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Low Expression of Iron–Sulfur Cluster Binding Protein RFESD Predicts Adverse Clinic Outcomes in Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) is a devastating hematopoietic malignancy with high heterogeneity and poor outcomes. In this study, we searched for the new biomarker to promote the efficacy of diagnosis, therapy and prognosis of AML by transcriptome and clinical information from the Cancer Genome Atlas (TCGA) database, Gene Expression Omnibus (GEO) datasets, and Genotype-Tissue Expression (GTEx) projects. RFESD was found to be significantly downregulated in AML, and was confirmed as an independent prognostic factor by univariate Cox regression. A survival prediction nomogram was established with a C-index of 0.762, which indicated reliable prognostic prediction. Function pathway enrichment analysis of differentially expressed genes (DEGs) indicated immune response related pathways in low RFESD expression group. Through gene co-expression and protein–protein interaction (PPI) network analysis, we identified KLF1 as the hub gene, which was linked to adverse clinical outcomes. Furthermore, we found that low expression of RFESD tended to be positively correlated with immune cells and immune check-points. Finally, drug sensitivity prediction indicated some inhibitors associated with cell-cycle and kinases. Accordingly, our research illustrated that the low expression level of RFESD predicts an adverse prognosis in AML patients.

Introduction

Acute myeloid leukemia (AML) is a rapid and aggressive heterogeneous blood cancer that originates from the blocked differentiation and clonal expansion of myeloid progenitor cells[1, 2]. AML patients are often accompanied by many complications involving splenomegaly, anemia, and infection. The adverse prognosis and leukemia recurrence are common after allogeneic hematopoietic stem cell transplantation (HSCT)[3, 4]. The efficacy of conventional anthracycline and cytarabine chemotherapy (7 + 3 regimen) is limited as well[5, 6]. In recent years, the exploration of new AML therapies based on specific biomarkers has owned the vital role in enhancing the efficacy of systemic treatment and improving the survival rate of patients[7]. Nevertheless, there is still inherent and acquired drug resistance in AML patients. Since given the complexity and heterogeneity of AML, the identification of reliable and robust biomarkers has remained a challenge.

RFESD is located on 5q15, which encodes an iron–sulfur cluster binding protein with oxidoreductase activity[8, 9]. RFESD could bind to iron-sulfur clusters and participate in mitochondrial oxidative metabolism[10]. Abnormal expression of RFESD promotes the metabolic reprogramming, which indicates the sign of cancer [11]. Lately, targeting metabolic factors has become an innovative treatment regime[12, 13]. In addition, studies have shown that the expression of RFESD is closely associated with folate metabolism[14]. Nevertheless, the role of RFESD in AML remains elusive.

In this study, we performed survival predicted functions, cox regression analysis, and nomogram model establishment to assess the prognosis of AML patients based on RFESD expression. In addition, we performed pathways enrichment, PPI analysis, and correlation analysis of immune infiltration or immune checkpoints to detect the pathogenic molecular mechanisms of RFESD. Finally, we predicted the

potential medicines sensitive to low expression of RFESD. Our findings revealed the prognostic value of RFESD and might propose original views into the original biomarker of leukemogenesis.

Materials and Methods

Data source

We collected the matrix of mRNA expression from TCGA LAML cohorts (RNA-seq, n = 151)[15] and GTEx normal samples (n = 70)[16]respectively. The RNA-seq and clinical information from the TCGA LAML and GTEx datasets were acquired using the UCSC XENA browser (https://xenabrowser.net/datapages/)[16–18]. To maintain the comparability of data from different databases, transcripts per kilobase million (TPM) values from RNA-Seq were determined for intrasample comparison after log2 transformation. In our study, specimens with no survival data were excluded.

Differentially expressed genes (DEGs) analysis

Patients from TCGA-LAML database were divided into two groups by the median RFESD expression level. DESeq2 was used for identifying DEGs with default parameters and the following thresholds: p-adjust < 0.05, |log2FC| ≥ 0.585[19].

Identification of overall survival-related genes

The LAML cohort was used to investigate the potential prognostic significance of the selected genes in AML patients. AML patients were divided into two groups (RFESD low and RFESD high groups). Overal survival (OS) related genes with the p value < 0.05 were selected using univariate Cox hazard regression analysis for further research. The external cohort GSE12417(n = 163) was used to validate our results[20].

Functional pathway analysis

We carried out functional GO annotations analysis to investigate the underlying functions of DEGs in AML, including biological process (BP), cellular component (CC), and molecular function (MF) performed with DAVID Bioinformatics Resources[21, 22]. A Benjamin–Hochberg adjusted p value < 0.05 was interpreted as statistically significant. Heatmap of clustered DEGs was generated using R package. To further evaluate the gene function, GSEA enrichment was performed with the gene sets obtained from the Molecular Signatures Database. Results were considered significant when |NES | >1, p value < 0.05, and FDR < 0.25[23].

Comprehensive protein–protein interaction analysis

Associations between RFESD and the expression of other genes were assessed using the LinkedOmics database (http://www.linkedomics.org/login.php). We derived the PPI network from the STRING database (https://string-db.org/) to estimate the interactional correlations of the DEGs[24]. A confidence score > 0.4 was considered significant. Hub proteins and key nodes in the constructed PPI network were identified using the Cytoscape plugin CytoHubba[25]. We investigated the association of RFESD expression with hub genes through correlation heatmaps by using the R package ggplot2.

