Original Article

Dipetidyl peptidase-4 and transferrin receptor serve as prognostic biomarkers for acute myeloid leukemia

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Background: Acute myeloid leukemia (AML) is the most common hematological malignancy in adult patients. Ferroptosis-related signatures have been shown to act as regulators of the progression of multiple cancer types, but the role of ferroptosis in AML remains to be elucidated. We performed the present study to preliminarily investigate the roles of ferroptosis-related genes (FRGs) in AML.

Methods: The transcriptome data of AML patients was downloaded from The Cancer Genome Atlas (TCGA) and the transcriptome data of normal samples was obtained from the Genotype-Tissue Expression (GTEx) database. FRGs were selected via public articles. Expression levels of FRGs between AML and normal samples were analyzed. The prognostic model based on FRGs was constructed via lasso regression. The expression levels and prognostic role of FRGs were identified from the risk model. We also performed validation experiments to verify the expression levels of the final selected genes via immunohistochemistry, polymerase chain reaction (PCR), and RNA-seq. Finally, we explored the associations between immune infiltration, drug sensitivity, and the selected FRGs.

Results: The transcriptome data of 151 AML samples were retrieved from TCGA and 70 bone marrow normal samples were retrieved from the GTEx database. Additionally, 23 FRGs were collected from the published articles. There were 22 differentially expressed FRGs, and among them, dipetidyl peptidase-4 (DPP4) (P= 0.011, HR =1.504), GPX4 (P=0.055, HR =1.569), LPCAT3 (P<0.001, HR =2.243), SLC7A11 (P=0.012, HR =2.243), and transferrin receptor (TFRC) (P=0.029, 0.774) had a significant influence on the prognosis of AML patients via lasso regression. The area under the curve (AUC) values of the 1-, 3-, and 5-year receiver operating characteristic (ROC) curves of the FRG signatures indicated that this model is novel and effective method for predicting the prognosis of AML patients. DPP4 (P<0.001) was overexpressed while LPCAT3 (P<0.001), TFRC (P<0.001), GPX4 (P<0.001), and SLC7A11 (P<0.001) were downregulated, further validation experiment results indicated that DPP4 was significantly downregulated but TFRC was upregulated in AML samples. Dysregulation of DPP4 and TFRC influence numbers of chemotherapy regimens sensitivity.

Conclusions: DPP4 and TFRC act as biomarkers for predicting and diagnosing AML, and their expression levels also have significant correlations with drug resistance in AML.

Keywords: Acute myeloid leukemia (AML); DPP4; TFRC; prognostics biomarker

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Introduction

Acute myeloid leukemia (AML) is characterized by a loss of control of myeloid precursor cell proliferation and undifferentiation (1). If AML patients do not undergo appropriate treatment, death can rapidly occur. Anthracycline and cytarabine have remained the standard therapy regimens for AML patients since the 1970s (2). Despite advances in diagnostic and therapeutic methods, the overall survival (OS) of AML patients has not significantly improved. Over the past decade, with the introduction of targeted therapy agents combined with traditional chemotherapy, the rates of complete remission (CR) have been improved, but the rate of relapse is still unchanged. Relapse of disease remains an obstacle for lengthening the OS of AML patients. For high-risk patients, the rate of disease relapse is more than 60% and results in a short median disease-free survival (DFS) of less than 1 year (range, 4 to 11 months) (3). To date, several driver mutations have been observed in AML patients, and these mutations have deep influence on the prognosis of AML patients. Kishtagari *et al.* study summarizes the driver mutations in AML, based on the functions of driver genes, they are divided into signal transduction (FLT3, NRAS, KRAS, and KIT), splicing mutations (SF3B1, ZRSR2, U2AF1, and SRSF2), tumor suppressors (TP53, WT1, and TET2), AML licensing mutations (NMP1), epigenetic modifiers (IDH1, IDH2, TET2, SRSF2, BCOR, BCORL, TET2, ASXL1, and EHZ2), transcription factors (RUNX1, CEBPA, and GATA2), and chromatin modifiers (Cohesin, ASXL1, and EHZ2) (4). Patients with the mutated NMP1, RUNX1, and TP53 lead to poor prognosis, but biallelic mutated CEBPA indicate favorable prognosis (5). Several studies have also demonstrated the occurrence of targeted therapy resistance (6,7). The target regimens enasidenib and ivosidenib have been used to treat IDH mutated AML patients (8). Sorafenib was used to therapy the with FLT3-ITD mutated AML patients (9). However, the resistance of these target therapy has been found (6,10). These indicated that some unique mutation can be sever as the diagnostic and prognostic biomarkers for AML patients, as well as assessing the drug resistance, relapse risk, and therapy targets markers. Drug resistance and disease relapse may be the main reasons leading to the poor outcomes of AML patients, but the underlying mechanisms are still unclear. It is therefore important to find novel biomarkers for diagnosis, assessing prognosis, monitoring drug resistance, and even supplementary therapy methods for AML patients.

Iron is a fundamental inorganic nutrient which has a critical role in multiple biological processes such as DNA and RNA synthesis, cellular respiration, immune responses, and detoxification processes, among others (11). Ferroptosis was introduced in 2012 and is defined as a unique iron-dependent form of cell death. The features of ferroptosis include smaller mitochondria with increased membrane density, and decreased mitochondrial cristae (12). Ferroptosis strike the death balance in common cells and tissues (13). Several studies have demonstrated that ferroptosis is a significant regulator of tumor progression (14-16). Ferroptosis is regulated via several factors, and ferroptosis-related genes (FRGs) may be the most significant regulators among them. FRGs have been observed to be differentially expressed and play key roles in the prognosis of various cancer types such as pancreatic cancer, glioma, and hepatocellular carcinoma (17-20). From these findings, it is clear that FRGs have been well investigated in solid tumors. In regards to AML, several studies have explored the mechanism of drug-induced ferroptosis (21-23). Du *et al.*'s study indicated that DHA can inhibit leukemia cell proliferation via inducing ferroptosis (21). Furthermore, Du *et al.* revealed that inhibition of ferroptosis can promote ATPR-induced AML cell differentiation by regulating the ROS-autophagy-lysosomal pathway (22). Zhu *et al.* showed that typhaneoside inhibited leukemia cell proliferation via inducing ferroptosis-related autophagy (23). These findings indicate that inducing ferroptosis may be a novel potential anticancer method for AML. However, there have been no studies that have investigated FRG expression levels, their prognostic role, and their association with the tumor microenvironment (TME) and drug resistance in AML patients. In the present study, we used bioinformatics to analyze FRG expression levels, their prognostic role, and their association with immune infiltration and drug sensitivity. Furthermore, we collected normal samples and AML patient samples to validate the gene expression levels via immunohistochemistry, polymerase chain reaction (PCR), and next-generation sequencing (NGS). We present the following article in accordance with the REMARK reporting checklist (https://dx.doi.org/10.21037/atm-21-3368).

Methods

Raw data

The transcriptome data and clinical data of 151 AML samples from The Cancer Genome Atlas (TCGA) database

and 70 bone marrow normal samples from the Genotype-Tissue Expression (GTEx) database were collected from the University of California Santa Cruz database (UCSC Xena, https://xenabrowser.net/datapages/). Subsequently, log2 (FPKM+1) normalization was performed on the transcriptome data. We searched and extracted 23 FRGs from PubMed (24-26).

