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Original Article

The role of SLC22A4 in Acute Myeloid Leukaemia

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Abstract. ***Background:*** **This study aimed to explore the role of SLC22A4 (encoding the organic cation transporter OCTN1) in acute myeloid leukaemia (AML) prognosis and therapy. Methods: RNA-sequencing data from 151 TCGA-AML samples and six Gene Expression Omnibus datasets (62 normal bone marrow and 520 AML samples) were analysed. Weighted gene co-expression network analysis identified immune-related gene modules. Differential expression analysis, survival analysis (Kaplan–Meier, Cox regression), methylation profiling, immune infiltration (xCell, EPIC), and drug sensitivity correlations were performed. Statistical methods included Wilcoxon rank-sum tests, ROC curves, and LASSO regression.**
Results**: SLC22A4 expression was significantly decreased in AML compared with normal samples. The high-expression group was associated with a better prognosis than the low-expression group. Gene set enrichment analysis revealed enrichment in metabolic transport, immune, and tumour-related pathways. SLC22A4 expression was negatively correlated with immune cells, and methylation of SLC22A4 was significantly negatively correlated with expression. Moreover, it was predicted that 5 miRNAs could regulate SLC22A4 expression. Drug-sensitivity analysis showed positive correlations with cyclobenzaprine, hydrochloride, SGX-523, and simvastatin, and negative correlations with fluorouracil, abexinostat, EMD-534085, hypothemycin, tamoxifen, and sunitinib.**
Conclusion**: SLC22A4 may be useful as a potent molecular-targeted agent in AML.**

Keywords: Bioinformatics; Acute myeloid leukaemia; Prognostic biomarkers; Therapy; Tumour microenvironment.

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Introduction. Acute myeloid leukaemia (AML) is a genetically heterogeneous haematologic malignancy characterised by the rapid growth of abnormal myeloid cells in the bone marrow.[1,2] Despite improvements in remission rates with intensive chemotherapy,[3] AML remains associated with poor outcomes due to high relapse rates and treatment-related mortality.[4,5] The 5-year overall survival rate of patients with AML from 2010 to 2017 was 28%.[6-8] The molecular mechanisms of AML are currently unclear. Studies have shown that overexpression or repression of genes may promote or inhibit the occurrence and progression of AML. Sperm-

associated antigen 1 (SPAG1) is highly expressed in patients with AML, and its high expression is associated with poor prognosis. It promotes the proliferation and survival of AML cells and activates the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling pathway. Inhibition of SPAG1 can affect the sensitivity of AML cells to venetoclax and may serve as a therapeutic target in this setting.[9] Low expression of carnitine transporter CT2 in AML cells impairs the transport of carnitine, reduces basic oxygen consumption, and subsequently inhibits the growth and viability of AML cells.[10] Overexpression of homeobox A10 (HOXA10) and interferon-stimulated exonuclease gene 20 are adverse prognostic factors for AML.[11,12] Overexpression of the MDS1 and EVI1 complex locus gene inhibits the myeloid differentiation of haematopoietic progenitor cells, leading to increased self-renewal and survival of transformed haematopoietic progenitor cells, which is an adverse prognostic factor for AML.[13]

Despite advancements in chemotherapy and targeted therapies, such as FLT3 and IDH inhibitors, long-term survival rates of patients with AML remain low, particularly in elderly patients, with 5-year survival rates below 30%.[14,15] Recent breakthroughs in immunotherapy, notably PD-1/PD-L1 inhibitors, have revolutionised treatment paradigms across cancers, including AML. For instance, clinical trials evaluating PD-1/PD-L1 blockade in relapsed/refractory AML demonstrate enhanced T-cell-mediated anti-leukemic activity and improved survival in subsets of patients.[16] These advances underscore the critical role of immune dysregulation in AML progression and highlight the need to identify biomarkers that bridge molecular aberrations with immune microenvironment dynamics.

Solute carrier family 22 member 4 (SLC22A4) (a member of the SLC22 family, encoding OCTN1, an organic cation transporter) emerges as a novel candidate in this context. A research study showed that decreased expression of the uptake transporter OCTN1 (SLC22A4) leads to poor prognosis in paediatric patients with AML.[17] SLC22A4 increased the transport of imatinib, thereby enhancing the sensitivity of chronic myeloid leukaemia cells to this agent.[18] Unlike conventional AML biomarkers that mainly reflect genetic instability, such as FLT3-ITD or NPM1 mutations, SLC22A4 uniquely integrates metabolic transport, immune regulation, and chemoresistance. Even though SPAG1 and HOXA10 are established prognostic markers associated with proliferation and differentiation arrest, the role of SLC22A4 in regulating nucleoside analogue transport (e.g., cytarabine) offers unique mechanistic advantages. SLC22A4-mediated drug efflux directly impacts intracellular cytarabine concentrations, the cornerstone of AML therapy.[17] Mechanistically, SLC22A4 dysfunction may drive AML pathogenesis through multiple axes: (1) drug resistance, whereby reduced SLC22A4 expression impairs cytarabine uptake and promotes leukaemia cell survival; and (2) epigenetic regulation, in which hypermethylation of cytosine-phosphate-guanine (CpG) sites (e.g., cg05231888) inhibits SLC22A4, linking epigenetic silencing to adverse outcomes.[17]

In this study, we analysed differentially expressed genes (DEGs) between AML and normal samples and screened genes associated with immune infiltration and survival prognosis. This study aimed to investigate the function of SLC22A4 in AML and its value as a new tumour marker for predicting the prognosis of AML.

Materials and Methods.

Data Acquisition and Pre-processing. The study included data from 151 de novo AML samples from The Cancer Genome Atlas (TCGA) database (AML NEJM 2013; https://portal.gdc.cancer.gov/), with survival data available for 142 of those (GSE68833). This chip does not overlap with any of the other GEO datasets used in this study. The AML RNA-sequencing (RNA-seq) dataset was downloaded from the UCSC Xena database (https://xenabrowser.net/datapages/).

RNA-seq data from 151 TCGA-AML samples and six Gene Expression Omnibus (GEO) datasets (GSE9476, GSE12662, GSE30029, GSE34577, GSE12417, GSE37642) were obtained as raw counts.[16] GSE12417 was used to evaluate the relationship between SLC22A4 expression and prognosis. GSE12417 includes RNA-seq expression profiles and survival data of 163 patients with cytogenetically normal AML. Two peripheral blood mononuclear cell samples and one myeloproliferative sample were removed from the analysis. GSE37642 includes 422 patients with AML; 417 samples with available information on survival were selected for analysis. GSE9476 contains 26 AML and 18 normal tissue samples derived from the bone marrow. GSE34577 includes 48 AML and 18 normal tissue samples derived from the bone marrow.[17,19-28] The abundance of immune cell subsets in patients with AML was analysed using the GSE12417 and GSE37642 datasets. Raw counts were normalised using DESeq2's median-of-ratios method to correct for library size differences, followed by variance-stabilising transformation to minimise heteroscedasticity. Batch effects arising from platform heterogeneity were adjusted using the ComBat algorithm from the sva R package (v3.46.0), which harmonises expression distributions across datasets while preserving biological variation.

CellMiner Database. The CellMiner database (https://discover.nci.nih.gov/cellminer/) provides resources for the exploration of pharmacogenomics using cancerous cell lines.[29] The effect of SLC22A4

expression on drug sensitivity was analysed using this database.

Weighted Gene Co-expression Network Analysis for the Identification of Key Genes Co-associated with Immune-characteristics. Co-expression networks and hub genes associated with CD8_T_Cells_Danaher_et_al_PCA, CD8_T_Cells_EPIC, Immune Score_Xcell, and Microenvironment Score_Xcell were identified using the weighted gene co-expression network analysis (WGCNA) package (version 1.72-1) in R.[30] To determine the optimal soft thresholding power (β), we evaluated scale-free topology fit indices ($R^2 \geq 0.90$) and mean connectivity across a range of β values (1–20).[31] A β of 7 was selected for both GSE12417 and GSE37642 cohorts, as it achieved the highest scale-free topology fit ($R^2 = 0.93$ and $R^2 = 0.91$, respectively) while maintaining moderate mean connectivity (<200 edges per gene), balancing network specificity and biological interpretability. Dynamic tree cutting (minimum module size = 30, deepSplit = 2) identified co-expression modules, which were correlated with immune scores (xCell, EPIC) (https://xcell.ucsf.edu/) to prioritise modules for downstream.

Thereafter, a scale-free network was constructed based on the gene expression profile using the pickSoftThreshold function (correlation coefficient $R^2 > 0.90$). Modules were identified using the Dynamic Tree Cut algorithm. R software (version 4.2.2, https://www.R-project.org/) was used to draw a module–immune feature correlation heat map. Modules and immune features with the highest correlation and smallest *p*-value were selected as candidate genes for subsequent analysis.

Immune Infiltration Analysis. The ggpubr (version 0.6.0) and ggsci (version 3.0.0) packages in R were used for visualisation.[32,33] Immune cell infiltration levels were quantified using xCell (v1.1.0) and EPIC (v1.1.5) algorithms via the IOBR R package (v0.99.9).[34] Single-sample gene set enrichment analysis (ssGSEA) was performed with the GSVA package (v1.46.0), using hallmark gene sets from MSigDB (v7.5.1).[35-37] Pathway enrichment for SLC22A4-associated genes was conducted with clusterProfiler (v4.0.0) and ReactomePA (v1.38.0), applying the Benjamini–Hochberg correction (FDR < 0.05).[35]

Gene Set Enrichment Analysis. Gene set enrichment analysis examines the molecular signalling pathways of SLC22A4 at the gene set level by analysing the distribution trend of the SLC22A4 gene using phenotype-related microarray data.[38] According to the median levels of SLC22A4, the gene expression profiles in TCGA samples were divided into two groups (i.e., SLC22A4 low- and high-expression). Subsequently, DEGs were analysed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/) pathway and Reactome pathway analyses. All detected genes were analysed using R packages; the ReactomePA, org.. Hs.eg.db, ClusterProfiler, and enrichplot packages were utilised to display the results.[39] Pathways with meaningful enrichments were identified based on *p*-values below 0.05 and the normalised enrichment score ranking.

