in the absence of exogenous human cytokines, inhibited the engraftment and growth of AML CD34+CD38− cells (28). Together, these results indicated that CD34+CD38− LSCs are more quiescent than bulk AML cells, but can still respond to hematopoietic growth factors thereby entering the cell cycle.

MicroRNA (miR)-126 was identified as a critical regulator of LSC quiescence (**Table 1**). Overexpression of miR-126 expanded primitive CD34-positive cells, delayed the G_0 exit of CD34+CD38− LSCs, while the G_0 status of CD34+CD38+ and CD34-negative cell populations remained unaffected. Moreover, miR-126 overexpression enhanced resistance to the combination treatment of daunorubicin and cytarabine by preserving LSCs in a quiescent state (42). Thus, miR-126 can keep CD34+CD38− AML cells in a more primitive state by increasing the proportion of quiescent CD34+CD38− cells, thereby decreasing the overall proliferative output and differentiation of AML blasts. miR-126 is highly expressed in LSCs as compared to leukemic progenitors, and high miR-126 expression in AML is associated with poor OS and a higher chance of relapse. Targeting miR-126 in LSCs reduced their clonogenic capacity, in the absence of an inhibitory effect on normal BM cells (43). Multiple components of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway are repressed by miR-126 (42), consistent with previous mouse studies demonstrating that the PI3K/Akt pathway plays a key role in governing quiescence and regulating HSC and LSC self-renewal (44, 45) (**Table 1**). Although most known self-renewal regulators have comparable functions in HSCs and LSCs, regulation of the cell cycle by miR-126 is opposite. Reduced miR-126 levels resulted in HSC expansion, while the maintenance of LSC was impaired (42). Accordingly, expression of a constitutively active form of Akt in HSCs (45) or loss of the negative regulator PTEN (44) resulted in HSC exhaustion and LSC expansion, suggesting that targeting the PI3K/Akt signaling pathway, like targeting miR-126, will differentially affect quiescence and self-renewal in HSCs and LSCs.

Another promising therapeutic target to eliminate quiescent AML LSCs is FOXM1 (**Table 1**). *Foxm1* upregulation activated the Wnt/β-catenin signaling pathway by direct binding to β-catenin and stabilizing β-catenin through inhibition of its degradation, thereby preserving LSC quiescence, and promoting LSC self-renewal in MLL-rearranged AML. Targeting FOXM1 inhibited the survival, quiescence, and self-renewal of MLL-AF9-transformed LSCs (46).

The WT1 gene and HCK, a member of the Src family of tyrosine kinases, were identified as being more highly expressed in cell cycle-quiescent primary AML LSCs than in normal HSCs. Moreover, comparison of cell surface markers expressed on LSCs and normal HSCs revealed that CD32 and CD25 are promising therapeutic targets to specifically eradicate LSCs. While CD32 and CD25 are not or less expressed on normal HSCs, these markers are expressed in a large fraction of primary human AML LSCs (52.5% of the AML cases express CD32, CD25, or both on the LSCs), stably located on the LSC cell surface after chemotherapy treatment and present on cell cycle-quiescent AML-initiating cells residing within the endosteal niche (30).

Although several studies suggested that LSCs withstand chemotherapy regimens due to quiescence and dormancy (7, 41), this concept was recently challenged. In a AML PDX mouse model, residual AML cells were neither enriched in immature quiescent cells, nor in LSCs after treatment with cytarabine, suggesting that cytarabine similarly depleted quiescent G_0 AML cells and proliferating cells (36). LSCs were shown to have a variety of sensitivities to cytarabine, and treatment with cytarabine reduced the frequency of leukemia-initiating cells by inducing an exit from G_0, increasing proliferation and subsequent depleting part of the LSCs. Transcriptional profiling of cytarabine residual AML cells that contained leukemia-regenerative capacity showed that cytarabine sensitive and -resistant LSCs have a distinct transcriptome (12). Together, these results suggested that part of the LSC pool is not inherently resistant to chemotherapy, and also showed that quiescence or dormancy is not sufficient to protect LSCs from chemotherapy-induced cell death. Moreover, these results suggested that LSCs can acquire genetic and epigenetic alterations and exhibit phenotypic plasticity to adjust to environmental changes, highlighting the dynamic properties of AML LSCs during the course of therapy.

EPIGENETICALLY-DRIVEN DRUG RESISTANCE IN AML

Epigenetic changes contribute to resistance to chemotherapy and targeted treatments (89–91) (**Figure 2**). Epigenetic mechanisms driving sensitivity to therapy in the individual leukemia cell could be established by genetic aberrations, by signaling from the microenvironment but also by the leukemia cell of origin. Currently, it is increasingly recognized that therapeutic resistance in the absence of a genetic aberrancy is a major cause of recurrence and metastasis in several cancers, including AML (91–94). As epigenetic modifications, such as DNA methylation and post-translational modifications on histone tails, are reversible, these epigenetic marks provide great opportunities to target non-genetic therapy resistance using specific epigenetic inhibitors.

Since LSCs and leukemic blasts share a common set of genetic mutations in most AML cases, functional properties that differ between these two cell compartments are likely driven by epigenetic differences. Indeed, multiple differentially methylated regions were identified between LSC-containing and non-LSC-containing cell populations. In LSCs, these regions were predominantly hypomethylated and largely associated with transcriptional upregulation. The DNA methylation signature differentially present in LSCs compared to leukemic progenitors consisted of 71 genes, which were enriched for HOX genes, and which were associated with a poor prognosis independent of other known risk factors (10). In addition to DNA methylation, histone tail modifications, including acetylation, methylation, and ubiquitination contribute to transcriptional

TABLE 1 | Therapeutic targets and drugs to overcome therapy resistance mechanisms in AML (stem) cells.

AML (stem) cell therapy resistance mechanism	Target protein or process	Drug	Preclinical studies	Clinical trial for AML	References
Quiescence	miR-126	N/A	Reduction of clonogenic capacity of LSCs in the absence of an inhibitory effect on normal BM cells.	N/A	(42, 43)
	PI3K/Akt; PTEN	Rapamycin	Depletion of leukemia-initiating cells and restoration of normal HSC function.	- Phase I: rapamycin + decitabine in r/r AML - Phase I: rapamycin + chemotherapy in newly diagnosed AML, r/r AML and secondary AML - Phase II: rapamycin in r/r AML	(44, 45)
	FOXM1	Thiostrepton	Reduction of self-renewal capacity of LSCs in MLL-rearranged AML, synergistic effects with chemotherapy on induction of apoptosis in LSCs, and prolonged survival *in vivo*.	N/A	(46)
Epigenetically driven drug resistance	LSD1	Iadademstat (ORY-1001)	LSD1 target gene specific increase of H3K4me2, induction of AML blast differentiation and reduction of LSC self-renewal capacity, while sparing normal CD34+ cells.	Phase I/IIa in r/r AML (non-M3) and r/r MLL-rearranged AML	(47–49)
		TCP	Induction of differentiation of AML blasts and inhibition of AML cell growth.	Phase I/II: ATRA + TCP in r/r AML (non-M3)	(50, 51)
		GSK-LSD1	Myeloid differentiation in MLL-rearranged AML cells, causing global gains in chromatin accessibility, with an enrichment of PU.1 and C/EBPα at these open sites.	N/A	(52)
	EZH2 and/or EZH1	DZNEP	Reduced EZH2 and H3K27me3 levels, resulting in reduced CD34+CD38- LSC numbers. In combination with panabinostat, synergistic induction of apoptosis in AML cells, while sparing normal CD34-positive BM progenitor cells.	N/A	(53, 54)
		OR-S1	Reduction of LSC numbers, impaired AML progression and prolonged survival *in vivo*. Priming AML cells for chemotherapy-induced cell death.	N/A	(55)
		Valemetostat (DS-3201)	Recruitment of quiescent AML LSCs into cell cycle.	Phase I in r/r AML	(56)
	G9a	CM-272	Activation of interferon response, inhibiting proliferation and promoting apoptosis. Prolongation of OS in AML xenogeneic mouse models.	N/A	(57)
		Pinometostat (EPZ5676)	Tumor growth suppression, reduced colony-forming capacity, and terminal differentiation in DNMT3A-mutated AML cells.	Phase I in MLL-rearranged AML	(58, 59)
	BET proteins	JQ1	Anti-leukemic effects accompanied by terminal differentiation and elimination of LSCs. Reduction of BCL2 and c-myc levels, inducing apoptosis in NPM1c+ with or without FLT3-ITD or MLL-rearranged AML. In combination with panabinostat, synergistic induction of apoptosis in AML, while sparing normal CD34-positive BM progenitors.	N/A	(60, 61)
		Birabresib (OTX015)	Inhibition of cell growth, cell cycle arrest and apoptosis in AML cells.	Phase I in r/r AML	(62)
		Molibresib (GSK525762)	Downregulation of BCL2, c-myc and IRF8, reduction in clonal growth and induction of apoptosis in AML cells, and survival advantage *in vivo*.	Phase I/II in r/r AML and secondary AML	(63)
Hypoxia and metabolism	BCL2	Venetoclax	Inhibition of OXPHOS and impairing energy homeostasis, upregulation of myeloid differentiation genes, and downregulation of cell cycle and proliferation genes.	Phase Ib and phase III: venetoclax + azacitidine in elderly AML Phase Ib: venetoclax + azacitidine or decitabine in elderly AML Phase II: venetoclax in r/r AML Phase III: venetoclax + cytarabine in newly diagnosed AML ineligible for intensive chemotherapy	(13–17, 64, 65)
	MCL-1	(–)BI97D6	Induction of mitochondrial apoptosis in AML, due to disrupted MCL-1/BIM and BCL2/Bax interactions, while sparing normal hematopoietic stem/progenitor cells.	N/A	(66)

(Continued)

TABLE 1 | Continued

AML (stem) cell therapy resistance mechanism	Target protein or process	Drug	Preclinical studies	Clinical trial for AML	References
		VU661013 + venetoclax	Destabilization of BIM/MCL1 and induction of apoptosis in AML. Synergistic reduction in tumor burden after combination therapy.	N/A	(67)
		AZD5991	Induction of apoptosis in AML by activation of Bak-dependent mitochondrial apoptosis, and anti-tumor activity.	Phase I/II: AZD5991 monotherapy or in combination with venetoclax in r/r AML	(68)
		AMG176	Rapid induction of apoptosis in AML, growth inhibition of human AML *in vivo*.	Phase I: AMG176 + azaciditine in r/r AML	(69)
		MIK665 (S64315)	Induction of AML cell death, induction of antitumor responses after combination treatment with a BCL2 inhibitor.	Phase I: S64315 in r/r AML (non-M3) Phase I: VOB560 (BCL2 inhibitor) + S64315 in r/r AML	(70)
	HIF-1α	Echinomycin	Inhibition of colony formation, induction of apoptosis of CD34+CD38- AML cells. Elimination of leukemia initiating cells and reduction in human leukemic burden.	N/A	(71)
		TH-302	Hypoxia-dependent apoptosis in AML cells, by reducing HIF-1α expression, decreasing proliferation, inducing a cell-cycle arrest, and enhancing double-stranded DNA breaks. Prolongation of residual disease after chemotherapy treatment *in vivo*.	N/A	(72, 73)
Bone marrow micro-environment	CXCR4	Plerixafor (AMD3100)	Mobilization of AML blasts from the BM niche into peripheral circulation, sensitization of leukemic blasts to cytarabine and decreased tumor burden *in vivo*.	Phase I/II: plerixafor + mitoxantrone, etoposide and cytarabine in r/r AML Phase I/II: plerixafor + decitabine in newly diagnosed elderly Phase I/II: plerixafor + fludarabine, idarubicin, cytarabine and G-SCF in r/r AML	(74–76)
		ARV-825	CXCR4 and CD44 downregulation, impairment of CXCL12-directed migration, increased oxidative stress, downregulation of gene signatures associated with stemness, Wnt/β-catenin and Myc pathways, and decrease in number of LSCs.	N/A	(77)
	TGFβ	1D11	Enhanced cytarabine-inducted apoptosis of AML cells in hypoxic conditions. Combination treatment with plerixafor and cytarabine decreased leukemia burden in FLT3-mutated mice.	N/A	(78)
	VEGF-C	VGX-100	Reduction of clonogenic capacity and induction of differentiation of AML blasts, *via* suppression of FOXO3A and inhibition of MAP/ERK proliferative signals.	N/A	(79)
		Bevacizumab	N/A	Phase I: bevacizumab n r/r AML Phase II: bevacizumab + mitoxantrone + cytarabine in r/r AML Phase II: bevacizumab + daunorubicin + cytarabine in newly diagnosed elderly	(80) (81, 82)
Adipocytes	FABP4	BMS309403	Inhibition of AML blast survival, while sparing nonmalignant CD34-positive cells.	N/A	(83)
	FAO	Etomoxir	Disruption of metabolic homeostasis in AML cells, induction of ROS production and ATF4. Inhibition of CPT1a and subsequent sensitization of AML cells to cytarabine. Induction of an energetic shift towards low OXPHOS and increase in anti-leukemia effects of cytarabine.	N/A	(36) (84) (85)
		Avocatin B	Upregulation of ATF4 and synergistic induction of ROS production and apoptosis in AML cells after combination treatment with cytarabine.	N/A	(84)
Stress response	Autophagy	VSP34 inhibitors	Inhibition of autophagy and cell proliferation abolishes acquired FLT3 inhibitor resistance.	N/A	(86)
	PERK/eIF2α pathway	Atovaquone	Phosphorylation of eIF2α, enhancing ATF4 protein expression and ATF4-specific target genes, inhibiting OXPHOS, and inducing growth arrest and apoptosis in AML cells.	N/A	(87)
		GSK2606414 + BIX-01294	Synergistic induction of apoptosis in AML cells, while sparing normal HSCs.	N/A	(88)

LSC, leukemic stem cell; HSC, hematopoietic stem cell; BM, bone marrow; r/r AML, relapsed and refractory acute myeloid leukemia; OS, overall survival; ROS, reactive oxygen species; oxidative phosphorylation, OXPHOS, N/A, not applicable.

output. The methylation of histones is dynamically regulated, and depending on the position and nature of the methylated residues, can either promote or repress transcription. Histone methyltransferases (HMTs) add methyl groups to specific histone residues, while histone demethylases (HDMs) remove methyl groups from specific residues on histone tails (95). In general, the methylation on histone 3 (H3) lysine-4 (H3K4), H3K36, and H3K79 activate gene expression, whereas methylation on H3K9, H3K27, and H4K20 is associated with transcriptional repression. LSCs in MLL-rearranged leukemia were characterised by high levels of H3K4me3 and low H3K79me2, resulting in aberrant expression of HOX genes and Meis1 (96). The H3K4 lysine specific demethylase 1 (LSD1; KDM1A) is highly expressed in AML, and associated with transcriptional repression (97). LSD1 can demethylate mono- and di-methylated H3K4 and H3K9, but also has a scaffolding activity that facilitates recruitment of histone deacetylases to chromatin sites where transcription factors such as GFI and GFIB are bound (98). Moreover, LSD1 sustains the differentiation block in certain molecular subtypes of AML, particularly in MLL-translocated AML, and is required for the self-renewal potential of LSCs (97, 99). Targeting LSD1 in AML promoted differentiation (100), and compromised the self-renewal capacity of LSCs in (pre)clinical models of AML (**Table 1**). For example, iadademstat (ORY-1001), a covalent and highly specific LSD1 inhibitor, induced a gene-specific increase of H3K4me2, resulting in induction of AML blast differentiation and reduction of LSC self-renewal capacity, while sparing normal CD34-positive cells (47). In phase-I clinical trials, treatment of relapsed/refractory (r/r) AML patients with iadademstat resulted in the induction of differentiation of the leukemic blast cells, and reduction of blast percentages in peripheral blood and BM. In some individual patients there was a relation between response and induction of *CRISP9* (48, 49), *CD86*, *VCAN*, *S100A12* and *LY96* (48). However, In a phase-I/II clinical trial using the LSD1 inhibitor tranylcypromine (TCP) in combination with all-trans retinoic acid (ATRA) for r/r AML patients, there was an overall response rate of only 20%. Molecular markers associated with response were not identified and a global increase in H3K4me2 upon TCP was only observed in two patients (50). In non-responders to TCP and ATRA combination therapy there was enrichment for expression of genes involved in mTOR signaling and for expression of higher basal histone deacetylase 2 (*HDAC2*) (51). Moreover, treatment of MLL-rearranged AML with the LSD1 inhibitor GSK-LSD1 caused global gains in chromatin accessibility, with a strong enrichment of PU.1 and C/EBPα at these open sites. Depletion of PU.1 or C/EBPα generated resistance to LSD1 inhibition (52).

Next to LSD1, expression of other HDMs, including the H3K27 demethylase KDM6B, the H3K36 demethylase KDM2B, and the H3K9/36me3 demethylase KDM4A correlated to treatment response. KDM6B is increased in AML as compared to normal BM, and positively correlated with poor survival. Treatment with the KDM6 inhibitor GSK-J4 enhanced the global levels of H3K27me3 and showed synergistic effects with cytarabine. GSK-J4 treatment decreased the expression of cell-cycle related pathways and HOX genes in AML cells (101). KDM2B interacts with active chromatin, is overexpressed in LSCs, and can function as the DNA binding subunit of the polycomb repressive complex 1 (PRC1). Knockdown of KDM2B was shown to impair the self-renewal capacity of LSCs (102). Inhibition of KDM4A by JIB-04 restored the levels of H3K36me3 but also induced sensitivity to chemotherapy (103). Together, these studies indicate crucial roles for several HDMs in non-genetic therapy resistance in AML, and show their potential as therapeutic targets to deplete LSCs and/or MRD.

PRC2 is one of the two classes of polycomb-group (PcG) protein complexes. PRC2 contains histone methyltransferase activity and primarily trimethylates H3K27, leading to silencing of target gene transcription, while PRC1 is able to condense nucleosomes, inducing stable gene silencing. The PcG proteins are required for long term epigenetic silencing and have an important role in maintaining "stemness" (104). The enhancer of zeste homolog 2 (EZH2) is a member of the PRC2 complex, mediating transcriptional silencing through H3K27me2/3 (105). In AML, quiescent LSCs express the highest levels of EZH1 and EZH2 (55). In about 45% of relapsed AML, loss of EZH2 and consequently a reduction in H3K27me3 occurs. This EZH2 loss resulted in resistance toward multiple drugs, including tyrosine kinase inhibitors (90), and is associated with a poor OS (106). EZH2 loss may be mediated by the interaction between cyclin-dependent kinase 1 and heat shock protein 90, that induces EZH2 proteasomal degradation, and drug resistance *via* deregulation of HOX gene expression (90). Loss of EZH2 in AML can also be due to a 7/7q chromosomal deletion, as EZH2 is located on chromosome 7q36.1, or can be caused by splicing dysfunction as a result of mutations in U2AF1 or SRSF2. Genetic aberrancies in the U2AF1 and SRSF2 genes have been shown to decrease EZH2 mRNA levels in about 10-25% of AML patients (107, 108). AML and myelodysplastic syndrome patients with a 7/del7q are largely refractory to chemotherapy and have a particular poor prognosis (109). Also presence of U2AF1 and SRSF2 mutations in AML is associated with adverse outcome (110), which might be explained by loss of EZH2-driven resistance to treatment (90). Interestingly, low H3K27me3 levels in AML samples, potentially as a result of low EZH2, is also a parameter for a poor prognosis (90, 106). However, the role of EZH2 in therapy resistance in AML is complex and may depend on the context. Indeed, a stage-specific and opposite function for EZH2 at the early and late stages of the disease was suggested; EZH2 acted as a tumor suppressor at the stage of AML induction, while it exerted an oncogenic function during leukemia maintenance (111). Moreover, the therapeutic effect of EZH2 inhibition may be dependent on the treatment with which it is combined. The EZH2 inhibitor 3-Deazaneplanocin A (DZNEP) in combination with the HDAC inhibitor panabinostat synergistically induced apoptosis in primary AML cells, while not affecting the survival of normal CD34-positive BM progenitor cells (53). DZNEP reduced EZH2 and H3K27me3 levels, resulting in a reduction in the number of CD34+CD38- LSCs (54) (**Table 1**). Quiescent LSCs are highly dependent on both EZH1 and EZH2, and dual inhibition of EZH1 and EZH2 by OR-S1 primed AML

cells for chemotherapy-induced cells death (**Table 1**). Moreover, OR-S1 reduced the number of LSCs, impaired leukemia progression and prolonged survival of AML PDX mice (55). In a phase-I clinical trial, inhibition of EZH1/2 with valemetostat drove quiescent AML LSC into the cell cycle (56). Based on these results, it would be of interest to investigate if EZH1/2 inhibition reduces MRD load and/or LSCs after chemotherapy- and/or venetoclax treatment.