Screening differentially expressed FRGs

We screened differentially expressed FRGs between the TCGA-LAML cohort (tumor) and the GTEx cohort (normal) for further analysis. Differential analysis was carried out with the Wilcoxon test in R software. A heatmap plot of differentially expressed genes was generated via the ggplot2 package. P<0.05 was considered statistically significant.

Construction of the ferroptosis-related prognostic signature

We obtained prognostic FRGs via univariate cox regression based on differential expression of FRGs, then used lasso regression to obtain a more refined signature by constructing a penalty function. Multivariate cox regression (stepwise) was used to construct the final prognostic signature. KM survival analysis was used to generate the survival curves based on median values, and log-rank P<0.05 was considered statistically significant. The receiver operating characteristic (ROC) curves, nomogram, and calibration curve of the prognostic signature were generated via the R packages survivalROC, survminer, and rms, respectively. P<0.05 was considered as statistically significant.

Tumor immune infiltration analysis

We used the CIBERSORT algorithm of tumor immune cell infiltration to calculate the abundance of 22 immune cells in the TCGA-LAML cohort. The correlation analysis of immune cells was carried out via the Spearman method.

Immunohistochemistry

Bone marrow smears of AML and normal cases were collected, fixed with 10% neutral formalin, dehydrated with gradient alcohol, and stained with hematoxylin and eosin (HE). The following antibodies were used for immunostaining: dipetidyl peptidase-4 (DPP4) (Abcam, ab187048), GPX4 (Proteintech, 14432-1-AP), LPCAT3 (Abcam, ab239585), SLC7A11 (Proteintech, 26864-1-AP), and transferrin receptor (TFRC) (Proteintech, 10084-2-AP).

PCR

EDTA anticoagulant tubes were used to collect the peripheral blood of healthy adults and AML patients, and Trizol (Invitrogen, China) was used to extract total RNA. Then, the concentration of total RNA was detected by a nucleic acid analyzer. GeneRuler DNA Ladder Mix and Maxima Reverse Transcriptase were used to reverse transcribe RNA into cDNA, and gene expression levels were detected according to the 2X SG Fast qPCR Master Mix (High Rox, B639273, BBI, ABI) kit instructions. GAPDH was used as an internal reference, and the results were calculated using the $2^{-\Delta\Delta Ct}$ method.

RNA-sequence (RNA-seq)

EDTA anticoagulant tubes were used to collect the peripheral blood of healthy adults and AML patients, and Trizol (Invitrogen, China) was used to extract total RNA. RNA samples were used to perform NGS. The library construction and transcriptome sequencing were completed by Shenggong Bioengineering (Shanghai) Co., Ltd.

Drug sensitivity analysis based on risk score

The R package pRRophetic was used to perform the drug sensitivity analysis.

Statistical analysis

The differential FRGs were screened through the Wilcoxon method. Kaplan-Meier (KM) plots were used to analyze the differential survival between groups, and log-rank P<0.05 was considered statistically significant. Univariate cox regression, lasso regression, and multivariate (stepwise) cox regression were used to construct the prognostic signature. Wilcoxon and Spearman tests were used for difference analysis and correlation analysis, respectively. P<0.05 was considered statistically significant.

Ethical statement

The study was conducted in accordance with the

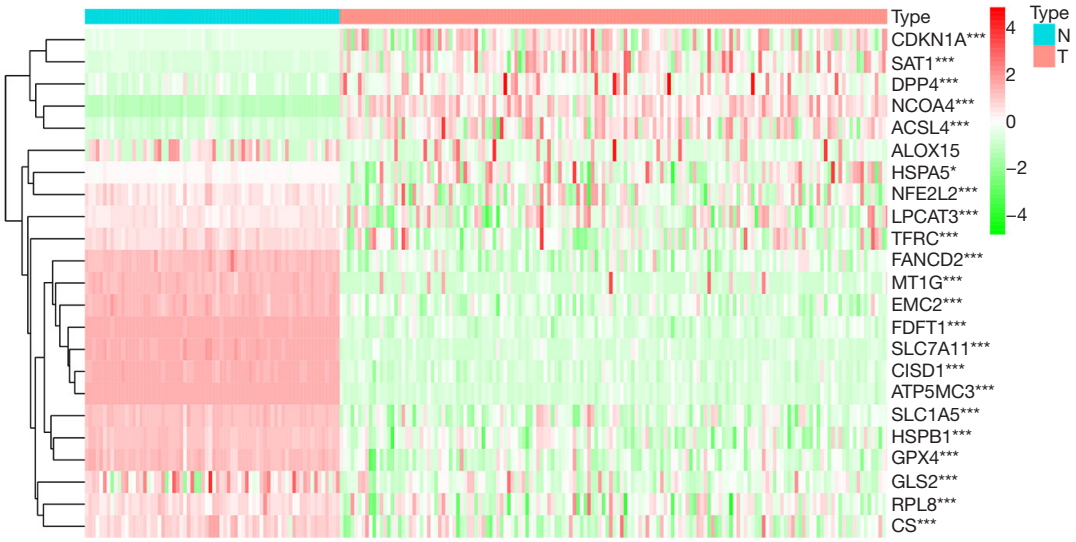

Figure 1 Differential expression of ferroptosis-related genes in acute myeloid leukemia patients. Red represents genes with high expression, and green represents genes with low expression. *, *** represent P<0.05, and P<0.001, respectively.

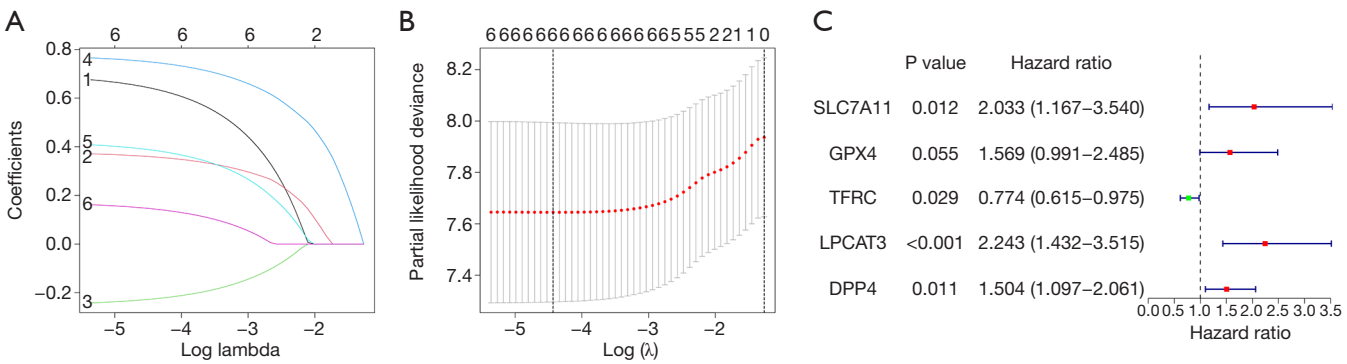

Figure 2 Construction of the FRG prognostic signature for acute myeloid leukemia. (A,B) Selection of the optimal λ threshold for lasso regression. (C) The forest graph of the FRG prognostic signature. FRG, ferroptosis-related gene.

Declaration of Helsinki (as revised in 2013).

Results

Differential expression of FRGs in AML patients

We retrieved 23 FRGs from PubMed and analyzed the differential expression of FRGs between AML (n=151) and normal bone marrow (n=70). The heatmap plot showed that there were 22 differentially expressed FRGs (*Figure 1*).