Methylation Analysis. In this study, the level of SLC22A4 methylation was determined using the Shiny Methylation Analysis Resource Tool (SMART app; http://www.bioinfo-zs.com/smartapp), an interactive web application for DNA methylation analysis based on the TCGA database.[40]

Statistical Analysis. All statistical analyses were performed using R (version 4.2.2, https://www.R-project.org/). Differential expression analysis of RNA-seq data between AML and normal samples was conducted using the Wilcoxon rank-sum test. To mitigate the risk of false positives due to multiple comparisons, the Benjamini–Hochberg false discovery rate (FDR) correction was applied across all differential expression analyses, with significance defined as FDR-adjusted $p < 0.05$. Survival outcomes were analysed via Kaplan–Meier curves, and differences between groups were assessed using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using univariate and multivariate Cox proportional hazards regression models. Variables with $p < 0.2$ in univariate analysis were included in multivariate models to avoid overfitting.

To account for multiplicity in immune cell subtype analyses, the Bonferroni correction was applied, with significance thresholds adjusted to $p < 0.0025$ (0.05/20). Methylation-expression correlations were evaluated using linear regression, and FDR correction was similarly applied to CpG site analyses. A receiver operating characteristic (ROC) curve was drawn using the pROC package (https://www.R-project.org/) to evaluate the diagnostic performance of SLC22A4 expression. The relationship between clinicopathological features and SLC22A4 expression was analysed using the Student's *t*-test (satisfies normal + homogeneity of variance), Welch's *t*-test (satisfies normal + does not satisfy homogeneity of variance), and the Wilcoxon rank-sum test (does not satisfy normal, nonparametric test). Survival curves were calculated using the Kaplan–Meier method. Univariate and multivariate analyses of the risk of mortality were performed using the Cox proportional hazards regression model. All tests were two-tailed, and statistical significance was defined as $p < 0.05$.

Results.

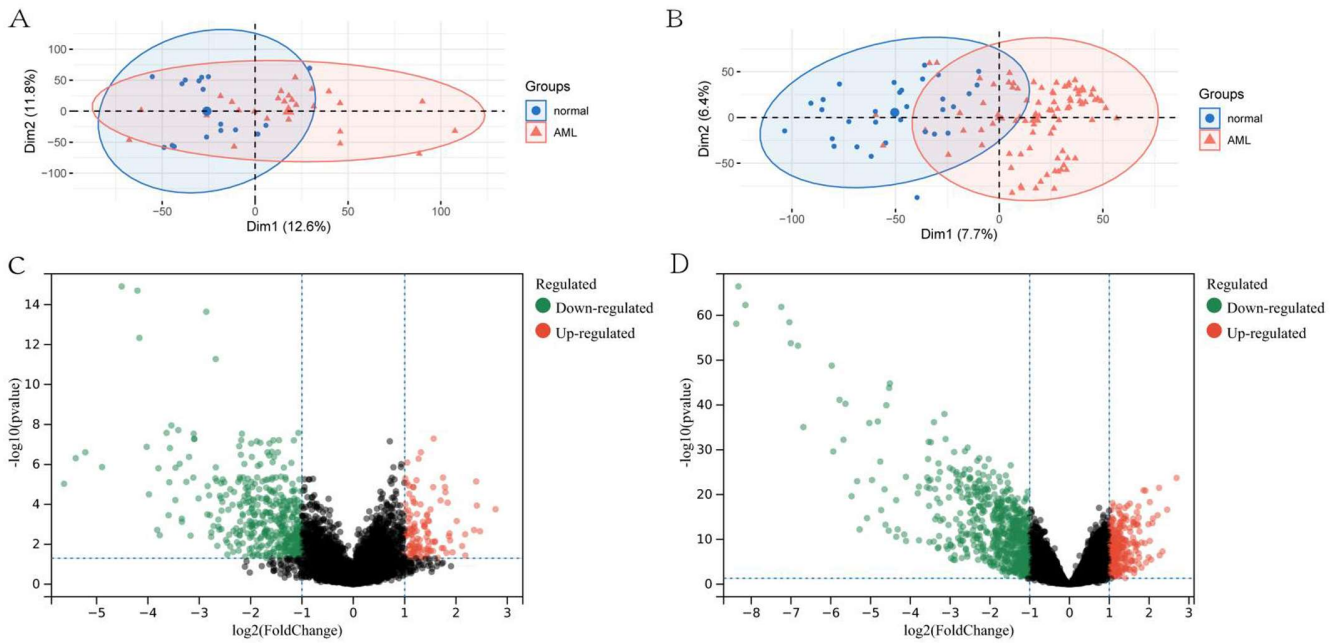

Figure 1. Differential analysis using GEO data for the identification of DEGs. (A) Confidence ellipses of the PCA grouping results for GSE9476 dataset. (B) Confidence ellipses of the PCA grouping results for GSE30029 dataset. (C) Volcano plot of the differential analysis of the GSE9476 dataset. (D) Volcano plot of the differential analysis of the GSE30029 dataset. DEGs: differentially expressed genes; GEO: Gene Expression Omnibus; PCA: principal component analysis; AML: Acute Myeloid Leukemia.

Analysis of Abnormally Expressed Genes in AML. Differential analysis of the GSE9476 and GSE30029 datasets was performed to identify DEGs between the AML and normal groups. Group differences were displayed using principal component analysis (PCA) (**Figures 1A, B**). Differential expression analysis of GSE9476 and GSE30029 datasets identified 523 (385 down-regulated and 138 upregulated) and 925 (713 down-regulated and 212 upregulated) DEGs in AML compared with normal samples, respectively (|log2 fold change| > 1, FDR < 0.05; **Figures 1C, D**). Volcano plots explicitly demarcated significance thresholds (FDR < 0.05, dashed horizontal line) and fold-change cutoffs (|log2FC| > 1, vertical lines), ensuring transparent criteria for DEG selection. This evidence indicates that these genes were abnormally expressed across the normal and AML samples.

Analysis of Prognostic Genes Associated with Immune Markers. We sought to identify immune markers related to the survival prognosis of patients with AML. Therefore, PCA, EPIC, and xCell analyses were performed in the GSE12417 and GSE37642 datasets using the IOBR package in R, and survival curves were used for representation. The results showed that four immune markers (CD8_T_cells_Danaher_et_al_PCA, CD8_Tcells_EPIC, immune score xCell, and MicroenvironmentScore_xCell) were related to the survival prognosis. Higher expression levels of immune markers were linked to a better survival prognosis (p < 0.05; hazard ratio < 1; **Figures 2A, B**). This finding suggests that these four immune markers play an inhibitory role in the development and progression of AML.

Weighted Gene Co-expression Network Analysis for the Identification of Gene Modules Related to Immune Markers. The GSE12417 and GSE37642 datasets were used for WGCNA analysis. For the construction of a scale-free network, a soft thresholding power (β) of 7 was estimated under the condition of Cex1 = 0.9 for both GSE12417 and GSE37642 (**Figures 3A, B**). In GSE12417, hierarchical clustering identified 12 co-expression modules clustered into one class (**Figures 3C, E**). Of the 12, the brown module showed the strongest positive correlation with the xCell immune score (Cor = 0.89, p < 1e−200; **Figure 3G**). In GSE37642, 10 co-expression modules clustered into one class were identified (**Figures 3D, F**). Of the 10, the brown module again showed the strongest positive correlation with the xCell immune score (Cor = 0.76, p < 1e−200; **Figure 3H**). Therefore, the brown modules in these two datasets were further analysed. In the GSE12417 and GSE37642 datasets, the brown module contained 981 and 1,469 genes, respectively. The intersection of the brown module genes in GSE12417 and GSE37642 and the DEGs identified from the GSE9476 and GSE30029 yielded 13 genes: interferon regulatory factor 8 (IRF8); tetraspanin 13 (TSPAN13); carboxypeptidase vitellogenic-like (CPVL); mitochondrial calcium uniporter dominant negative subunit beta; tissue inhibitor of metalloproteinase-1; glutaminyl-peptide cyclotransferase (QPCT); terminal nucleotidyltransferase 5C (TENT5C); SLC22A4; biliverdin reductase A (BLVRA); tumour necrosis factor