Apart from PRC2, other HMTs can regulate methylation on histone tails, including DOT1L and G9a. G9a catalyzes mono- and di-methylation of H3K9 and induces changes in redox homeostasis (112). G9a was shown to accumulate under hypoxic conditions (113), and as AML LSCs are known to reside in hypoxic BM niches, therapeutic targeting of G9a may efficiently eliminate AML LSCs (**Table 1**). Indeed, loss of G9a impaired AML progression and reduced LSC frequency (114). CM-272, a small molecule simultaneously inhibiting G9a and demethyltransferase (DNMT)-1 activity, inhibited proliferation, promoted apoptosis in AML cells, and prolonged the OS in AML xenogeneic mouse models (57). The HMT DOT1L plays a key role in initiation and maintenance of MLL-rearranged leukemia, because of its role in H3K79 methylation and subsequent upregulation of Meis1 and HOXA (115). Inhibition of DOT1L by EPZ5676 suppressed tumor growth, reduced colony-forming capacity, and induced terminal differentiation in DNMT3A-mutant AML cells (58). In a phase-I study, treatment with EPZ5676 resulted in a significant reduction in H3K79me2 levels, while CR was only achieved in two of the 51 r/r MLL-rearranged AML patients (59), indicating that anti-DOT1L monotherapy is not sufficient to achieve clinical benefit in r/r AML patients.

Other epigenetic modifiers playing a critical role in the maintenance of AML are bromodomain and extra-terminal motif (BET) proteins, which sustain Myc expression to promote aberrant self-renewal (116). BET family proteins, including bromodomain-containing 4 (Brd4), facilitate gene transcription by binding to acetylated lysines in histones and transcription factors. Brd4 was identified as a promising therapeutic target for AML (60, 61, 117) (**Table 1**), and targeting Brd4 using small hairpin RNAs or small molecule inhibitors resulted in a strong anti-leukemia effect, terminal myeloid differentiation and elimination of LSCs (60). Despite these promising preclinical results, monotherapy with BET inhibitors showed limited efficacy and CR was only induced in a few AML patients (62, 63). Resistance to BET inhibitors emerges from LSCs in the absence of new genetic mutations (93), and is acquired through adaptive transcriptional plasticity and the conversion of AML cells to a more immature LSC phenotype. Regulators of enhancer formation were identified as key mediators of the resistant state (92), but also chromatin remodelling, leading to activation of the Wnt signalling pathway, was shown to be involved in BET inhibitor resistance (93, 118). BET inhibitor resistant AML cells use available factors, such as PU.1 and interferon regulatory factor 8 (IRF8), to nucleate the different enhancers, facilitating remodelling of regulatory pathways that rapidly restore expression of survival genes (92, 118). Reversion of the BET inhibitor resistant phenotype was accomplished by targeting the mechanisms whereby AML cells use alternative enhancers. LSD1 inhibition is able to re-sensitize stable BET inhibitor resistant AML cells, by facilitating enhancer switching mediated by PU.1 and IRF8 (92). Although it has yet to be seen whether resistance to other (epigenetic) drugs work *via* similar mechanisms, these results suggest that rather than aiming at reversion of the transcriptional state of resistant cancer cells, it may be more effective to disable the process of enhancer remodeling.

AML THERAPY SENSITIVITY AND THE CELL OF ORIGIN

Epigenetic states conferred by cell of origin shape the molecular classification across a diverse array of tumor subtypes. The cell of origin of leukemic transformation is also a determinant of therapy sensitivity. Analysis of genetic abnormalities in paired AML samples at diagnosis and relapse indicated that leukemia-initiating cells within MRD can originate from rare LSCs, from a dominant subclone with a HSC phenotype, or from subclones of immunophenotypically committed leukemia cells that retained "stemness" (94). Leukemia initiated from HSC demonstrated to exhibit higher disease penetrance, aggressiveness, and resistance to chemotherapy and have higher expression of the transcription factor Mecom (EVI-1) than leukemia arising from more differentiated progenitor cells (119–122) (**Figure 1**). High expression of EVI-1 is a prognostic factor associated with inferior OS among AML patients harbouring MLL gene rearrangements (123). Moreover, HSC-derived leukemia exhibit decreased apoptotic priming, attenuated p53 transcriptional output, and resistance to LSD1 inhibitors. The expression of EVI-1 modulates the abundance and activity of the p53 protein. Interestingly, EVI-1^{high} AML cells are sensitized to LSD1 inhibition by venetoclax (122), suggesting that immature AML cases, which have their origin in the HSC or MPP, can be sensitized for anti-LSD1-induced apoptosis by therapeutic targeting of BCL2. The level of response to the combination of venetoclax and azacitidine is also related to the differentiation stage of the AML. More immature, primitive AML, likely originating from a more immature cell, such as the HSC or MPP, was more sensitive to venetoclax than differentiated monocytic AML. Venetoclax-resistant monocytic AML had a distinct transcriptomic profile, reduced expression of BCL2, and showed to rely on MCL1 for oxidative phosphorylation (OXPHOS) and survival. Consequently, there was outgrowth of monocytic subpopulations of AML cells after venetoclax treatment at relapse (18). Targeting these venetoclax-resistant monocytic leukemia cells might be accomplished by therapies that are highly efficient in eradicating more differentiated as compared to immature AML.

HYPOXIA, METABOLISM, LSCs AND THERAPY RESISTANCE

Hypoxia, a condition in which the normal tissue oxygen level is reduced, has been identified as a major contribution to resistance

to many drugs and an enhanced tumorigenicity of cancer stem cells (124). The transcriptional regulator hypoxia-inducible factor 1 alpha (HIF-1α) responds to hypoxia by binding to hypoxia-response elements and by regulating expression of hypoxia-response genes, finally resulting in activation of enzymes involved in DNA repair, cell differentiation and apoptosis (125). In hypoxic tumor cells, the accumulation of chemotherapeutic drugs is reduced, and induction of drug resistance by increased genomic instability, suppression of DNA repair and suppression of a cell cycle arrest can occur (126).

HSCs reside in hypoxic niches in the BM, in where HIF-1α signaling regulates the maintenance of their quiescent and pluripotent state by promoting HIF-1α-driven gene expression, including vascular endothelial growth factor (VEGF), CXC chemokine receptor-type 4 (CXCR4) and stem cell factor (SCF) (127). Since LSCs are well adapted to hypoxic conditions, there is a protective effect of the niche on the persistence of these LSCs, which results in their reduced sensitivity to therapy. Under hypoxic conditions, reactive oxygen species (ROS) are generated by mitochondria. In contrast to HSCs, LSCs are able to reduce generation of ROS and induce the activation of ROS removing pathways such as autophagy, resulting in enhanced survival of LSCs compared to HSCs (128).

Through glycolysis, glucose is metabolized to pyruvate, and in the presence of oxygen pyruvate can be further metabolized to acetyl-CoA, that is oxidized in the tricarboxylic acid (TCA) cycle to drive OXPHOS and the generation of ATP. Although AML LSCs are mainly in a low oxidative and quiescent cell state, they often lack the ability to enhance glycolysis and therefore switch from anaerobic glycolysis to mitochondria-mediated OXPHOS as their major pathway to generate energy, while HSCs rely mainly on anaerobic glycolysis (64). Also, residual AML cells after cytarabine treatment showed an increased mitochondrial mass, and retained active polarized mitochondria, reflecting a high OXPHOS status. Moreover, presence of a high OXPHOS gene expression signature was predictive for a worse treatment outcome in AML patients (36). The deacetylating mitochondrial protein sirtuin-3 (SIRT3) protected AML cells from cytarabine-induced apoptosis by inhibiting ROS production and by enhancing OXPHOS. Increased SIRT3 activity in AML cells resulted in resistance to chemotherapy (129). Together, these results showed that enhanced mitochondrial OXPHOS plays a major role in therapy resistance in AML.

Inhibition of BCL2 by venetoclax efficiently affected LSC survival by inhibition of OXPHOS and impairing energy homeostasis, resulting in upregulation of myeloid differentiation genes, and downregulation of cell cycle and proliferation genes (64) (**Table 1**). However, AML cells with high expression of the anti-apoptotic protein MCL1 showed resistance to BCL2 inhibitors (18, 64). To further reduce AML MRD load after venetoclax it may be an efficient strategy to use MCL1 inhibitors. Indeed, inhibition of MCL1 resulted in elimination of venetoclax-resistant AML (stem and progenitor) cells (18, 66–68) (**Table 1**). Several clinical trials are currently investigating the combination of BCL2 and MCL1 inhibitors, including AZD5991, AMG176 and MIK665 (68–70). In a clinical trial, treatment of elderly previously untreated AML patients with venetoclax in combination with the hypomethylating agents azacitidine or decitabine led to high overall response rates (13). This efficient response was due to targeting LSCs by inhibiting their amino acid uptake and catabolism, resulting in suppression of OXPHOS (65). Due to a more complex metabolic profile, LSCs derived from relapsed AML patients were less sensitive to this combination (130), highlighting again the importance of understanding the metabolic dynamics of LSCs during the course of disease.

In AML patients, the presence of mutations in IDH1 and IDH2 dysregulated mitochondrial function due to accumulation of 2-hydroxygluterate (2-HG). This resulted in increased ROS levels and activation of HIF-1α (131). LSCs residing within IDH1/2 mutated patients showed an increased dependency on BCL2, and were more sensitive to venetoclax, due to inhibition of cytochrome *c* oxidase by 2-HG (132). However, in a phase 2 clinical trial treating high-risk r/r AML patients with venetoclax, only 33% of the IDH1/2 mutated AML patients showed a CR. The effect of venetoclax treatment on the survival of LSCs was not determined in this trial (15).

The synthesis of HIF-1α in the hypoxic BM microenvironment induces upregulation of CXCR4 on the membrane of LSCs, thereby enhancing their migration ability, their anchorage in the BM niche and their resistance to therapy (7). Although targeting hypoxia and HIFs has been considered as potential therapeutic approaches for AML, several studies showed contrasting results (**Table 1**). CD34+CD38− LSCs have the highest levels of *HIF-1α*, and loss of HIF-1α led to the elimination of LSCs (71). In contrast to this result, loss of HIF-1α accelerated conversion of pre-leukemic cells to LSCs and shortened AML latency. Moreover, deletion of HIF-1α gave rise to faster progression of chemotherapy-treated MLL-AF9 AML cells (133).

Hypoxia-activating prodrugs (HAPs) are able to specifically target cells in hypoxic niches, as the active form of the drug is released under hypoxic conditions (134). The HAP TH-302 induced hypoxia-dependent apoptosis in AML cells, by reducing HIF-1α expression, thereby decreasing proliferation, inducing a cell-cycle arrest, and enhancing double-stranded DNA breaks (72). Administration of TH-302 after chemotherapy to mice with residual disease prolonged their survival (73), suggesting that specifically targeting of HIF-1α in the hypoxic niche may be a successful therapeutic strategy to specifically eliminate chemotherapy resistant AML (stem) cells.

LSCs, THE BONE MARROW MICROENVIRONMENT AND THERAPY RESISTANCE

There is emerging evidence that AML LSCs can remodel the BM niche into a leukemia-permissive microenvironment, thereby suppressing normal hematopoiesis. The complex interplay between LSCs and their microenvironment, including adhesion molecules, chemokines and cytokines, contribute to LSC survival, therapy resistance and disease relapse. Understanding

these interactions is crucial for the development of effective drugs to overcome niche-mediated AML drug resistance.

Leukemia cells express the β-1 integrin receptor family members very late antigen-4 (VLA-4) and VLA-5, and the β-2 integrin LFA-1, which interact with the stromal ligands vascular cell adhesion molecule 1 (VCAM-1), fibronectin and intracellular adhesion molecule 1 (ICAM-1) to support attachment to the niche, thereby activating prosurvival and proliferative pathways in the leukemic blasts (135–137). Gene expression profiling of BM mesenchymal stromal cells (MSC) co-cultured with leukemia cells revealed upregulation of nuclear factor (NF)-κB signaling, which reduced sensitivity to chemotherapy in the leukemia cells. Mechanistically, activation of the N*F*-κB signaling pathway was caused by the interaction of VLA-4 on the leukemia cells with ICAM-1 on the MSC (138).

HIF-1α signaling regulates LSC maintenance, quiescence and therapy sensitivity by promoting expression of VEGF, CXCR4, CXCL12 and SCF on both the AML blasts and the stromal cells (139). AML blasts and especially LSCs express CXCR4 on their surface and migrate in response to CXCL12 (140). The protective effects of the BM niche could be reduced by inhibition of the CXCL12–CXCR4 interaction (**Table 1**). For example, the CXCR4 antagonist plerixafor released HSCs and AML blasts from the BM niche, and the combination of plerixafor with cytarabine decreased tumor burden in an AML mouse model (74). Also in r/r AML patients, treatment with plerixafor enhanced the effect of chemotherapy (75). However, in newly diagnosed older AML patients, the clinical benefit of plerixafor was not shown, and mobilization of AML LSCs and progenitor cells after treatment was only observed in some patients (76). Targeting CXCR4 together with other membrane molecules involved in the attachment of AML (stem) cells to the BM niche may be an efficient strategy to release AML LSCs and enhance their sensitivity to therapy. Degradation of BET proteins with the BET proteolysis-targeting chimera ARV-825 resulted in downregulation of both CXCR4 and CD44 in AML cells, and as a result impairment of CXCL12-directed migration, increased oxidative stress, and downregulation of gene signatures associated with "stemness" and Wnt/β-catenin and Myc pathways. Importantly, treatment with ARV-825 alone and in combination with cytarabine decreased the number of LSCs (77).

Pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα), interferon (IFN)-α, IFN-β, IFN-γ, IL-1 and IL-6, influence the adhesion of AML cells with their BM microenvironment and consequently AML survival and sensitivity to therapy, potentially through modulation of NF-κB signaling. CD34+CD38- LSCs, but not normal HSCs or non-LSC AML blasts, showed constitutive NF-κB activity due to autocrine TNF-α secretion, resulting in their expansion (141). Targeting transmembrane TNF-α increased sensitivity to chemotherapy, inhibited AML cell growth, and impaired AML engraftment in secondary serial transplantations (142). In addition to TNF-α, most AML cells express the pro-inflammatory cytokine IL-1, and especially IL-1β, enhancing the production of other pro-leukemic chemokines and thereby generating a pro-inflammatory niche. This pro-inflammatory environment promotes LSC and AML blast survival, proliferation and apoptosis-resistance (143, 144). Furthermore, the IL1R co-receptor IL1RAP is highly expressed on LSCs but not on HSCs of most AML patient samples, and involved in LSC self-renewal (32). Targeting IL1RAP could eliminate both leukemic bulk cells as well as LSCs and progenitor-enriched cell fractions of primary AML patient samples, while normal HSCs and progenitor cells were spared (145).

Several members of the transforming growth factor β (TGFβ) family suppressed the growth of primary AML cells (144), and blockade of TGFβ was therefore thought to enhance chemotherapy sensitivity of AML cells (**Table 1**). Indeed, in co-culture with human MSC, treatment of primary AML samples with a neutralizing TGFβ1-antibody resulted in enhanced proliferation of both CD34+CD38− and CD34+CD38+ AML cell populations, and improved sensitivity to cytarabine (146). Blocking TGFβ signaling using the neutralizing TGFβ antibody 1D11 increased cytarabine-induced apoptosis of AML cells in hypoxic conditions. The combination of 1D11 with plerixafor and cytarabine decreased leukemia burden in a murine FLT3-mutated AML mouse model (78).

VEGF expressing endothelial cells in the BM niche protect VEGF receptor 3-expressing AML cells from chemotherapy-induced apoptosis, due to increased BCL2/Bax ratios (147). Moreover, treatment with a monoclonal VEGFC antibody reduced the clonogenic capacity of CD34-positive AML blasts, and induced their differentiation *via* the suppression of FOXO3A and inhibition of MAP/ERK (79) (**Table 1**). However, targeting VEGF signaling as novel therapeutic strategy has not been proven effective, as treatment with bevacizumab in AML patients showed controversial results in clinical trials. While bevacizumab after chemotherapy showed a favorable CR rate and duration in r/r AML patients that were resistant to the classical cytotoxic agents (80), in two other clinical trials with AML patients it showed not to be effective (81, 82).

ADIPOCYTES, AML LSCs AND THERAPY RESISTANCE

In addition to glucose, proteins and fatty acids can also be metabolized to acetyl-CoA to drive the TCA cycle and OXPHOS in the production of ATP (148). As a result of stimuli from the BM microenvironment, such as hypoxia and nutrient availability, AML cells can modulate their metabolic state. Adipocytes, the major stromal cells present in the BM, support the survival and growth of AML cells by stimulating fatty acid oxidation (FAO) and mitochondrial OXPHOS as a result of fatty acid transfer (149). Moreover, adipocytes in the BM showed to impair the efficacy of chemotherapeutic drugs, and relapse rates after chemotherapy were much higher in mice that were obese than in mice with a normal body weight (149, 150). LSCs can induce lipolysis in adipocytes to induce FAO in AML (stem) cells by abundant fatty acids, thereby evading chemotherapy-driven elimination of the AML cells (**Table 1**). Only part of the LSC population in the BM express the fatty acid transporter CD36 (151), and these CD36+ LSCs showed to be highly proliferative and distinct from the pool of CD69 expressing LSCs that contain self-renewal potential (152). Further research should investigate if

targeting the fatty acids uptake by CD36 would be a successful strategy to specifically eliminate AML LSCs. Furthermore, the lipid chaperone fatty acid binding protein 4 (FABP4) is involved in the interaction of adipocytes with leukemia cells, and its expression correlated with the activation of the peroxisome proliferator-activated receptor (PPAR) γ (153). FABP4 was increased in AML cells after culturing them with BM adipocytes (154), and downregulation of FABP4 resulted in increased survival of mice with Hoxa9/Meis1-driven murine leukemia (83).

BCL2 is directly reducing ROS generation (155), and BCL2 overexpression promoted the survival of low ROS-producing quiescent LSCs (64). Targeting FAO increased the production of ROS and caused apoptosis in AML cells (156). As targeting BCL2 by venetoclax eliminated low ROS-producing LSCs (64), targeting both FAO and BCL2 might be a successful synergistic approach to eliminate LSCs. FAO inhibition by etomoxir, an inhibitor of the FAO key rate-limiting enzyme carnitine O-palmitoyltransferase 1 (CPT1), disrupted metabolic homeostasis, increased ROS production, and subsequently induced expression of the integrated stress response (ISR) mediator activating transcription factor 4 (ATF4) in AML cells (84). Inhibition of CPT1 showed anti-AML effects (84, 85). CPT1 controls FAO by conjugating fatty acids with carnitine for translocation into the mitochondrial matrix. Expression of CPT1A is regulated by PPARs and the PPARγ coactivator-1 (157), and inhibition of CPT1A by etomoxir not only directly eliminated leukemia cells but also sensitized them to cytarabine (85). Etomoxir induced an energetic shift towards low OXPHOS and resulted in increased anti-leukemia effects of cytarabine (36). Moreover, avocatin B, another inhibitor of FAO, was synergistic with cytarabine in inducing apoptosis in AML cells that were co-cultured with adipocytes by causing an increase in ROS production (84).

