DNA methylation-based prognostic biomarkers of acute myeloid leukemia patients

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Background: Acute myeloid leukemia (AML) is a heterogeneous clonal disease that prevents normal myeloid differentiation with its common features. Its incidence increases with age and has a poor prognosis. Studies have shown that DNA methylation and abnormal gene expression are closely related to AML.

Methods: The methylation array data and mRNA array data are from the Gene Expression Omnibus (GEO) database. Through the GEO data, we identified differential genes from tumors and normal samples. Then we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses on these differential genes. Protein-protein interaction (PPI) network construction and module analysis were performed to screen the highest-scoring modules. Next, we used SurvExpress software to analyze the genes in the highest-scoring module and selected potential prognostic genes by univariate and multivariate Cox analysis. Finally, the three genes screened by SurvExpress software were analyzed using the methylation analysis site MethSurv to explore AML associated methylation biomarkers.

Results: We found three genes that can be used as independent prognostic factors for AML. These three genes are the low expression/methylation genes ATP11A and ITGAM, and the high expression/low methylation gene ZNRF2.

Conclusions: In this study, we performed a comprehensive analysis of DNA methylation and gene expression to identify key epigenetic genes in AML.

Keywords: Acute myeloid leukemia (AML); methylation; gene expression; biomarker

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Introduction

This study aims to analyze acute myeloid leukemia (AML); a group of genetically heterogeneous malignant clonal diseases that can block normal myeloid differentiation (1), as well as AML which is also the most common type of adult leukemia with the poorest prognosis.

Only in the United States of 2019, there have been 10,920 deaths, and 21,450 new cases of AML diagnosed (2). At present, induction therapy for AML is based primarily on cytotoxic drugs that enable complete remission (CR) in 70% of adult patients (3). However, the likelihood of the recurrence rate in AML patients is still high, especially in older patients with prognosis of having high-risk factors. Yet, the long-term cure rate was lower in the absence of allogeneic hematopoietic stem cell transplantation (Allo-HCT) in adult AML patients with first complete remission (CR1) (4).

With the emergence of various molecular targeted therapies for AML in the last two years, there are now several new drugs and other clinical trials currently underway; however, there is not enough data to know which newly approved drugs should be chosen or in which order or combination they should be used in (2). New early diagnostic biomarkers and therapeutic targets are therefore urgently needed to improve the diagnosis and risk management of AML in order to reduce the mortality rate of AML.

Epigenetic modifications include a class of heritable non-genetic changes in gene expression, usually including DNA methylation, histone modifications, and chromatin remodeling (5). In healthy hematopoietic stem cells, epigenetic processes play a key role in cell differentiation and hematopoiesis (6). Among them, DNA methylation affects the function of key genes. It is closely related to tumors by silencing tumor suppressor genes and activating oncogenes by high/low methylation (7). Abnormal DNA methylation is considered a hallmark of AML and is considered a powerful epigenetic marker in early diagnosis, prognosis prediction, and treatment decision making (8).

Abnormal gene expression is closely related to tumor prognosis. Studies have shown that *NPM1* (9), *FLT3* (10,11), C-*KIT* (12), AML1-*ETO* (13), *RUNX1* (3,14), *TP53* (3,15), *CBFB/MYH11* (13,16), *TET2* (17), *DNMT3A* (18), *JAK-STAT* (19), and *CXCR4* (20,21) ,*HOXA* family (22), *NAT10* (23) gene are associated with prognosis of AML. A few studies have shown altered DNA methylation in cancer, but the roles of key differentially methylated genes (DMGs) and differentially expressed genes (DEGs) in AML remain unclear.

In this study, we performed a comprehensive analysis of DNA methylation and gene expression to identify key epigenetic genes in AML. The methylation genes and differential genes of AML patients and normal individuals were downloaded from the GEO database. After data preprocessing, we identified differential genes between tumors and normal samples and performed KEGG and GO analyses on these genes. Protein-protein interaction (PPI) network construction and module analysis were then performed, and the highest-scoring modules were screened. SurvExpress software and analyzed the genes to be assigned the highest-scoring module with a P value <0.05 were selected to perform survival analysis and risk assessment in the cancer dataset. Finally, MethSurv analyzed the three genes screened by SurvExpress software to explore methylation biomarkers associated with AML survival.

Methods

Microarray data

We extracted gene expression (GSE114868) and methylation (GSE63409) profiling data from the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information.

The AML-associated dataset GSE63409 submitted by Jung N based on the GPL13534 platform was obtained from the GEO database and included 15 AML samples and 5 normal samples. The AML-associated dataset GSE114868 submitted by Huang H based on the GPL17586 platform was obtained from the GEO database and included 194 AML samples and 20 normal samples (*Figure 1*).