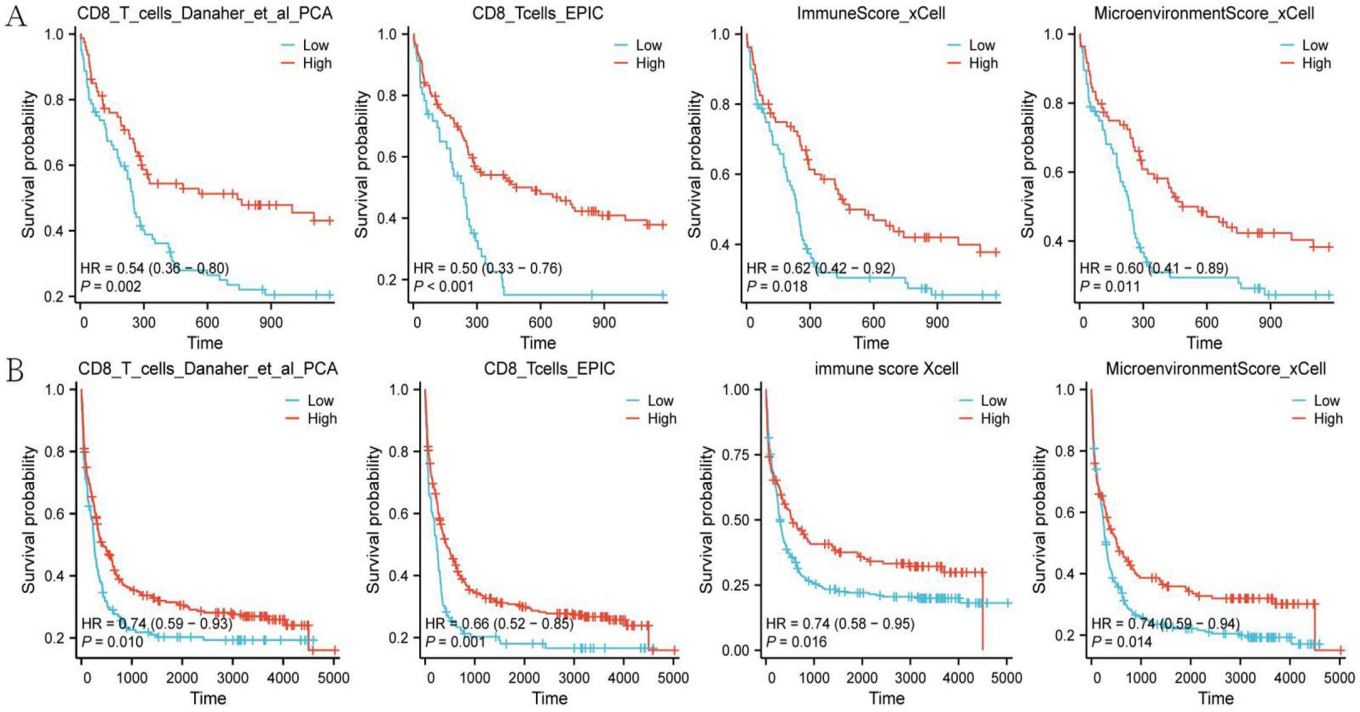

Figure 2. Analysis of immune markers related to the prognosis of patients with AML. (**A**) GSE12417 dataset. (**B**) GSE37642 dataset. AML: acute myeloid leukemia.

Table 1. Baseline Clinical and Demographic Characteristics of All Enrolled Patients Stratified by SLC22A4 expression.

Characteristic	Low expression of SLC22A4	High expression of SLC22A4	*p*-value
Patient, n	75	75	
Age, n (%)			0.869
≤60 years	44 (58.7%)	43 (57.3%)	
>60 years	31 (41.3%)	32 (42.7%)	
Cytogenetic risk, n (%)			0.003
Favorable	8 (10.7%)	23 (30.7%)	
Intermediate/normal	44 (58.7%)	39 (52%)	
Poor	23 (30.7%)	13 (17.3%)	
WBC count (×10^9/L), n (%)			0.286
≤20	35 (46.7%)	42 (56%)	
>20	40 (53.3%)	33 (44%)	
BM blasts (%), n (%)			0.012
≤20	22 (29.3%)	37 (49.3%)	
>20	53 (70.7%)	38 (50.7%)	
PB blasts (%), n (%)			0.624
≤70	34 (45.3%)	37 (49.3%)	
>70	41 (54.7%)	38 (50.7%)	
FAB classifications, n (%)			0.017
M0	12 (16%)	5 (6.7%)	
M1	19 (25.3%)	17 (22.7%)	
M2	25 (33.3%)	14 (18.7%)	
M3	3 (4%)	11 (14.7%)	
M4	11 (14.7%)	18 (24%)	
M5	5 (6.7%)	10 (13.4%)	
Sex, n (%)			0.139
Female	38 (50.7%)	29 (38.7%)	
Male	37 (49.3%)	46 (61.3%)	
FLT3 mutation, n (%)			0.314
Negative	48 (64%)	55 (73.3%)	
Positive	27 (36%)	20 (26.7%)	
RAS mutation, n (%)			0.267
Negative	68 (90.7%)	73 (97.3%)	
Positive	7 (9.3%)	2 (2.7%)	

Abbreviations: BM, bone marrow; FAB, French–American–British; FLT3, fms related receptor tyrosine kinase 3; PB, peripheral blood; SLC22A4, solute carrier family 22 member 4; WBC, white blood cell.

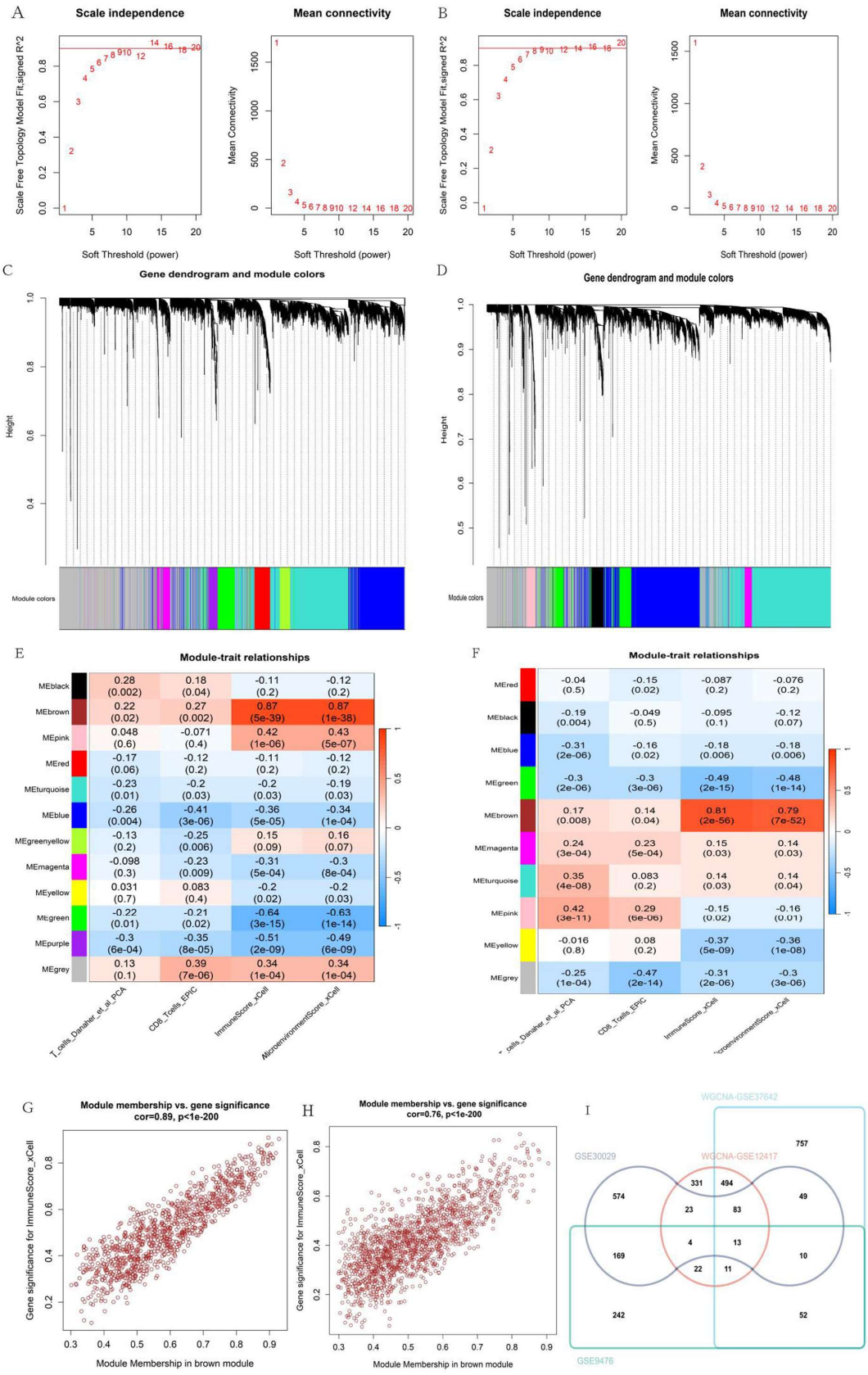

Figure 3. WGCNA for the identification of genes related to immune markers. (**A, B**) According to the scale-free topology criterion in GSE12417 (**A**) and GSE37642 (**B**), a soft threshold (β) of 7 was selected. (**C, D**) In GSE12417 (**C**) and GSE37642 (**D**), the clustering trees of genes with similar expression patterns were clustered into co-expression modules. (E, F) The module-immune marker relationship shows the correlation between the characteristic genes of each gene module in GSE12417 (**E**) and GSE37642 (**F**) and the phenotype. (**G, H**) Scatter plots of the number of module members and importance for each gene in the brown module of GSE12417 (**G**) and the brown module of GSE37642 (**H**). The x-axis represents the correlation between the gene and the co-expression module, and the y-axis represents the correlation between the gene and immune score xCell. (**I**) Venn diagram of the genes in the brown modules of GSE12417 and GSE37642 and the DEGs identified from GSE9476 and GSE30029. DEGs: differentially expressed genes; WGCNA: weighted gene co-expression network analysis.