Establishment of the FRG prognostic signature for AML

We obtained 7 FRGs that affected the OS of AML patients via univariate cox regression of differentially expressed FRGs. The results of lasso regression indicated that λ=−4.4 was the optimal value, then 6 FRGs were obtained for further analysis (*Figure 2A,2B*). Finally, a 5-FRG prognostic signature was established for AML (*Figure 2C*).

A heatmap was generated showing the FRG signature's gene expression in low-risk and high-risk samples (*Figure 3A*). The risk score curve and survival status plot

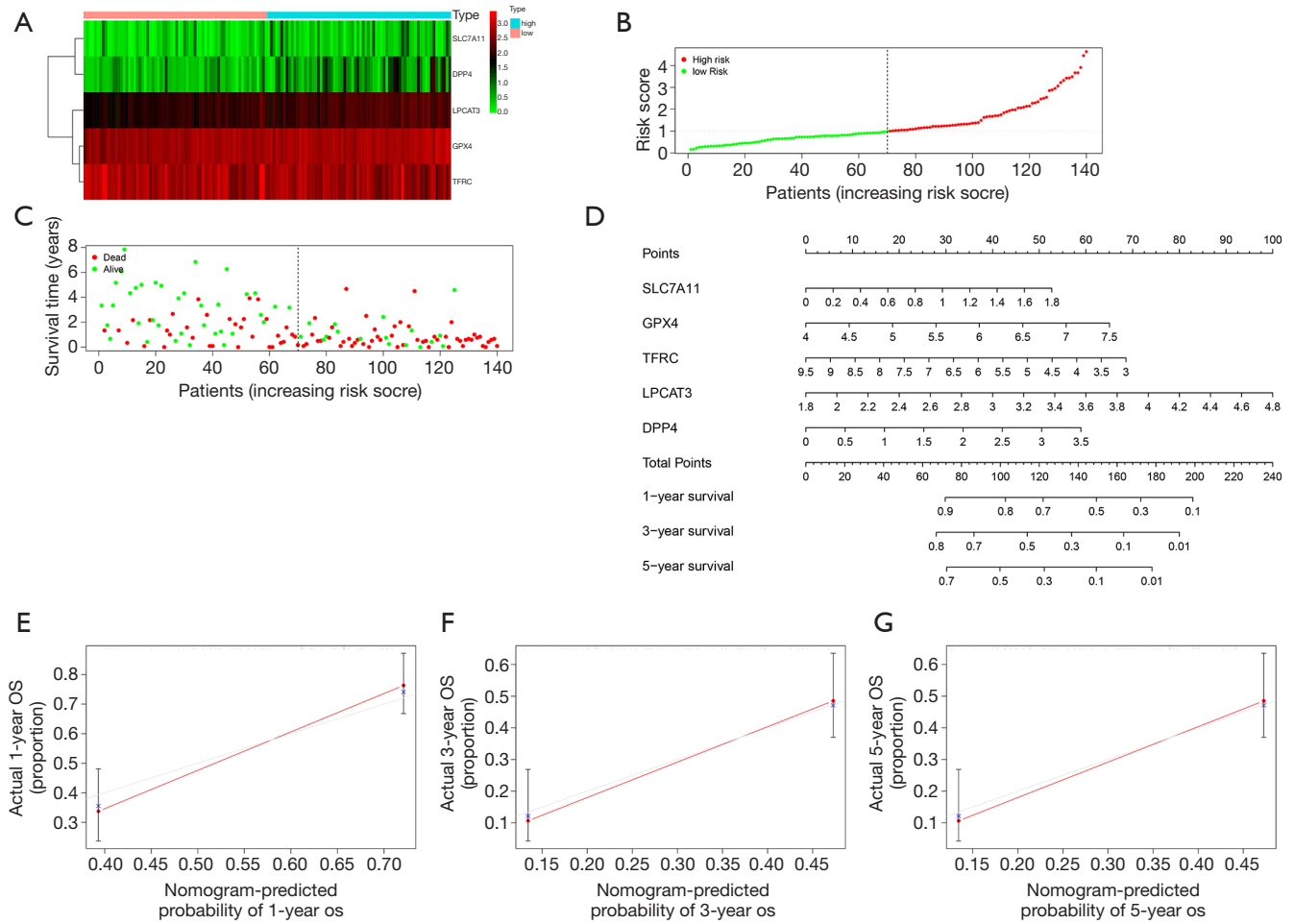

Figure 3 The expression of the signature genes, risk score curve, survival status, nomogram, and calibration curve of the FRG prognostic signature. (A) Heatmap of the expression of FRG signature genes in low- and high-risk samples. Red represents high expression and green represents low expression. (B) Risk score curve of the FRG prognostic signature. Dotted lines represent the boundaries between high- and low-risk groupings. (C) Survival status plot of the FRG prognostic signature. (D) Nomogram of the FRG prognostic signature. The 1-year (E), 3-year (F), and 5-year (G) calibration curves of the nomogram. X-axis and Y-axis represent the predicted survival and actual survival probability of patients' overall survival, respectively. FRG, ferroptosis-related gene. FRG, ferroptosis-related gene.

indicated that low and high-risk could well distinguish between surviving and dead patients (*Figure 3B,3C*). The nomogram and calibration curves demonstrated that the FRG prognostic signature had perfect predictive ability (*Figure 3D-3G*).

KM survival analysis and ROC curve of the FRG signature

The area under curve (AUC) values of the 1-, 3-, and 5-year ROC curves of the FRG signature were 0.804, 0.785, and 0.930, respectively (*Figure 4A*). KM survival analysis indicated that patients with low risk had a better OS for AML (log-rank P<0.001) (*Figure 4B*).

FRG expression levels and their association with prognosis

There were 5 genes in the FRG signature, namely *DPP4*, *LPCAT3*, *TFRC*, *GPX4*, and *SLC7A11*. *DPP4* was highly expressed in tumors compared with normal samples (*Figure 5A*), while *LPCAT3*, *TFRC*, *GPX4*, and *SLC7A11* were lowly expressed in tumor samples (*Figure 5B-5E*). In terms of prognosis, high expression of *DPP4*, *LPCAT3*,

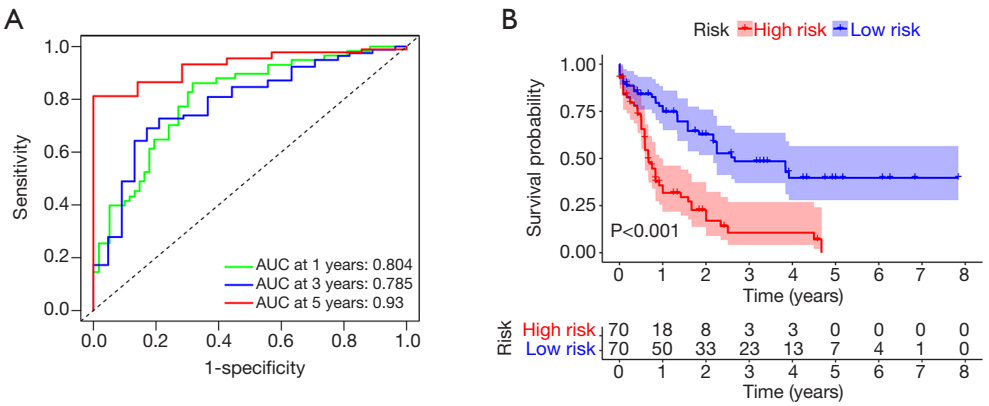

Figure 4 KM survival analysis and ROC curve of the FRG signature. (A) The ROC curves of the FRG signature. Green, blue, and red represent 1-year, 3-year, and 5-year ROC curves, respectively. (B) KM survival analysis of high and low risk of FRG signature. KM, Kaplan-Meier; ROC, receiver operating characteristic; AUC, area under curve; FRG, ferroptosis-related gene.