Identification of DEGs

GEO provides users with a useful tool called GEO2R that can be used to analyze microarray data. GRO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) were used to analyze gene expression in GSE114868 and GSE63409. Gene expression (GSE114868) and methylation (GSE63409) data set genes were considered statistically significant as |t| ≥2.0 and adj P<0.01. Then, we identified hypomethylated/upregulated genes via overlapping the hypomethylated and overexpression gene lists and identified hypermethylated/downregulated genes via overlapping the hypermethylated and low-expression gene lists. Finally, Then, we use the
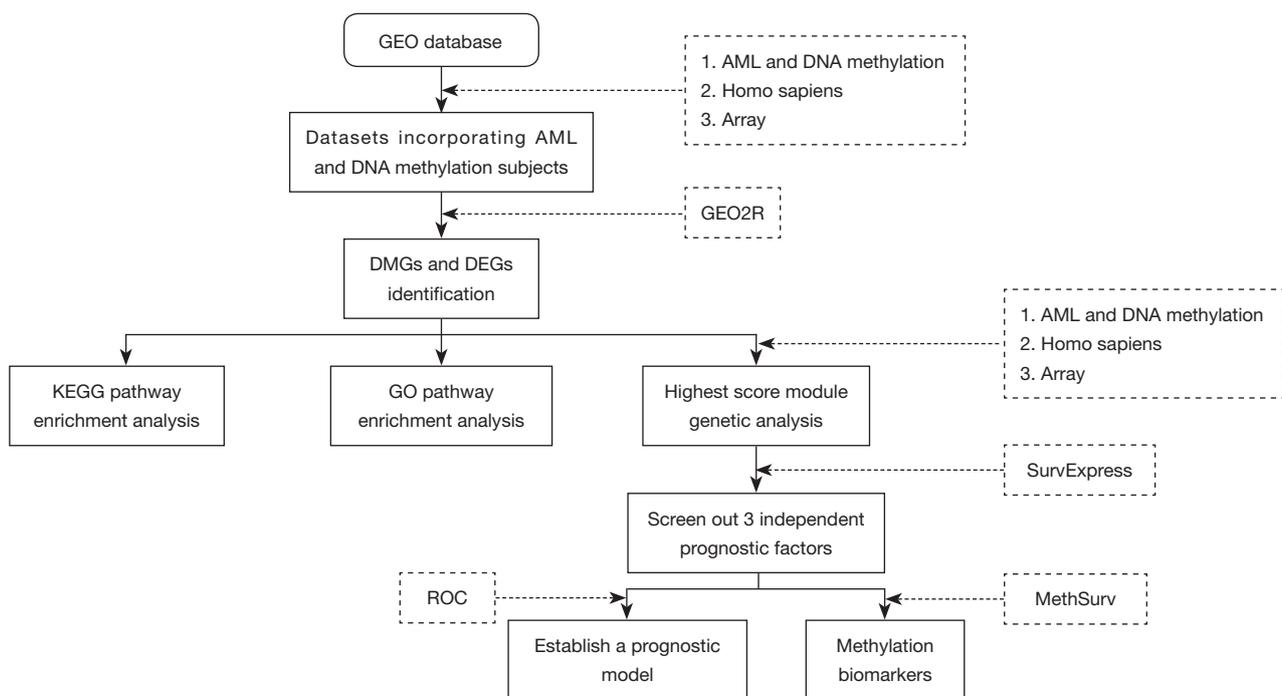
Figure 1 Graphical abstract.

Venn diagram network tool to draw the Venn diagram. (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Functional enrichment analyses for module genes

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and utilities of the biological system from molecular-level information. The Gene Ontology (GO) could be used to perform enrichment analysis. We used DAVID (https://david.ncifcrf.gov/) to make KEGG pathway analysis and GO enrichment analysis, including Biological Process, Cellular Component, and Molecular Function for the hypomethylated/upregulated genes and hypermethylated/downregulated genes.

PPI network construction and module analysis

PPI analysis is used to search core genes and gene modules related to carcinogenesis. In this study, the PPI network analysis of the hypomethylated/upregulated genes and hypermethylated/downregulated genes were performed using the search tool for the Retrieval of Interacting Genes (STRING) database. The interaction score was set at 0.7.

Then, MCODE plugin in Cytoscape is used to find clusters with the degree cut-off, haircut on, k-core, node score cut-off, max depth set as 10, 0.2, 2, 0.2 and 100 in PPI network and the module with the highest score would be picked out to make survival analyses.

Survival analysis and Cox regression, ROC curve

SurvExpress is a comprehensive gene expression database and web-based tool that uses biomarker gene lists as input to provide survival analysis and risk assessment in cancer data sets (24). We selected genes in the highest-scoring module of the hypermethylated low expression and low methylation high expression overlapping genes, respectively. The univariate Cox model was used to calculate the association between gene expression levels and patient overall survival (OS). When the P value was <0.05, its modular gene was used as an independent prognostic factor for patient survival. AML samples are divided into two groups: (I) high-risk group; (II) low-risk group. Survival analysis was performed by Kaplan-Meier survival plots, log-rank P value, and hazard ratio (HR, 95% confidence interval). By comparing the sensitivity and specificity of risk-based survival predictions, the accuracy of prognostic
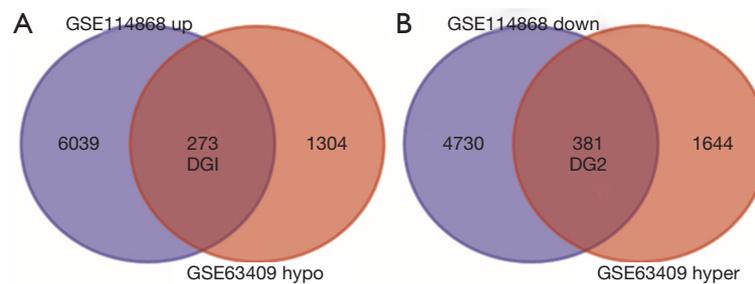
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Figure 2 Identification of aberrantly methylated differentially expressed genes in gene expression datasets (GSE114868) and gene methylation datasets (GSE63409). (A) Hypomethylation and upregulated genes; (B) hypermethylation and downregulated genes.
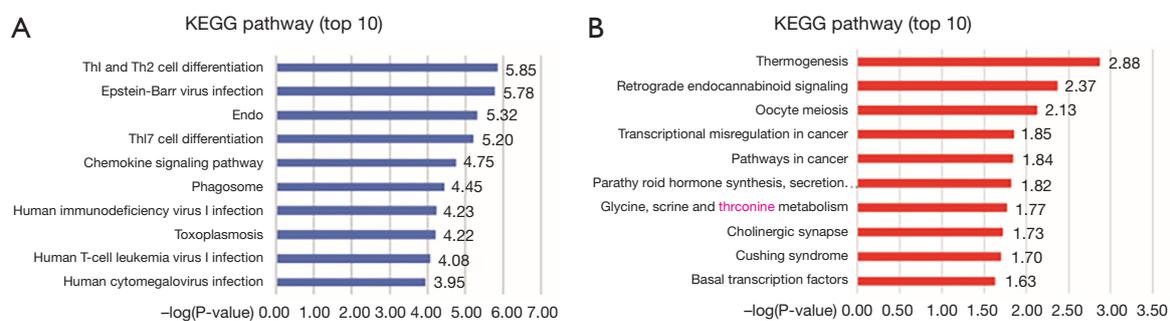