Hub-gene identification

Three algorithms of maximum neighborhood component (MNC), maximal clique centrality (MCC) and edge percolated component (EPC) in Cytoscape were applied to recognize and visualize the pivotal hub genes. Statistical comparisons were performed by Spearman correlation. Correlations between transcriptional level of RFESD and those of hub genes in AML subjects were visualized using ggplot2[25].

Immune infiltrate analysis

By using Single Sample GSEA (ssGSEA) in the R package gsva, Spearman correlation coefficients were computed between the expression level of RFESD and ssGSEA-based immune cell infiltration levels[26, 27]. The involved immune cells were of 22 immune cell subtypes, including B cells, monocytes, macrophages, neutrophils, NK cells, DC cells and all subtypes of T-cells. The relationships between RFESD expression and the expression of immune checkpoint molecules, including CD40LG, CD274, TIGIT, LAIR1, CD160, C10orf54 and HAVCR2, were identified through correlation heatmaps by using the R package ggplot2.

Drug sensitivity prediction analysis

The median inhibition concentration (IC50), calculated using the pRRophetic package in R[28, 29], was used for drug sensitivity prediction.

Survival and statistical analysis

All statistical analyses were performed with R, version 4.4.1 (https://www.r-project.org/). The Wilcoxon rank-sum test and Kruskal–Wallis test were used to detect differences among continuous variables. The correlation of clinical features with RFESD expression was detected with Pearson's correlation $\chi 2$ test. For survival analysis, Cox proportional hazards analysis was conducted by using "survival" and "survminer" in R. Variables significant in Cox univariate analysis were selected for multivariate analysis. The Kaplan-Meier method was used for univariate analyses of OS. Receiver operating characteristic (ROC) curves and AUC values were generated by pROC in R to assess the diagnostic efficacy of RFESD

for AML. All tests were two-sided, and the p value < 0.05 was considered to indicate statistical significance.

Results

Low expression of RFESD in AML implicated adverse clinical features

To explore the expression landscape and potential indication of RFESD, the mRNA levels in AML and normal tissues were extracted from databases respectively. The flow chart of analysis with GTEx and TCGA-LAML cohort was shown in Supplemental Table 1. Pan-cancer analysis revealed that RFESD was widely and highly expressed in most malignancies (Fig. 1a), especially in AML ($p < 0.0001$, Fig. 1b). We also explored the expression level of RFESD in another GEO datasets (GSE13159, $p < 0.0001$, Fig. 1c). Intriguingly, RFESD expression distinguished AML from healthy individuals with a predictive power of 1.000 (95% confidence interval, CI = 1.00–1.00), as shown by AUC values analyzed by the ROC curve (Fig. 1d). Therefore, we doubted whether RFESD expression in AML patients is clinically relevant. We observed RFESD expression is linked to clinical characteristics. The lower expression of RFESD owned higher BM blasts percentage(BM blasts > 20%, $p < 0.05$, Fig. 1e) and WBC counts(WBC counts > $20\times10^{\wedge9}$, $p < 0.01$, Fig. 1f) in AML patients. Furthermore, similar trends were observed when patients were divided by low or high RFESD expression; more details are shown in Supplemental Table 2.

(a). Levels of RFESD expression in different tumors from TCGA and GTEx database. **(b)** Expression levels of RFESD in AML (n = 151) and normal samples (n = 70). **(c)** Expression levels of RFESD in GSE13159, AML (n = 501) and normal samples (n = 73). **(d)** ROC of RFESD in AML. Clinical characteristics including **(e)** BM blasts, **(f)** WBC counts. Analysis between two groups of unpaired samples: Wilcoxon rank sum test, analysis among multiple groups of samples: Kruskal–Wallis rank sum test (ns $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Low expression of RFESD predicts unfavourable prognosis in AML

We further investigated the prognostic value of RFESD in AML. Firstly, patients with low RFESD expression had shorter OS than high RFESD expression group (p value = 0.024, Fig. 2a), which indicated that low RFESD expression level was associated with the adverse prognosis in AML patients. Furthermore, we validated results in an independent external validation cohort GSE12417 (p value = 0.03, Fig. 2b). In addition, univariate and multivariate logistic regression analyses were performed to determine whether low expression of RFESD was an independent prognostic factor for AML. Univariate Cox regression analysis was showed in Fig. 2c, which demonstrated that low level of RFESD expression was associated with poor OS (hazard ratio, [HR]: 2.133; 95% confidence interval [CI]: 1.2587–3.616; p value = 0.0049). Meanwhile, unfavourable cytogenetics (Intermediate cytogenetic risk, HR: 5.610, 95% CI: 2.6050–12.081, $p < 0.001$, Poor cytogenetic risk, HR: 4.5052, 95% CI: 1.9217–10.548, $p < 0.001$),