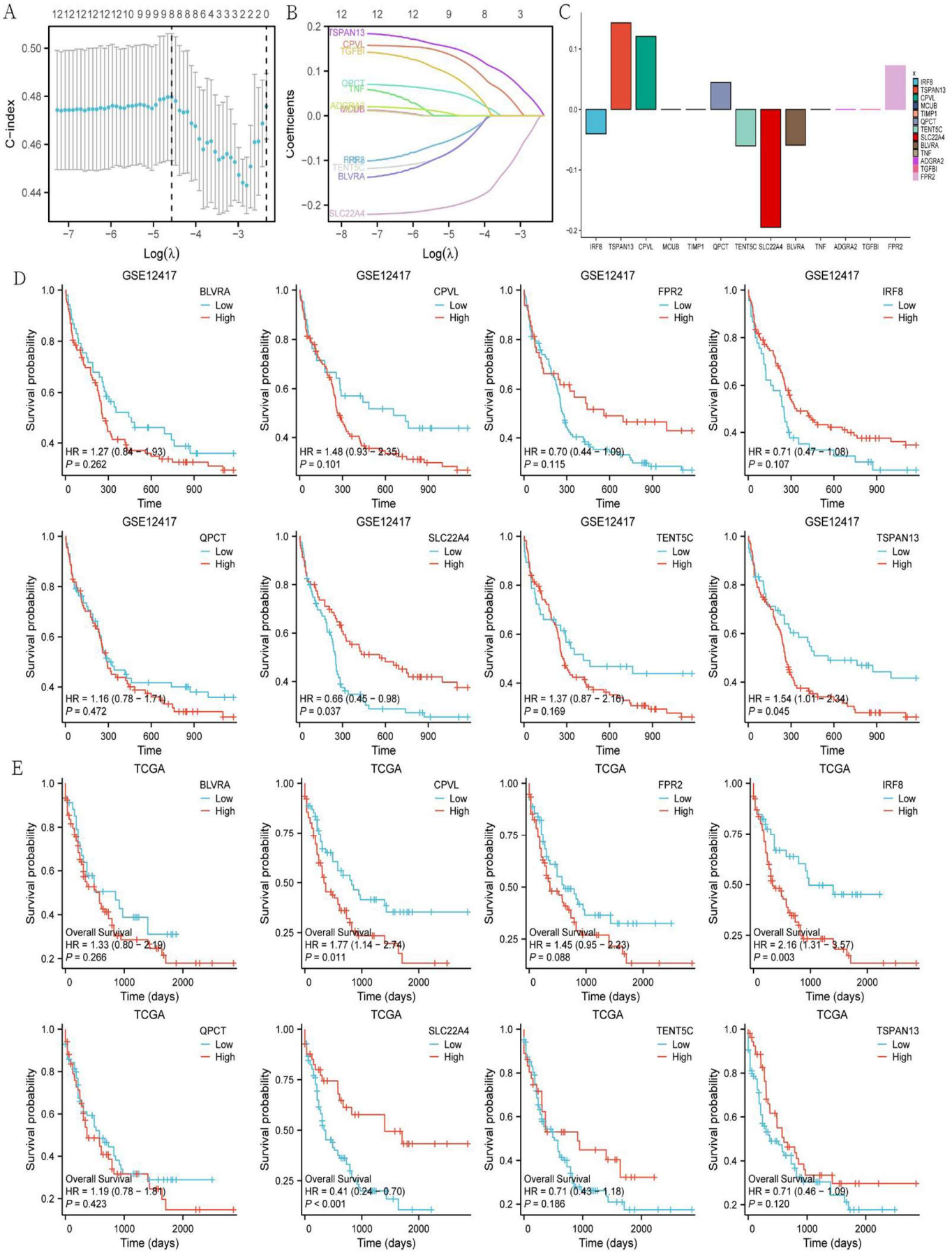

Figure 4. Further screening of genes related to AML prognosis. (**A**) LASSO coefficient screening plot. (**B**) LASSO variable trajectory plot. (**C**) LASSO variable coefficient bar plot. (**D**) Survival curves of significant genes in GSE12417. (**E**) Survival curves of significant genes in TCGA. AML: acute myeloid leukemia; LASSO: least absolute shrinkage and selection operator; TCGA: The Cancer Genome Atlas.

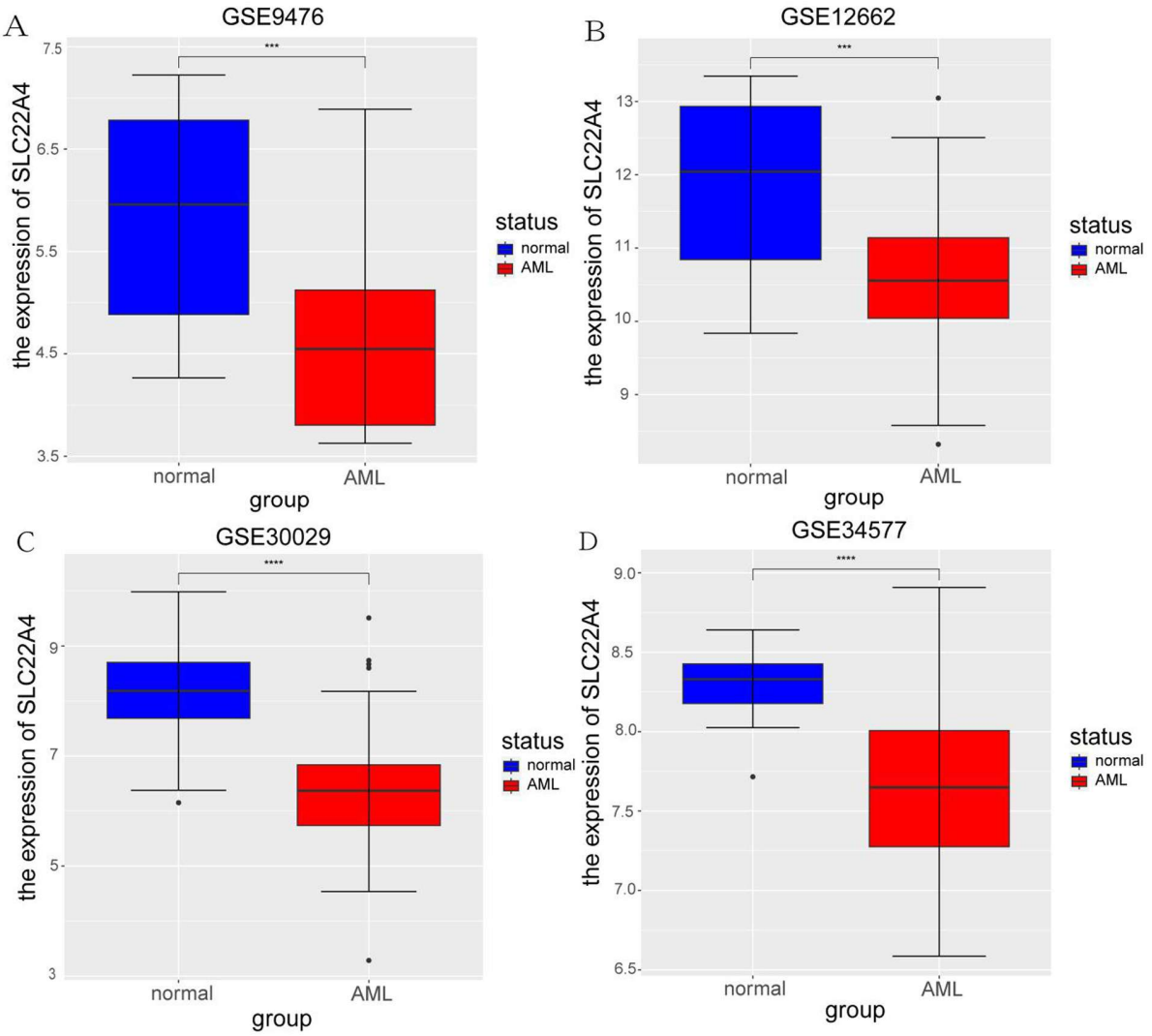

Figure 5. Expression of the SLC22A4 gene in tumor and normal samples. Grouped bar plot of SLC22A4 gene expression in GSE9476 (**A**), GSE12662 (**B**), GSE30029 (**C**), and GSE34577 (**D**). SLC22A4: solute carrier family 22 member 4.

(TNF); adhesion G protein-coupled receptor A2; transforming growth factor beta induced; and formyl peptide receptor 2 (FPR2) (**Figure 3I**). Of the 13 candidate genes derived from the WGCNA and DEG intersections (**Figure 3I**), survival analysis in the TCGA cohort revealed SLC22A4 as the most robust prognostic marker, with high expression correlating with prolonged overall survival (HR = 0.41, 95% CI = 0.24–0.70, $p < 0.001$; **Figure 3E**). Notably, IRF8 (HR = 2.16, 95% CI = 1.31–3.57, $p = 0.003$) and CPVL (HR = 1.77, 95% CI = 1.14–2.74, $p = 0.011$) also demonstrated significant associations with survival, albeit with opposing risk profiles. In contrast, BLVRA, FPR2, TSPAN13, TENT5C, and QPCT lacked prognostic relevance ($p > 0.05$), underscoring the specificity of SLC22A4 in AML prognosis.

Analysis of Genes Related to AML Prognosis. The GSE12417 dataset was used for this analysis. Least absolute shrinkage and selection operator (LASSO) regression analysis was performed on the 13 obtained genes. Eight genes with significant LASSO coefficients were detected: IRF8, TSPAN13, CPVL, QPCT, TENT5C, SLC22A4, BLVRA, and FPR2 (**Figures 4A–C**). The significance of these eight genes in relation to survival was further verified in the GSE12417 and TCGA datasets. The results were displayed using survival curves (**Figures 4D, E**). SLC22A4 expression was significantly altered ($p < 0.05$) in both datasets. A higher expression of SLC22A4 indicated a better survival prognosis for patients with AML (HR < 1).

Levels of SLC22A4 Expression in AML and Normal Samples. Four datasets containing normal and AML samples (GSE9476, GSE12662, GSE30029, and GSE34577) were used to analyse SLC22A4 expression in AML and normal samples. The results showed that, compared with normal samples, SLC22A4 expression

was significantly decreased in AML samples in the four datasets (**Figures 5A–D**). This evidence suggests that SLC22A4 acts as a tumour suppressor gene in AML.

Effect of SLC22A4 Gene Expression on Prognosis. Using TCGA and Genotype-Tissue Expression datasets, ROC analysis was performed to evaluate the prognostic ability of SLC22A4 in AML. As shown in **Figure 6A**, the area under the ROC curve (AUC) was greater than 0.8, suggesting that SLC22A4 can be used to distinguish AML from normal samples. The time-dependent ROC curve showed that the AUC for predicting 5-year survival was greater than 0.7 (**Figure 6B**), suggesting that SLC22A4 is effective in predicting 5-year prognosis.