STRESS RESPONSE, LSCs AND THERAPY RESISTANCE

Human HSCs are sensitive to environmental stress and prone to programmed cell death. HSCs ensure their persistence by using the ISR, also known as the unfolded protein response, in order to survive low-levels of stress caused by metabolic processes during normal homeostasis (158, 159). The ISR pathway balances the activation of apoptosis due to stress signals with survival pathways that protect the cell from dying (160). In response to stress stimuli, the eukaryotic translation initiation factor 2α (eIF2α) is phosphorylated by the stress-responsive eIF2α kinases general control non-derepressible 2 (GCN2), heme-regulated inhibitor, protein kinase R, and protein kinase RNA-like ER kinase (PERK). Phosphorylated eIF2α reduces global protein synthesis while allowing translation of specific genes, including ATF4, ATF5 and C/EBP Homologous Protein (161). Components of the eIF2α pathway, including GCN2 and ATF4, specifically contributed to survival of therapy-resistant cells during hypoxia. Activation of the ISR protects against ROS (162), and is therefore thought to be an important contributor to the survival of AML LSCs residing in hypoxic niches after therapy.

In response to ER stress human HSCs maintain their functionality preferentially through activation of the PERK-eIF2α IRS pathway (158). This pro-survival pathway was also shown to modulate the stress response of LSCs, thereby affecting their sensitivity to therapy and their survival (**Table 1**). In primary AML, CD34+CD38− LSCs contained lower eIF2α and elevated ATF4 levels as compared to the more differentiated CD34+CD38+ AML cell populations (159), highlighting that there is an increased activity of the ISR in LSCs, and implicating that ATF4 is a potential therapeutic target to eliminate LSCs. A mutation in FLT3, the FLT3-ITD, was shown to positively control ATF4 levels and enhanced autophagy in FLT3-ITD-mutated AML patient cells. Inhibition of ATF4 in FLT3-ITD-positive AML inhibited autophagy-dependent AML cell proliferation and tumor burden (**Table 1**). Moreover, inhibition of autophagy by VPS34 inhibitors abolished resistance to FLT3 inhibitors in murine xenograft models (86). The antiparasitic drug atovaquone enhanced the phosphorylation of eIF2α, increased ATF4 protein levels and transcription of ATF4 target genes, and inhibited mitochondrial OXPHOS, which resulted in growth arrest and apoptosis of AML patient cells (87). Moreover, activation of the PERK signaling pathway and subsequent activation of autophagy induced resistance to G9a inhibition in AML LSCs. Combination treatment of PERK and G9a inhibitors induced apoptosis in LSCs (88). Together, these studies suggest that therapeutic targeting of the PERK-eIF2α-ATF4 ISR pathway may be an efficient approach to eradicate AML LSCs.

DISCUSSION AND CONCLUSION

One of the biggest challenges in treating AML is the development of relapse after initial treatment. Even with high remission rates, therapy resistance and relapse are often appearing and are the major obstacles to a cure. AML "persisters" (MRD) after initial therapy are caused by various mechanisms that co-exist, including epigenetic, transcriptional, and metabolic processes. Successful therapeutic strategies targeting AML MRD and LSCs will increase efficacy of treatment and finally the survival of patients. Increasing the knowledge on the mechanisms driving this persistence, and also on the changes in identity of MRD and LSCs during the course of the disease is crucial for the development of successful therapeutic strategies to overcome therapy resistance and to inhibit leukemia-initiating potential. Similarities and differences between normal HSCs and AML LSCs and studying LSC heterogeneity and intra- and interpatient plasticity are key for therapy development specifically eradicating LSCs at any time during the disease course. Persistent LSCs are characterized by their quiescent state and might exist prior to drug treatment; however, they might also become resistant upon exposure to therapy. At relapse, there are substantially more LSCs and phenotype diversities of LSCs than at diagnosis, indicating that the current therapeutic treatments induce dramatic changes in the LSC and relapse-initiating cell compartment. This indicates that there is a high degree of phenotypic plasticity to impose "stemness" on leukemia cells and shows the importance of studying leukemia re-initiating cells after the initial therapy. Future characterization of leukemia

blasts and LSCs should therefore be performed after treatment to identify specific leukemia-relapse initiating markers that could be used not only to prognostically detect MRD, but also to early detect relapse and to be used as therapeutic targets delaying or even preventing relapse.

There is a complex interplay between AML (stem) cells, their BM niche, and the outcome of treatment. This interplay is significantly influenced by signaling events from the BM niche affecting metabolism, epigenetic processes, stress responses and transcriptomes of the AML cells, and all subsequently affecting the level of leukemia (stem) cell death induced by therapy. Therefore, targeting the BM microenvironment by novel therapeutic strategies will also be crucial to overcome AML drug resistance. For example, targeting the energy consumption of LSCs in combination with inhibition of BCL2 can potentially eradicate residual chemotherapy-resistant LSC populations. Both FAO and BCL2 directly reduce ROS generation, promoting the survival of low ROS-producing quiescent LSCs. The combination of venetoclax and a FAO inhibitor might be a successful approach to eliminate LSCs with adapted energy homeostasis. Furthermore, the differentiation stage of the AML cells plays an important role in the response to therapy. AML cases originating from HSCs or MPPs, thus immature AML cases with high EVI-1 expression, may be successfully treated with venetoclax, while more differentiated AML cases may be successfully treated with alternative therapies that are more efficient in the eradication of differentiated AML cells.

In conclusion, characterization of AML (stem) cells at the single cell level during the course of the disease and especially at MRD will provide valuable insights into the mechanisms of AML persistence and relapse-initiation and is key for development of successful treatment strategies that reduce or prevent relapse.

AUTHOR CONTRIBUTIONS

NV, FD, and LS wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Complexity of the Tumor Microenvironment and Its Role in Acute Lymphoblastic Leukemia: Implications for Therapies

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The microenvironment that surrounds a tumor, in addition to the tumor itself, plays an important role in the onset of resistance to molecularly targeted therapies. Cancer cells and their microenvironment interact closely between them by means of a molecular communication that mutually influences their biological characteristics and behavior. Leukemia cells regulate the recruitment, activation and program of the cells of the surrounding microenvironment, including those of the immune system. Studies on the interactions between the bone marrow (BM) microenvironment and Acute Lymphoblastic Leukemia (ALL) cells have opened a scenario of potential therapeutic targets which include cytokines and their receptors, signal transduction networks, and hypoxia-related proteins. Hypoxia also enhances the formation of new blood vessels, and several studies show how angiogenesis could have a key role in the pathogenesis of ALL. Knowledge of the molecular mechanisms underlying tumor-microenvironment communication and angiogenesis could contribute to the early diagnosis of leukemia and to personalized molecular therapies. This article is part of a Special Issue entitled: Innovative Multi-Disciplinary Approaches for Precision Studies in Leukemia edited by Sandra Marmiroli (University of Modena and Reggio Emilia, Modena, Italy) and Xu Huang (University of Glasgow, Glasgow, United Kingdom).

Keywords: microenvironment, ALL, bone marrow, angiogenesis, HIF, hypoxia, therapies

INTRODUCTION

Tumors have a small subpopulation of cells responsible for cancer initiation and relapses (1–3). Leukemic stem cells (LSC), like normal hematopoietic stem cells (HSC), depend on the interaction with the microenvironment or on the niche for their own self-renewal and maintenance.

The microenvironment consists not only of tumor cells but also of physiological tissues which include the stroma (supporting scaffold) with fibroblasts, blood vessels and white blood cells, especially T lymphocytes and macrophages, whose precursors are monocytes deriving from the myeloid lineage. Some tumors have a rich T lymphocyte infiltration (4, 5). Innate immunity cells such as macrophages, granulocytes and immature myeloid cells from which monocytes or

macrophages derive are also found in different quantities (6). Today, it is becoming increasingly clear that the immune system can have a double significance, that is, to manifest antitumor activity or to offer a significant contribution to the tumor development, therefore constituting a barrier that opposes the efficacy of anticancer drugs by creating chemoresistance (7). The paradoxical role that the immune system plays in tumor control can be explained by its plasticity that leads to pro or antitumor properties (8–10).

Therefore, considering the role of the tumor microenvironment in neoplastic diseases, it is clear that in the treatment of numerous tumors it is necessary not only to target the tumor cells, but also to control the inflammatory microenvironment that causes resistance to anticancer therapies (11–14). In this regard, clinical studies aim at blocking the recruitment of inflammatory cells into the tumor environment by neutralizing attractive chemokines with specific antibodies, and by taking advantage of suitable strategies to inhibit the activity of cells. A possible antitumor therapeutic strategy therefore is targeting the inflammatory microenvironment in combination with the blocking of immune checkpoints.

In recent years, the interaction of LSC with other cells located in the BM, the so-called medullary microenvironment, has a leading role in these phenomena. In this context, it contributes decisively to the development of the disease and to drug resistance mechanisms and is also decisive in the process that leads stem cells to transform into malignant cells.

In particular, the so-called mesenchymal progenitors, stem cells derived from the BM, constitute a particular population, since they support the niche of both normal and leukemic HSC (15, 16). The rationale is therefore to explore this network of relationships that feeds and protects LSC, in order to develop therapeutic and personalized strategies that could effectively interfere with these communication routes or with the "interlocutors". In the intertwining of these relationships the process of angiogenesis also represents a crucial aspect, favoring the malignant progression of cancer, both as regards solid tumors and hematological ones (17, 18).

The complexity of interactions within the leukemic microenvironment has been explored for many years. In this review, the aim is to summarize the current understanding of the cellular and molecular network of the tumor microenvironment in ALL, and therefore its ability to modulate the leukemogenesis process, leading to the development of future treatment strategies that can intervene in the remodeling of the leukemic microenvironment.

ACUTE LYMPHOBLASTIC LEUKEMIA

ALL is one of the most common malignancy in child population (19–22). In pediatric patients, modern protocol therapies are associated with overall success, while the adult population that develops this disease has an unfavorable prognosis: the survival rate in patients from 25 to 59 years old is around 40%, while in the aging the survival rate is usually below 20%.

ALL is more frequent in males, and specifically adolescents and young adults display a greater number of T-cell acute lymphoblastic leukemia (T-ALL) subtype rather than the other ALL progenitors. The B-cell subtype accounts for about 75-80% of ALL cases and it mainly develops in children with a peak incidence of around 2-5 years (23, 24). B-ALL is characterized by an arrest in lymphoid differentiation and therefore in the lymphoid development as pro or pre-B, with recurrent genetic alterations that involve cell cycle and lymphogenesis control genes (21). In adults, prognosis of B-ALL is more consistent at least partially for the BCR-ABL1 fusion protein onset, which is usually found in 2-10% of cases in children. T-ALL subtype is characterized by the accumulation of undifferentiated thymocytes that have acquired multiple genomic aberrations affecting critical transcriptional and signaling networks (25). It represents 12.7% of pediatric cases, and survival rates at 5 years for children and adolescents affected by T-ALL are 70–75%, while in adults the rates are 35–40%. A morphological and structural profile between two ALL cell line subtypes is shown in **Figure 1**, that reports the morphology of a B cell precursor leukemia cell line, SEM, and a T cell leukemia, Jurkat cell line. The images were acquired by Transmission Electron Microscopy (TEM). Observing TEM images, SEM cell nucleus is delimited by a nuclear envelope and contains more than one nucleolus (n) and some areas enriched in heterochromatin (he). Jurkat cells have a well-defined rounded nucleus with envelope, and a larger and more distributed cytoplasm. Around the nucleus, structures such as mitochondria (m) can be appreciated (**Figure 1**).

Chemoresistance and relapse mechanisms are driven by a pool of rare leukemia initiating cells (LICs). These cells are able to escape chemotherapeutic drugs and are mainly involved in self-renewal and in new LICs generation. Moreover, this cellular class has in common several properties with HSCs, that normally leads to the formation of a HSC cell identical to the parent cell, and to a partially differentiated progenitor cell incapable of self-renewal (therefore not stem cells), but with a large replicative potential. The type of differentiation that the progenitor cell undergoes (also called TAC, Transit Amplifying Cell due to its replicative capacity) determines the distinction of blood cells into two large classes: cells from the myeloid lineage and cells of the lymphoid lineage, therefore T lymphocytes, B lymphocytes and Natural Killer (NK) cells (26, 27).

The optimal site for HSC is the BM, that includes specialized areas called niches consisting of different cell types that control the number, quiescence, self-renewal, differentiation and distribution of HSCs (23). The niches are divided into two distinct, but equally connected areas: the first in which the osteoblast plays a primary role (Osteoblastic Niche); the second where endothelial cells predominate and has been called Vascular Niche (23, 28, 29).

In particular, in the vascular niche, sinusoidal endothelial cells favor the survival, proliferation and differentiation of myeloid progenitors and megakaryocytes. HSCs in the Vascular Niche are exposed to hormones, growth factors,

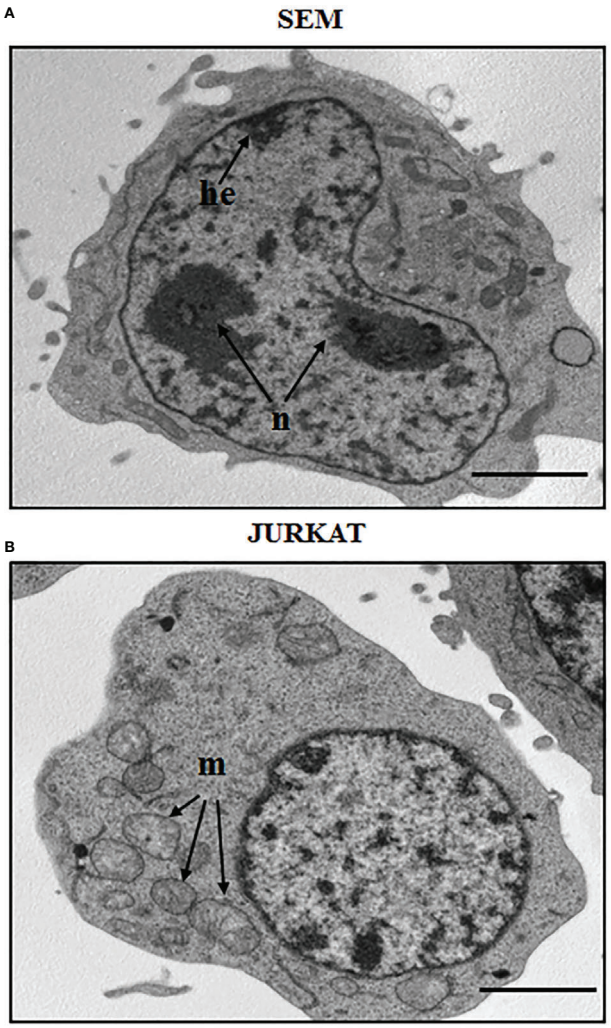

FIGURE 1 | Morphological aspect of a B cell precursor leukemia (SEM) and a T-ALL (Jurkat) cell lines. TEM images of: **(A)** SEM cells at 8000 magnification; **(B)** Jurkat cells at 8000 magnification. n: nucleolus, he: heterochromatin, m: mitochondria. Scale bars correspond to 2 μm. For TEM analysis, samples were fixed in 2.5% glutaraldehyde and post-fixed in 2% osmium tetroxide, dehydrated in acetone solutions and included in Araldite Durcupan ACM (Fluka). Samples were then counterstained with uranyl acetate in saturated solution and lead citrate and observed under transmission electron microscope Zeiss EM910 at 100 Kv.

oxygen and nutrients, therefore to detect signals and stimuli from the peripheral circulation. This favors self-maintenance, proliferation and/or differentiation (30, 31).

On the contrary, the osteoblastic niche could act as a reservoir since it favors the maintenance of the state of quiescence (32). However, not all the cells of the endosteal surface perform these functions; those that define the osteoblastic niche are represented by the N-cadherin$^+$ and CD45$^-$ (SNO) subpopulation.

The direct contact between the microenvironment and leukemic cells is therefore essential, and also the activation of different signaling pathways characterizes the microenvironment in a specific way (33, 34). Phosphatidylinositol-3-kinase (PI3K)/AKT-Bcl2 or NOTCH1 appear to be relevant pathways to be induced by microenvironment and leukemic cell connections (15).

Finally, long-term overactivity of reactive oxygen species (ROS) could have deleterious effects on stem cells and on leukemia incidence (35), and in this context several networks are activated in response to ROS increment such as P53, AKT, MAPK and ataxia telangiectasia mutation (ATM).

A better understanding of the contribution of microenvironmental stimuli in mediating leukemogenesis may provide a better scenario of new therapeutic targets in the treatment of leukemias.

The Role of Cytokines in Leukemic Microenvironment

The inflammatory microenvironment surrounding cancer cells is a complex network in which immune infiltration has long been considered a defense mechanism against malignant tumors. In fact, it can contribute to the development, spread of cancer, metastasis and the onset of resistance to anticancer therapies (36). Cytokines are essential for BM niche homeostasis, and the first studies on B-ALL reported a higher expression of these molecules when compared with healthy BM samples, suggesting an autocrine/paracrine regulation of leukemic cells and cytokines (37). Among the cytokines with more modulatory activity IL-7, IL-8 and IL-15 are the most involved.

Interleukin-7 (IL-7) is a cytokine produced by stromal cells and promotes hematological malignancy development and differentiation (ALL, T-cell lymphoma). It functions primarily as a growth factor and an antiapoptotic molecule, essential for the differentiation of T lymphocytes with TCR γ– δ. It also works as a cofactor during myelopoiesis and is capable of activating monocytes/macrophages and NK lymphocytes. Its receptor is a heterodimer consisting of an alpha chain, which binds IL-7, and a gamma chain, a common component of the IL-2, IL-4, IL-9, IL-15 receptor. Experimental data suggest that, even in humans, IL-7 could act as a factor stimulating leukopoiesis, given that it is of great importance in the treatment of bone marrow transplant patients (38). Like normal T and B progenitors, most T-ALL and B-ALL cells express functional receptors for IL-7 (IL-7R) which are able to promote leukemia survival and proliferation both *in vitro* and in patient- derived xenograft assays, suggesting a role for IL-7R/IL-7 pathway in ALL progression. IL-7 and IL-7R activate three main signaling networks: STAT5, PI3K/AKT/mTOR and MEK/ERK, leading to leukemia cell progression and survival, also with regard to T-ALL (25, 39, 40). An overview of the IL-7 signaling, upon activation IL-7R, is summarized in **Figure 2**. Upon IL-7 binding, IL-7 receptor α chain (IL-7Rα) and γ-common (γc) chains dimerize, with consequent activation of JAK3 and JAK1 kinases that bind to the intracellular domain of γc and IL-7Rα, respectively. JAK1 phosphorylates the IL-7Rα intracellular domain with activation of PI3K and STAT, resulting in STAT translocation to the nucleus and in the transcription of target genes involved in cell cycle progression or differentiation. Phosphorylated AKT promotes cell survival through degradation of pro-apoptotic proteins and glucose uptake (**Figure 2**).

The activity of IL-7 in supporting ALL development was firstly documented in *in vitro* models (41, 42). In has been shown that B-ALL primary cells co-cultured with stromal cells displayed a CXCL12-dependent proliferation which was concomitant to the presence of IL-7 and, to a lesser extent, to the expression of IL-3 (43). This synergistic/additive effect was partially correlated with the increased phosphorylation of components of CXCL12-activated signaling networks (e.g., PI3K/AKT and MEK/ERK). In *in vivo* models, mice with IL-7 receptor knocked out exhibited thymic atrophy, developmental arrest of double-stage positive T cells, and severe lymphopenia. Administration of IL-7 in mice leads to an increase in B and T cells and in T cell recovery after cyclophosphamide addition or after BM transplantation (44). Thymic epithelial cells increased survival of T-ALL primary cells through IL-7, suggesting a functional role for the thymic microenvironment in the acquisition of selective growth advantage of leukemic cells (23). Importantly, in BM stromal or thymic epithelial cells co-cultured with T-ALL cells enhanced survival specifically required IL-7/IL-7R interactions, while the blocking of either IL-7 or IL-7R significantly reduced apoptosis inhibition mediated by the leukemic microenvironment (45). Moreover, IL-7 receptor is upregulated in about 50% of T-ALL through mutational activation of NOTCH, considered an upstream regulator (42).