increasing age(> 60, HR: 4.350, 95% CI: 2.6180–7.228, p < 0.001), positive IDH1R140 mutation (HR:0.365, 95% CI: 0.1499–0.888, p = 0.0264) and positive NPM1 mutation (HR:0.401, 95% CI: 0.2111–0.763, p = 0.0054) were also risk factors associated with poor outcomes. Then, all significant variables in univariate Cox regression analysis were included in multivariate Cox regression analysis. Subsequently, unfavourable cytogenetics (Intermediate cytogenetic risk, HR: 4.465, 95% CI: 2.112–9.439, p < 0.001, Poor cytogenetic risk, HR: 3.531, 95% CI: 1.597–7.809, p = 0.0018), increasing age (> 60, HR: 3.587, 95% CI: 2.239–5.745 p < 0.001), positive IDH1R140 mutation (HR: 0.292, 95% CI:0.127–0.671 p = 0.0037) and low level of RFESD expression (HR: 2.289; 95% CI: 1.411–3.713; p < 0.001) were identified as independent prognostic factors for OS(Fig. 2d). Moreover, a nomogram including the prediction model was established based on multivariable logistic regression analysis. The established nomogram was well calibrated and had good discriminative power, with a concordance index (C-index) of 0.762 (95% CI: 0.713–0.811, p < 0.001, Fig. 2e) for OS prediction. Furthermore, to describe the clinical net benefit, we plotted the calibration curves, that showed the short-term (1 or 3 years) calibration curve presented high concordance between the nomogram predicted survival probability and observed fraction survival probability (Fig. 2f). In summary, the constructed survival-predictive model revealed excellent accuracy for predicting OS in AML patients.

GO and GSEA analysis for DEGs based on RFESD expression were enriched in immune-related signaling pathways

To explore the potential mechanisms of RFESD in leukemogenesis, we first identified the DEGs between two AML populations divided by high and low RFESD expression (Fig. 3a). In total, 385 DEGs (adjusted p value < 0.05, |log2(FC)| >1) were obtained and shown in volcano plots (Fig. 3b), including 155 upregulated genes and 230 downregulated genes. To elucidate the intracellular signaling pathways influenced by the DEGs, we performed GO and GSEA analysis. We extracted the top6 enriched terms from GO analysis (Fig. 3c-e, p < 0.05). Interestingly, many terms of BP were associated with immune response pathways, involving immune response, adaptive immune response, immunoglobulin mediated immune response, immunoglobulin production, innate immune response and inflammatory response.

Moreover, GSEA analysis was used to assess enrichment pathway of RFESD expression. Finally, 11 pathways were enriched in RFESD-low group (Supplemental Table 3), of which the major affected pathways included inflammatory response, IFNγ-response, TNF signaling via NF-κB, IL6-JAK-STAT3 signaling, oxidative phosphorylation, and IFNα response(Fig. 3f-k). Accordingly, we extrapolated that low RFESD expression impinged upon AML may be associated with immunoregulation.

Tumor-infiltrating lymphocytes have been reported to be prognostic in various tumors[30, 31], we performed correlation analysis between RFESD expression and 22 different types of infiltrating immune cell subtypes[32, 33]. The results showed that RFESD expression obtained the obvious positively correlation with the numbers of B cells naïve, Dendritic cells activated, Mast cells, T cells follicular helper, Blast cells, CD4 memory resting, and Monocytes (Supplemental Fig. 1a). The details of the quantitative analysis by Spearman's correlation coefficient are shown in Supplemental Fig. 1b-h. Furthermore, we

clarified the relationship between RFESD expression and immune checkpoints (CD40LG, TIGIT, CD274, HAVCR2, LAIR1, CD160 and C10orf54) expression (Supplemental Fig. 1i). Details of the correlation analysis are shown in Supplemental Fig. 1j-p.

Identification of hub genes connected with RFESD expression

To assess possible proteins directly interact with RFESD, we next performed protein-protein interaction (PPI) analyses and co-expression modules. As showed, most genes of AML were positively correlated with the expression of RFESD (Fig. 4a). Details of positively correlated genes of RFESD were displayed in Fig. 4b, and negatively correlated genes were showed in Fig. 4c. The DEGs-related PPI network was constructed to determine hub genes. The top 10 potential markers were calculated by the MNC, MCC and EPC respectively (Fig. 4d-f). We obtained 6 shared genes (AHSP, ALAS2, GATA1, GYPB, KLF1, SLC4A1) among above three gene molecules (Fig. 4g, h). Then we confirmed that there were 4 genes expressing significantly differently (Fig. 4i). Finally, we examined the relationship between the expression levels of potential hub genes and prognosis, and found that only KLF1 was positively related to RFESD expression and linked to poor clinical outcomes in patients with AML (Fig. 4j).

(a) Co-expression analysis of RFESD in the TCGA-LAML dataset. **(b–c)** The top 10 positively/negatively correlated genes are displayed. **(d-f)** The top 10 hub genes were selected on the basic of **(d)** MNC, **(e)** MCC, and **(f)** EPC. **(g)**Venn diagram of hub genes from 3 modules. **(h)** The association of RFESD with hub genes (AHSP, ALAS2, GATA1, GYPB, KLF1 and SLC4A1). **(i)** Expression levels of hub genes in AML patients (n = 151) and normal samples(n = 70). **(j)** The difference in OS between patients with high and low KLF1 expression levels shown by Kaplan-Meier curves. (****, $p < 0.0001$).