Figure 3 Analysis of the top ten pathways of KEGG for aberrant methylation differentially expressed genes in AML. (A) Hypermethylation and downregulated genes pathway names; (B) hypomethylation and upregulated genes pathway names.

performance was assessed using ROC curves of patients over time, 10, 30, 50, and 70 months, respectively.

DNA methylation data in MethSurv

MethSurv is used to explore methylation biomarkers associated with the survival of various human cancers (25). MethSurv is freely available at https://biit.cs.ut.ee/methsurv. Through the MethSurv website, we will analyze the DNA methylation analysis of the selected AML-related genes in the TCGA database.

Results

Identification of DEGs and DMGs in AML

After obtaining DEGs and DMGs, 6,039 upregulated genes and 4,730 low-expression genes in GSE114868 were found from the gene expression microarray analysis. There were 1,644 hypermethylated genes and 1,304 hypomethylated genes in GSE63409 identified by the gene methylation microarray analysis. Further analysis of overlapping genes

revealed 273 hypomethylated/upregulated genes and 381 hypermethylated/downregulated genes (*Figure 2A,B*).

KEGG pathway analysis

KEGG analysis was performed with hypomethylated/high-expression genes and hypermethylated/low-expression genes, and the first ten pathways were selected. Hypermethylated/low-expression genes were enriched in Th1 and Th2 cell differentiation, Epstein-Barr virus infection, Endocytosis, Th17 cell differentiation, Chemokine signaling pathway, Phagosome, Human immunodeficiency virus 1 infection, Toxoplasmosis, Human T-cell leukemia virus 1 infection, Human cytomegalovirus infection (*Figure 3A*). The hypomethylated/high-expression genes were significantly enriched in the thermogenesis, retrograde endocannabinoid signaling, oocyte meiosis, transcriptional misregulation in cancer, pathways in cancer, parathyroid hormone synthesis, secretion and action, glycine, serine and threonine metabolism, cholinergic synapse, Cushing syndrome, basal transcription factors
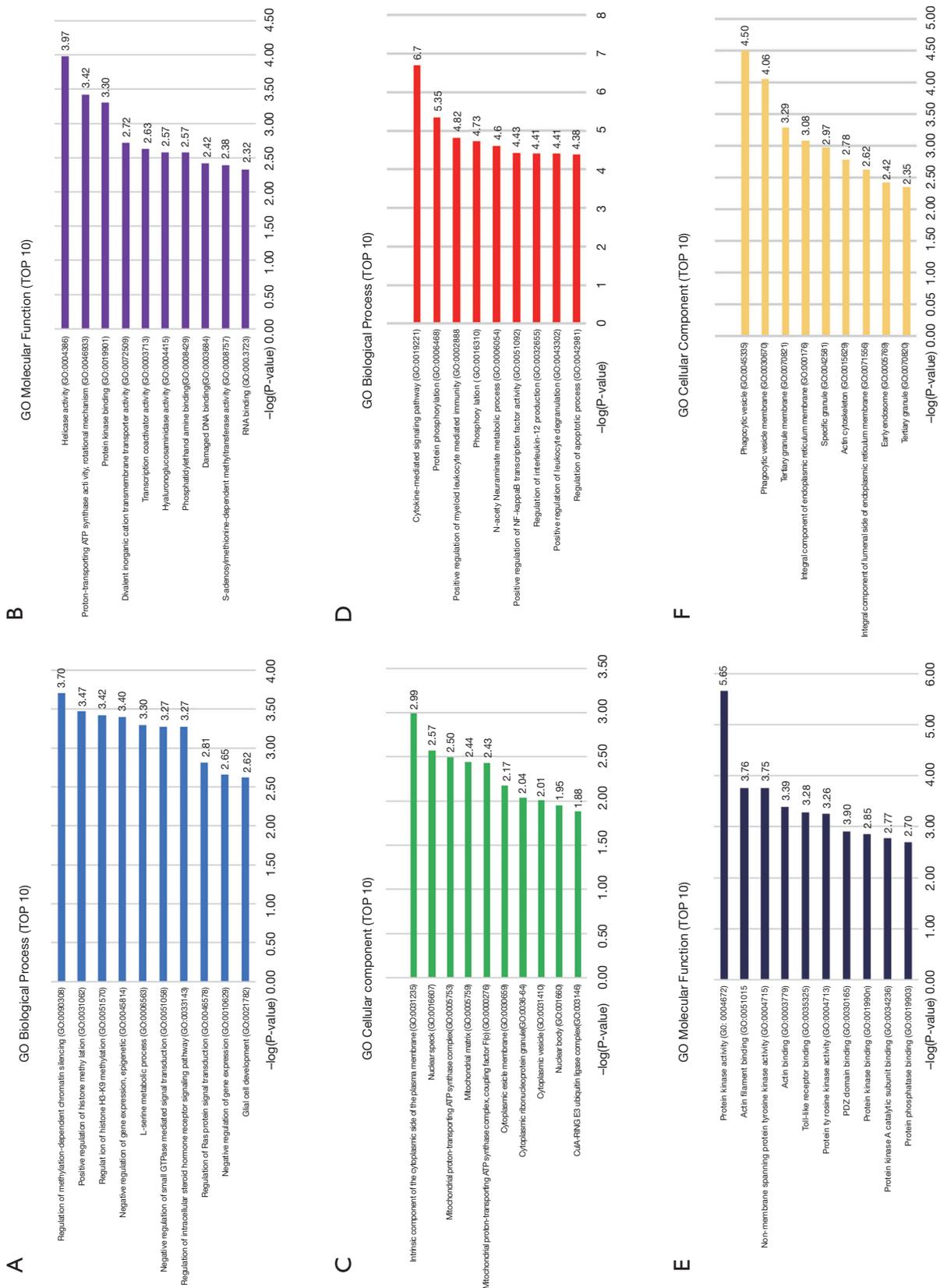
Figure 4 Analysis of the top ten pathways of GO for aberrant methylation differentially expressed genes in AML. (A,B,C) Hypomethylation and upregulated genes pathway names; (D,E,F) hypermethylation and downregulated genes pathway names.