It has also been shown that C-C Motif Chemokine Ligand 2 (CCL2) and IL-8 were able to increase the adhesion of ALL cells to BM stromal cells, in a stromal-dependent manner, and to promote survival and proliferation of BM stromal cells, while they seem not to have effect on most precursor B-cell ALL survival or migration. Indeed, it was found that the BM plasma levels of CCL2 and IL-8 in ALL children at diagnosis were significantly higher than in healthy controls. Moreover, in T-ALL IL-8 production is physiologically regulated by a specific signaling network, represented by CXCL12/CXCR4 axis and the nuclear factor-κB (NF- κB) and JNK/AP-1 pathways. Therefore, the analysis of the molecular mechanisms that intervene in the modulation of IL-8 could be important to further hypothesize possible alternative therapeutic strategies (46, 47).

The role of IL-15 has been reported in a xenograft model of human B cell precursor (BCP)-ALL in immunocompromised mice, characterized by infiltration of ALL blasts into the CNS. Blasts and brain resident cells induced aberrant activation of host cells in the brain microenvironment, with a consequent cytokine and exosomes release, including IL-15 that binds to astrocytes and brain vessel endothelial cells. It has been shown that the silenced expression of either IL-15 in the NALM6 cell line significantly decreased CNS infiltration in engrafted mice, thus providing important points by which lymphoblasts modulate the brain microenvironment (48).

The Role of Signaling Networks in Leukemic Microenvironment

LICs and BM niches are characterized by specific signal transduction pathways which, if expressed in an aberrant way, influence the mechanisms of survival, proliferation, drug resistance and cellular invasion. Therefore, the aim is to identify the molecular mechanisms that promote and support tumor proliferation and downstream survival of important signaling pathways critical for the onset and progression of ALL. Two important signaling networks are CXCL12-CXCR4 and NOTCH.

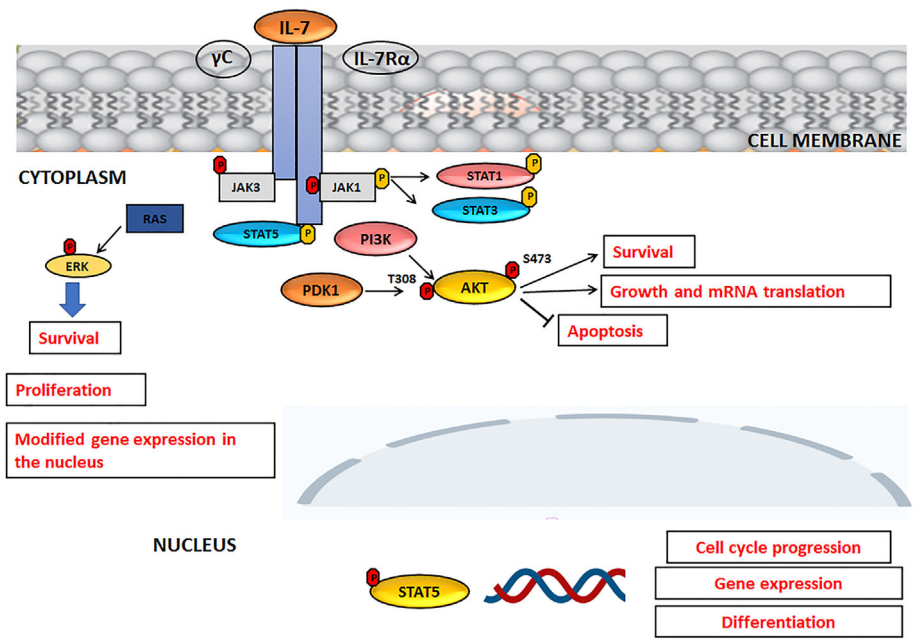

FIGURE 2 | IL-7 signaling pathway. For details, please see the text.

CXCL12-CXCR4 Network

The activation of the CXCR4 receptor by the chemokine CXCL12 (also known Stromal cell-Derived Factor-1, SDF-1) represents one of the most studied transductional axes, in the modulation of the HSC migratory behavior and in the localization of the leukemic cells to their niches (49, 50). As a demonstration of the important biological function performed by CXCL12, the amino acid sequence of this chemokine is extremely conserved among the different species during evolution. CXCL12 plays a role of primary importance already during embryonic development; experimental mouse models of gene knockout for CXCL12 or its CXCR4 receptor showed defects in the heart, intestines, circulatory and nervous systems, which are already lethal in the early stages of development (51). Furthermore, the lack of ability of the hematopoietic progenitors to colonize the bone marrow is significant, and this phenomenon can be related to the role of CXCL12 in regulating the migration of HSC and their trafficking between the different hematopoietic niches. The mechanisms that regulate the activity of CXCL12 remain largely still to be characterized.

It is currently believed that the expression of CXCL12, as well as the CXCR4 receptor, is extremely dynamic and regulated by mechanisms of autocrine and paracrine secretion (52). In particular, CXCR4 is widely expressed within the bone marrow by immature or maturing hematopoietic cells, of which it finely regulates the processes of homing, bone marrow retention or migration and entry into the circulation (53). Worthy of note is also the high expression of both CXCL12 and CXCR4 by stromal cells and medullary endothelial cells, which seems to confirm the existence of autocrine and paracrine stimulation circuits within the medullary microenvironment (54).

The activation of CXCR4 induces the up-regulation of different signaling networks such as p38 mitogen-activated protein kinase (p38 MAPK), MEK/ERK, PI3K/AKT, and protein kinase C (PKC) (50). It has been reported that PI3K/AKT network could control cell cycle progression by regulating the cyclin B1 and stathmin expression/phosphorylation (55). Moreover, both MEK/ERK and p38 MAPK kinase promoted T-ALL migration through interactions with $\alpha 2\beta 1$ integrin, suggesting that this blockade could be a valuable target for T-ALL therapeutic treatment (56).

CXCL12 and CXCR4 could be also important in B-ALL. Indeed, an elevated expression of CXCR4 in B-ALL blasts has been related to a worse patient outcome, and the expression of CXCR4 active form has been associated with a poor survival for B-ALL patients (23, 57). Further evidence emerged from studies conducted on a CXCR4 receptor antagonist, AMD3100 (Plerixafor). It was reported that a single injection of AMD3100 is able to induce rapid mobilization of hematopoietic progenitors in mouse models, as well as in humans (58). The molecular mechanism underlying this effect could be the binding of the antagonist to the receptor expressed by stem cells and hematopoietic progenitors, or to the receptor present on the surface of stromal cells, or both. In particular, it was seen that AMD3100 was able to block chemotaxis both in the B-ALL cells NALM6 and in primary B-ALL cells, therefore enhancing the effects of cytokines and growth factors found in the BM microenvironment (59).

CXCR4 is also highly expressed in T-ALL cells (60), reporting also CXCR7 as the second receptor for CXCL12 that is able to augment CXCR4 response in ALL, therefore contributing to leukemia maintenance by initiating cell recruitment to BM niches (60). A genetic targeting of CXCR4 in mouse T-ALL after disease onset could lead to a rapid, sustained disease remission, and CXCR4 antagonism is able to block human T-ALL in primary xenografts. Loss of CXCR4 decreased leukemia initiating cell activity *in vivo* and modulated targeted key T-ALL regulators, such as the MYC pathway (61).

It was also found that BM stromal cells are able to up-regulate IL-8 mRNA in T-ALL cells through the activity of CXCR4, and exogenous CXCL12 induced IL-8 mRNA synthesis in primary T-ALL cells, with consequent activation of nuclear factor –κB (NF-κB) and c-Jun (47).

NOTCH Network

NOTCH pathway is one of the most evolutionarily conserved signaling cascade across multiple species and it is involved in the regulation of essential cellular functions, from cell fate decision during embryo development, to the regulation of cell proliferation, cell growth and survival (62, 63). NOTCH is a surface receptor protein and binding of its cognate ligands, such as Jagged 1 and 2 (JAG1-2) or Delta-Like 1-4 (DLL1-4), promotes the proteolytic cleavages of the extracellular domain by the Tumor Necrosis Factor-Alpha Converting Enzyme metalloproteinase (TACE), followed by the cleavage mediated by a γ-secretase which facilitates the release and nuclear translocation of the NOTCH intracellular domain (ICN1), where it recruits coactivators to form a transcription-activating complex, finally mediating its biological functions (64). Importantly, the activity of NOTCH family members is context-dependent, exerting sometimes even opposite roles (promoting cell growth or apoptosis) in different cell types and/or depending on the expression of specific transcriptional programs. Considering its pleiotropic activities and the spectrum of cellular processes in which it is involved, aberrant expression/activity of NOTCH signaling components has been associated with a variety of cancers, from solid tumors to hematological malignancies where, given its dependency on the cellular context (genetic and/or molecular variabilities), NOTCH can act either as an oncogene or as a tumor-suppressor (65, 66). Gain-of-function mutations as well as non-mutational hyperactivation of NOTCH1 and NOTCH2 have been described in B-cell specific lymphoproliferative disorders, including Chronic Lymphocytic Leukemia (CLL) and different Non-Hodgkin lymphoma subtypes (i.e., Diffuse Large B cell lymphomas, Mantle Cell lymphoma, Burkitt's lymphoma) (67). NOTCH1 mutations represent one of the most frequent genetic modifications in T-ALL, often in association with mutations/activation of the Hedgehog signaling pathway, suggesting cooperation between those pathways, even though this genetic interaction has not been described yet. In both CLL and T-ALL, interference with

NOTCH activity has been shown to have a negative impact on the growth of cancer cells, confirming the oncogenic potential of NOTCH in those diseases (68). Whether NOTCH plays a similar role in B-ALL is still a matter of debate. Several studies suggested a contribution of genes involved in developmental signaling pathways, i.e., Hedgehog and NOTCH, in promoting survival and chemoresistance of leukemic cells, including B-ALL (69–71). Additionally, there are evidence showing that inhibition of NOTCH activity in B-ALL cell lines results in enhanced sensitivity to cytotoxic treatments, suggesting protumoral properties for NOTCH in B-ALL. However, conflicting data suggest that NOTCH activation can be deleterious for leukemic cells, as enforced expression of NOTCH in human or mouse B-ALL cells results in growth arrest and apoptosis (72). This is in line with a study reporting the activity of NOTCH in inducing apoptosis in several B-ALL cell lines: here, the activation of NOTCH resulting in NOTCH target Hairy/Enhancer of Split1 (HES1)-mediated PARP1 activation and subsequent apoptosis-inducing factor (AIF)-induced apoptosis may in part explain the contrasting effects of NOTCH signaling in B-ALL versus T-ALL, representing a mechanism of cell-specific consequences of NOTCH activation. In a recent work, the characterization of a new *in vitro* B-ALL model has been proposed. The model is called VR-ALL and is a common-type B-ALL cell line with mutations in some components of NOTCH signaling, including NOTCH1 and NOTCH2. This mutated cell line gives rise, once injected into immunodeficient NOG mice, to a mouse xenograft model of B-ALL. VR-ALL has reported to be sensitive to γ-secretase inhibitors (GSIs) as well as conventional anti-leukemic drugs, therefore helping to deepen further the role of NOTCH signaling in B-ALL (71). NOTCH activity is highly contextual and thus, it can be significantly influenced by the genetic landscape and/or the transcriptional program characterizing the cellular system used in the experimental settings. In this view, the use of genetically and biologically distinct cell lines might explain, at least in part, the discrepancy between the different studies. Importantly, several inhibitors of NOTCH pathway have been developed and entered clinical trials for the treatment of CLL and T-ALL and could be rapidly extended for the treatment of aggressive B-ALL, if supported by consistent pre-clinical evidence.

Making a connection between the IL-7 signaling pathway and NOTCH1 in the characterization of the leukemic tumor microenvironment, another important and aberrant signaling pathway is worthy of note, and it is represented by PI3K/AKT/mTOR. Overactivation of the PI3K/AKT/mTOR signaling pathway is a common occurrence in many patients with ALL, portends a poor prognosis and for many years literature highlighted that this network, constitutively active in ALL, increases cell proliferation, survival and drug resistance (22, 25, 73–77). The activation of the PI3K/AKT/mTOR signaling pathway also results from the close correlation between the inputs deriving from IL7Rα/JAK signaling, NOTCH1-mediated upregulation of IL7Rα, NOTCH1 and the modulation of specific microRNAs (miRNAs) that induce the inhibition of PTEN tumor suppressor gene expression, reduction of PTEN induced by phosphorylation of casein kinase 2 (78), inactivation of phosphatase SHIP1, or elevated ROS concentration (76).

THE ROLE OF ANGIOGENESIS IN ALL

Many tumors have a noticeable increase in vessel density, which causes the tumor to transition from benign to malignant, in a process referred to as "angiogenic switch". The formation of new vessels is a complex process involving many types of cells including macrophages. In fact, the overexpression of colony stimulating factor 1 (CSF-1), in a mouse model, resulted in a premature accumulation of macrophages in the hyperplastic lesion, an early angiogenic switch and consequently accelerated the transition to malignancy (79).

The angiogenesis process in hematological malignancies is similar to that reported in solid tumors (80).

During tumor development and progression, cancer and stromal cells often are in a poor oxygen condition and have poor access to nutrients. Indeed, in addition to the cellular components, other physiological factors regulate the stem cell niche. A physiological modulator of particular interest is hypoxia (17, 81). Some studies suggest that hypoxia allows for a series of events in the tumor microenvironment that lead to the expansion of aggressive clones from heterogeneous tumor cells and promote a lethal phenotype. The hypoxic response is mainly attributed to hypoxia inducible factors (HIFs).

HIF Signaling

HIF-dependent signaling can induce changes that promote cancer progression, survival, stem cell maintenance and vascular remodeling (82, 83). The HIF family of transcription factors includes HIF1, HIF2, and HIF3. These factors all have a HIF-α subunit, that is extremely sensitive to oxygen (HIF1-α, HIF2-α, or HIF3-α, respectively). Each of these subunits contains two proline residues (HIF1-α: P402/P564 and HIF2-α: P405/P531), which are hydroxylated in normoxia condition by proteins containing prolylhydroxylase (PHD). Hydroxylation of proline residues promotes binding to the von Hippel-Lindau tumor suppressor (pVHL), which leads to ubiquitination and degradation of HIF1-α (**Figure 3**) (84). High expression of HIF-α was detected in 67% of patients with acute myelogenous leukemia (AML) (85), 66.7% of patients with pediatric ALL (86) and in 100% of patients with B-CLL patients (87).

The importance of HIF-1α in leukemia microenvironment is given by the fact that it has an important role in differentiation and growth of stem cells, maintaining also the ability of HSC to regulate ROS and to keep a quiescence state (35). It was demonstrated that in T-ALL NOTCH1 is required for HIF-1α stabilization, contributing to HIF-1α- dependent proliferation, invasion and chemoresistance (88). Moreover, it has been reported that in Jurkat cells HIF-1α could be significantly regulated by a protein, called seven in absentia homolog 2 (SIAH2), involved in invasion and metastasis processes (89).

In T-ALL Jurkat cells, SIAH2 knockdown led to increased apoptosis and decreased proliferation. Moreover, PHD, P27 and Caspase3 were upregulated and HIF-1α, Vascular Endothelial Growth Factor (VEGF), VEGF Receptor 2 (VEGFR2), Matrix Metallopeptidase 13 (MMP-13), CyclinE1, C-myc and BCL2 were downregulated in the same cell model, making SIAH2 an attractive therapeutic target, also in correlation with HIF-1α modulation (89). In another work β-catenin transcription was reported to be upregulated by hypoxia HIF-1α stabilization, and deletion of the same transcription factor was able to reduce LIC frequency. Of note, the deletion of β-catenin or HIF-1α did not impair the growth or viability of bulk tumor cells, suggesting that components of the Wnt pathway and HIF specifically support leukemia stem cells (90). HIF-1α is also correlated to mTOR signaling, frequently aberrant in ALL models: coculture of ALL with stromal cells in hypoxic condition was able to induce HIF-1α and AKT/mTOR networks, and mTOR inhibition decreased HIF-1α, inducing ALL cell chemosensitization (91). HIF-1α expression has also been reported to be associated with worse overall and event-free survival in a Ph-negative pre-B ALL model, implicating that inhibition of AKT signaling or blockade of HIF-1α-mediated pro-survival signaling events may improve clinical outcomes in pre-B ALL (91). However, further studies are certainly needed to clarify HIF oncogenic and/or tumor suppressor activity, in particular in the hematological cancer stem cells (HCSCs).

Therefore, it appears extremely interesting to target hypoxia mechanism, the tumor tissue and the microenvironment, mainly due to the point that hypoxia leads to resistance to anticancer therapies. Some of the therapy-resistance mechanisms include (i) increased expression of drug efflux pumps (such as P-glycoprotein, Pgp), (ii) oxygen-dependent cytotoxicity of some molecules, (iii) mutations conferring resistance, (iv) selection of insensitive cells to p53-mediated apoptosis and (v) decreased cell proliferation rate in hypoxic regions (92).

VEGF and Thrombospondin-1 (TSP-1)

In addition to HIF-1α, numerous other angiogenic regulators in the hypoxic environment are signal proteins and bind to stimulatory or inhibitory receptors located on the surface of vascular endothelial cells. The VEGF-A and TSP-1 are well known angiogenesis modulators: the first one is an inducer, while the latter is an angiogenesis inhibitor. The VEGF-A gene encodes ligands that are involved in governing the growth of new blood vessels during embryonic and postnatal development, and then in the adult in regulating the survival of endothelial cells and neoangiogenesis (93). Three tyrosine kinase receptors (VEGFR-1 -2 -3) regulate this signal pathway at several levels, demonstrating its complexity. Therefore, the expression of the VEGF gene can be hyper-regulated both by hypoxia and by oncogenes signals (94). Moreover, VEGF ligands can be sequestered in the extracellular matrix in latent form, and be subjected to release and activation by a protease degrading the extracellular matrix (e.g., MMP-9) (95).