GPX4, and *SLC7A11* resulted in a shorter OS, while high expression of TFRC resulted in a better OS in AML patients (*Figure 5F-5J*).

The relative abundance and correlation of 22 immune cells in the TCGA-LAML cohort

The histogram shows the relative abundance of 22 immune cells in the TCGA-LAML cohort (*Figure 6A*). The heatmap of correlations between the 22 immune cells indicated that M2 macrophages were negatively correlated with other immune cells, and resting mast cells were positively correlated with other immune cells (*Figure 6B*).

Prognostic immune cells in AML patients

KM survival analysis indicated that high infiltration of resting mast cells resulted in a better OS in AML patients (*Figure 7A*). Nevertheless, high infiltration of M2 macrophages resulted in a poor prognosis (*Figure 7B*).

Correlation between the FRG signature biomarker and the abundance of resting mast cells and M2 macrophages

Spearman correlation analysis demonstrated that DPP4 was negatively correlated with resting mast cells and M2 macrophages (*Figure 8A*). GPX4 was positively correlated with resting mast cells but negatively correlated with M2 macrophages (*Figure 8B*). LPCAT3 was positively correlated with resting mast cells but negatively correlated with M2 macrophages (*Figure 8C*). SLC7A11 was positively correlated with resting mast cells but negatively correlated with M2 macrophages (*Figure 8D*). TFRC was negatively correlated with resting mast cells but positively correlated with M2 macrophages (*Figure 8E*).

The results of validation experiments

The results of immunohistochemistry indicated that DPP4, GPX4, LPCAT3, SLC7A11, and TFRC had higher expression in AML bone marrow samples (*Figure 9*). Furthermore, PCR results showed that TFRC (P<0.01) was significantly overexpressed, but DPP4 (P <0.01), GPX4 (P<0.01), LPCAT3 (P<0.01), and SLC7A11 (P<0.01) were significantly downregulated in AML samples (*Figure 10*). To further validate these selected gene expression levels between normal and AML samples, RNA-seq was performed, and the results showed that TFRC was significantly overexpressed in AML samples (P=2.13E-6), while DPP4 (P=0.016) was significantly downregulated in AML samples (https://cdn.amegroups.cn/static/public/atm-21-3368-1.xls).

Drug sensitivity

The ultimate goal of cancer research is finding novel or complementary therapy regimens for cancer patients. We used TFRC and DPP4 to divide AML patients into high- and low-risk score groups, and explored the association between risk score and drug sensitivity. The results showed that patients with downregulation of TFRC were resistant

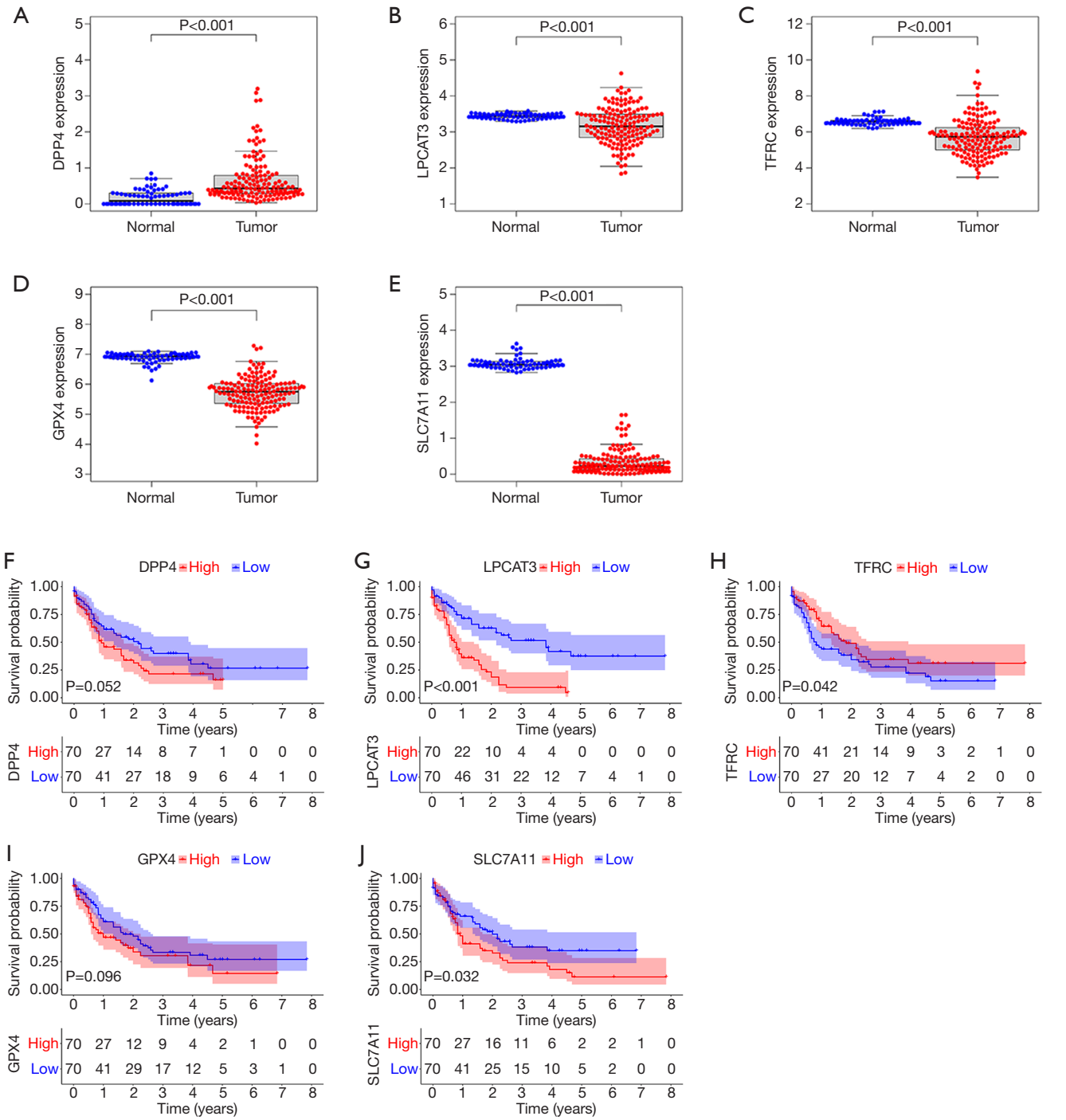

Figure 5 FRG expression levels and their association with prognosis. Expression levels of (A) DPP4, (B) LPCAT3, (C) TFRC, (D) GPX4, and (E) SLC7A11 in tumor and normal samples. Kaplan-Meier survival analysis of expression levels and overall survival based on (F) DPP4, (G) LPCAT3, (H) TFRC, (I) GPX4, and (J) SLC7A11. FRG, ferroptosis-related gene.