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(*Figure 3B*).

GO term analysis

GO analysis was performed with hypomethylated/high-expression genes and hypermethylated/low-expression genes, and the first ten pathways were selected. Regarding the biological processes (BP), the hypomethylated/upregulated genes were significantly enriched in regulation of methylation-dependent chromatin silencing, positive regulation of histone methylation, regulation of histone H3-K9 methylation, negative regulation of gene expression, epigenetic, L-serine metabolic process, regulation of intracellular steroid hormone receptor signaling pathway, negative regulation of small GTPase mediated signal transduction, regulation of Ras protein signal transduction, negative regulation of gene expression, glial cell development (*Figure 4A*). In the molecular function (MF), the hypomethylated/high-expression genes were enriched in helicase activity, proton-transporting ATP synthase activity, rotational mechanism, protein kinase binding, divalent inorganic cation transmembrane transporter activity, transcription coactivator activity, phosphatidylethanolamine binding, hyaluronoglucosaminidase activity, damaged DNA binding, S-adenosylmethionine-dependent methyltransferase activity, RNA binding (*Figure 4B*). In the cellular component (CC), the analysis revealed that enrichment mainly occurred at intrinsic component of the cytoplasmic side of the plasma membrane, nuclear speck, mitochondrial proton-transporting ATP synthase complex, mitochondrial matrix, mitochondrial proton-transporting ATP synthase complex, coupling factor F(o), cytoplasmic vesicle membrane, cytoplasmic ribonucleoprotein granule, cytoplasmic vesicle, nuclear body, Cul4A-RING E3 ubiquitin ligase complex (*Figure 4C*).

The BP enriched by the hypermethylated/low-expression genes included cytokine-mediated signaling pathway, protein phosphorylation, positive regulation of myeloid leukocyte mediated immunity, phosphorylation, N-acetylneuraminate metabolic process, positive regulation of NF-kappaB transcription factor activity, positive regulation of leukocyte degranulation, regulation of interleukin-12 production, regulation of apoptotic process, interferon-gamma-mediated signaling pathway (*Figure 4D*). In the MF, the hypomethylated/high-expression genes were enriched in protein kinase activity, actin filament binding, non-membrane spanning protein tyrosine kinase activity, actin-binding, Toll-like receptor binding, protein

tyrosine kinase activity, PDZ domain binding, protein kinase binding, protein kinase A catalytic subunit binding, protein phosphatase binding (*Figure 4E*). In the CC, the hypermethylated/low-expression genes were enriched in the phagocytic vesicle, phagocytic vesicle membrane, tertiary granule membrane, is an integral component of endoplasmic reticulum membrane, specific granule, actin cytoskeleton, which is integral component of lumenal side of endoplasmic reticulum membrane, early endosome, tertiary granule, Golgi membrane (*Figure 4F*).

PPI network construction and module choice

PPI network analysis was performed through the STRING database and Cytoscape software. We obtained a PPI network map of hypomethylation/upregulation genes and hypermethylation/downregulation genes. The PPI network map of the hypomethylated/upregulated gene has 256 nodes and 176 edges (*Figure 5*), and the PPI network map of the hypermethylated/downregulated gene has 373 nodes and 309 edges (*Figure 6*). The above two PPI network diagrams were respectively imported into the Cytoscape software to construct a module through the MCODE plug-in, in which the hypomethylation/upregulation gene was constructed into 9 modules, and the hypermethylation/downregulation gene was constructed into 12 modules. We selected the highest scoring modules (*Figure 7A,B*).