Drug sensitivity analysis of AML cells with low RFESD expression

The prediction of drug susceptibility was executed for the two AML populations with low expression of RFESD. Collectively, RFESD low expression group might be sensitive to Ataxia-telangiectasia mutated(ATM) kinase inhibitor(KU-55933), phosphoinositide 3-kinase(PI3K) inhibitors(Pictilisib, GNE-317, AZD6482), Aurora A/B/C kinases inhibitor(Tozasertib), mitogen-activated extracellular signal-regulated kinase(MEK) inhibitor(Trametinib, Selumetinib), cell cycle inhibitors(Epirubicin, NU7441), apoptosis induced molecule(MG-132), mTOR inhibitor(AZD2014), and extracellular regulated protein kinases(ERK) inhibitor(ERK-2440) (Fig. 5a-l). These results implied that low expression of RFESD was significantly correlated with chemotherapy and targeted therapy regimens for AML.

(a) KU − 55933. **(b)** NU7441. **(c)** Pictilisib. **(d)** Tozasertib. **(e)** Trametinib. **(f)** AZD2014. **(g)** Epirubicin. **(h)** ERK_2440. **(i)** Selumetinib. **(j)** MG − 132. **(k)** GNE − 317. **(l)** AZD6482. (*** $p < 0.001$, **** $p < 0.0001$).

Discussion

AML with unrestrained myeloid cell production is one of the most threatening hematological malignancies[34]. Although many new targeted agents have been approved for the clinical management of AML, remission rates and adverse prognosis have improved[35]. The highly heterogeneous nature of the disease has led an urgent need for more comprehensive and effective prognostic as well as therapeutic targets for AML. Evaluating molecular genetic damage as predictors and identifying prognostic markers is currently a relatively active area of research[36].

Hypoxic microenvironment promotes the occurrence of oxidative stress in leukemia cells, and produces peroxidation pressure on the lipid of cell membrane[37]. Iron-sulfur cluster (Fe-S) has been proved to rescue ferroptosis process through regulating iron homeostasis[38]. As a new non-apoptotic cell death model, ferroptosis has a profound impact on various pathological processes and cancers[39, 40], which also provides a new strategy for AML treatment and overcoming apoptosis resistance.

Based on mentioned above, we explored the TCGA database to confirm the relationship between AML clinical features and RFESD expression. As expected, down-regulation of RFESD was discovered to be significantly associated with poor clinical features and low survival rates. Therefore, it is suggested that low expression of RFESD may be consistent with the poor prognosis in AML patients.

Furthermore, we preliminarily explored the pathogenic molecular mechanisms of RFESD by bioinformatics analyses. DEGs enrichment pathways were focused on the immune response by GO analysis, while GSEA pathways also enriched in inflammatory response, IFNγ-response, TNF signaling via NFκB, IL6-JAK-STAT3 signaling, oxidative phosphorylation, and IFNα response. Inflammatory factors were associated with the proliferation of blast cells and the dysregulation of HSCs functions[41]. Therefore, we hypothesed that RFESD might work as the potential biomarker to regulate the immune signaling pathways.

Finally, PPI network analysis was performed to detect the potential hub genes. KLF1 was obviously linked to poor prognosis in AML. The RFESD expression was related to immune infiltration cells in AML, especially by B cells naïve, Dendritic cells activated, Mast cells activated, Follicular helper T, Blast cells and CD4 cells. The immune infiltration analysis illustrated that low expression of RFESD promoted the differentiation blockage and the immune response. We also found RFESD expression obtained the positive correlation with checkpoint genes (CD274, TIGIT, CD40LG, and CD160), which were markers of T cells and considered a dysfunction of antitumor immunity. These results may lead the deep exploration of RFESD in basic and clinical research for AML. Moreover, it has been identified that genetic aberrations lead to drug tolerance in AML with the advancements in next-generation sequencing technology[42, 43]. Drug sensitivity prediction was performed to provide the therapeutic strategy focus on RFESD, which suggested that patients with low expression of RFESD might be more sensitive to Pictilisib, Tozasertib, Trametinib, Epirubicin, and Selumetinib. Therefore, in considering combinations of AML drugs, clinical drugs selection should be made in conjunction with these results.

In summary, our research demonstrated low RFESD expression has a detrimental effect on overall survival in AML patients and may serve as the independent prognostic biomarker and immunotherapy

target for AML. This study may help clinicians explore and determine new targeted regimens for patients.

Declarations

Competing interests

Authors have no relevant financial or non-financial interests to disclose.

Author Contribution

JJY and HS designed the study. Xiaodan Wang collected the data. JJY and YZ performed the data analysis. HS and Xiaodan Wang designed the research and give an approval of the final mauscript. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The datasets presented in this study can be found in the GEO, TCGA and GETx repositories:

https://www.ncbi.nlm.nih.gov/geo/;

https://portal.gdc.cancer.gov/projects/TCGA-LAML; https://xenabrowser.net/datapages/.

The accession numbers can be found in the article.