VEGFR-1 and -2 are mostly expressed on vascular endothelial cells, and the activation of VEGFR-2 is predominantly responsible for mediating VEGF-dependent angiogenesis and the induction of vascular permeability (96). VEGFR-1 is also expressed on HSCs and leukemic cells, vascular smooth muscle cells, different solid tumors such as colon cancer, and in monocytes (97, 98), while VEGFR-2 is expressed on endothelial progenitor cells and megakaryocytes (99). VEGFR-3 is largely restricted to lymphatic endothelial cells (100) and was analyzed in the plasma concentrations in comparison and in combination with CA 15-3 in some cancers in relation to the control groups, to better define its role as a tumor marker (101). The first finding that leukemia progression was accompanied by increased bone marrow vascularization was provided by Judah Folkman's and collaborators (102) who demonstrated that the BM of ALL

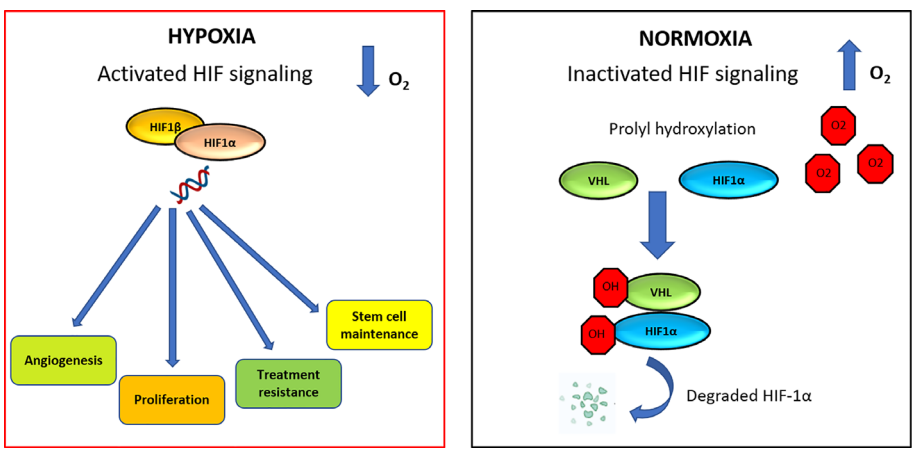

FIGURE 3 | HIF signaling network in normoxic and hypoxic conditions. In hypoxia condition, HIF-1α is able to increase angiogenesis process, proliferation, therapy resistance mechanisms and stem cell maintenance. Under normoxic condition, VHL is the recognition component of an E3 ubiquitin-protein-ligase, target for the proteasomal degradation of HIF-1α. As a result, HIF-1α is modified and it is recognized by a proteasome and rapidly degraded.

patients had a higher number of blood vessels compared with controls, and this was assessed immunohistochemically by factor VIII staining of bone marrow biopsies. Moreover, VEGF and basic fibroblast growth factor (bFGF) were also detected in urine and peripheral blood samples from ALL patients and correlated with the increase in bone marrow angiogenesis (103). VEGF can be regulated by signaling pathways such as PI3K/AKT, as demonstrated in B-ALL cell lines and patients, where an overexpression of heme oxygenase-1 (HO-1) was found in the bone marrow stromal cells (BMSCs) (104). It was found that B-ALL cell lines became resistant to the chemotherapeutic agent vincristine (VCR) in presence of VEGF recombinant protein induced by HO-1 expression. Therefore, VEGF may promote VCR resistance in B-ALL cells, and this mechanism is stimulated by PI3K/AKT network.

On the other hand, TSP-1 exerts an anti-angiogenic activity and could be important in the maintenance therapy of ALL patients, whose effect is anti-endothelial. In ALL children in the maintenance phase consisting in daily oral mercaptopurine, weekly oral methotrexate and VCR once a month for 12 months and dexamethasone for 5 days/month for 12 months, circulating endothelial cells (CEC), endothelial progenitor cells (EPC) and endothelial microparticles (EMP), but also VEGF, VEGFR-1 and Ang-2, TSP-1 and regulatory T lymphocytes (Treg) were monitored. It was reported a statistically significant decrease in EPC and EMP counts throughout the maintenance phase associated with a significant increase in TSP-1 levels, therefore pointing out an antitumor maintenance therapy activity with involvement of anti-angiogenic effects (105). TSP-1 and other epi-driver genes such as LYN, TRAF3 or FLT1 were also analyzed in B-ALL cells and in patients, underlining their use as prospective biomarkers in ALL progression or as targets for innovative therapeutic agents that can be involved in altered DNA methylation and gene expression (106).

THERAPEUTIC OPTIONS TARGETING THE MICROENVIRONMENT IN ALL: FOCUS ON SOME RECENT THERAPIES

As mentioned in the previous sections, leukemia cell microenvironment involves several factors and processes including expression of adhesion molecules, interactions between cancer and stromal cells, cytokine and growth factors, and angiogenesis. It is therefore extremely important to adopt and at the same time to find innovative therapeutic strategies that can slow down, at least in part, the progression of ALL, the cell-cell interactions at the level of the tumor microenvironment and the angiogenesis process, which as previously mentioned, is intrinsically involved in the characterization of the leukemic microenvironment, in the development of stem cells but above all in the scaffolding of the entire vascular network that determines the profile of the neoplasm. Consequently, it appears to be relevant to block the interactions between malignant cells and their niche, and to identify innovative and selective Small Molecule Inhibitors (SMIs) able to inhibit most of the components forming part of the signaling pathways that affect the niche and the BM directly.

In the following sections the most recent evidences regarding the inhibitory activity of some classes of compounds at the level of the leukemic microenvironment are shown. These drugs are also summarized in **Table 1**.

Prodrugs

A prodrug is commonly defined as a substance that is inactive in its initial form. Once administered, the compound turns into an active state (138). The drug then takes effect to treat a specific disorder or condition at a desired time interval. Metabolization changes the molecular structure of a prodrug and this process divides this drug substance into one in which the molecules break down into fragments and into another that becomes soluble after being metabolized.

TABLE 1 | A summary of the most recent evidences of drugs targeting the microenvironment in ALL.

Drug	Relevant effects	Clinical Trials	Reference(s)
Prodrugs			
PR104	Hypoxia condition: hypoxia-selective cytotoxicity *via* DNA crosslinking	–	(107–109)
Nelarabine	Normoxia condition: Cell growth blockage ALL sensitization improvement to other drugs (AraG)	NCT00501826 NCT03020030	(110–117)
OBI-3424	Cytotoxic activity Prolongation the event-free survival Disease regression	NCT04315324	(118)
HIF inhibitors			
Echinomycin	Improvement of the survival of mouse models Cell growth blockage	–	(119)
Proteasome inhibitors			
Bortezomib (Velcade™)	Apoptosis induction Activation of caspase-3, -8 and -9 Angiogenesis pathway inhibition Stimulation of HDAC inhibitors activity Alteration of mitochondrial transmembrane potential	–	(126–133)
Carfilzomib	Apoptosis and autophagy induction Minimal Residue Disease response improvement		(134–137)

Remaining in the context of the normoxic or hypoxic condition, a hypoxia activated prodrug (HAP) is represented by PR104, that is a phosphate ester, rapidly hydrolyzed *in vivo* to the corresponding alcohol, PR104A, therefore acting as a HAP. In hypoxia condition, PR104 is reduced to the amines PR104H and PR104M, which induce DNA cross-linking in hypoxic cells (107). PR104, that could be activated also by the enzyme aldo-keto reductase 1C3 (AKR1C3) showed remarkable activity in *in vivo* B-ALL mouse models, suggesting that sensitivity is correlated with AKR1C3, whose expression could be used as a biomarker to select patients for this treatment in future clinical trials (108). In detail, an important aspect highlighted by this study was the great expansion of the hypoxic zones in the BM of leukemic mice, with a consequent loss of vessels integrity with increased leukemic cell burden. BM samples from B-ALL patients, immunostained for HIF1α expressed also in stromal cells, showed a strong positivity at diagnosis, which was impressively reduced when patients achieved a remission (108). The efficacy of PR104 has been reported also in preclinical models of pediatric T-ALL (109).

Several studies are ongoing to generate new HAPs that could be stimulated and activated by hypoxia condition for new molecularly targeted inhibitors having potential benefits in ALL treatments.

As regards instead the non-hypoxic prodrugs, one example is Nelarabine, a water-soluble prodrug of 9-β-Darabinofuranosylguanine (ara-G), a purine nucleoside analogue preferentially accumulating in T-lymphoblasts (110). Nelarabine is indicated for the treatment of patients with T-ALL (111–113) and T-cell lymphoblastic lymphoma (T-LBL) (115) that have not responded or have relapsed after treatment with at least two chemotherapy regimens.

Focusing more in detail on clinical trials currently active, Nelarabine was recently well tolerated in combination with the chemotherapy regimen Hyper-CVAD in the treatment for T-ALL (115), and its activity is involved significantly in the modulation of different signaling networks, mainly by targeting aberrant PI3K/AKT/mTOR signaling pathway (111, 116). Phase II recruiting study of effects of Hyper-CVAD and other drugs with Nelarabine in previously untreated T-ALL and Lymphoblastic Lymphoma is also active (please see www.clinicaltrials.gov, Identifier: NCT00501826). This Interventional Clinical Trial involves the enrollment of approximately 160 participants, and the crucial objectives of the study include the determination of complete remission (CR) following treatment, the safety and overall survival of previously untreated patients with T-cell ALL and T- cell lymphoblastic lymphoma, as well as the efficacy of two other antineoplastic drugs, pegaspargase and venetoclax. Nelarabine would be administered for several days and pegaspargase in multiple treatment steps. The study is recruiting and a further objective foresees how the combination of different chemotherapeutic agents can influence the different phases of cell growth, blocking it as much as possible, also influencing protein synthesis in terms of inhibition, considering the role of pegaspargase. Pegaspargase and Nelarabine are included in treatments with other chemotherapeutic agents also in newly diagnosed ALL children and adolescents (please see www.clinicaltrials.gov, Identifier: NCT03020030).

In a recent study, a combined pharmacogenomics data combined with data from an ALL cell line panel and patients reported the sensitivity differences between T-ALL and B-ALL. It was seen that the depletion of the deoxynucleotide triphosphate (dNTP) hydrolase SAMHD1, that is able to cleave and inactivate AraG, sensitized ALL cells to AraG, underlining the role as a therapeutic target to improve nelarabine therapies in ALL patients (117).

Recently, a novel AKR1C3-prodrug, OBI-3424, has shown significant antitumor activities in preclinical models of pediatric T-ALL, in relapsed/refractory T-ALL and also in solid tumors (118). A relevant reduction in BM infiltration was observed in ALL patient-derived xenografts (PDX) characterized by aggressive and fatal disease (118). Toxicity of OBI-3424, as well as the evaluation of the complete remission (CR), the event-free survival (EFS), the relapse-free survival (RFS) and the overall survival (OS) in patients with relapsed/refractory T-ALL are also the objectives reported in a phase II recruiting trial (please see www.clinicaltrials.gov, Identifier: NCT04315324).

Targeting HIF

Targeting HIF-1α represents another promising approach to block hypoxia-dependent progression, cell growth and drug resistance (92).

Although HIF represents an excellent and promising target in the characterization of the leukemic tumor microenvironment, there are currently no recent drugs capable of selectively inhibiting this transcription factor in ALL. The goal is therefore to develop more specific SMIs in ALL in order to make a significant breakthrough in the reduction of angiogenesis. In previous studies, echinomycin inhibiting HIF activity in lymphoma and leukemic stem cell models has been demonstrated, significantly improving the survival of mouse models and limiting the progression and growth of cancer cells (119). A very recent study instead reports the possible correlation between the activity of this transcription factor and the signaling pathway of PI3K/AKT/mTOR (120). In fact, it has been shown that in T-ALL cells the mTOR inhibitor rapamycin in normoxia is able to mimic the effects of the hypoxic condition, decreasing cell growth and increasing quiescence. Knocking down (KD) HIF-1α, a key regulator of the cellular response to hypoxia, the effects observed in hypoxic T-ALL were antagonized with a return to chemo-sensitivity. Therefore, the inhibition of mTOR in HIF1α KD T-ALL protected leukemic cells from chemotherapy. This mechanism may help to suppress the drug resistance of T-ALL in hypoxia, suggesting new therapeutic protocols (120).

Proteasome Inhibitors

The potential effects of proteasome inhibitors as anticancer therapy as well as for the treatment of other diseases have long been studied (121–123). Studies have shown that these agents could induce programmed death in leukemia cell lines, Burkitt's

lymphoma cell and other blood cancers and solid tumors (122, 124, 125).

Proteasome inhibitors are a relatively new class of cancer-targeted therapy. An example is Bortezomib (Velcade™) that is known to induce apoptosis, blockage of cytokine effects of myeloma and also on ALL models (126–129). Bortezomib works by activating two upward activating kinases of NFκB (RIP2 and IKKβ) and causes non-proteasomal degradation of IκB and stimulates DNA binding of NFκB (130). Moreover, it acts at the medullary level by stimulating the osteoblastogenetic process and inhibiting the angiogenic pathway (127).

The most recent studies reported how this drug could be effective in ALL relapsed and refractory pediatric patients, in addition with reinduction chemotherapy based on combinations with mitoxantrone, vincristine, pegaspargase or dexamethasone (131). Response rates were most significant in T-ALL. Notably, no patient in the study demonstrated neurological toxicities, despite this may represent a side effect induced by bortezomib, together with pulmonary complications. In ALL models, Bortezomib is able to enhance the activity of HDAC inhibitors, enzymes that normally regulate the structure and function of chromatin (132). In detail, treatment of pre-B ALL cells with the HDAC inhibitor panobinostat influences cell viability, induces apoptosis at increasing time treatment and leads to an increase in the expression levels of cyclin-dependent kinase inhibitors p21 and p27, correlating with the reduction of c-Myc and CDK4 mRNA expression level. Apoptotic cell death through the alteration of apoptosis-related genes is further enhanced by administration to cells with Bortezomib. Indeed, this drug led to an enhanced cytotoxicity of the HDAC inhibitor through decreasing the mRNA expression levels of anti-apoptotic target genes (132).

In Jurkat and Molt-4 T-ALL cells, the co-treatment of bortezomib with the glycosidic antibiotic daunorubicin enhanced the activation of caspase-3, -8 and -9, and the effect was reversed by the pan-caspase inhibitor, Z-VAD-FMK (133). Moreover, cotreatment with bortezomib and daunorubicin enhanced the collapse of mitochondrial transmembrane potential and upregulated the proapoptotic protein, B-cell lymphoma 2 (Bcl-2)-interacting mediator of cell death (Bim). These findings can help to deepen preclinical and clinical investigations (133).

The activity of Bortezomib was recently compared with a second recent proteasome inhibitor, Carfilzomib, whose efficacy was reported both in B and T-ALL models (134, 135). It was in fact seen that Carfilzomib showed relevant activity in the majority of ALL cell lines except for the P-glycoprotein-positive t (17, 19) ALL cell lines, and the knockout of the IKZF1 gene was associated with a favorable response to Carfilzomib treatment (136), making the association of Carfilzomib with the current chemotherapy protocols a new therapeutic option for refractory ALL with P-glycoprotein-negative leukemia cells. In a Phase I study, increasing doses of Carfilzomib were associated with Hyper CVAD in Patients with Newly Diagnosed ALL, showing promising results, also in terms of safety, feasibility and Minimal Residue Disease (MRD) response improvement (137).

CONCLUSIONS

The tumor microenvironment represents a key factor in the maintenance and metastasis of different type of tumors. Therefore, the comprehension of the interactions between the microenvironment and the tumor constitutes a fundamental element for the study of new promising therapies, considering also the fact that the cellular and molecular connections that are established within the microenvironment itself strongly influence neoplastic growth and dissemination.

Focusing on hematological malignancies, the ultimate goal in the characterization of the leukemic microenvironment, therefore of the niches, is the realization of new pharmacological therapies targeting the BM in connection with actual optimized protocols. This could be achieved by hitting pharmacologically the interactions between the malignant cells and the hematopoietic cell-intrinsic networks that define and influence the niche or the BM microenvironment, such as CXCL12/CXCR4, NOTCH, MEK/ERK or PI3K/AKT signaling networks, or the interactions between interleukins, essential for BM niche homeostasis and functionality.

The formation of new vessels is also important for the growth, progression and maintenance of leukemic cells. An extremely important modulator in tumor microenvironment is hypoxia. This condition triggers a process that leads to the development of new blood vessels, generally exploited by tumor tissues to grow and create metastasis. Indeed, exploiting the distinctive hypoxia aspects and the hypoxia-dependent component such as HIF-1α or the angiogenesis factor VEGF in the context of hematological neoplasms and, in this specific manuscript, in ALL, represents a promising challenge to improve the current treatment scenario, selecting the optimal drug doses to reduce side effects and overcoming drug resistance.

The complete characterization of the leukemic tumor microenvironment for the formulation of innovative and specific therapies has not yet reached the highest level of completeness. However, the rapid discovery of microenvironment-ALL interactions increasingly aims at relevant clinical advances.

Comprehensive strategies are therefore needed for the characterization of the leukemic tumor microenvironment and leukemogenesis, further expanding proteomic and genomic methodologies, imaging and sequencing technologies and immunotherapeutic approaches, which can then consequently broaden the spectrum for ALL cure, both in pediatric and in adult patients.

AUTHOR CONTRIBUTIONS

CS, GV and LMN: idealization, literature search, intellectual input, manuscript editing and writing; IC, CB and EC: literature search and writing initial version of the manuscript. All authors

contributed to the article final revision and approved the submitted version.

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The Role of Metabolism in the Development of Personalized Therapies in Acute Myeloid Leukemia

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Despite significant recent advances in our understanding of the biology and genetics of acute myeloid leukemia (AML), current AML therapies are mostly based on a backbone of standard chemotherapy which has remained mostly unchanged for over 20 years. Several novel therapies, mostly targeting neomorphic/activating recurrent mutations found in AML patients, have only recently been approved following encouraging results, thus providing the first evidence of a more precise and personalized approach to AML therapy. Rewired metabolism has been described as a hallmark of cancer and substantial evidence of its role in AML establishment and maintenance has been recently accrued in preclinical models. Interestingly, unique metabolic changes are generated by specific AML recurrent mutations or in response to diverse AML therapies, thus creating actionable metabolic vulnerabilities in specific patient groups. In this review we will discuss the current evidence supporting a role for rewired metabolism in AML pathogenesis and how these metabolic changes can be leveraged to develop novel personalized therapies.

Keywords: acute myeloid leukemia, metabolism, personalized therapy, leukemic stem cell, drug resistance

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INTRODUCTION

Acute myeloid leukemia (AML), the most prevalent acute leukemia in adults (1), is a highly heterogeneous disease. Clinically, patients present with symptoms due to blood cytopenias secondary to the bone marrow failure due to the marrow expansion of immature myeloid progenitors with a concurrent block in normal maturation. The accumulation of blasts often causes high peripheral white cell counts and can also lead more rarely to the seeding of leukemic cells in extramedullary tissues (2). AML is a disease of the elderly, with a median age at presentation of over 65 (3). Remission induction treatment in AML consists of standard chemotherapy with a combination of cytarabine and daunorubicin generally followed by further consolidation chemotherapy and possibly a hematopoietic stem cell transplant according to patients risk profile. Such intensive treatment is often precluded to elderly/unfit patients because of its inherent toxicity. Moreover, despite high remission rates with current therapies, the majority of

Abbreviations: 5-aza, 5-azacitidine; AraC, cytarabine; IDH, isocitrate dehydrogenase; FLT3, Fms Related Receptor Tyrosine Kinase 3; 3-PG, 3-phosphoglycerate; α-KG, α-ketoglutarate; BCAA, branched-chain amino acids; CH3-THF, methyltetrahydrofolate; CoA, coenzyme A; CPT, carnitine palmitoyltransferase; DHAP, dihydroxyacetone phosphate; GA-3-P, glyceraldehyde 3-phosphate; GLUT; glucose transporter; OAA, oxaloacetate; P, phosphate; SLC, solute carrier transporter; SucCoA, succinyl-coenzyme A; THF, tetrahydrofolate.

patients will suffer disease relapse which means that only 35 to 40% of adult patients younger than 60 and 5 to 15% of patients who are older than 60 years of age are cured. The outcome in older patients who are unable to receive intensive chemotherapy remains particularly poor, with a median survival of only 5 to 10 months (2).