Survival analysis of genes

To assess whether the identified prognostic markers are valuable for predicting patient survival, we focused on the genes in the highest-scoring modules. We used SurvExpress software to analyze hypomethylated/upregulated, and hypermethylated/downregulated genes for univariate and multivariate Cox regression analysis. AML samples were divided into two groups: (I) high-risk group; (II) low-risk group. First, we performed the univariate Cox regression analysis of the highest-scoring module gene. The hypermethylation/downregulation gene module 1 has 20 genes, and the hypomethylation/upregulation gene module 1 contains 7 genes (*Table 1*). We screened for genes with P values <0.05 (*ATP11A*, *ITGAM*, *ZNRF2*). These three genes were then subjected to multivariate Cox regression analysis and found to have P values <0.05 (*Table 2*). The three genes were independent prognostic factors, and then we use three genes to establish a prognostic model (*Figure 8A,B*). The ROC curves assessed the accuracy of the
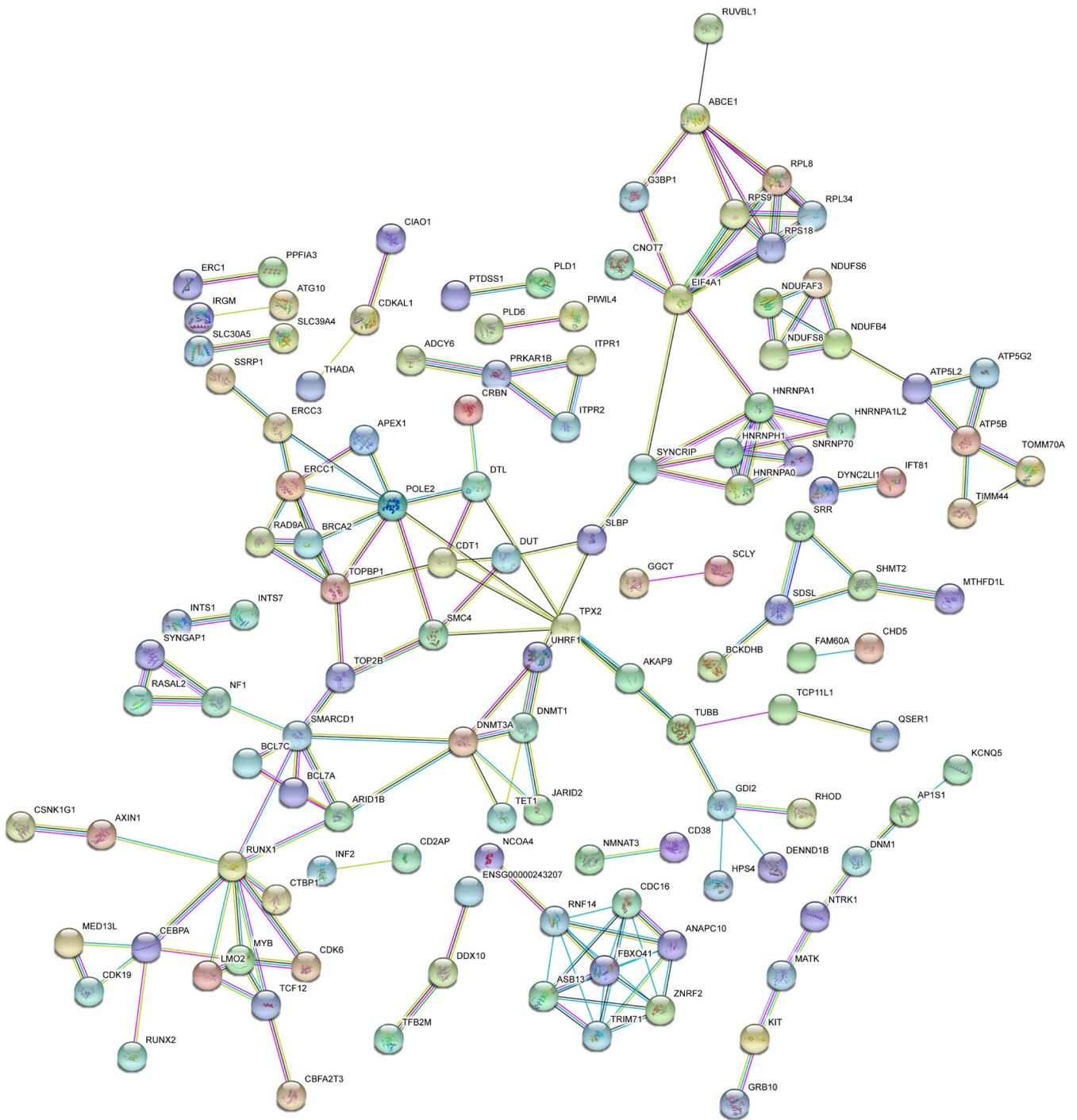
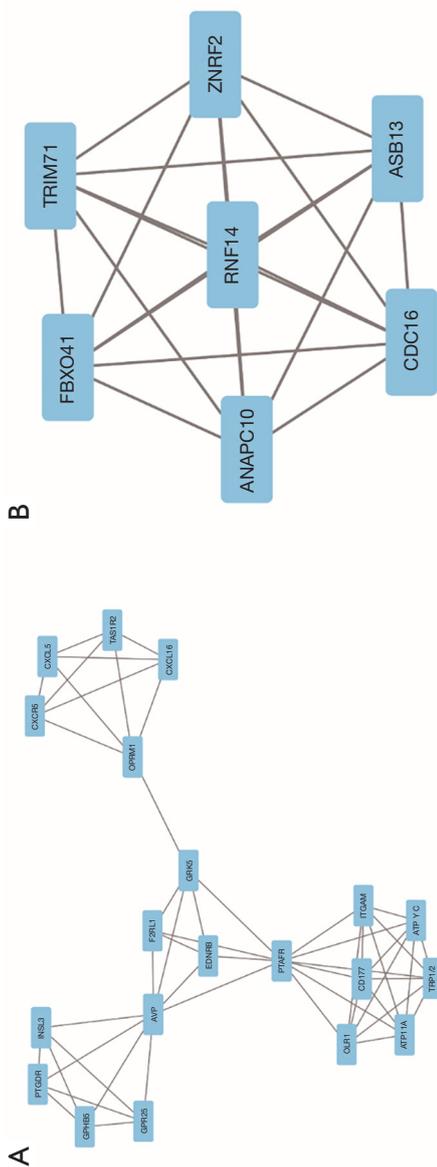**Figure 5** PPI network of hypomethylation/upregulation genes.