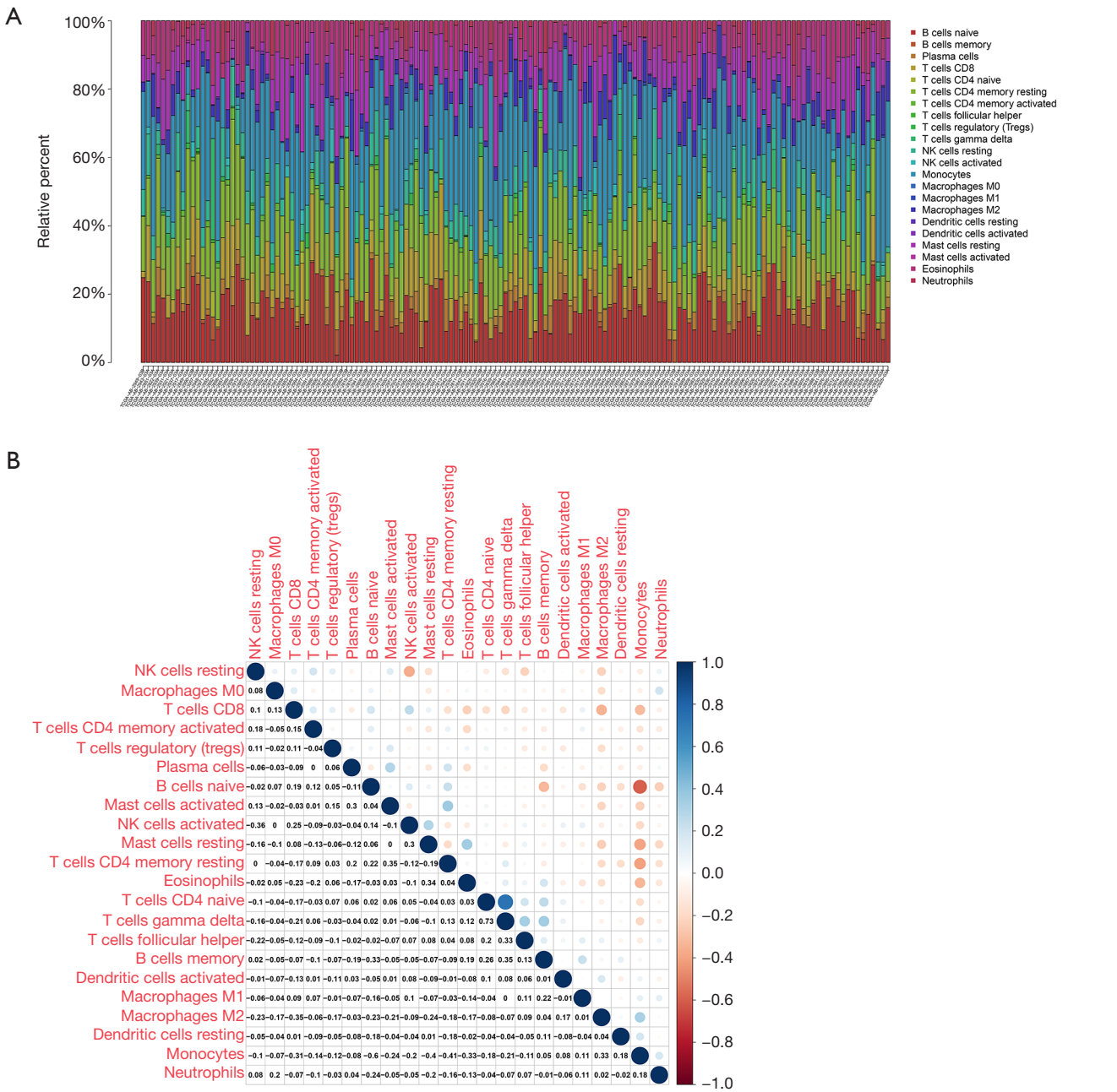

Figure 6 The relative abundance and correlation of 22 immune cells in the TCGA-LAML cohort. (A) Histogram of the relative abundance of 22 immune cells. (B) Heatmap of correlations between the 22 immune cells. Blue and red represent positive and negative correlation, respectively.

to many drugs such as ATRA, axitinib, and vinorelbine, among others, but sensitive to dasatinib, bryostatin.1, and so on (*Figure 11*). According to DPP4, the sensitivity analysis revealed that patients with scores based on the DPP4 group were resistant to CMK and cytarabine, and among others, but sensitive to dasatinib *Figure 12*).

Discussion

With the better learning the critical role of ferroptosis in

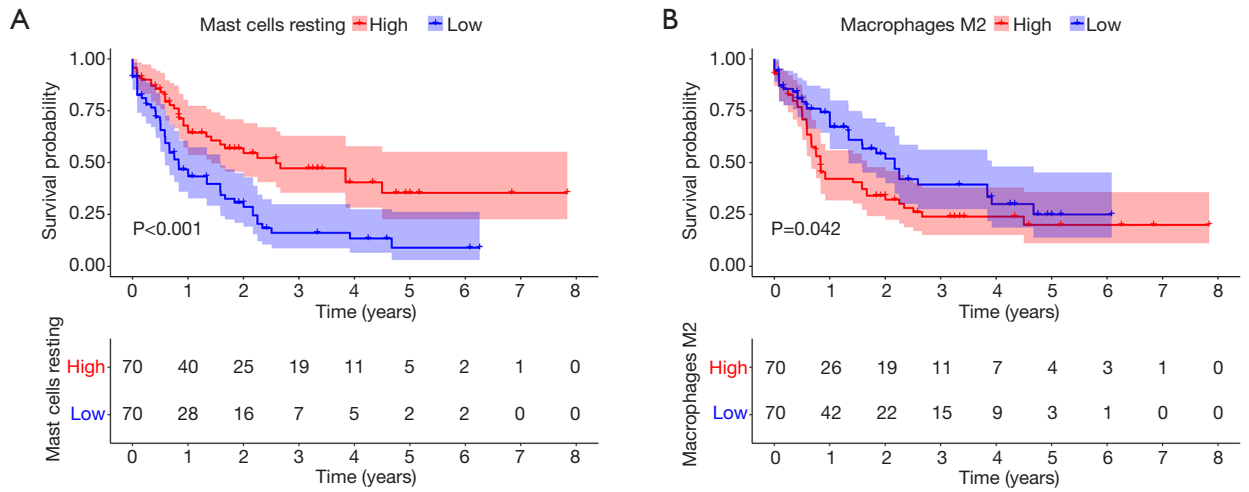

Figure 7 Immune cells that affected the overall survival in AML patients. Kaplan-Meier survival analysis of (A) resting mast cells and (B) M2 macrophages. AML, acute myeloid leukemia.

tumorigenesis, therapy response, drug resistance in various cancer types. FRGs have been shown to be important factors that significantly influence tumor progression in multiple cancer types such as hepatocellular carcinoma, clear cell renal cell carcinoma, and breast cancer (27-29). AML, as the most common hematological malignancy in adult patients, is still an incurable disease and poses a big challenge for public health. A number of studies have shown that ferroptosis-related signatures take part in several important processes in solid cancer, but no study has revealed the underlying mechanism and role of FRGs in AML. We therefore attempted to investigate their expression levels, prognostic role, influence on the TME, and the effect of drug resistance in AML.