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Figures

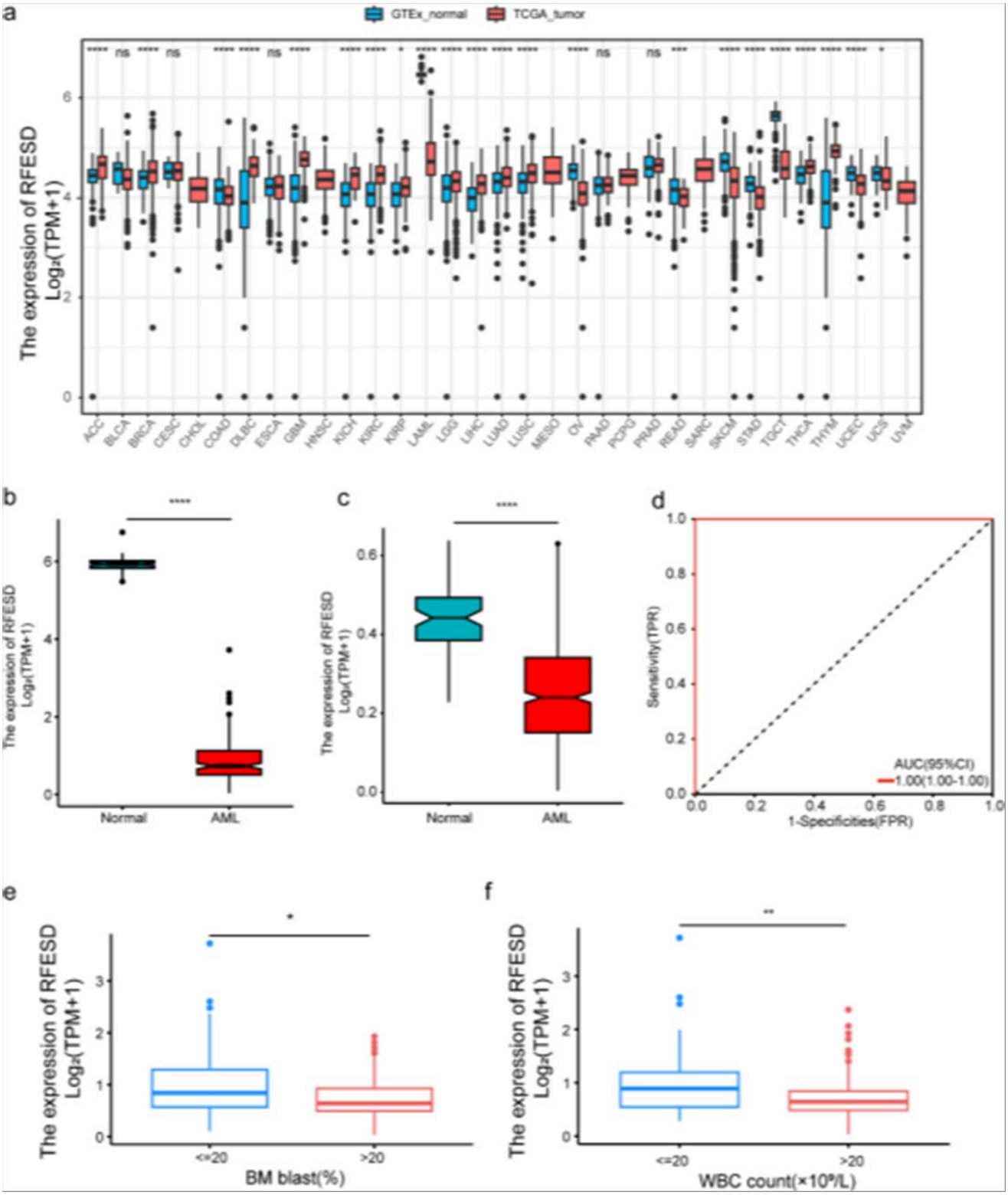


Figure 1

Low expression of RFESD in AML implicated adverse clinical features.

(a). Levels of RFESD expression in different tumors from TCGA and GTEx database. **(b)**Expression levels of RFESD in AML (n = 151) and normal samples (n = 70). **(c)**Expression levels of RFESD in GSE13159, AML (n = 501) and normal samples (n = 73). **(d)** ROC of RFESD in AML. Clinical characteristics including **(e)** BM blasts, **(f)** WBC counts. Analysis between two groups of unpaired samples: Wilcoxon rank sum test, analysis among multiple groups of samples: Kruskal–Wallis rank sum test (ns p ≥0.05, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