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Figure 6 PPI network of hypermethylation/downregulation genes.

Table 1 The highest scoring module gene

Hypermethylation/downregulation module 1 genes
PTGDR
TRPM2
OLR1
ATP11A
GPR25
F2RL1
CXCR5
CD177
ATP6V0C
EDNRB
TAS1R2
AVP
GRK5
INSL3
ITGAM
CXCL16
CXCL5
OPRM1
GPHB5
PTAFR
Hypomethylation/upregulation module 1 genes
FBXO41
CDC16
ASB13
ANAPC10
RNF14
ZNRF2
TRIM71

Figure 7 The highest scoring module. (A) Hypermethylation/downregulation gene module 1; (B) hypomethylation/upregulation gene module 1.

Table 2 Three survival-related genes based on multivariate Cox regression analysis

Genes	HR	95% CI	P value
ATP11A	0.599	0.441–0.813	0.001
ITGAM	1.305	1.144–1.488	<0.001
ZNRF2	0.579	0.408–0.822	0.002

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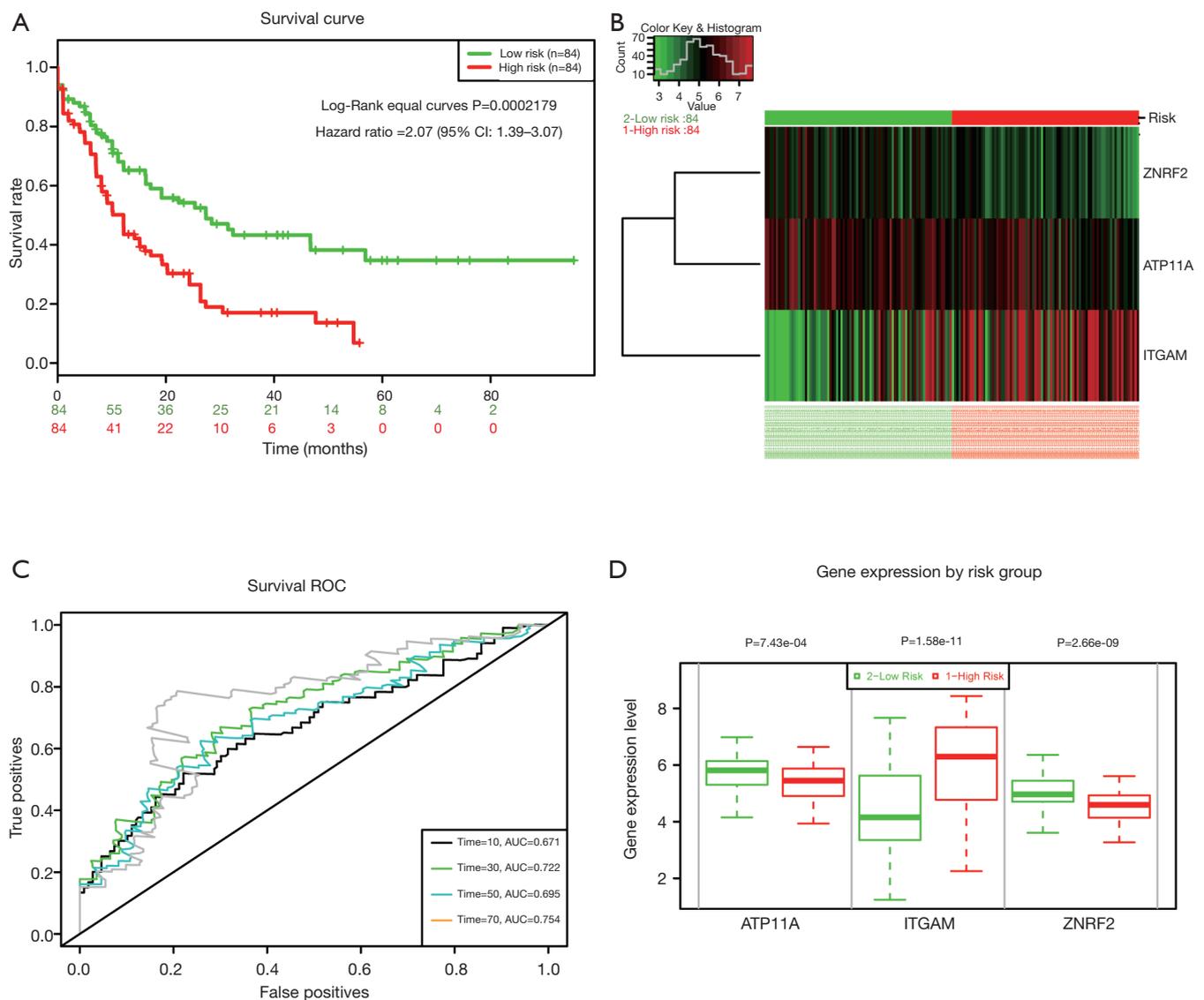Hu et al. Prognostic markers of AML methylation genes

Figure 8 Multivariate Cox Regression analysis of three genes. (A) Kaplan-Meier survival curves for overall survival outcomes according to the risk cutoff point; (B) the heatmap of three independent AML-related prognostic genes. 2 genes were Hypermethylation/downregulation genes, and 1 gene were Hypomethylation/upregulation genes; (C) time-dependent ROC curves analysis for survival prediction by the three key genes; (D) Box plot of three key genes expression by risk groups.

prognostic model at 10, 30, 50, and 70 months (*Figure 8C*). These three genes were used to evaluate the prognosis of AML cytogenetic risk typing and cell morphological typing and found that these three genes differed in the intermediate/Normal and M2 prognostic analysis (*Table 3*). Finally, we show the expression levels and risk assessment of three key genes (*Figure 8D*).