AML arises either in a hematopoietic stem cell (HSC) or a more differentiated progenitor which has acquired the ability to self-renew indefinitely as a result of specific mutations (4). This cell of origin named leukemic stem cell (LSC) is able to recapitulate disease and generate relapse due to its resistance to standard chemotherapies. AML LSCs can reside in different phenotypically characterized populations in each patient and are able to evolve clonally through the acquisition of subsequent mutations (5). Recent sequencing efforts to identify novel gene mutations have led to further refinement of AML categorization in different subtypes based on their mutation profile and its putative effect on AML pathogenesis. Among the subgroups identified are those carrying mutations in transcription factors/epigenetic regulators, those carrying mutations in genes encoding for components of the spliceosome machinery and cohesin complexes, and cases carrying mutations in signaling genes (6, 7). In parallel with these sequencing efforts, further research in AML biology has highlighted altered transcription/epigenetic dysregulation and abnormal signaling as common themes in many AML subtypes (8). Thanks to the above efforts, we have recently witnessed the development of multiple novel therapies in AML with already nine being FDA approved for use since 2017 (see **Table 1**). Many of these novel therapies, although often not curative, have led to improved outcomes and increased our ability to treat less fit patients more effectively (23). This is because they either specifically target recurrent mutations found in some AML patients or are more effective in specific subgroups thus making them more tailored and less toxic and a first step towards a more personalized therapeutic approach. However, currently only a minority of patients can access true personalized precision therapy based on their genomic profile. Moreover, many of the recurrent mutations found in AML are not ideal therapeutic targets, either because it is not possible to design small molecules interfering with their function (i.e. transcription factors), or because they are loss-of-function mutations whose wild-type function is difficult to restore with a small molecule. As a result, it is important to identify other targetable biological features associated with unique AML subtypes which will allow the development of specific, highly effective and non-toxic therapy for most if not all AML patients. Rewired metabolism has emerged as a new hallmark of cancer (24) and recently there has been increasing evidence supporting its role in AML initiation and maintenance (25). Perhaps more importantly, unique metabolic rewirings have been shown to develop in specific AML subtypes (26), in response to therapy (27) or in specific AML cell populations including LSC (28). Metabolic reactions are carried out by specific enzymes which are targetable with small molecules and many metabolic inhibitors are already in clinical use or clinical development (29). Targeting rewired metabolism might therefore become a precision medicine

TABLE 1 | Agents recently approved for treatment of AML (9, 10).

Approval (FDA)	Name	Mechanism of action	AML patient group	Possible interactions with metabolic pathways
2020	Oral azacitidine (11)	Hypomethylating agent	Maintenance therapy for adults in first remission	Multiple: Reports of suppression of TCA cycle and OxPhos in combination with venetoclax; as single agent downregulates several metabolic patways in AML cell lines (12)
2018/2019	Ivosidenib (13)	IDH1 inhibitor	Relapsed/refractory or Age ≥75 years or comorbidities precluding intensive therapy	Inhibitor of cytosolic isocitrate dehydrogenase 1
2018	Glasdegib [in combination with low dose AraC (14)]	Hedgehog pathway inhibitor	Age ≥75 years or comorbidities precluding intensive therapy	No major known interactions
2018	Venetoclax [in combination with hypomethylating agent (15) or low dose AraC (16)]	Bcl-2 inhibitor	Age ≥75 years or comorbidities precluding intensive therapy	Reports of suppression of TCA cycle and OxPhos
2018	Gilteritinib (17)	Multiple TK inhibitor including FLT3	Relapsed/refractory	Reports of synergistic activity of this class of compounds with inhibition of glycolysis, glutaminolysis and ROS scavenging
2017	Midostaurin [in combination with standard chemotherapy (18)]	Multiple TK inhibitor including FLT3	Newly diagnosed	Reports of synergistic activity of this class of compounds with inhibition of glycolysis, glutaminolysis and ROS scavenging
2017	Enasidenib (19)	IDH2 inhibitor	Relapsed/refractory	Inhibitor of cytosolic isocitrate dehydrogenase 2
2017	Gemtuzumab ozogamicin [monotherapy (20) or in combination with standard chemotherapy (21)]	Anti-CD33 monoclonal antibody (gemtuzumab) conjugated to cytotoxic agent (ozogamicin)	Relapsed/refractory, Newly diagnosed	No major known interactions
2017	CPX-351 (22)	Liposomal formulation of daunorubicin and cytarabine (1:5 ratio)	t-AML or AML with MRC (no age restriction)	No major known interactions

therapeutic approach based on our knowledge of the specific metabolic vulnerabilities associated with AML subtypes, cell population and/or therapy combinations. Here we will discuss how targeting metabolism might help in fulfilling the promise of personalized medicine in AML.

METABOLIC CHANGES IN PATIENTS WITH ACUTE MYELOID LEUKEMIA

The association between changes in metabolic status and prognosis or severity of disease in patients with AML has been long known. In hospitalized patients, hyperglycemia is associated with adverse prognosis independently of their cytogenetics profile or advanced disease status, and this effect can be only partially explained by higher risk of sepsis (30). Additionally, higher glucose levels and increased glycemic variability result in lower remission rates and increased mortality in older patients with AML (31). Modified glucose metabolism, presenting as enhanced glycolysis and truncated TCA cycle monitored in serum of cytogenetically normal patients by a panel of six metabolites, could predict inferior prognosis independently of other molecular markers (32). This evidence of a global metabolic shift is in accordance with observations that AML cells modify the systemic regulation of glucose metabolism by inducing insulin resistance and hyperglycemic phenotype to allow for an increased availability of glucose necessary for their own growth (33). However, despite being a good indicator of the relevance of metabolic changes in AML biology, the use of such a generalized approach as the measurement of serum metabolites does not allow for development of any targeted therapeutic strategies, apart from supportive measures like controlling glycemia.

METABOLIC PLASTICITY OF ACUTE MYELOID LEUKEMIA CELLS

It's been almost a century since Otto Warburg first described aerobic glycolysis as one of the hallmarks of the metabolism of cancer cells (34). AML is not different from other malignancies in its dependency on glycolysis and increased glucose uptake (32, 35, 36). In BCR-ABL and MLL-AF9 leukemia models, already a modest inhibition of glycolysis performed by the deletion of M2 pyruvate kinase isoform (PKM2) resulted in inhibition of leukemogenesis, while sparing the physiological hematopoiesis (37). Inhibition of glycolysis, either through pharmacological inhibition by the use of 2-deoxyglucose (2-DG) or downregulation of glucose transporter 1, increased the sensitivity of AML cells to classically used chemotherapeutics like cytarabine (32, 38, 39). Additionally, 2-DG alone demonstrated strong antileukemic effects in both AML cell lines and primary samples harboring FLT3-ITD and KIT mutations also through inhibition of N-glycosylation and surface expression of the mutant receptor tyrosine kinases (40). Further confirmation for the relevance of glycolysis in AML cell viability comes from a report which showed that in conditions of glucose deprivation, AML cells switch to fructose utilization to fuel the glycolytic pathway by upregulation of fructose transporter GLUT5 which leads to more malignant phenotype and increased chemoresistance (41). Another possible strategy in targeting glycolysis could be through modulation of lactate metabolism, as a recent report has shown that inhibition of lactate transporters MCT1 and MCT4 induced cell death and increased sensitivity to chemotherapy (42).

However, typical descriptions of the Warburg effect, as well as the use of 18-fluorodeoxyglucose as a marker of cancerous cells in positron emission tomography, often lead to a common misconception of cancer cells switching their energy metabolism to glycolysis on the expense of tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OxPhos), while in reality, both pathways are dysregulated and often increased in comparison to healthy cells of origin, which makes them both interesting for potential therapeutic targeting (34). Infact, in AML LSC a specific dependency on OxPhos has been demonstrated. This can be specifically targeted using venetoclax, a Bcl-2 inhibitor which suppresses both TCA cycle and OxPhos, most likely by inhibiting amino acid metabolism which fuels TCA cycle in AML stem cells (28, 43, 44). This is in stark contrast to metabolic properties of healthy hematopoietic stem cells and bulk AML cells that show a far higher glycolytic reserve rendering them less sensitive to agents that inhibit mitochondrial activity (28). Metabolic plasticity of AML LSC nevertheless allows for an escape from metabolic pressure of venetoclax treatment by an increase in fatty acid oxidation (FAO) that continues to drive enhanced OxPhos when amino acid metabolism is inhibited leading to resistance to venetoclax/azacitidine treatment protocol (44, 45), confirming previous experimental results that simultaneous pharmacological inhibition of FAO enhances sensitivity of AML cells to Bcl-2 inihibiton (46). Further support for the dependency of AML LSC on OxPhos has been lent by a recent report showing that chemoresistant AML LSC are better defined by their metabolic characteristics than their immunophenotype, with cytarabine resistant cells being highly dependent on OxPhos and FAO (47). The strong reliance on FAO in AML cells, even independently of exposure to treatment, can be at least partially explained by downregulation of prolyl hydroxylase domain 3 protein (PHD3), an upstream regulator of acetyl-coA carboxylase 2 (ACC2). In physiological conditions, PHD3 and ACC2 activation suppresses FAO in the presence of other nutrients and therefore a prominent downregulation of PHD3 observed in AML patients could lead to this described dependence of AML cells on FAO (48).

Glutamine is a highly abundant non-essential amino acid that can serve as a source of precursors for TCA cycle through conversion to glutamate and α-ketoglutarate (αKG). Glutamine metabolism thus carries a great potential for therapeutic targeting in AML. Indeed, inhibition of glutamine utilization, either through uptake inhibition (49, 50) or a blockade in glutaminolysis (51), resulted in marked anti-leukemic effects in several AML models. Cellular roles of glutamine are, though, much broader then just

serving as a metabolic precursor of αKG. Glutaminolysis supplies cells with precursors for synthesis of glutathione, one of the most abundant cellular regulators of redox status, and *de novo* nucleotide synthesis. When used in combination with drugs that increase ROS formation, like arsenic trioxide or homoharringtonine, inhibitors of glutaminase (GLS), the first enzyme in glutaminolysis, significantly decreased the viability of AML cells and leukemia burden in treated mice (52). Glutamine role in *de novo* nucleotide synthesis has been less investigated, although many of classical chemotherapeutics are nucleoside analogues, and there is evidence that resistance to demethylation agents decitabine and 5-azacytidine, both nucleoside analogue prodrugs, could be circumvented by pharmacological inhibition of *de novo* pyrimidine synthesis (53). Targeting of *de novo* purine synthesis can also be achieved through inhibition of one carbon folate pathway (54) or pyrimidine synthesis through inhibition of dihydroorotate dehydrogenase (DHODH) (55–57), which induces differentiation of AML cells. Several clinical trials are currently assessing the efficacy of DHODH inhibitors as potential therapies in AML (clinicaltrials.gov: NCT03760666, NCT04609826, NCT03761069, NCT03404726).

The greatest success in metabolic targeting, and a proof of concept that specific modulation of metabolic pathways could result in therapeutic benefit for specific subgroups of AML patients, is differentiative therapy of patients carrying isocitrate dehydrogenase (IDH) mutations with IDH inhibitors. IDH1 and IDH2 mutations are present in 15–20% of patients with AML (58). The product of the neoezymatic activity of the mutated protein, 2-hydroxyglutarate (2-HG), competitively inhibits αKG-dependent enzymes, such as epigenetic regulator TET2, and induces a hypermethylated phenotype in AML cells that results in differentiation blockade (59). Small molecule inhibitors of both IDH2 (60) and IDH1 (61, 62) reduce the levels of 2-HG and induce differentiation of AML cells, and were able to produce durable remissions in patients when used as single agents (13, 19, 63). IDH2 inhibitor enasidenib and IDH1 inhibitor ivosidenib hence became first FDA-approved metabolism targeting drugs for AML.

Based on this, it is clear that the number of potential metabolic targets in AML is large. The question that naturally arises is how this extensive knowledge of metabolic changes in AML cells can help us identify the subgroups of patients who could benefit the most from modulation of specific metabolic pathways, thus leading to the development of tailored metabolic therapeutic approaches.

PERSONALIZED APPROACHES TO METABOLIC TARGETING OF AML

A recent metabolomics study on bone marrow samples of patients with *de novo* AML showed that when stratified by morphologic properties, genetic markers, differentiation status and European LeukemiaNet (ELN) 2017 risk groups, AML patients demonstrated a strong diversification of their metabolic properties (64). This underlines the potential of molecular markers already in use to serve as a guide to define patient subgroups with specific metabolic vulnerabilities.

Beside the previously mentioned success of targeting dysregulated metabolism of αKG in patients carrying IDH1 and IDH2 mutations, potential metabolic targets have been identified in AML subgroups with other molecular signatures. One of the main regulators of cellular αKG pools, and hence the activity of TET-family of DNA demethylases, is branched-chain amino acid transaminase 1 (BCAT1), the enzyme involved in branched-chain amino acids (leucine, isoleucine and valine) catabolism by transferring α-amino groups to αKG, producing glutamate and corresponding α-ketoacids. In $IDH^{WT}TET^{WT}$ patients overexpression of BCAT1 phenocopies the effects of IDH mutation and presents with a decrease in αKG levels and subsequent DNA hypermethylation. Patients with high levels of *BCAT1* showed enrichment for leukemia stem cell signatures and relapsed patients present with an increase in *BCAT1* levels (65). Interestingly, BCAT1 is also involved in leukemic transformation of EZH2-mutated myeloproliferative neoplasms (66), as well as blastic transformation of chronic myeloid leukemia (67), which further emphasizes the potential of this enzymatic pathway for the development of targeted therapies in specific AML subtypes.

The research in amino acid metabolism in AML is currently mostly focused on glutamine because it is a known vulnerability of AML cells, and specific inhibitors of GLS are reported to be safe and well tolerated in a Phase Ib/II clinical trial in patients with advanced myelodysplastic syndrome (68). AML cells carrying the poor prognosis internal tandem duplication (ITD) activating mutation in the FLT3 tyrosine kinase are specifically dependent on glutamine metabolism, both as a fuel for TCA cycle and precursor of gluthatione necessary for ROS scavenging, and glutaminolysis inhibition rescues resistance to FLT3 inhibitors (27). Moreover FLT3-ITD AML models demonstrate increased levels of aerobic glycolysis and similar effects in enhancement of the activity of FLT3 inhibitors can be achieved by glycolysis inhibition (69). FLT3-inhibition induces a marked upregulation of cellular oxidative stress which is circumvented through the activity of glucose 6-phosphate dehydrogenase. The inhibition of this enzyme and the use of compounds that increase mitochondrial ROS, like phenformin, greatly increased the sensitivity to FLT3-inhibition (70). In addition, it was recently described that patients with FLT3-ITD mutation present with dysregulated serine metabolism and inhibition of *de novo* serine synthesis, most likely through decrease in purine synthesis for which serine is a physiological precursor, decreases proliferation of FLT3-ITD mutated AML cells and increases their sensitivity to cytarabine (26).

Many other molecular markers appear to be associated with specific dysregulation in metabolism. Patients with t(8;21) translocation often present with mutations in transcription factor ZBTB7A (71), which has been described as transcriptional repressor of several critical glycolytic genes (72). AML cells with mutated *EVI1*, an oncogene associated with particularly poor clinical outcome, depend on mitochondrial creatine kinase CKMT1. Inhibition of CKMT1 induced a blockade in arginine–creatine pathway and impaired

mitochondrial respiration and ATP production. Interestingly, the gene sets that were most enriched in AML cells overexpressing *EVI1* were purine and pyrimidine synthesis pathways (73). Specific alteration in polyamine, purine and pyrimidine metabolism have also been described in patients with *NPM1*-mutations, a marker associated with favorable prognosis (74), again pointing out to a more widespread and under-investigated role of nucleotide synthesis in AML. A preliminary report in a mouse model demonstrated that induction of *ASXL1* mutation in hematopoietic stem and progenitor cells induced hematopoietic dysfunction followed by an increase in mitochondrial activity and ROS production (75), which might indicate for a potential role of metabolic dysregulation in pathogenesis of *ASXL1*-mutated AML. It is also worth noting that cancer cells with mutations in *SF3B1*, a gene which is frequently mutated in myelodysplastic syndrome and chronic lymphocytic leukemia, demonstrated dysregulated serine synthesis pathway *via* downregulation of enzyme phosphoglycerate dehydrogenase (PHGDH) and increased dependency to extracellular serine (76), which is interesting because of the role serine plays in one carbon metabolism which contributes to folate metabolism, synthesis of nucleotides and several amino acids, as well as methylation reactions and redox balance (77). Finally, one of potentially most intriguing patient subgroups for metabolic targeting is the one with *TP53* mutations. *TP53* mutations are commonly observed in solid tumors, but are present in only 5–10% of AML patients and, in spite of some recent progress in targeted therapy (78), they still represent a very unfavorable prognostic marker without an adequate treatment option (79). *TP53* has been shown to regulate a multitude of metabolic pathways in solid tumors, including glucose metabolism, oxidative phosphorylation, lipid metabolism, nucleotide metabolism etc. (80), but very little is known on its potential role in metabolic regulation in AML and further work is needed to elucidate if metabolic targeting could result in novel therapeutic strategies for AML patients harboring *TP53* mutations.

The group of patients that could most likely benefit from metabolic modulations are patients resistant to available therapies. As already mentioned, resistance to both cytarabine (47) and venetoclax (44, 45) are mediated by increased OxPhos and metabolic switch to FAO, while resistance to FLT3 inhibitors is mediated through alterations in metabolism of glutamine (27) and serine (26), as well as glycolysis (69). During intensive chemotherapy, AML cells appear to pass through a form of metabolic bottleneck producing resistant cells with a distinctive metabolic signature consisting of a switch in glutamine utilization primarily for nucleotide synthesis and gluthatione production and a modification of pyrimidine synthesis pathway to a higher dependency from extracellular aspartate provided by the bone marrow stroma (81). A targeted metabolic disruption could, thus, serve as a manner to prevent this metabolic plasticity of leukemic cells, overcome the survival advantage it provides, and reduce the establishment of resistance and consequent relapses. Metabolic pathways that could be relevant for resistance to pharmacologic agents in use, together with molecular signatures potentially associated with certain metabolic dysregulations, are summarized in **Figure 1**.

No compounds directly targeting an unmutated metabolic pathways has been approved for the treatment of AML, but there are several currently in clinical trials. Phase I clinical trials are underway for the glutaminase inhibitor CB-839 (NCT02071927) and oxidative phosphorylation inhibitor IACS-010759 (NCT02882321) in patients with relapsed or refractory AML (82), as well as safety and tolerability of adding HMG-CoA inhibitor pitavastatin to venetoclax in subjects with newly diagnosed AML (NCT04512105). After promising preclinical data that showed reduction in AML burden upon depletion of extracellular arginine levels using pegylated arginine deiminase (83), a phase II study in 43 relapsed/refractory/poor-risk patients demonstrated only a modest response (84) and a randomized trial of pegylated-recombinant arginase I BCT-100 in elderly AML patients is currently ongoing in the United Kingdom (unpublished correspondence).

Another intriguing therapeutic strategy for targeting metabolic pathways in AML could be through pharmacological modulation of their upstream regulatory signaling pathways, many of which have inhibitors already in clinical use (85). As one example, the mTOR pathway, which is a long recognized regulator of glucose, lipid and amino acid metabolism, has been recently re-evaluated for AML treatment in combination with standard chemotherapy when given in specific timed manner (86). Due to the breadth of the field of signaling in metabolic regulation, we refer the readers to other dedicated reviews on the topic (85, 87, 88).

Finally, the potential of dietary modifications and nutritional supplementation in unmasking the vulnerabilities of AML cells is recently being increasingly investigated. High fructose diet is known to contribute to cancer development. However it can also unmask novel vulnerabilities as AML cells upregulate *de novo* serine synthesis pathway in high fructose conditions, and the inhibition of one of the components of this pathway, phosphoglycerate dehydrogenase, can block the reliance on fructose utilization (89). It is yet to be fully determined whether the reduction of fructose in the diet of AML patients could also prove to be of clinical efficacy (41), or it is a wider epidemiological target in the attempt to reduce the general burden of cancer (90). Additionally, specific dietary intervention can also be devised to modulate metabolic pathways which can enhance the therapeutic efficacy of standard therapies. As an example, preclinical evidence has shown that supplementation with methyl-tetrahydrofolate (91) or histamine (92) show beneficial anti-leukemic effects by modulation of folate pathway which enhances the the efficacy of MYC-targeted therapies and methotrexate, respectively.