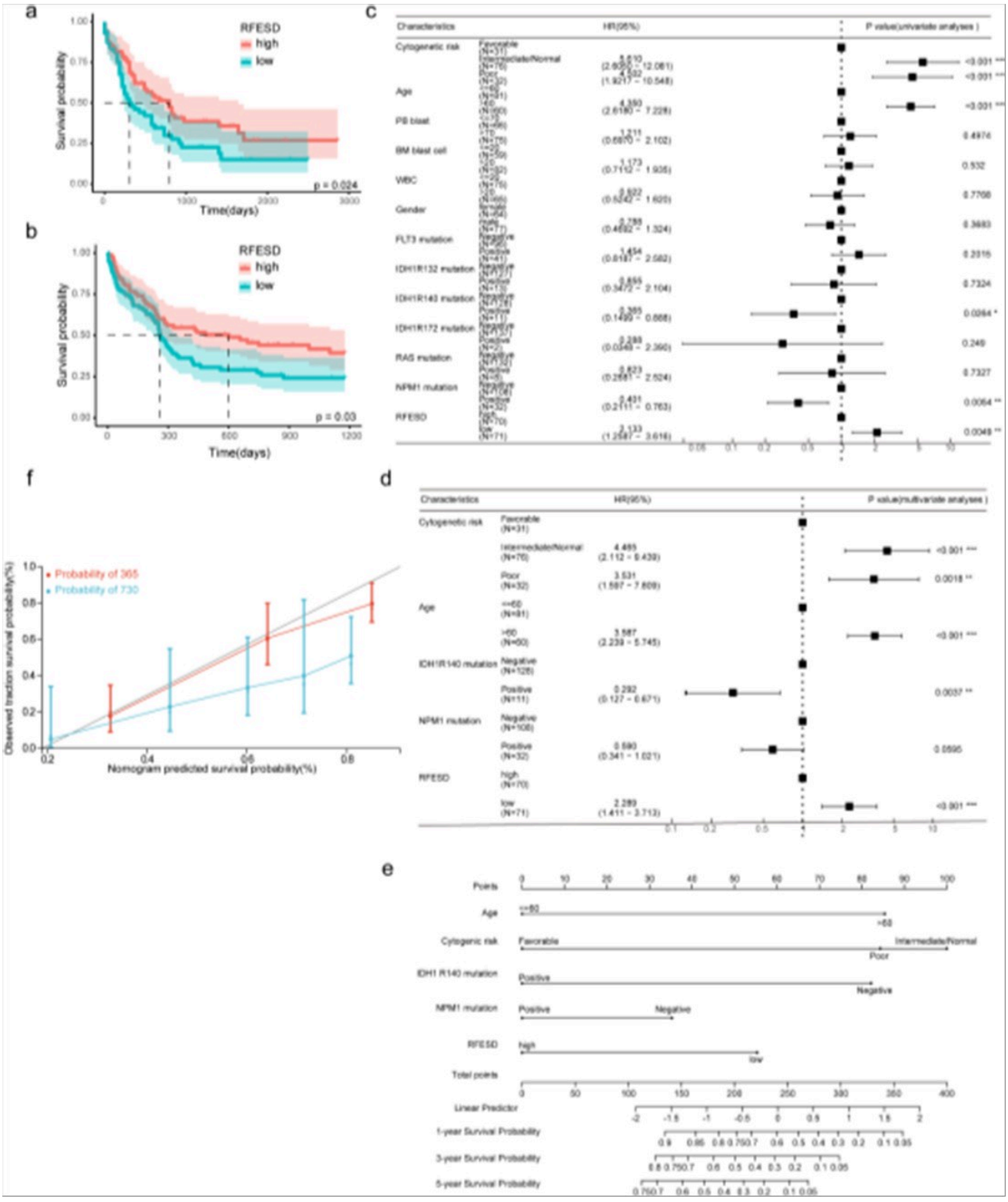


Figure 2

Low expression of RFESD predicts worse prognosis in AML. KM curve analysis of OS between high and low RFESD expression groups, **(a)** TCGA-LAML cohort, **(b)** GSE12417. **(c)** Univariate analyses of OS showed by forest plot. **(d)** Multivariate analyses of OS showed by forest plot. **(e)**Nomogram integrates RFESD and other prognostic factors in AML. **(f)** Calibration curve of nomogram.