DNA methylation data in MethSurv

MethSurv is used to explore methylation biomarkers associated with the survival of various human cancers. MethSurv analyzed DNA methylation in TCGA. We performed methylation analysis of three genes (*ATP11A, ITGAM, ZNRF2*) that can be used as independent

Table 3 Cytogenetic risk typing and morphological cell typing of these three genes based on multivariate Cox regression analysis in AML

Class	HR	95% CI	P value
CYTO-RISK-Favorable	2.750	0.570–13.300	0.190
CYTO-RISK-Intermediate/normal	1.750	1.050–2.900	0.029
CYTO-RISK-Poor	1.360	0.570–3.250	0.475
MORPHO-CODE-M0 undifferentiated	1.090	0.300–3.910	0.895
MORPHO-CODE-M1	1.750	0.740–4.100	0.195
MORPHO-CODE-M2	2.850	1.220–6.660	0.010
MORPHO-CODE-M3	3.990	0.410–38.940	0.198
MORPHO-CODE-M4	1.530	0.680–2.440	0.295
MORPHO-CODE-M5	0.880	0.240–3.200	0.849

AML, acute myeloid leukemia.

Table 4 The significant prognostic value of CpG in three key genes

Gene-CpG	HR	LR test P value
ATP11A-Body-Open_Sea-cg00990020	2.207	<0.001
ATP11A-Body-N_Shelf-cg04656015	2.273	<0.001
ATP11A-Body-N_Shelf-cg24199463	2.224	0.001
ATP11A-Body-S_Shelf-cg04229372	2.130	<0.001
ATP11A-Body-Open_Sea-cg00717219	1.837	0.001
ATP11A-Body-Open_Sea-cg25347801	1.826	0.002
ATP11A-Body-Open_Sea-cg21649349	1.975	0.004
ATP11A-Body-N_Shelf-cg03353885	1.881	0.002
ATP11A-Body-S_Shelf-cg09804528	1.866	0.002
ATP11A-Body-Open_Sea-cg17218041	1.884	0.008
ITGAM-Body-Island-cg05625471	1.558	0.036
ITGAM-Body-Island-cg02256631	1.523	0.045
ZNRF2-Body-Open_Sea-cg07510230	0.581	0.014
ZNRF2-Body-Open_Sea-cg07568841	0.655	0.023
ZNRF2-TSS1500-N_Shore-cg21557180	1.556	0.046

prognostic factors for AML in SurvExpress software. In the MethSurv software, we found that the P value of 73 CpG sites in the hypermethylated/down-regulated *ATP11A* in AML was <0.05, which we considered statistically significant. The top ten sites were shown in *Table 4*. Similarly, the hypermethylation/downregulation gene *ITGAM* has two CpG sites with a P value of <0.05, and the hypomethylation/upregulation gene *ZNRF2* has three CpG sites with a P value of <0.05, which is statistically significant (*Table 4*). We found that the difference in DNA methylation between cg00990020 of *ATP11A*, cg05625471 of *ITGAM*, and cg07510230 of *ZNRF2* was most pronounced (*Figure 9A,B,C*).