Patients were divided into high- and low-SLC22A4 expression groups based on median levels to analyse the association of SLC22A4 expression with clinical characteristics of AML. Clinical parameters of the two groups are shown in **Table 1**. In the TCGA dataset, the expression of SLC22A4 was statistically different across bone marrow blast percentage, RAS mutation, and cytogenetic risk groups ($p < 0.05$) (**Figure 6C**). Variables with $p < 0.2$ in univariate analysis (SLC22A4 expression, age, cytogenetic risk, and French–American–British classification) were included in the multivariate analysis. The results showed that SLC22A4 expression might be an independent prognostic factor for patient survival in AML ($p = 0.032$; **Figures 6D, E**). A nomogram also confirmed this finding (**Figure 6F**). The ROC curve was used to verify the accuracy of the established model, achieving AUC values below 0.7 for 1- and 3-year survival rates and below 0.8 for 5-year survival (**Figure 6G**), which indicates high accuracy in predicting patient survival.

Biological Insights into Aberrant SLC22A4 Expression in AML. To analyse the biological functions of the SLC22A4 gene, we performed differential analysis on TCGA-AML samples stratified by SLC22A4 expression. We then performed pathway enrichment analysis for SLC22A4 using GSEA. Under the KEGG signature, enrichment was observed in metabolic transport and immune- and tumour-related pathways, including the Fc epsilon receptor I signalling pathway, choline metabolism in cancer, herpes simplex virus 1 infection, *Salmonella* infection, and galactose metabolism (**Figures 7A, D**). Similarly, Reactome analysis identified enrichment in pathways such as *Leishmania* infection, reactive oxygen and nitrogen species production in phagocytes, SARS−CoV−2−host interactions, and Rab regulation of trafficking (**Figures 7B, C**).

Effect of SLC22A4 Expression on Immune Infiltration in AML. Single-sample GSEA analysis was performed to analyse the effect of SLC22A4 expression on immune infiltration in AML. SLC22A4 expression was positively correlated with dendritic cells (DCs), interstitial DCs, macrophages, neutrophils, effector memory T, and T helper 17 (Th17) cells. In contrast, negative correlations were observed with activated dendritic cells (aDCs), CD8 T cells, natural killer (NK) CD56bright cells, Th cells, and T follicular helper cells ($p < 0.05$; **Figure 8A**). xCell analysis showed that SLC22A4 expression was positively correlated with Basophils_xCell, CD4+_memory_T−cells, CD8+_T−cells, Macrophages, Monocytes, MSC_xCell, Pericytes, ImmuneScore, and MicroenvironmentScore, whereas negative correlations were observed with CD8+_Tcm, Chondrocytes_xCell, Endothelial_cells_xCell, HSC_xCell, ly_Endothelial_cells, Mast_cells_xCell, Megakaryocytes_xCell, Melanocytes_xCell, MEP, Neurons, Osteoblast, Preadipocytes_xCell, and StromaScore_xCell ($p < 0.05$; **Figure 8B**).

Transcriptional Regulatory Effects of SLC22A4 Promoter Methylation on Its Expression. Methylation sites on the SLC22A4 gene were searched to analyse the relationship between methylation and expression. The methylation sites located in the CpG island region of the SLC22A4 gene included cg26229274, cg13654253, cg07354253, cg08833630, cg11387240, cg07810106, cg16700673, cg12912258, cg27372468, cg17111895, cg13373085, cg05231888, cg04470557, and cg16087826 (**Figure 9A**). The correlation between the degree of methylation at these sites and SLC22A4 expression was analysed, finding that the methylation levels at four sites, namely cg13654253 ($R = -0.16$, $p = 0.034$), cg07354253 ($R = -0.18$, $p = 0.022$), cg05231888 ($R = -0.23$, $p = 0.003$), and cg25350825 ($R = -0.25$, $p = 9e-04$), were significantly negatively correlated with gene expression (**Figure 9B**).

Molecular Signature Correlated with SLC22A4 Expression. To explore the biological network induced by abnormal SLC22A4 expression in AML, the transcriptomes of TCGA-AML samples with lower and higher SLC22A4 expression were compared. The results revealed differences in miRNA expression (|log2 fold change|>0, false discovery rate <1, and $p < 0.05$; **Figure 10A**). Subsequently, functional enrichment analysis of the 61 differentially expressed miRNAs showed that the enriched pathways were mainly related to blood and immunity (e.g., 11, 12, and 15 miRNAs enriched in the haematopoiesis, immune response, and inflammation pathways, respectively) (**Figure 10B**). Next, four datasets (miRWalk, TargetScan, miRDB, and DIANA) were used to predict miRNAs that regulate SLC22A4 gene expression. The results suggest that five miRNAs (hsa-miR-1299, hsa-miR-1910-5p, hsa-miR-3659, hsa-miR-5093, and hsa-miR-6831-3p) in all four datasets regulate SLC22A4 gene expression (**Figure 10C**).

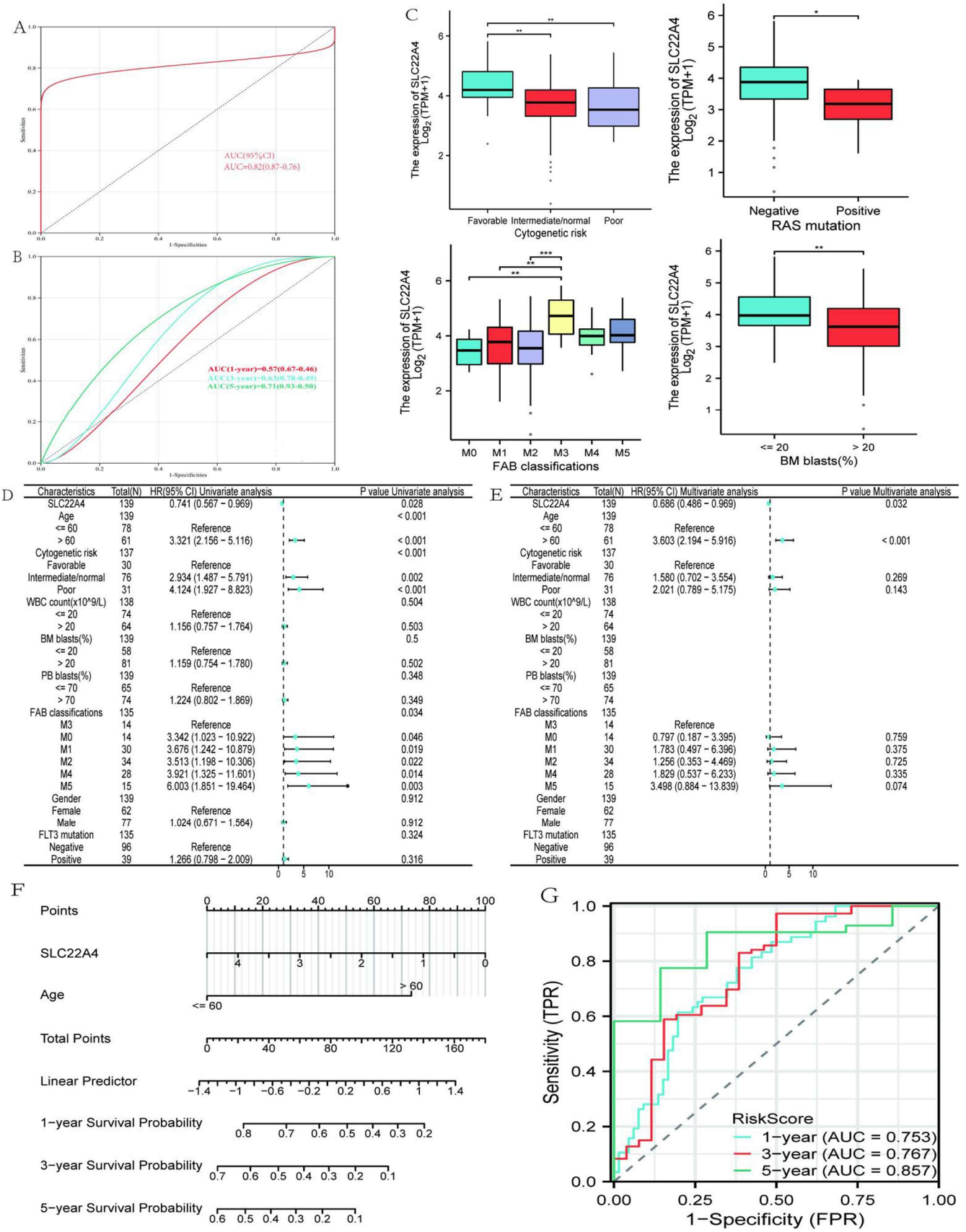

Figure 6. Clinical significance of SLC22A4 expression. (**A**) Discriminative capacity of SLC22A4 expression in patients with AML according to the ROC curve. (**B**) Predictive capacity of SLC22A4 expression for 1-, 3-, and 5-year survival according to the ROC curve. (**C**) Grouped bar plot of the effects of BM blasts (%), RAS mutation, and cytogenetic risk on SLC22A4 gene expression. (**D**) Forest plot of the univariate Cox proportional hazards regression analysis. (**E**) Forest plot of multivariate Cox proportional hazards regression analysis. (**F**) Nomogram model based on SLC22A4 expression. (**G**) ROC curve verifying the accuracy of the constructed model. AML: acute myeloid leukemia; BM: bone marrow; ROC: receiver operating characteristic; SLC22A4: solute carrier family 22 member 4; AUC: area under curve.