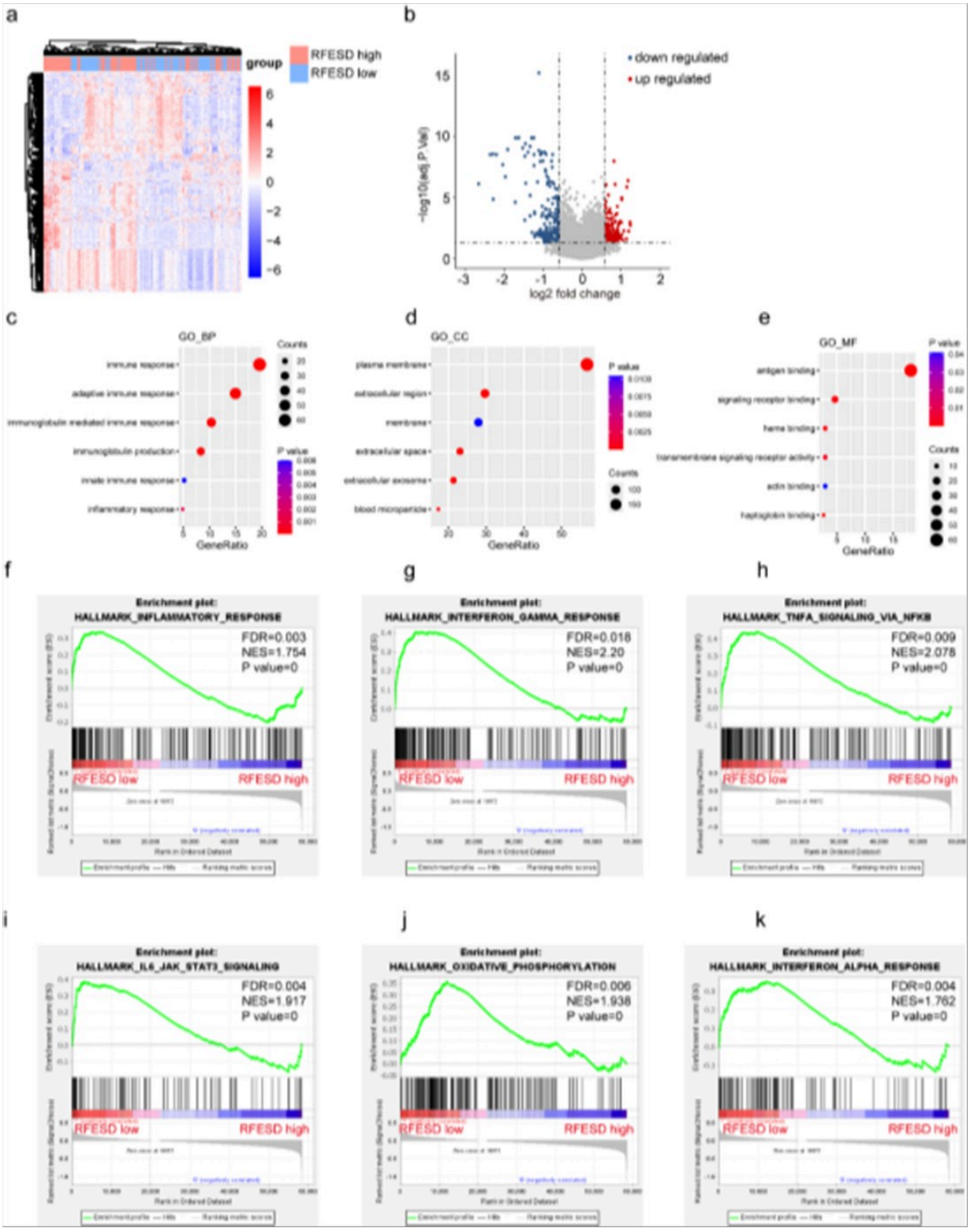


Figure 3

GO and GSEA analysis for DEGs based on RFESD expression were enriched in immune-related signaling pathways. (a) Heatmap of RFESD DEGs. **(b)** Volcano plot of DEGs. **(c-e)** Interactive analysis with result of GO analyses. **(f-k)** GSEA of DEGs.