CONCLUSIONS

Precision medicine has become an increasingly desirable goal in oncology. Using available knowledge banks of matched genomic-clinical data has already been shown to facilitate personally

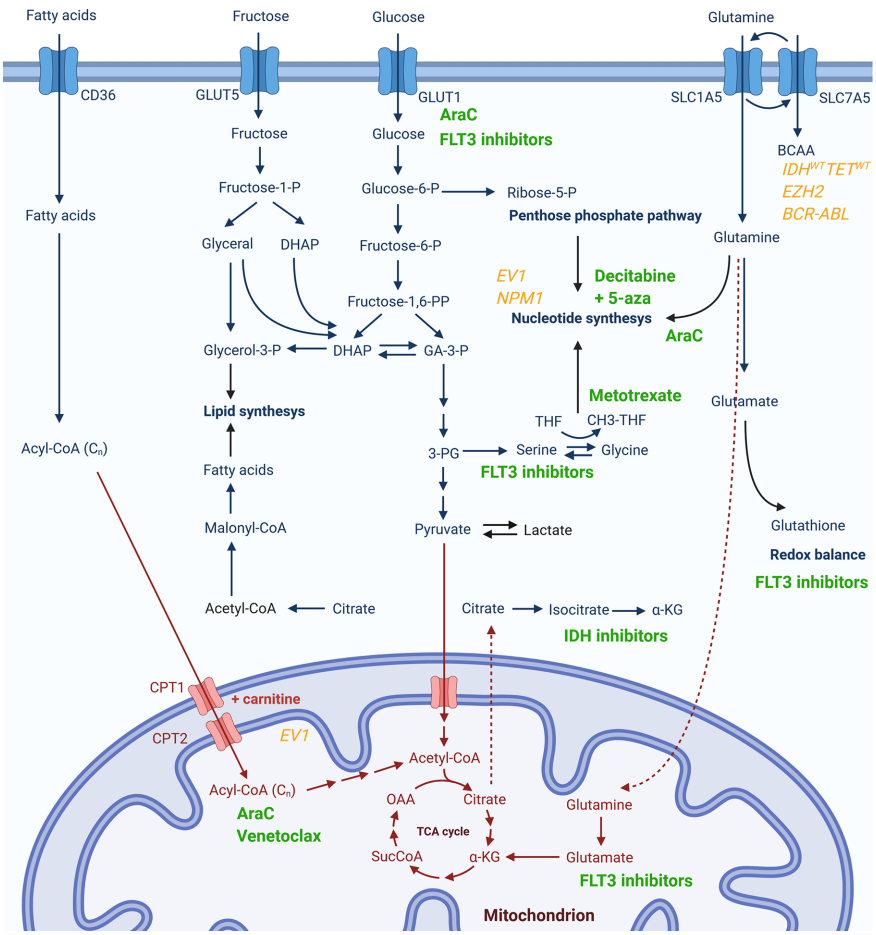

FIGURE 1 | Metabolic pathways potentially associated with resistance to currently used pharmacological agents or specific genetic signatures. Chemotherapeutics or targeted therapy drugs currently in use for AML (in green) are presented next to the cytosolic (in blue) or mitochondrial (in red) metabolic pathway whose activity has been reported to be modulated upon treatment with respective compound or which has been associated with drug resistance. Genetic markers associated to changes in certain metabolic pathways are given in yellow.

tailored therapeutic decisions (93). Moreover, the recent development of targeted therapies towards specific genetic subgroup will further increase the efficacy and reduce the toxicity of such personalized therapeutic approaches. Unique metabolic rewiring in several AML subtypes/cell populations and its role as therapeutic targets or in therapy resistance has increasingly come into focus. It is therefore possible to envision a near future in which we will be able to incorporate metabolic analysis in our knowledge banks to further refine our ability to devise personally tailored therapies. Moreover, the development of "targeted" metabolic inhibitors shown to be effective, specifically in certain AML subtypes, will also increase our therapeutic options for unique patient groups. Finally, our improved understanding of metabolic changes in AML can also be leveraged by using tailored dietary interventions likely to improve the efficacy of specific drugs thus providing a truly holistic approach to patient management.

AUTHOR CONTRIBUTIONS

VD and PG carried out primary literature search and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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"Modulating Phosphoinositide Profiles as a Roadmap for Treatment in Acute Myeloid Leukemia"

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Polyphosphoinositides (PPIns) and their modulating enzymes are involved in regulating many important cellular functions including proliferation, differentiation or gene expression, and their deregulation is involved in human diseases such as metabolic syndromes, neurodegenerative disorders and cancer, including Acute Myeloid Leukemia (AML). Given that PPIns regulating enzymes are highly druggable targets, several studies have recently highlighted the potential of targeting them in AML. For instance many inhibitors targeting the PI3K pathway are in various stages of clinical development and more recently other novel enzymes such as PIP4K2A have been implicated as AML targets. PPIns have distinct subcellular organelle profiles, in part driven by the specific localisation of enzymes that metabolise them. In particular, in the nucleus, PPIns are regulated in response to various extracellular and intracellular pathways and interact with specific nuclear proteins to control epigenetic cell state. While AML does not normally manifest with as many mutations as other cancers, it does appear in large part to be a disease of dysregulation of epigenetic signalling and many novel therapeutics are aimed at reprogramming AML cells toward a differentiated cell state or to one that is responsive to alternative successful but limited AML therapies such as ATRA. Here, we propose that by combining bioinformatic analysis with inhibition of PPIns pathways, especially within the nucleus, we might discover new combination therapies aimed at reprogramming transcriptional output to attenuate uncontrolled AML cell growth. Furthermore, we outline how different part of a PPIns signalling unit might be targeted to control selective outputs that might engender more specific and therefore less toxic inhibitory outcomes.

Keywords: phosphoinositides, PLCB1, PI3K, PIP4K, AML, epigenetic, transdifferentiation, bioinformatic

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a cancer of blood cells, in which myeloid progenitor cells lose their ability to differentiate while increasing their rate of proliferation giving rise to too many and/or too immature myeloid cells derivatives. These blast cells fail to differentiate into granulocytes or monocytes. Blast accumulation in the absence of proper haematopoiesis leads to impairment of the immune system and eventually to death. Although, compared to other types of cancers, AML is characterised by a low number of mutations, it is a highly heterogenous hematologic disease classified into many different subtypes, reflected in different clinical manifestations: understanding the relevance of this heterogeneity is critical to develop novel and more personalized clinical therapies (1). Today, the first line of treatment is still chemotherapy, with a 5-year survival rate of less than 30%: it is now clear that treating all patients with the same starting protocol (i.e. "one size fits all" strategy) benefits only a specific group of patients. Personalized therapies are aimed at using novel insights into patient specific genetic signatures of AML to define strategies to treat the AML (1, 2). Compared to the last 50 years, when administration of cytarabine and anthracyclines was the only standard therapy, the last 6 years has seen the introduction of many novel successful clinical trials, aimed to stratify and treat patients in subgroups tailored on patient specific genomic backgrounds (3). Although new molecular therapies are showing promising results, patients often relapse and therefore more therapeutic strategies are required. We propose that by more fully understanding how polyphosphoinositides (PPIns) profiles impact on cell behaviour, and by targeting the enzymes that modulate these lipids, a roadmap in the cell state could be generated to lead to new therapeutic strategies in AML treatments. This review will focus on how phosphoinositides participate in the regulation of cellular processes important in AML, i.e. cell growth, differentiation, apoptosis and epigenetic behaviour and how regulation of their modulating enzymes can be a beneficial additive for a more personalized AML treatment.

PHOSPHOINOSITIDES

Phosphoinositides are a family of phosphorylated lipid molecules that directly control several essential cellular processes, such as proliferation, survival, adhesion, vesicular trafficking and transcription (4). They are derived from phosphorylation of the parent precursor molecule, phosphatidylinositol (PtdIns) and can generate seven well characterised polyphosphoinositides (PPIns) (5). The structural basis of PtdIns consists of an inositol head group linked to diacylglycerol (DAG) by a phosphodiester bond. The inositol head group of PtdIns can be reversibly phosphorylated at the 3, 4 and 5 positions of the inositol ring giving rise to 7 different molecules: phosphatidylinositol 3-phosphate (PtdIns3P), PtdIns4P, PtdIns5P, PtdIns 3,4-bisphosphate [PtdIns(3,4)P_2], PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. PPIns can be interconverted by the activity of kinases and phosphatases whose activations can be controlled by extracellular and intracellular inputs (6, 7). Regulation of these kinases and phosphatases at different subcellular locations can lead to organelle specific PPIns profiles which in combination with their ability to interact with specific downstream signalling proteins enables the conversion of the chemical PPIns profiles into diverse function outputs (8) (**Figure 1**).

For instance, PtdIns(4,5)P_2 is mainly concentrated at the plasma membrane and it is involved in the regulation of integral membrane proteins such as ion channels which contain arginine lysine patches that bind to PtdIns(4,5)P_2 and induce a change in conformation and channel activity. PtdIns (3,4,5)P_3, which is synthesised by the phosphorylation of PtdIns (4,5)P_2 is also predominantly localised to the plasma membrane and can initiate many different signal cascades. In contrast PtdIns(4)P is predominantly found in the Golgi complex (9), where it modulates Golgi structure and function, whilst PtdIns (3)P and PtdIns(3,5)P_2 are found predominantly within early and late endosomes or lysosomes (10) (**Figure 1**). PtdIns(3,4)P_2 is another key molecule, when localized at the plasma membrane it can assist cytoskeletal rearrangements important for clathrin mediated endocytosis, macropinocytosis and lysosomal catabolism, cell migration and, in cancer cells, invasion (11). Moreover PtdIns(3,4)P_2 can mediate glucose uptake and insulin signalling and become a key second messenger (11).

After binding PPIn, proteins can change their localisation, conformation, interaction partners and also activity so that these interactions control various cellular processes (8): here we will describe some of them, relevant for AML.

PHOSPHOINOSITIDE 3-KINASES AND AML

Phosphoinositide 3-kinases are lipid kinases that phosphorylate one or more inositol phospholipids on the 3-position of the inositol ring (**Figure 2**). There are eight PI3Ks in mammalian cells that are sub grouped into three unique classes based on structural and enzyme-kinetic differences; four Class I isoforms (PI3K -α, -β, -γ, -δ), three Class II isoforms (PI3K-C2α,-C2β and -C2γ) and a single Class III isoform, known as vacuolar protein sorting 34 (Vps34) (12). In particular, PI3Kγ and PI3Kδ isoforms are abundant in hematopoietic cells, such as leukocytes (13, 14), whilst PI3Kα and PI3Kβ are mainly ubiquitously expressed. Class I PI3K can phosphorylate PtdIns(4,5)P_2 into PtdIns(3,4,5)P_3 and increased levels of PtdIns(3,4,5)P_3 are sensed by specific Plekstrin Homology (PH) domain containing proteins such as the serine threonine kinase AKT/PKB. PtdIns(3,4,5)P_3 can be degraded by a number of phosphatases such as PTEN, SHIP1 or INPP5D which are essential to maintain long term hematopoietic stem cells (15). Interaction with PtdIns(3,4,5)P_3 leads to activation of kinases and phosphatase that exert a wide spectrum of effects on downstream pathways which include cell proliferation, differentiation, apoptosis and metabolism (16). Class II isoforms have distinctive, non overlapping functions that regulate cell migration, proliferation and survival (17). PI3K-C2α is also involved in PtdIns(3,4)P_2 -mediated vesicular trafficking, membrane remodelling important for platelet formation and can be a scaffold protein important during mitosis. PI3K-C2β is an activator of Ca^{2+} flux and regulates many signalling pathways, whilst PI3K-C2γ is mainly involved in vesicular trafficking and in glucose homeostasis (17).

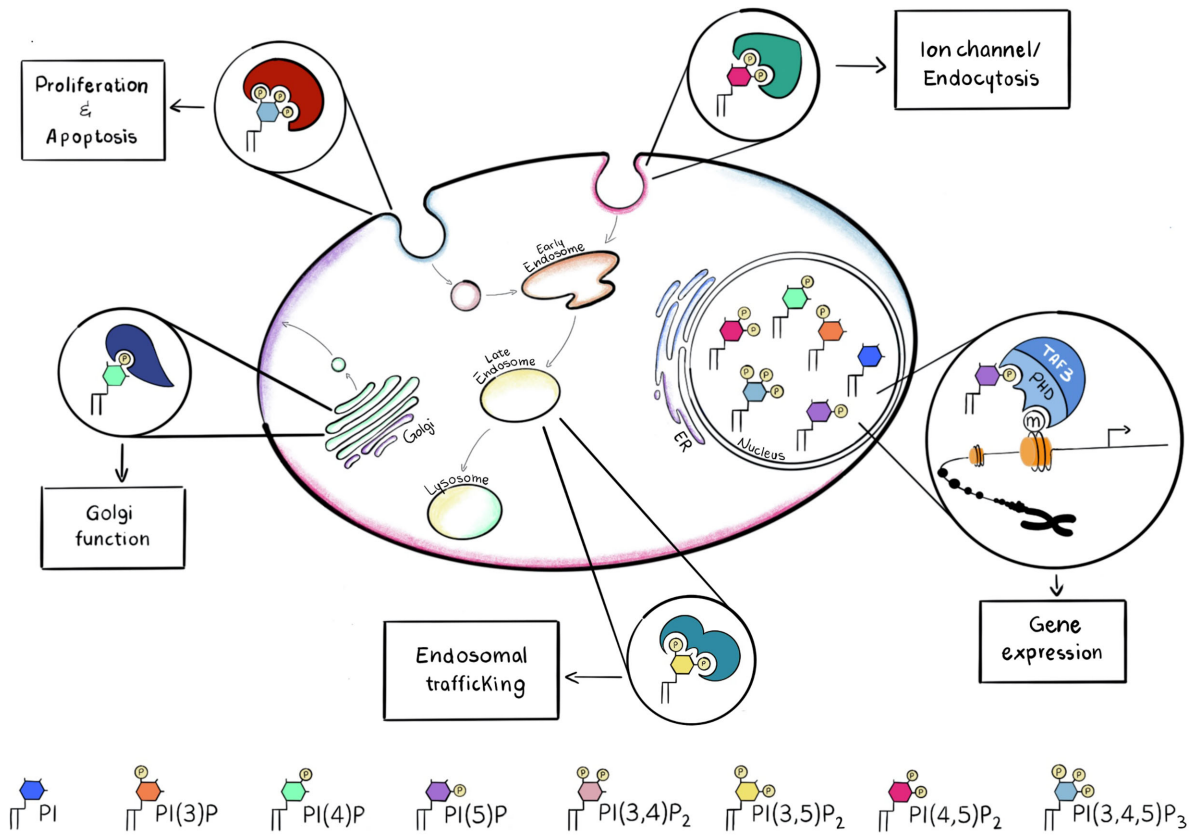

FIGURE 1 | PPIns may be phosphorylated on the 3, 4 and 5 positions to generate seven possible PPIns, each with a different spatial occupancy, that is recognized by different effector proteins. Recruitment of the necessary effector protein is guaranteed by the different accumulation of PPIns in the subcellular compartments. Here are some examples of how different PPIns interact with different effector proteins to convert chemical diversity into diverse functional functions.

The ubiquitous utilization of PI3K signalling by diverse receptor families together with the identification of mutations in multiple components of the PI3K signalling pathway in various cancers, has led to the development of compounds targeting this pathway. Many of these are under clinical investigation for cancer treatment showing varied levels of success (18).

In AML, the PI3K pathway dysregulation is a frequent event and correlates with poor prognosis (19). Constitutive activation of PI3K signalling is associated with hematologic malignancy and is probably triggered by abnormal activations of KIT, FLT3 and RAS, which are frequently muted in AML (20, 21).

Several preclinical trials using PI3K inhibitors have progressed to clinical trials but even though PI3K-related inhibition may target AML cells, including Leukemic Stem Cells (LSCs), they have met with a very limited success as monotherapies, probably as a consequence of compensatory activation of other survival pathways (22). For this reason, novel combinations and alternative pathway inhibitions may result in more efficacious and tolerable pharmacological regimens for AML treatment.

Firstly, PI3K/mTORC1/2 inhibition reached higher apoptosis rates than single inhibition or combined AKT/mTORC1 inhibition. Primary patient samples and cell lines carrying MLL rearrangements show higher sensitivity to PI3K/mTOR inhibition. In a larger cohort of MLL-AF9+ AML patients, a high incidence of additional mutations in genes involved in growth factor signalling pathways was identified, which could explain their preferential sensitivity (23). In THP-1 cells and patient-derived xenografted (PDX) cells, a combination treatment of the dual PI3K/mTORC1/2 inhibitor BEZ-235 with a MEK inhibitor showed highly synergistic effects on apoptosis. Using the MLL-AF9+ xenograft mouse model, Sandhöfer et al. highlighted the efficacy of PI3K/mTORC1/2 inhibition *in vivo*. Altogether, these data show a possible benefit of PI3K/AKT/mTOR inhibition as a therapeutic approach for MLL-rearranged leukaemia.

In the battle against AML, the effect of PI3K/AKT/mTOR inhibition can be augmented by BCL-2, which is an anti-apoptotic protein often overexpressed in several blood disorders, including AML. Increased expression of BCL-2 enhances survival by blocking apoptosis and is associated with increased chemoresistance and poor patient outcome (24).

The selective BCL-2 inhibitor Venetoclax has shown strong cytotoxic effects combined with a safe patient profile in AML (25) and Venetoclax has recently been approved by the FDA for CLL patients with the 17p deletion (26). Furthermore, it entered phase II clinical trials as a monotherapy in patients with refractory and relapsed AML (27).

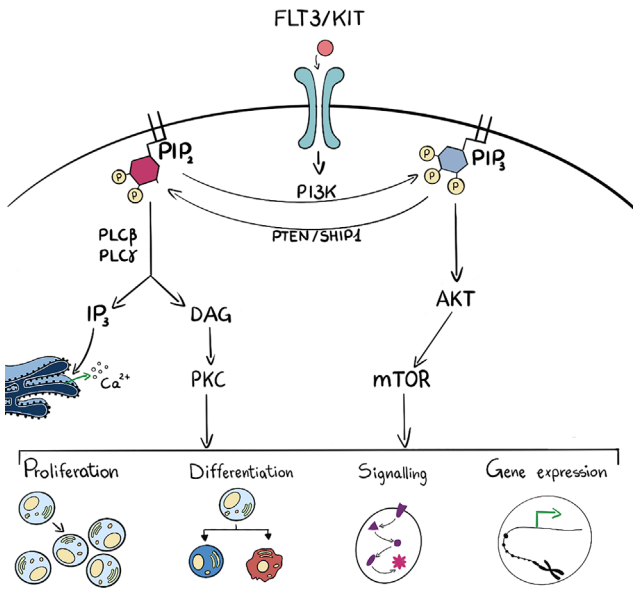

FIGURE 2 | Schematic representation (on the left side) of PLC activation which hydrolyses PIP_2 into DAG and IP_3. These last two are intermediate second messengers that can regulate many cell functions such as proliferation, differentiation, signal transduction and gene expression. On the right side, ligand binding to membrane receptor drives PI3K activation and consequent phosphorylation of PIP_2 into PIP_3 and subsequent activation of the AKT/mTOR pathway. These activated players can as well regulate many cell functions such as proliferation, differentiation, signal transduction and gene expression. PIP_3 can be dephosphorylated to PIP_2 by phosphatases such as PTEN and SHIP1. Many intermediate players are omitted for figure clarity.

Complete responses to BCL-2 are only observed in approximately 20% of patients suggesting that monotherapy does not reach durable responses. Thus, Venetoclax combined with inhibitors of survival pathways or classical chemotherapeutic drugs have been assessed for the treatment of AML, to increase the cytotoxic effects. Among these, the combined treatment of BCL-2 and PI3K inhibition enhanced leukemia cell death in AML cell lines, patient-derived blasts and xenograft models (28). The anti-leukemic effects of this drug combination can be further increased by ERK inhibition (29) although how toxicity profiles are affected is yet to be assessed. Downregulation of Mcl-1, a BCL-2 related antiapoptotic protein with PI3K/mTOR inhibitors may underlie the potentiation of the effect of Venetoclax, in leukemia cells (30).