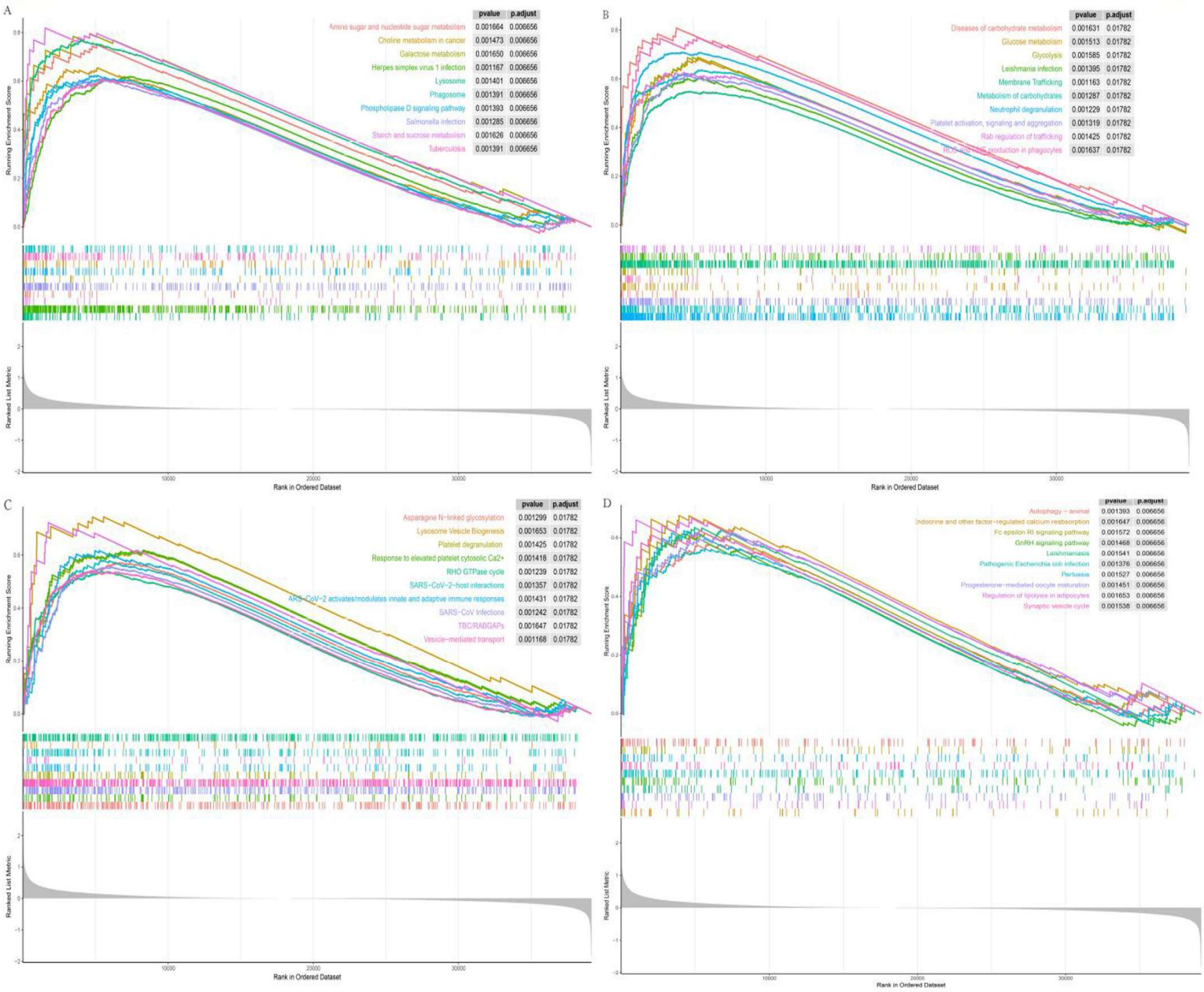

Figure 7. GSEA analysis. (**A, D**) GSEA analysis of SLC22A4 in KEGG. (**B, C**) GSEA analysis of SLC22A4 in Reactome. Different colors represent different functions or pathways. Rising and falling peaks indicate positive and negative regulation of SLC22A4. GSEA: gene set enrichment analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; SLC22A4: solute carrier family 22 member 4.

Effect of SLC22A4 on Drug Sensitivity. We used the CellMiner database to analyse the effect of SLC22A4 expression on drug sensitivity, finding that SLC22A4 expression was positively correlated with sensitivity to cyclobenzaprine hydrochloride, SGX-523, and simvastatin (**Figures 11A, H, I**). However, SLC22A4 expression was negatively correlated with sensitivity to fluorouracil, abexinostat, EMD-534085, hypothemycin, tamoxifen, and sunitinib (**Figures 11B–G**).

Discussion. We sought to identify key molecules relevant to AML by screening gene modules using WGCNA to detect DEGs related to immune markers. Ultimately, the analysis identified SLC22A4 as a target gene. The results of the survival analysis, baseline tables, and Cox proportional hazards regression analysis showed that SLC22A4 gene expression is significantly downregulated in AML and associated with various clinical features, such as bone marrow blast percentage, cytogenetic risk, and RAS mutation. Moreover, we found that decreased SLC22A4 expression is an independent prognostic factor for patients with AML, with lower expression levels correlating with poorer prognosis and shorter survival time. Buelow *et al.* reported that increased SLC22A4 expression was associated with improved overall survival and event-free survival in AML (OCTN1 is a high-affinity carrier of nucleoside analogues).[17] This evidence is consistent with the results of our study and highlights the important role of SLC22A4 in the development of AML.

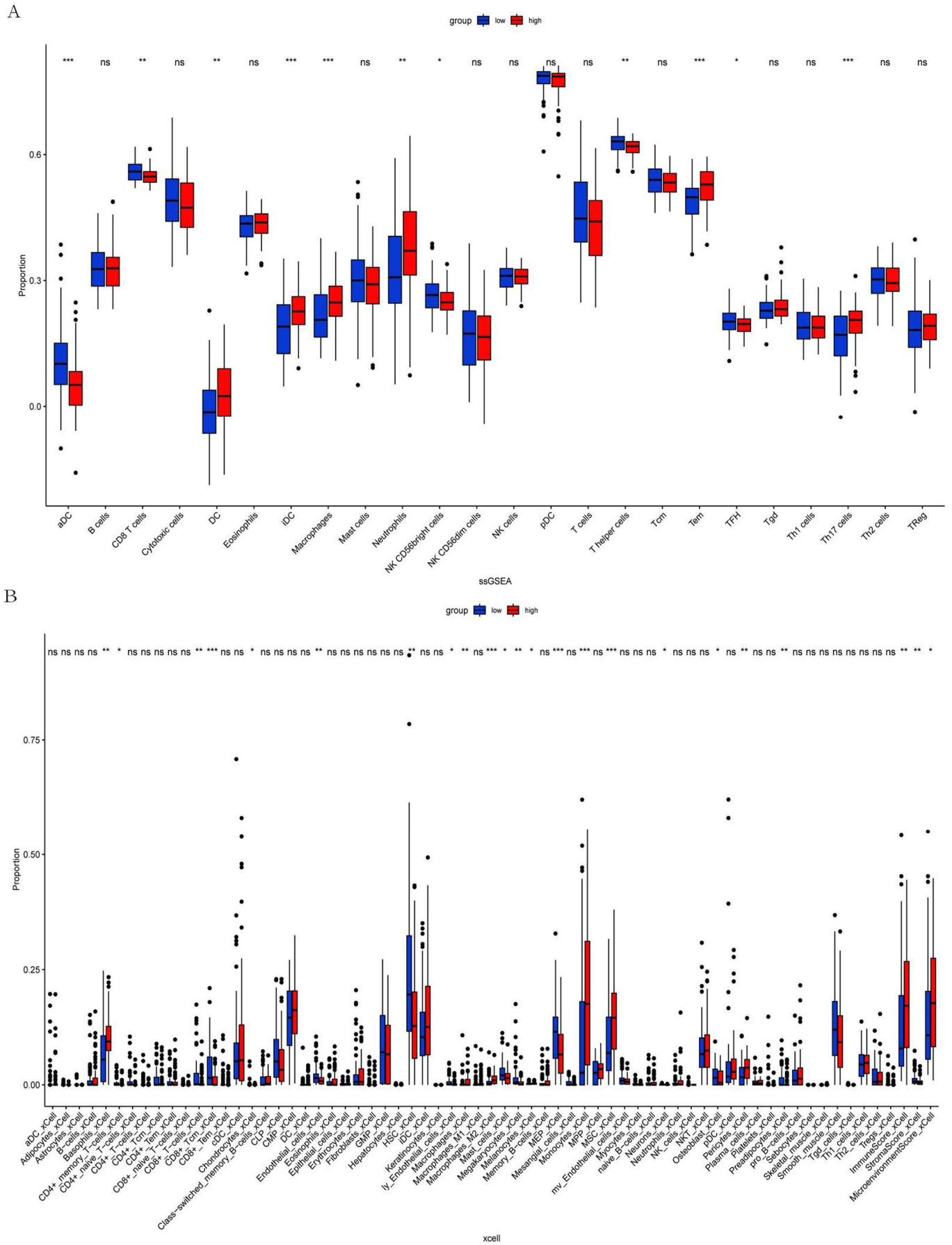

Figure 8. Role of SLC22A4 expression in immune infiltration. (**A**) Grouped bar plot of the relationship between SLC22A4 gene expression and immune cell infiltration under the ssGSEA algorithm. (**B**) Grouped bar plot of the relationship between SLC22A4 gene expression and immune cell infiltration under the xCell algorithm. SLC22A4: solute carrier family 22 member 4; ssGSEA: single-sample gene set enrichment analysis.