The transcriptome data of AML patients was downloaded from TCGA and the transcriptome data of normal samples was obtained from the GTEx database, and FRGs were selected via public articles. We analyzed the expression levels of FRGs between AML and normal samples. A prognostic model based on FRGs was constructed via lasso regression. Among the genes, SLC7A11, GPX4, TFRC, LPCAT3, and DPP4 were further investigated in terms of their expression levels and prognostic role in AML. We performed validation experiments to verify the final selected gene expression levels via immunohistochemistry, PCR, and RNA-seq. Finally, we explored whether there was an association between immune infiltration and drug sensitivity, and finally selected FRGs.

Recently, more and more studies have revealed the significant role of ferroptosis in cancer. Apart from being a unique form of cell death, ferroptosis has been shown to play important roles in cancer stem cells and the TME (30-32). As the most important regulators in the ferroptosis process, FRGs have been confirmed to play critical roles in the prognosis and resistance of glioma (33,34). In our study, DPP4 was overexpressed, while LPCAT3, TFRC, GPX4, and SLC7A11 were downregulated in AML samples compared to normal samples. Interestingly, several gene expression levels were inconsistent in the public dataset analysis. DPP4, GPX4, LPCAT3, SLC7A11, and TFRC all had higher expression in AML bone marrow samples. TFRC was significantly overexpressed, but DPP4, GPX4, LPCAT3, and SLC7A11 were significantly downregulated in AML samples via PCR analysis. RNA-seq results showed that TFRC was significantly overexpressed while DPP4 was significantly downregulated in AML samples. The prognostic model showed that SLC7A11, GPX4, TFRC, LPCAT3, and DPP4 significantly influenced the prognosis of AML patients. DPP4, LPCAT3, GPX4, and SLC7A11 may act as adverse biomarkers, while controversially, TFRC may act as a protective factor for AML patients. DPP4 acts as an adverse signature for breast, prostate, and pancreatic cancer, and inhibition of DPP4 can improve the prognosis of these patients (35). Zhang *et al.*'s study indicated that overexpression of glutathione peroxidase 4 (GPX4) could enhance cisplatin resistance *in vitro* (36). Guerriero *et al.* revealed that GPX4 was significantly overexpressed in human hepatocellular carcinoma, further indicating that

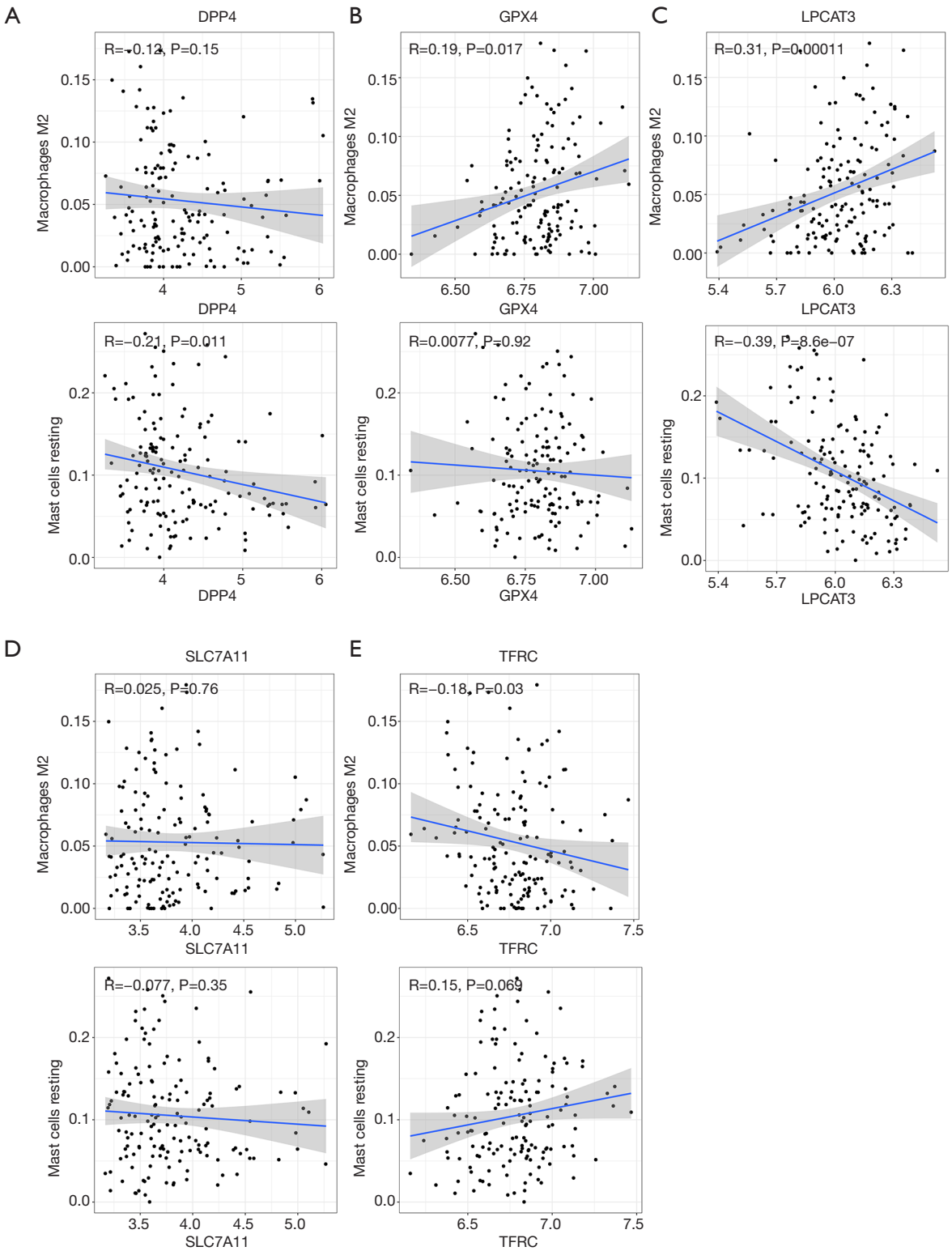

Figure 8 Correlation between the FRG signature biomarker and the abundance of resting mast cells and M2 macrophages. (A) DPP4, (B) GPX4, (C) LPCAT3, (D) SLC7A11, and (E) TFRC. FRG, ferroptosis-related gene.