PHOSPHOLIPASE AND AML

Phospholipases C (PLCs) hydrolyse PtdIns(4,5)P_2 into DAG and Ins(1,4,5)P_3: DAG can be bound by many proteins, i.e. protein kinase C (PKC) that transduce changes in DAG levels into phosphorylation and regulation of downstream targets, whilst Ins(1,4,5)P_3 can be bound by its cognate receptor on the endoplasmic reticulum and regulate Ca^{2+} efflux (**Figure 2**). Phospholipases are localized mainly at the plasma membrane but also in cell organelles: for example PLCβ1 is present in the nucleus where it is involved in transcriptional regulation (31–35).

Interestingly, PLCβ1 is involved in haematological malignancies by regulating haematopoiesis, especially in Myelodysplastic Syndromes (MDS), where it acts on both erythropoiesis and myelopoiesis (36–38), with possible implications in transformation into AML. PLCβ1 modulation is clinically relevant in leukemogenesis of MDS, as its mono-allelic deletion is associated with increased risk of AML progression and its expression is inversely correlated with AKT/ mTOR activation in higher-risk MDS (39–42). Moreover, specific mutations in inositide regulating enzymes including PLCγ2, AKT3 and PIK3CD were associated with Azacytidine and Lenalidomide therapy failure in MDS leading to a higher risk of AML progression (43). In addition to PLCβ1, also PLCβ3 plays a crucial role in haematopoiesis, since PLCβ3-deficient mice develop myeloproliferative disease, lymphoma, and other tumors (44): these mutant mice have increased numbers of hematopoietic stem cells with increased proliferative, survival and myeloid-differentiative abilities. Particularly, PLCβ3 exerts this function, not by its lipase activity, but by being a scaffold protein that hold together SHIP1 and the transcription factor Stat5, that in turn regulates the above processes.

Recent studies have observed also that survival of Leukemic Stem Cells from the bone marrow of AML patients is dependent on ORP4L, a protein that acts to scaffold PLCβ3 into a complex at the plasma membrane. ORP4L is able to extract PtdIns(4,5)P_2 from the plasma membrane and presents it as a substrate to PLCβ3 for hydrolysis, mediating Ins(1,4,5)P_3-induced endoplasmic reticulum Ca^{2+} release (45, 46). Importantly, genetic or pharmacological inhibition of ORP4L leads to LSCs death in AML and to defective bioenergetics, autophagic death and abrogation of T-ALL engraftment *in vivo* (47).

Finally, by a peptide microarray profiling array, in a t (8, 21) AML, PLCγ1 was found hyper-expressed and PLCγ1 KD showed a decreased in AML cell growth, increase of apoptosis and a higher chemosensitivity to the chemotherapeutic drug treatments upon hypoxic stress (48).

PHOSPHATIDYLINOSITOL-5-PHOSPHATE 4-KINASE AND AML

In a recent study a sh-RNA library targeting modulators of PPIns was used to identify novel targets essential in AML proliferation in at least three different AML cell lines. From the screen common modulators emerged as essential for proliferation and included three PPIns phosphatases (INPP5J, INPP5B, SYNJ), subunits of the PI3K pathway (PI3K-C2α, PI3K-R3, PI3K-R6), PLCβ2 and PIP4K2A (49). PIP4K2A is a kinase that phosphorylate PIns or Pins5P on the 4-position of the inositol ring, thus regulating the levels of both PtdIns(4,5)P_2 and PtdIns5P. Silencing PIP4K2A attenuated growth of primary human AML cells, while sparing healthy Hematopoietic Stem Cells HSCs (49). In AML cells, PIP4K2A regulates cell cycle progression and apoptosis dependent on the activation of mTOR and represents a novel potentially druggable target for the treatment of AML. The pro-leukemic role played by PIP4K2A was also

demonstrated in paediatric acute lymphoblastic leukaemia, where its expression correlated with chemoresistance (50). Moreover, susceptibility for development of acute lymphoblastic lymphoma has been associated with SNPs in both intronic and exonic regions of the PIP4K2A gene (51, 52).

The revelation that many different PPIns modulators are essential for AML cell growth suggest that understanding their individual mechanism of action might lead to the development of patient specific therapies that could be generated through combinations of molecules that deregulate these pathways. Importantly the enzymes that modulate PPIns are highly druggable and many inhibitors are already available. Given that many of these enzymes are also found in the nucleus where they regulate a specific pool of PPIns, which impact on transcriptional output we next describe their potential roles as epigenetic regulators in AML.

THE POTENTIAL FOR TARGETING NUCLEAR PHOSPHOINOSITIDES AS EPIGENETIC REGULATORS IN AML

PPIns and in particular PtdIns, PtdIns4P, PtdIns5P, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 are localized in the nucleus (53), in the nuclear envelope and in the nucleoplasm. Within the nucleoplasm PtdIns (4,5)P_2 and PtdIns4P have been localised by immunostaining to splicing speckles, nucleoli and to nuclear lipid islets (54). In the nucleus, the levels of PPIns respond to specific stimuli, such as cell stress, DNA damage, cell cycle progression or cell differentiation (55). These changes occur distinctly and independently of changes in the cytoplasmic profile of PPIns. Changes in nuclear PPIns appear to be particularly prevalent during control of differentiation or proliferation. The levels of nuclear PLCβ1 decrease during hematopoietic differentiation and increase during liver regeneration (34, 56). Surprisingly, increased nuclear PLCβ1 is required during myogenic differentiation which may be related to the initial phase of differentiation.

How exactly nuclear PPIns control differentiation is not completely clear. Nuclear PPIns can be bound by specific nuclear protein domains found in enzymes that control epigenetic signalling. For example the PHD finger domain is a nuclear receptor for PPIns, found mainly in nuclear proteins which are involved in all aspects of epigenetic signalling (57, 58). These domains also mediate interaction with modified and unmodified histone tails and act as protein dimerization domains which are likely modified by PPIns interaction. For example, the PHD fingers of ING2 (INhibitor of Growth protein 2) and TAF3 (TATA-Box Binding Protein Associated Factor 3) both act as sensors for H3K4me3 and for nuclear PPIns. ING2 regulates p53 acetylation and transcriptional output and TAF3 is a component of the basal transcription complex. In both cases, loss of PPIns interaction through mutation of the PHD finger, leads to protein loss of function, even if the interaction with H3K4me3 is unchanged. In the case of ING2 this leads to decreased acetylation of p53 and aberrant transcriptional output (57) while in the case of TAF3, which is involved in myogenic differentiation, there is a decrease in myogenic gene transcription and differentiation (59). Nuclear PPIns actually interact with a much wider variety of nuclear proteins involved in transcriptional output (60). For example, nuclear PPIns interact and allosterically regulate the Ubiquitin-like PHD and RING finger domain-containing protein 1 (UHRF1) (61). UHRF1 is a multidomain protein that regulates DNA methylation in response to changes in histone modification through its ability to interact with the DNA methylase DNMT1 and bind modified histones. The interaction between UHRF1 and PPIns occurs through a polybasic region (PBR) which are abundant in nuclear proteins. In fact, mass spectrometry revealed that PBRs were the most highly enriched domain after affinity purification of nuclear proteins on PtdIns(4,5)P_2 beads (62). Moreover nuclear PtdIns(4,5)P_2 also regulates the activity of the histone lysine demethylase PHF8 to control ribosomal RNA transcription (63) and the activity of chromatin remodeling complexes (64, 65). These data suggest that targeting nuclear PPIns signalling could be specifically used to control epigenetic signalling to impact on transcriptional output.

Despite having a low mutation burden, AML is highly heterogenous due to deregulation of the epigenetic landscape suggesting that epigenetic modulators could be leveraged to target and treat AML (66). On average 70% of patients have mutations in genes encoding epigenetic regulators including chromatin modifying genes or genes involved with the regulation of DNA methylation (67, 68). In Myelodysplastic syndromes (MDS), mutations are often associated with increased DNA methylation and demethylation therapies, such as azacytidine and more recently decitabine (DNA methylation inhibitors) are used to treat this disease. Interestingly, expression of nuclear PLCβ1 is increased during demethylation therapy in MDS patients and is associated with a good response to the drug (69) while monoallelic deletion of PLCβ1 is associated with poor prognosis of MDS patients (70). Other broad-based epigenetic therapies include inhibition of Histone deacetylases (HDAC). Newer more specific targets include IDH1 (isocitrate dehydrogenase 1) (71–75), DOT1, (a H3K79 methylase) and KDM1A (LSD1) a nuclear amine oxidase homolog that demethylates mono- and dimethylated Lys 4 and Lys 9 of histone H3 (76–82).

Epigenetic reprogramming can also be used to unlock the potential of well-established AML therapies such as All Trans Retinoic Acid (ATRA) for its broader use as a therapeutic in AML. ATRA is highly successful in treating Acute Promyelocytic Leukaemia (APL) but has little effect in other AML subtypes. Recent studies have shown that treatment with either KDM1A or GCN5 (a histone h3K9 acetylase) inhibitors reprogram non-APL AML cells to become sensitive to ATRA induced differentiation (83, 84). Identifying novel small molecules that can induce differentiation is a difficult, lengthy and stochastic process which can be assisted by predictive computational algorithms, such as Mogrify, that combine data from RNA expression and epigenetic landscape to predict perturbations necessary to change cell state (85). Given that nuclear PPIns metabolism is intimately linked with control of epigenetic signalling and the enzymes that regulate PPIns are highly druggable it might be possible to identify combinations of inhibitors aimed at targeting

PPIns metabolism to establish a particular quiescent cell state (**Figure 3**) given a deep understanding of how perturbing PPIns metabolism impact on gene expression.

Targeting epigenetic signalling enzymes, such as methylases or demethylases, may turn out to have toxicity issues and uncovering how they are controlled would allow for further fine tuning. Remarkably it appears that nuclear PPIns interaction with downstream targets regulates selective output signalling. For example, mutations that attenuate the interaction of the PHD finger of either ING2 or TAF3 with PPIns do not completely inhibit their function but rather lead to a more selective inhibition that impacts on the transcriptional output of a subset of gene targets (59, 86). PPIns interact with many different PHD and PBR containing proteins that span epigenetic writers, erasers and readers and the demonstration that therapeutically useful small molecules can be generated to block interactions between lipids and their cognate interaction domains (87) suggest that highly specific allosteric regulators that modulate the interaction of nuclear PPIns with specific proteins could be generated. These molecules are likely to selectively modulate the function of nuclear PPIns binding proteins enabling more specific targeting compared to inhibition of the PPIns modulating enzymes or of the epigenetic signalling activity itself. Combining this understanding with bioinformatic analysis as described above might enable the development of combination therapies that can subtly tune transcriptional output to drive AML tumour cells to express differentiation specific genes to attenuate proliferation.

CONCLUSION AND PERSPECTIVES

PPIns regulate a vast array of cellular process impacting on nearly every aspect of biology. They are controlled by a panel of kinases, phosphatases and phospholipases which generate distinct sub-cellular PPIns profiles that impact on downstream signalling through interaction with specific target proteins. Key to the possibility of targeting PPIns in AML is that many of the enzymes that modulate PPIns are essential for growth and proliferation of AML cells but have much less impact on the growth and differentiation of normal hematopoietic stem cells. Interestingly the level of expression of many PPIns modulating enzymes also stratify AML patients in terms of overall survival. We interrogated the TCGA data base for AML patients with all PPIns modulating enzymes and found twenty-two modulators that significantly (<0.05) stratify patient overall survival. For example and in accordance with our previous studies (49), high expression levels of PIP4K2A associate with poor survival (**Figure 4A**). PIP4K2A is one of three isoforms of PIP4K that phosphorylate PtdIns5P to generate PtdIns(4,5)P_2. Interestingly, the other two isoforms, 2B and 2C also stratify patient survival with 2C showing similar characteristics as 2A. However, PIP4K2B, which is predominantly localised in the nucleus, shows an inverse correlation with survival compared to 2A and 2C, such that low levels of 2B associate with poorer survival. This is in accord with our previous studies in breast cancer patients which revealed that low PIP4K2B is associated with poor survival (88). Similar stratification differences between PPIns modulating enzymes from the same enzymatic family are also observed in the MTMR family, which dephosphorylate PtdIns3P and PtdIns(3,5)P_2 to generate PtdIns and PtdIns5P respectively (**Figure 4A**). While these data are difficult to interpret, they suggest the presence of exploitable complexity within the system.

Another key aspect in targeting PPIns pathways, is that the enzymes that modulate them are highly druggable and many of them have already been under intense development for the identification of small molecular weight inhibitors. For example, there are multiple inhibitors targeting the PI3K pathway which are in various stages of clinical development. In many respects the PI3K

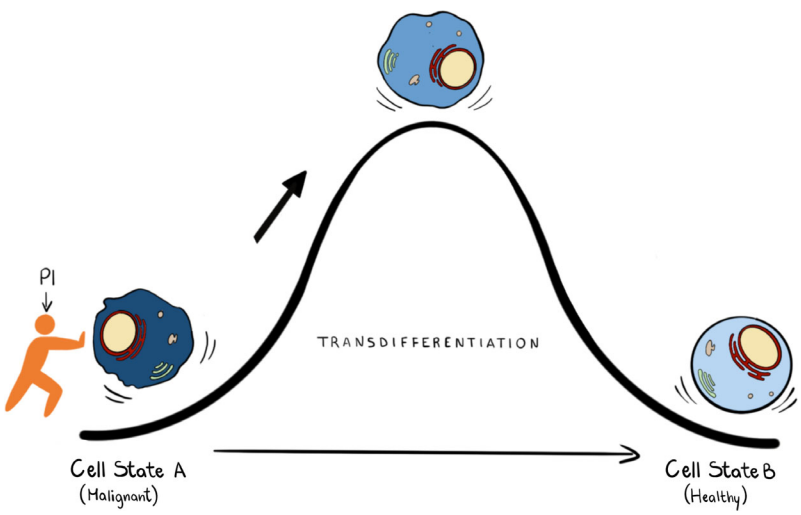

FIGURE 3 | In transdifferentiation a somatic cell can pass from a somatic differentiated change to: this process can be achieved by knowing the transcription factors and the epigenetic regulators that can drive this transition. We propose that PPIns, by being able to regulate epigenetic and transcription factors, could help this transition.

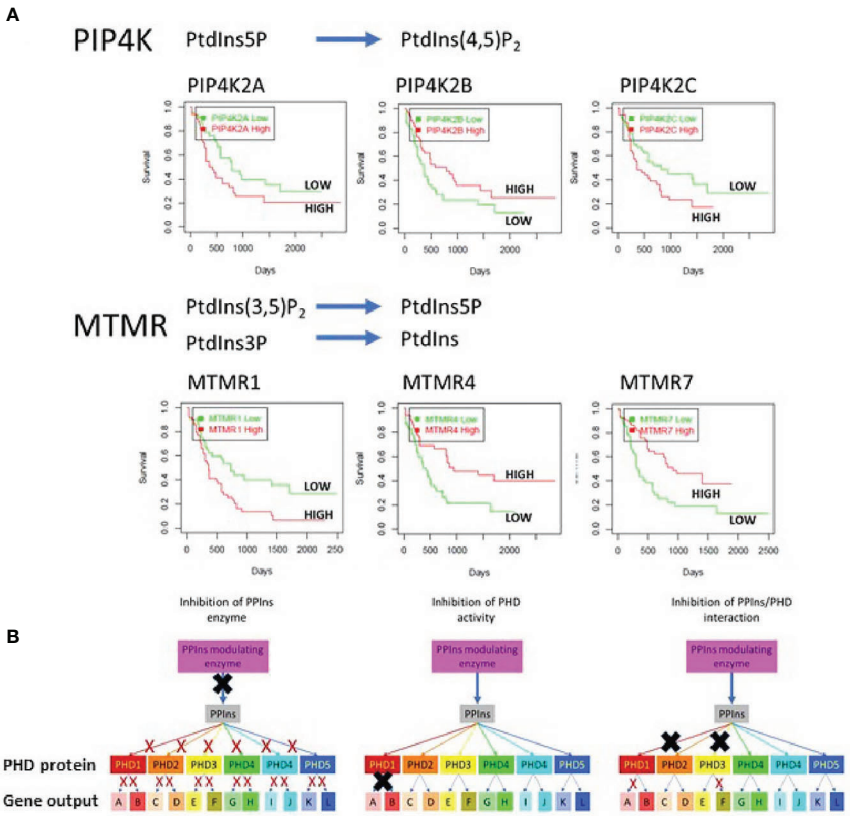

FIGURE 4 | **(A)** The TGCA AML patient data base was used to assess survival outcomes with respect to low or high expression of various PPIns modulating enzymes to demonstrate complex signalling and outcomes within enzymes from the same family. As examples we shown that low expression of PIP4K2A, PIP4K2C or MTMR1 is associated with increased survival. Surprisingly however the expression of PIP4K2B, a family member with PIP4K2A and 2C shows survival outcomes that are opposite with respect to its expression. Similar data are observed MTMR4 and 7 where high expression is associated with increased survival in AML. These data suggest that the complexity in PPIns signalling could be exploited to generate modulatory pathways that could be beneficial for AML treatment. **(B)** A schematic diagram illustrating that PPIns inhibitors could act at 3 points within a PPIns signalling unit: (i) inhibition of the PPIns modulating enzyme will impair all downstream targets and associated gene expression programs (A:L) (ii) inhibition of the activity of a PHD containing protein will likely impair all the genes that the protein regulates (gene A and gene B) or (iii) allosteric inhibition of in the interaction of PPIns with a specific interacting domain will impair selected output which might be tuned to specific pathways and therefore engender less toxic outcomes. In this example inhibition of two PHD domain interactions is shown which impair expression of gene A and gene F.

pathway holds a special place in PPIns metabolism as flux through the pathway is generally very low but is strongly stimulated by oncogenic pathways. While this imparts a therapeutic potential, the involvement of the PI3K pathway in normal cellular processes ultimately leads to potential for on target toxicities. For example the PI3K pathway is intimately involved in insulin signalling and on target issues with glucose homeostasis are often seen in therapeutic treatments (89). As signalling through one PPIns can impact on multiple downstream events, there are several points within a particular PPIns pathway that could be the focus for intervention, all of which likely would yield different outcomes (**Figure 4B**). We illustrate this using a specific nuclear PPIns modulator which changes the levels of its PPIns product to impact on epigenetic signalling proteins PHD1-PHD5. Each of these then can impact on the transcription of at least two genes. The system behaves similarly to what was observed with nuclear PIP4K2B, PtdIns5P and the downstream PHD finger containing proteins ING2 (57, 58, 86) and TAF3 (59). We envisage three points for therapeutic intervention. Inhibition of the PPIns modulating enzyme attenuates signalling through all the downstream PHD finger proteins effecting transcriptional output of all genes A-L. Inhibition instead at the level of the PHD finger protein attenuates only output of gene A and B. Finally, targeting the interaction site specifically between a PHD finger and the PPIns has the potential to attenuate selective transcriptional output. In this instance two different PHD finger/PPIns interactions are targeted to selectively block the transcriptional output of only gene A and F. Similar selective outputs were observed using mutants of ING2 and TAF3 that are unable to interact with PPIns but still interact with H3k4me3 (59, 86).

Exploiting PPIns signalling complexity, however, requires deep level understanding of how these interventions impact output, the gathering of which has become much more feasible with the advent of CRISPR gene editing tools. Combining this knowledge with bioinformatic network analysis using tools such as Mogrify and knowledge of patient specific transcriptional landscapes might allow the complexity within PPIns signalling pathways to be

exploited by intervening at various levels to convert an AML cell to a differentiated and non-proliferative cell state.

AUTHOR CONTRIBUTIONS

RF and ND contributed to the conception of the work. All authors contributed to the article and approved the submitted version.

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