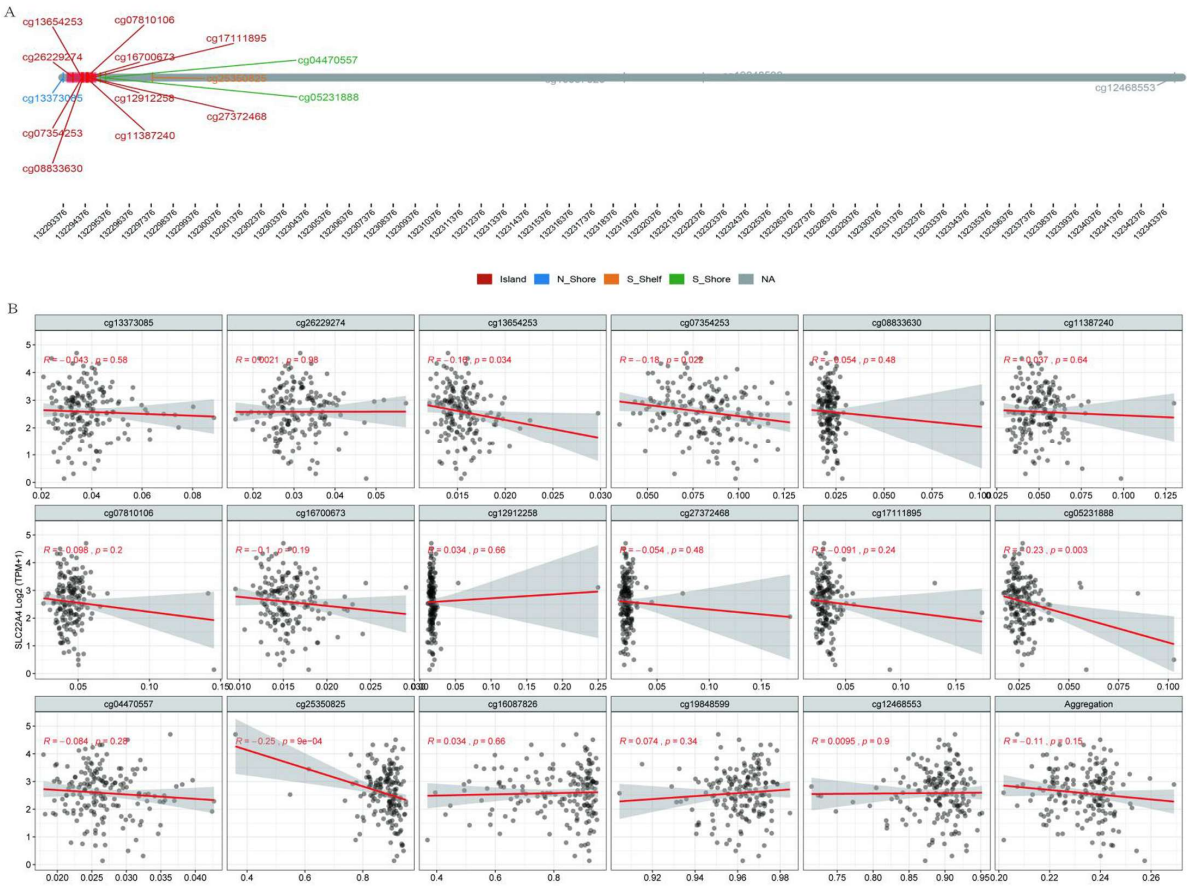

Figure 9. Relationship between SLC22A4 methylation and expression. (**A**) SLC22A4 gene methylation sites. (**B**) Effect of the methylation degree on SLC22A4 gene expression. SLC22A4: solute carrier family 22 member 4.

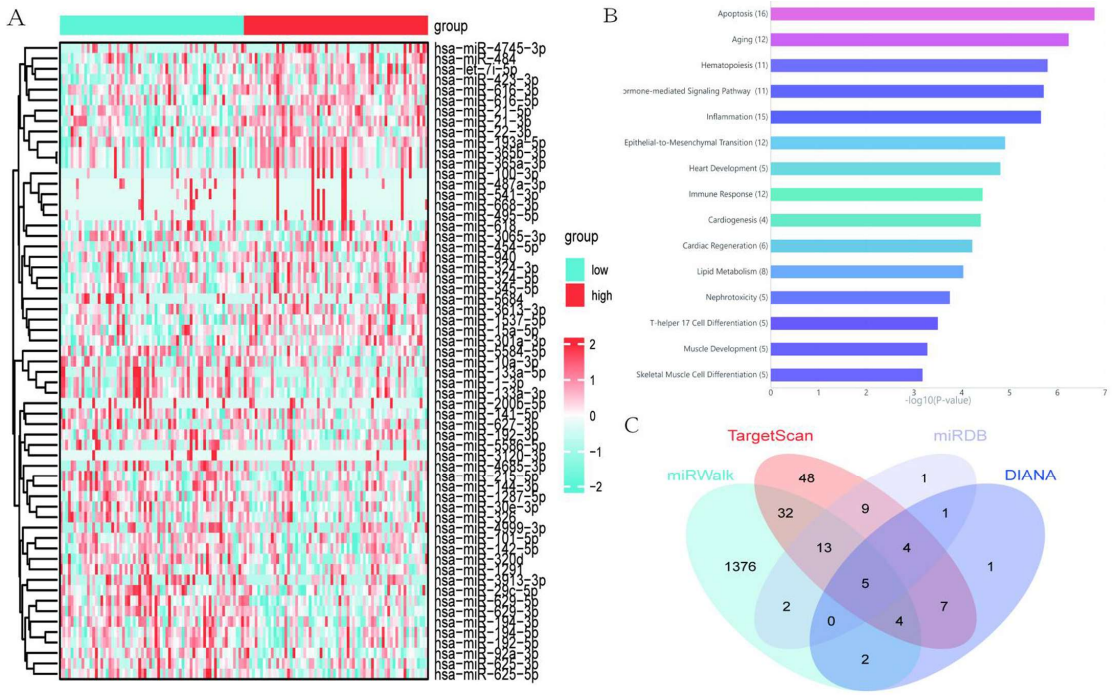

Figure 10. Molecular signatures associated with SLC22A4. (**A**) Heatmap of differentially expressed mRNAs between AML patients with low and high SLC22A4 expression (|log2 FC|>1, FDR<0.05 and p<0.05). (**B**) Bar plot of miRNA functional enrichment results. (**C**) Venn diagram of predicted miRNA targets of the SLC22A4 gene through miRWalk (http://mirwalk.umm.uni-heidelberg.de/), TargetScan (https://www.targetscan.org/vert_80/), miRDB (http://www.mirdb.org/), and DIANA (http://diana.imis.athena-innovation.gr/). AML: acute myeloid leukemia; FC: fold change; FDR: false discovery rate; SLC22A4: solute carrier family 22 member 4. ssGSEA: single-sample gene set enrichment analysis.

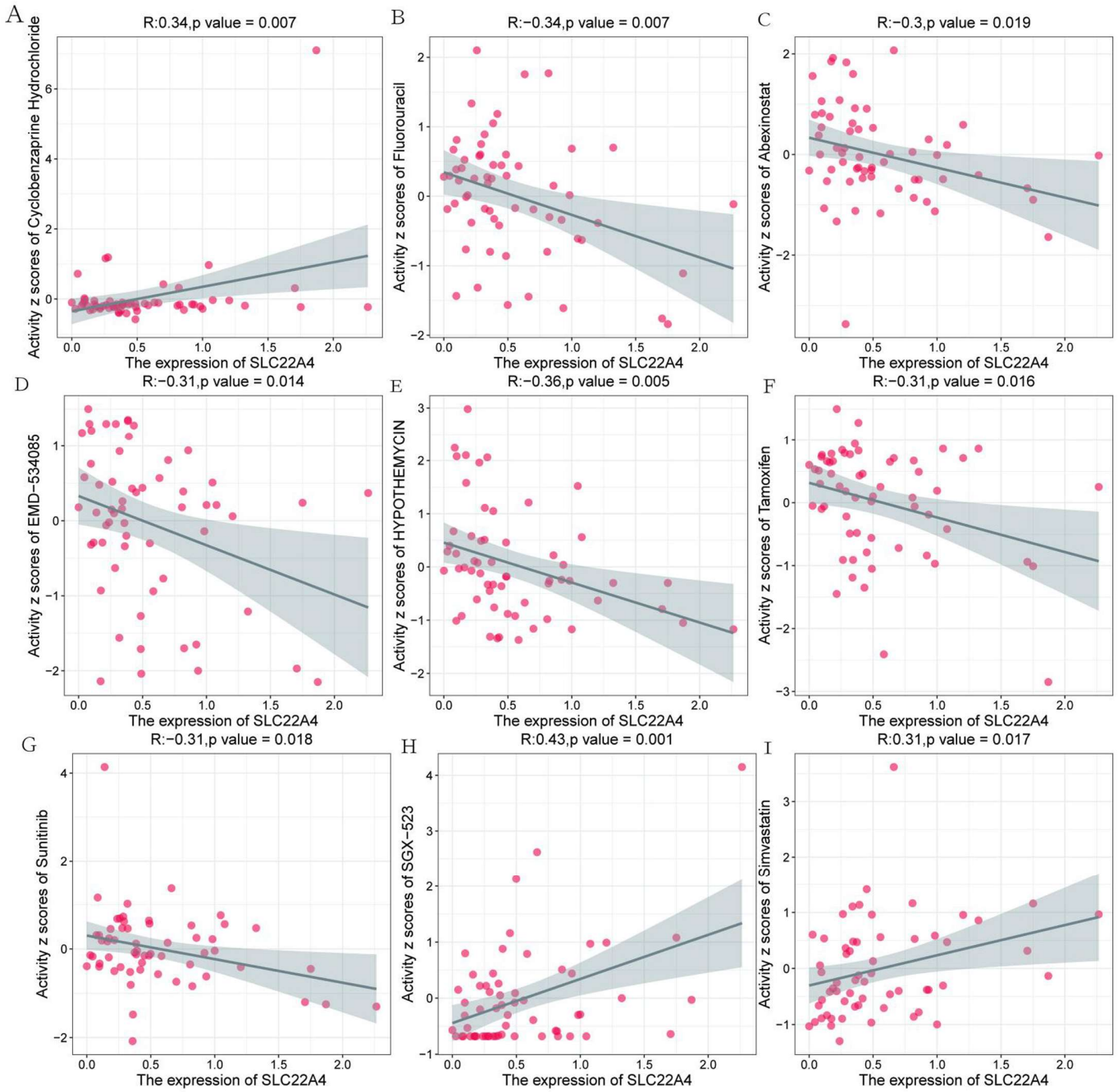

Figure 11. Effect of SLC22A4 on drug sensitivity. The expression of SLC22A4 was associated with the sensitivity to cyclobenzaprine hydrochloride (**A**), fluorouracil (**B**), abexinostat (**C**), EMD-534085 (**D**), hypothemycin (**E**), tamoxifen (**F**), sunitinib (**G**), SGX-523 (**H**), and simvastatin (**I**). SLC22A4: solute carrier family 22 member 4.