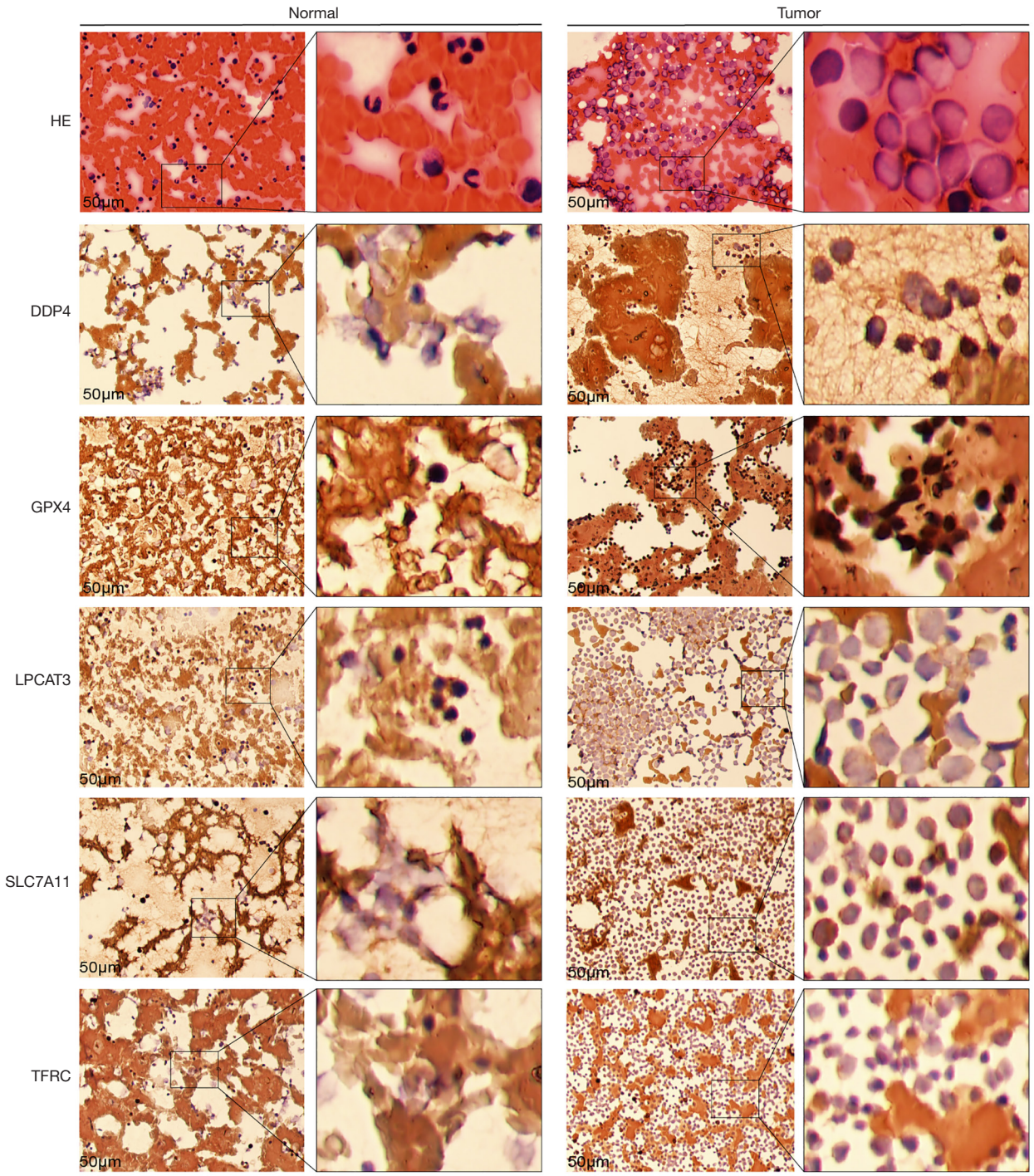

Figure 9 Immunohistochemistry findings of DPP4, GPX4, LPCAT3, SLC7A11, and TFRC expression.

expression levels may be impacted by cancer status (37). Ma *et al.* revealed that SLC7A11 was overexpressed in laryngeal squamous cell carcinoma, and the upregulation of SLC7A11 promoted tumor progression (38). From these findings, we can conclude that DPP4, LPCAT3, GPX4, and SLC7A11 have essential biological functions in multiple

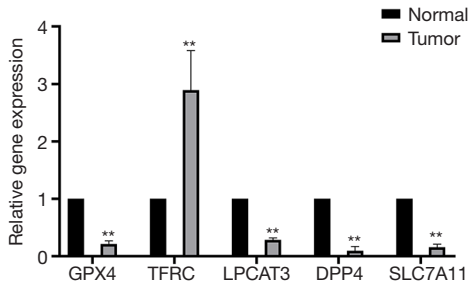

Figure 10 The PCR results of DPP4, GPX4, LPCAT3, SLC7A11, and TFRC expression. **, P<0.01. PCR, polymerase chain reaction.

cancer types, and most of them act as tumor promoters. In regards to AML, only GPX4 has been investigated in terms of its expression and prognostic role. Wei *et al.* showed that GPX4 was significantly downregulated in AML patient samples, and overexpression of GPX4 indicated a better outcome (39). In regards to TFRC, Huang *et al.* revealed that TFRC accelerated the progression of epithelial ovarian cancer via upregulating AXIN2 expression (40). In another study, TFRC also acted as a promoter of liver cancer cells, and inhibition of TFRC could suppress cancer cell growth and survival (41). From these findings, TFRC may be an oncogene for liver cancer and epithelial ovarian cancer, which is inconsistent with its prognostic role in AML patients. There has been no study that has explored the role of TFRC in AML.

Based on the fundamental function of ferroptosis in immune responses, we also performed an analysis of the relationship between final selected FRGs and immune cell infiltration. Based on the validation experiment results, we finally selected DPP4 and TFRC for this analysis. The results showed that TFRC and DPP4 were negatively correlated with resting mast cells but positively correlated with M2 macrophages. The TME is one of the critical regulators of immunotherapy, chemotherapy response, and tumor progression (42-44). Research on the TME in solid tumors has been prosperous, but the underlying mechanisms of the TME in therapy response, prognosis, and tumor progression are still unclear. Based on the complexity of the microenvironment of AML, only a few studies have preliminarily investigated the TME of AML (45-48). Carter *et al.* revealed that the TME can significantly influence the drug sensitivity of AML (45). Furthermore, our results showed that resting mast cell infiltration resulted in a better OS, but high infiltration of M2 macrophages resulted in a poor prognosis for AML patients. Lan *et al.* revealed that M2 macrophage-derived exosomes promoted the invasion and migration ability of colon cancer cells (49). M2 macrophages also served as promoters of multiple cancer types such as breast, gastric, and bladder cancer (50,51). The fundamental biological function of resting mast cells in cancer still remains to be elucidated, but several studies have shown that they may have a strong influence on cancer (52-54). Xu *et al.* indicated that M2 macrophages were enriched in AML, and led to poor outcomes (55). The other type of macrophages, M1 macrophages, may serve as protective factors in AML (56). These results also highlight the important role of the TME in AML, but there is still a long way to go.

The ultimate goal of the present study was to find a reasonable novel or complimentary therapy regimen for AML patients. We analyzed the association between DPP4, TFRC, and drug sensitivity in AML patients. The results showed that patients with downregulation of TFRC lead to resistant to ATRA, AZD.2281, CMK, and metformin, and upregulated TFRC induce resistant to bexarotene, bicalutamide, and dasatinib. According to DPP4, patients with high-risk scores were resistant to CMK and cytarabine, and among others, but sensitive to dasatinib. The dysregulated expression of DPP4 can influence the sensitivity to cytarabine, and cytarabine is one of the first-line therapy regimens in AML. Therefore, more reasonable chemotherapy regimens can be selected via this analysis.

Conclusions

In our study, we found that FRGs can serve as diagnostic and prognostic biomarkers for AML patients. FRGs not only have a strong influence on the TME of AML, but also drug resistance. The findings of this study provide useful information for clinicians to select therapy regimens based on FRG expression levels, and pave the way for future fundamental research to understand the underlying mechanisms of ferroptosis in AML.