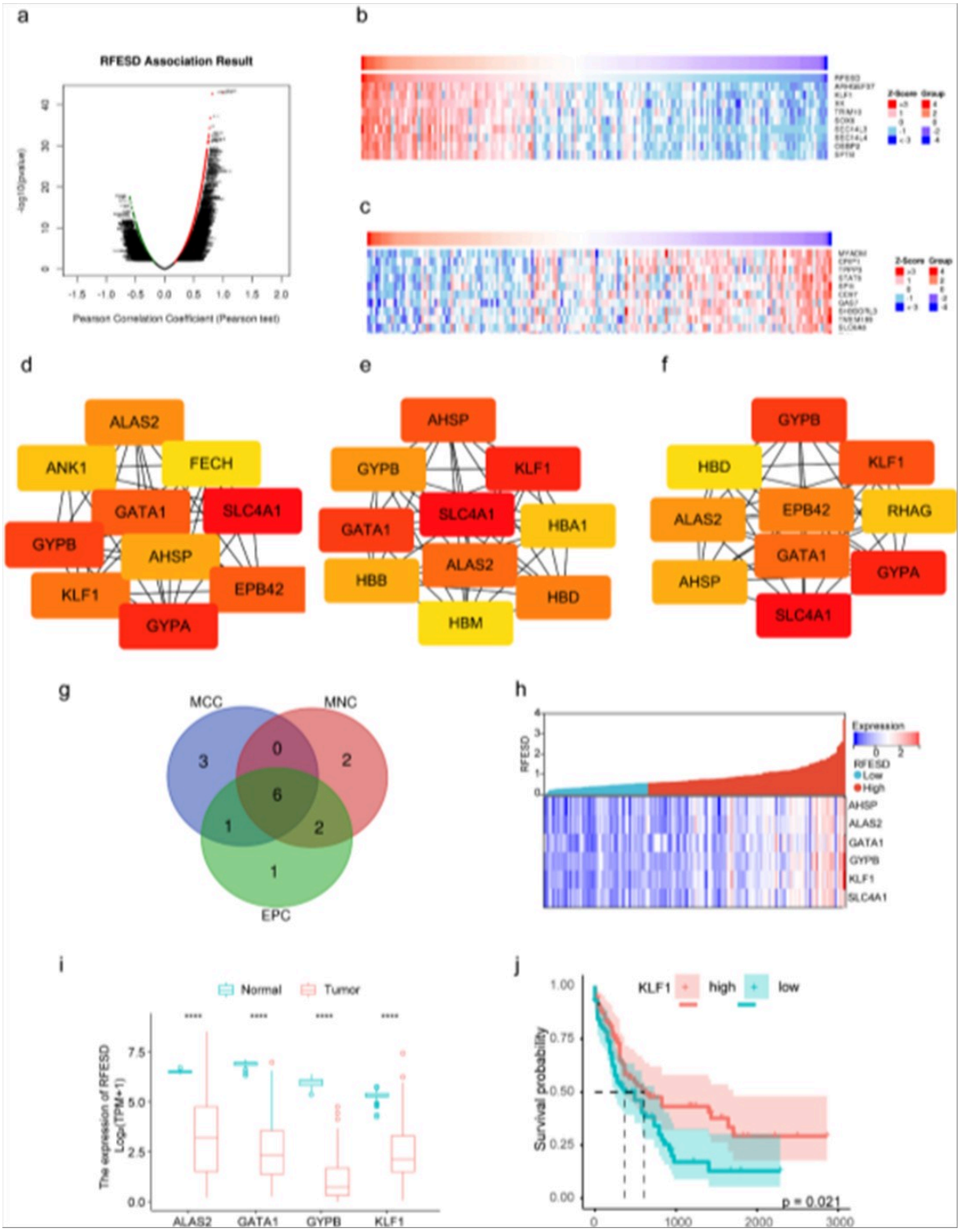


Figure 4

Identification of KLF1 as the hub gene connected with RFESD expression.

(a) Co-expression analysis of RFESD in the TCGA-LAML dataset. **(b–c)** The top 10 positively/negatively correlated genes are displayed. **(d-f)** The top 10 hub genes were selected on the basic of **(d)** MNC, **(e)** MCC, and **(f)** EPC. **(g)**Venn diagram of hub genes from 3 modules. **(h)** The association of RFESD with hub genes (AHSP, ALAS2, GATA1, GYPB, KLF1 and SLC4A1). **(i)** Expression levels of hub genes in AML patients (n = 151) and normal samples(n = 70). **(j)**The difference in OS between patients with high and low KLF1 expression levels shown by Kaplan-Meier curves. (****, p < 0.0001).

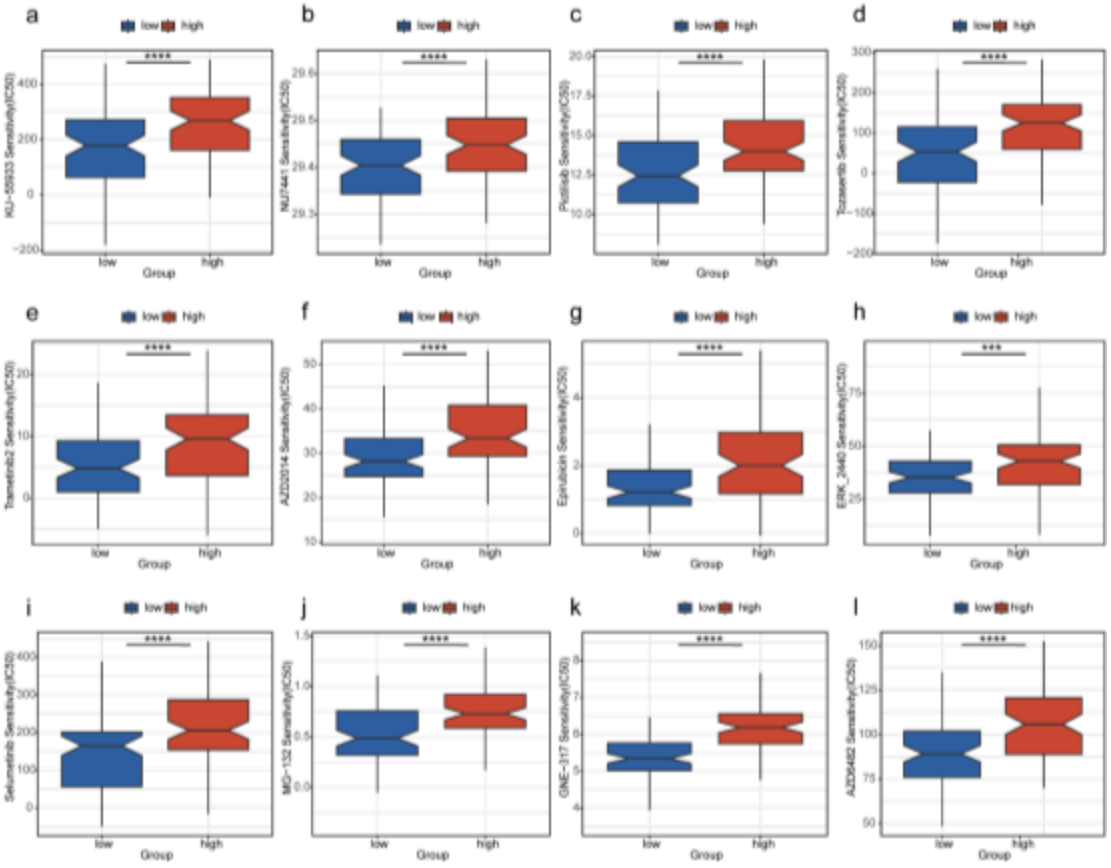


Figure 5

Drug sensitivity analysis of AML cells with low RFESD expression.

(a)KU−55933. **(b)** NU7441. **(c)** Pictilisib. **(d)** Tozasertib. **(e)**Trametinib. **(f)** AZD2014. **(g)** Epirubicin. **(h)** ERK_2440. **(i)**Selumetinib. **(j)** MG−132. **(k)** GNE−317. **(l)** AZD6482. (*** p < 0.001, **** p < 0.0001).

Supplementary Files

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