In this study, we conducted a pan-omics analysis of large public data sets (TCGA-AML and six GEO series; >600 samples). First, WGCNA identified a brown module whose eigengenes co-vary with immune scores; intersecting this module with DEGs nominated SLC22A4. LASSO-Cox modelling subsequently verified SLC22A4 as the strongest prognostic factor. We next observed that low SLC22A4 expression correlated with higher bone-marrow blast percentages, RAS mutations, and adverse cytogenetic risk, and we built a nomogram whose AUC exceeded 0.8. By integrating methylation arrays, miRNA-target prediction, ssGSEA/xCell immune-infiltration algorithms, and the CellMiner drug-sensitivity database, we demonstrated for the first time that SLC22A4 expression is positively and negatively associated with multiple immune subsets, including DCs, Th17, $CD8^+$ T cells, and M2 macrophages, proposing a "metabolism–immune dual-axis" that may influence therapeutic efficacy. We further identified five miRNAs and several CpG sites that participate in its epigenetic regulation and screened out potential sensitisers, such as cyclobenzaprine, SGX-523, and simvastatin, thus expanding the spectrum of combinatorial options.

Furthermore, the present study revealed a strong relationship between SLC22A4 gene expression and

immune cell infiltration in AML. Both xCell and EPIC analyses demonstrated a correlation between SLC22A4 gene expression and T cells and macrophages, with numerous positive correlations. Enrichment analyses (GSEA and miRNA) also showed that SLC22A4 gene expression is associated with certain infection- and immune-related pathways, suggesting that overexpression of SLC22A4 may enhance immune cell infiltration by upregulating certain substance transport pathways, thereby exerting an anti-tumour effect.

With the enhanced understanding of the molecular mechanisms of AML, the use of immunotherapy has achieved notable results. For example, treatment of CD34+ AML cells with vascular endothelial growth factor C monoclonal antibody can promote their differentiation into myeloid cells by inhibiting forkhead box O3a and ERK/MAPK proliferation signals, thereby improving patient prognosis.[41] Mesothelin-targeted bispecific antibodies may also be a promising immunotherapeutic option for mesothelin-positive paediatric patients with AML.[42] The ssGSEA algorithm is useful in predicting tumour immune-related genes and actual situations.[43,44]

In this study, SLC22A4 expression was positively correlated with DCs, interstitial DCs, macrophages, neutrophils, effector memory T cells, and Th17 cells, but negatively correlated with aDCs, CD8 T cells, NK CD56bright cells, and Th cells. These findings suggest that low SLC22A4 expression may inhibit the activation and function of pro-inflammatory immune cells (e.g., Th17 cells), weaken antigen presentation and T-cell activation, and affect the production and function of inflammatory factors (e.g., IFN-γ, TNF-α, and cytokines). The xCell algorithm was used to verify the results of ssGSEA. This method showed that SLC22A4 expression was positively correlated with CD4+_memory_T−cells, CD8+_T−cells, and macrophages, among others. These results indicate that downregulation of SLC22A4 expression may weaken the effect of immunotherapy through multiple effects, such as inhibiting immune effector cells, enhancing immunosuppressive cells, influencing cytokine networks and the microenvironment, and affecting stem cell functions. Both algorithms showed that SLC22A4 was significantly correlated with multiple immune cells, such as T cells, DCs, and macrophages. SLC22A4 was positively correlated with pro-inflammatory cells (e.g., Th17 cells and DCs) and negatively correlated with cytotoxic cells (e.g., aDCs and CD8 T cells). It was also positively correlated with immunosuppressive cells (e.g., M2 macrophages). These results suggest that SLC22A4 is involved in regulating the function and activity of these cells, while playing a role in the regulation of inflammation and immune balance. Moreover, it may play a role in the immunosuppression process. In summary, SLC22A4 gene expression may guide the prognosis of immunotherapy in AML.

The prognostic significance of SLC22A4 likely stems from its dual roles in drug transport and immune modulation. As a high-affinity transporter of nucleoside analogues, reduced SLC22A4 expression may limit intracellular drug accumulation, enabling leukaemic-cell survival – a hypothesis supported by its positive correlation with sensitivity to cytarabine derivatives.[17] This duality may reflect context-dependent interactions; for instance, SLC22A4's link to M2 macrophages (a tissue-repair phenotype) could indicate a compensatory mechanism in which chronic inflammation fosters immunosuppression.[45] Early-stage SLC22A4 loss could impair effector immune responses (e.g., CD8+ T-cell activation), and compensatory recruitment of M2 macrophages might occur in later stages to mitigate tissue damage, a phenomenon also observed in solid tumours.[46]

Furthermore, SLC22A4 encodes the transporter OCTN1, which is associated with drug transport.[15] OCTN1 also contributes to the transport of cytarabine in AML cells.[47] We found that SLC22A4 expression was positively correlated with sensitivity to cyclobenzaprine hydrochloride, SGX-523, and simvastatin, and negatively correlated with sensitivity to fluorouracil, abexinostat, EMD-534085, hypothemycin, tamoxifen, and sunitinib. These results suggest that SLC22A4 expression is a marker for drug treatment.

We analysed the epigenetic factors that affect SLC22A4 gene expression, including gene methylation and miRNAs. We identified four methylation sites that are negatively related to SLC22A4 gene expression (cg13654253, cg07354253, cg05231888, and cg25350825) and five miRNAs (hsa-miR-1299, hsa-miR-1910-5p, hsa-miR-3659, hsa-miR-5093, and hsa-miR-6831-3p).

Although this study provides valuable insights into the role of SLC22A4 in AML, it has several limitations. First, investigation of the molecular mechanisms involved in the process is lacking. Our analyses were entirely bioinformatics-based and lacked experimental validation, despite the use of multiple datasets to minimise potential bias. Although bioinformatics analysis plays an important role in the management of AML, experimental validation using in vitro models is required to advance this research. Specifically, knockdown and overexpression models can be employed to assess the impact of SLC22A4 on drug sensitivity, such as cytarabine LC50, and its role in immune cell crosstalk, including macrophage polarisation. Additionally, preclinical testing in murine AML models is essential to evaluate the efficacy of OCTN1 agonists or demethylating therapies in restoring SLC22A4 function.

Second, the correlations between SLC22A4 expression and drug IC50 values were mined from the CellMiner database. However, NCI-60 comprises predominantly solid-tumour cell lines and lacks representative acute myeloid leukaemia (AML) models. Consequently, the observed associations should be viewed as exploratory hypotheses rather than definitive predictors of chemosensitivity in AML. Follow-up studies in HL-60, KG-1, OCI-AML3 (AML cell lines), and primary patient blasts are essential to validate whether modulating SLC22A4 truly alters the potency of the identified compounds.

Third, all conclusions in this work derive from publicly available omics data; no cellular, animal, or clinical specimens were experimentally interrogated. Key assertions, such as SLC22A4-mediated control of cytarabine uptake, immune-cell polarisation, or methylation-driven silencing, therefore, remain correlative. CRISPR knock-out/overexpression models, patient-derived xenografts, and leukaemia-initiating-cell assays are required to establish causality and to quantify the translational value of SLC22A4 as a therapeutic target. Finally, immune-cell fractions were estimated with xCell and EPIC algorithms. Although convenient, these tools infer relative abundance solely from bulk transcriptomes and are sensitive to tumour purity, batch effects, and the choice of signature genes. Without orthogonal validation by flow cytometry, immunohistochemistry, or multiplex immunofluorescence on paired bone-marrow biopsies, the reported immune landscapes reflect statistical associations rather than true cellular localisation or functional status. Future investigations should integrate experimental immunophenotyping to confirm the link between SLC22A4 expression and specific leukemic micro-environmental compositions.

Translational Implications: From Biomarker to Therapeutic Target. To translate SLC22A4 into clinical practice, two strategies are proposed. The first strategy involves the use of OCTN1 agonists, such as small molecules that enhance SLC22A4/OCTN1 activity. These molecules, including ergothioneine analogues, have the potential to restore cytarabine uptake in SLC22A4-low AML, thereby synergising with conventional chemotherapy. The second strategy focuses on demethylating agents, such as azacitidine. These agents may reverse SLC22A4 silencing, as there is a strong negative correlation between promoter methylation and expression. This approach could potentially reactivate SLC22A4 expression and improve treatment outcomes in patients with AML.

Conclusions. Through differential expression analysis, WGCNA screening of immune-related gene modules, and prognostic analysis, we identified SLC22A4 as an immune-associated gene signature and a factor of poor prognosis. SLC22A4 expression was significantly decreased in AML, and higher expression was associated with a good prognosis of survival. The degree of methylation and some miRNAs may participate in the regulation of SLC22A4 expression. Moreover, SLC22A4 expression was correlated with drug sensitivity. Whether restoring SLC22A4 expression (e.g., via hypomethylating agents) or modulating its transport activity (e.g., with OCTN1 ligands) will alter leukaemic-cell survival or immune recognition remains an open question that must be tested in functional assays and in prospective preclinical models.

Availability of Data and Materials. Data for a total of 151 de novo AML samples from The Cancer Genome Atlas (TCGA) database (AML NEJM 2013; https://portal.gdc.cancer.gov/) were included in this study; survival data were available for 142 of those. The AML RNA-sequencing dataset was downloaded from the UCSC Xena database (https://xenabrowser.net/datapages/).

Authors' Contributions . Li YY and Li Y conceived and designed the study. Li YY and Cheng WG collected the data. Li Y helped with the data analysis and statistics. All authors took part in drafting the manuscript. All authors read and approved the final manuscript.

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