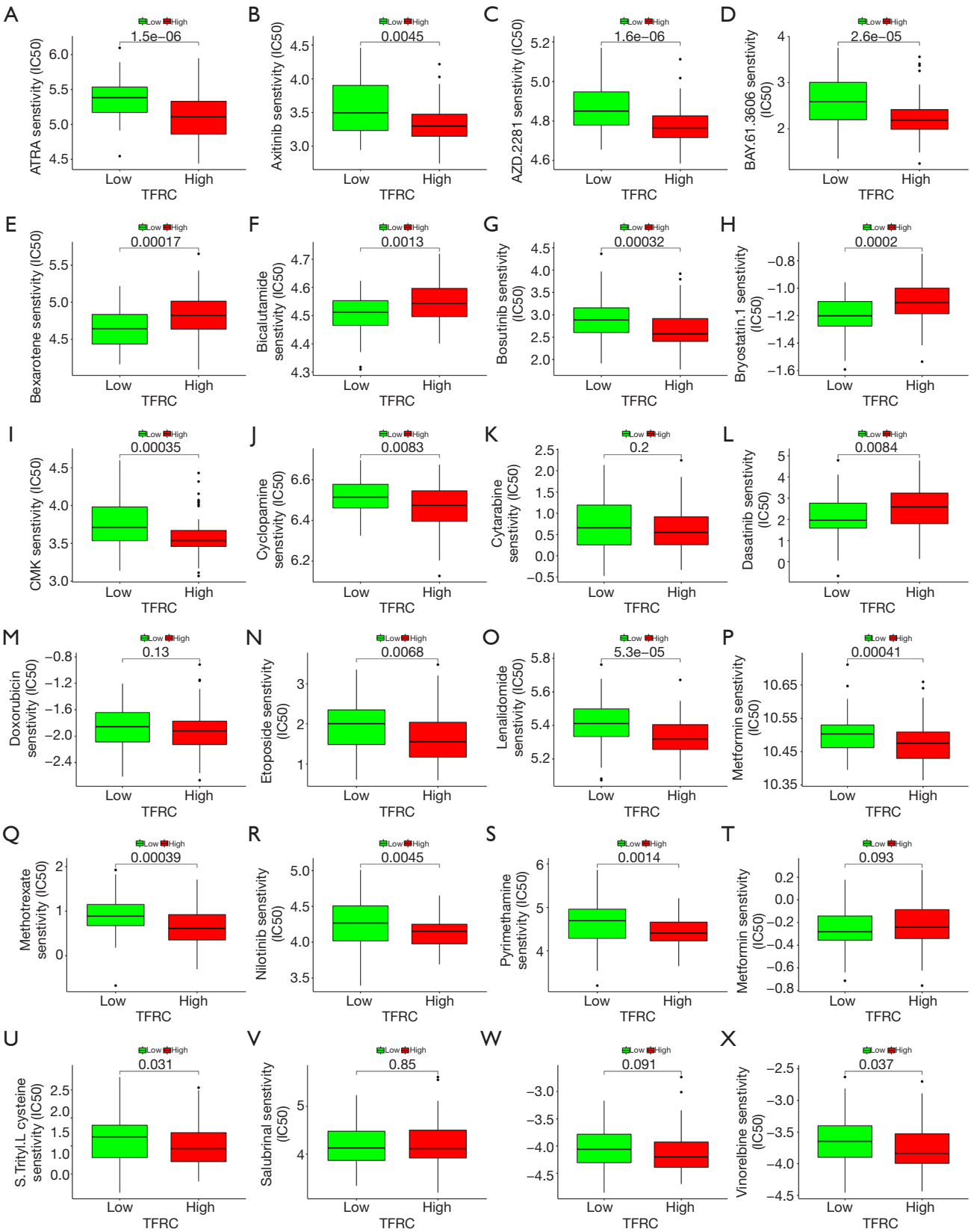

Figure 11 Relationship between risk score and drug sensitivity via the R package pRRophetic (TFRC).

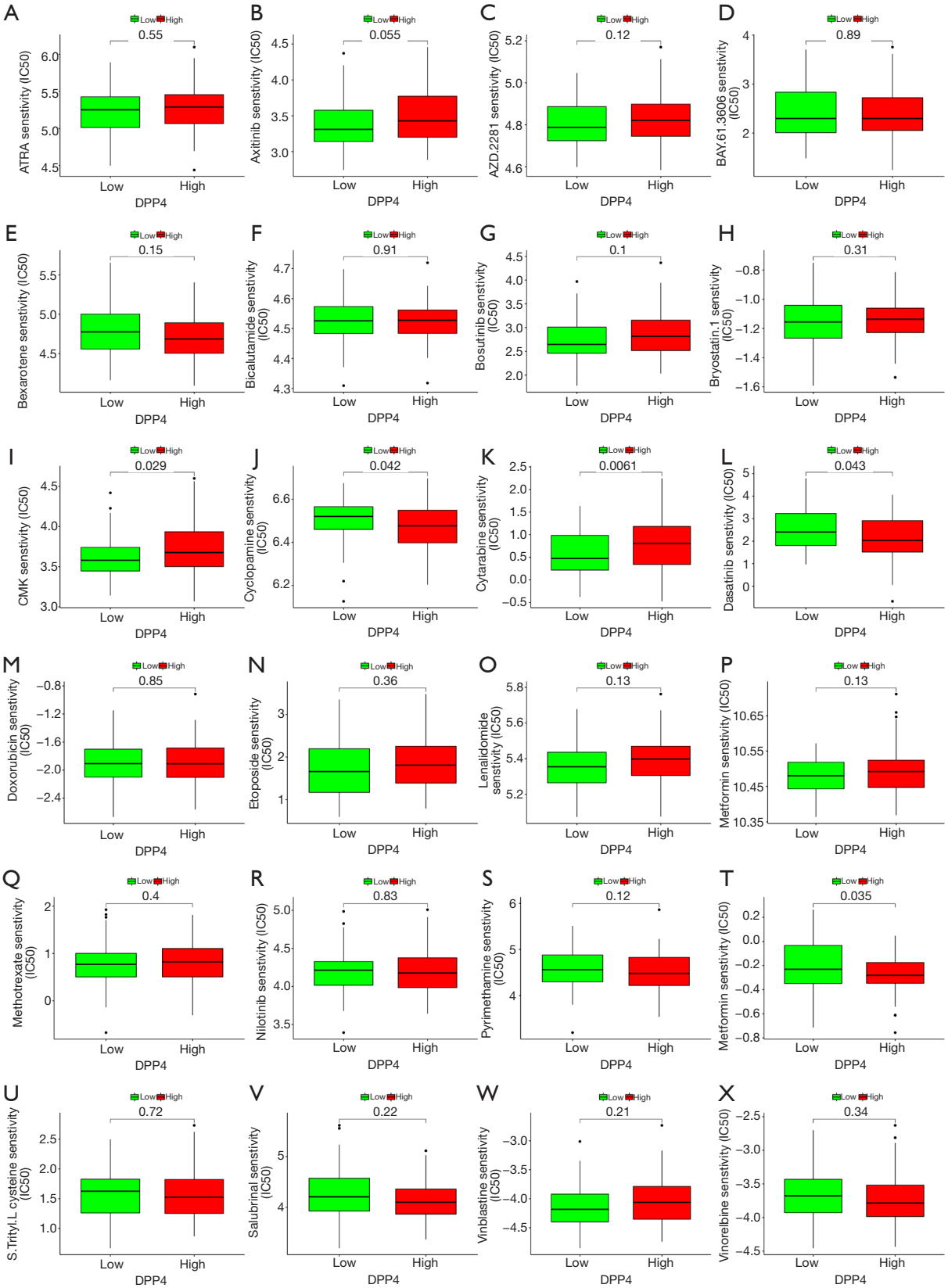

Figure 12 Relationship between risk score and drug sensitivity via the R package pRRophetic (DPP4).

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Footnote

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