Discussion

AML is the most common acute leukemia in adults and is

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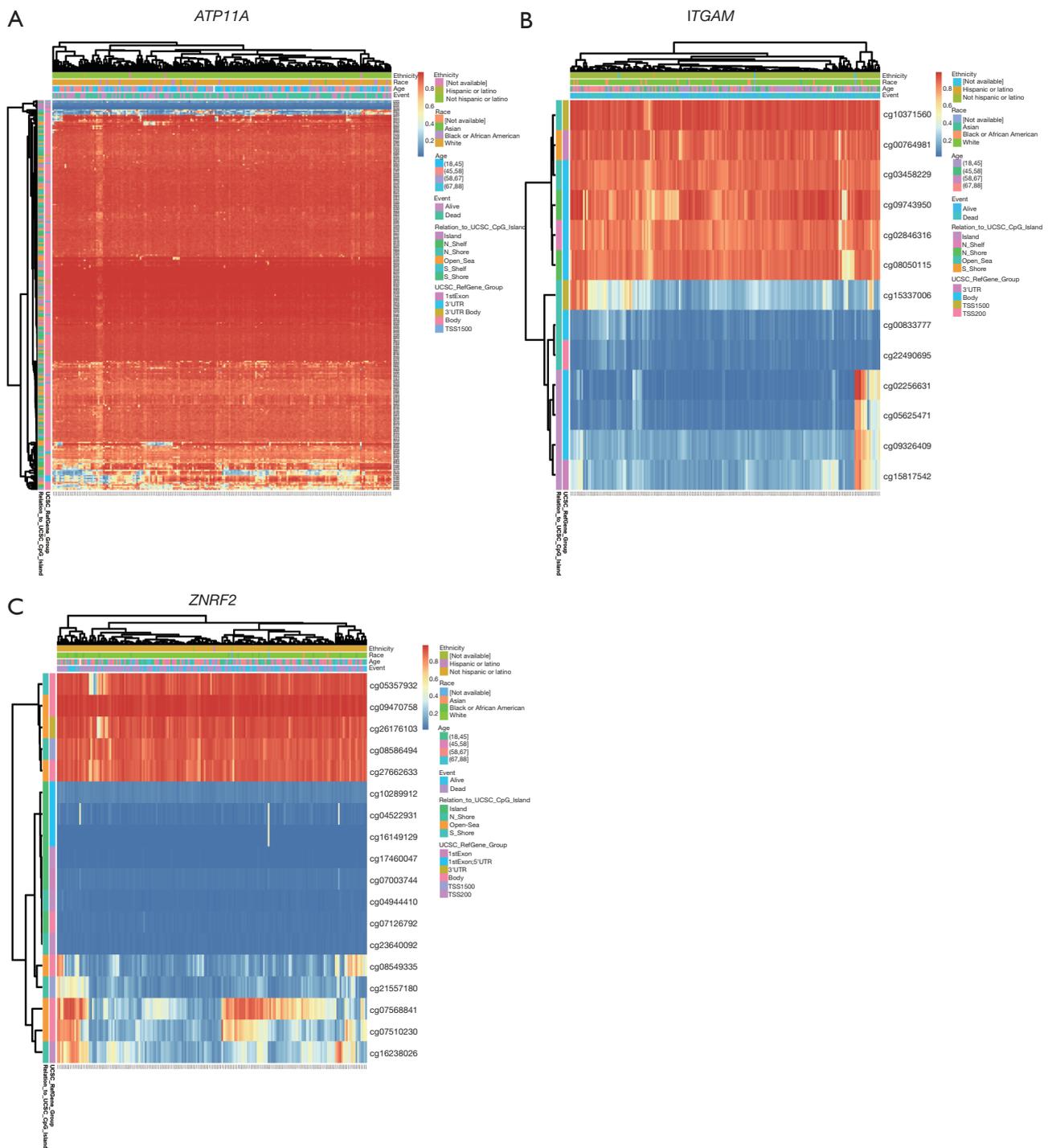

Figure 9 DNA methylation of three key genes in MethSurv. (A) *ATP11A*; (B) *ITGAM*; (C) *ZNRF2*.

a highly heterogeneous and fatal (1). DNA methylation is the most studied epigenetic modification and is essential in the promotion of many important BP (7). Abnormal DNA methylation can lead to a variety of pathological conditions, including carcinogenesis. (8) Certain DNA methylation engages in the initial stages of carcinogenesis, such as RASSF1A in ovarian cancer (26). In addition, DNA methylation is stable over some time and can be detected non-invasively in the blood (27). Therefore, DNA methylation has the exciting potential to become an early diagnostic biomarker for cancer. Multiple studies have shown that gene expression abnormalities are closely related to AML (10). Identification of novel biomarkers will aid in early diagnosis and improved prognosis. In this study, we performed a comprehensive analysis of DNA methylation and gene expression to identify key epigenetic genes in AML.

This study is the first to report bioinformatics studies and its association between gene methylation of AML and the corresponding mRNA expression. Through our research, we found three genes that can be used as independent prognostic factors for AML. These three genes are the low expression/methylation genes *ATP11A* and *ITGAM*, and the high expression/low methylation gene *ZNRF2*. *ATP11A* is an adenosine triphosphate binding cassette (ABC) transporter homolog gene and belongs to an extended family of ABC transporters that confer multidrug resistance to cancer cells. For example, in lymphocytic leukemia, cancer cells are resistant by increasing *ATP11A* expression (28). In previous studies, it was found that the expression level of *ATP11A* gene in colorectal cancer tumor tissues was significantly higher than that in corresponding normal tissues, and it was important for the prognosis evaluation of colorectal cancer (29). Studies have shown that the *ATP11A* gene is a methylation biomarker for prostate cancer and is expressed in patients with metastatic and lethal PCA (30). *ITGAM* is a major non-human leukocyte antigen associated with the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE) and IgA nephropathy (31). Recent reports that SNP rs4597342 in *ITGAM* 3'UTR affect miR-21 binding may be considered a risk factor for psoriasis development (32). However, the above two genes have not been reported in AML. *ZNRF2* is a ubiquitin ligase of the RING superfamily. It has been shown that membrane-associated E3 ubiquitin ligase *ZNRF2* is involved in mTor activation and regulation through protein interactions, and *ZNRF2* depletion reduces cell size and

cell proliferation (33). *ZNRF2* also plays a crucial role in tumorigenesis. For example, *ZNRF2* enhances the mTor and its downstream targets CyclinD1 and CDK in NSCLC cells, and the negative correlation between *ZNRF2* and miR-100 in osteosarcoma specimens, low miR-100 is associated with poor prognosis in OS patients (34,35). To date, the role of the *ZNRF2* gene in AML and how it regulates AML through aberrant methylation is unclear. These three genes may be good indicators for assessing the prognosis of AML. We used these three genes to set up an independent prognostic model with high accuracy, which can be used to assess the prognosis of patients with AML and as a good target for AML treatment.

This study had several limitations: (I) the small number of cases evaluated; (II) the results of the study have not been validated on clinical samples.

In summary, our research identified many aberrantly expressed genes and pathways that can be regulated by aberrant methylation in AML through a comprehensive analysis of gene expression and methylation microarrays. We identified some new markers and pathways through multi-database analysis that could be an accurate diagnosis and treatment for AML; however, determining the role of these identified genes in the AML process requires more research.

Acknowledgments

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work, and the questions related to the accuracy

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or integrity of any part of the work are appropriately investigated and resolved.

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