High expression of *FLT3* is a risk factor in leukemia

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Abstract. Several studies have shown that internal tandem duplication (ITD) of FMS-like tyrosine kinase 3 (*FLT3*) can result in the failure of leukemia treatment and contribute to a poor prognosis. However, the role of the overexpression of *FLT3* in leukemia remains to be fully elucidated. By mining public database, the present study first identified that the expression of *FLT3* in leukemia was markedly higher, compared with that in other types of tumor and cell lines, indicating that *FLT3* is important in leukemia. In leukemia, *FLT3* was found to be significantly upregulated in acute myeloid leukemia and acute lymphoblastic leukemia, and a high expression of *FLT3* contributed to reduced survival rates. By analyzing Gene Expression Omnibus and The Cancer Genome Atlas data, it was found that genetic alterations and modification of DNA methylation increased the expression of *FLT3* in leukemia. *FLT3*-ITD and *FLT3* tyrosine kinase domain point mutations increased the expression of *FLT3* in four independent datasets. In addition, the status of *FLT3* gene methylation was negatively correlated with the expression of *FLT3*, and haploinsufficiency of DNA methyltransferase 1 increased the expression of *Flt3* in mouse leukemia cells. By analyzing the enrichment of differentially-expressed genes in chemical and genetic perturbation datasets, it was found that genes, which were upregulated in the *FLT3* high expression group had myeloid lymphoid leukemia- and nucleophosmin 1-like signatures, indicating that the overexpression of *FLT3* may use the same mechanism to promote leukemia. Collectively, the results of the present study showed that the overexpression of *FLT3* is a potential risk factor in leukemia.

Introduction

The FMS-like tyrosine kinase 3 (*FLT3*) gene, encoding a membrane-bound receptor tyrosine kinase, is crucial in normal hematopoiesis (1,2). It has been reported that *FLT3* has two mutation types in leukemia, the most common form of *FLT3* mutation is an internal tandem duplication (ITD) within the juxtamembrane domain, which occurs in 15-35% of patients with acute myeloid leukemia (AML) (3-13) and 5-10% of patients with myelodysplasia (MDS) (14,15). Another mutation type is the missense point mutation on the tyrosine kinase domain (TKD), which occurs in 5-10% of patients with AML, 2-5% of patients with MDS and 1-3% of patients with acute lymphoblastic leukemia (ALL) (9,16,17). *FLT3*-ITD can promote ligand-independent dimerization, autophosphorylation and constitutive activation of the receptor, which lead to the aberrant activation of multiple signaling pathways, including phosphatidylinositol 3-kinase/AKT, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and signal transducer and activator of transcription 5 (STAT5) (18-20). *FLT3*-TKD also promotes constitutive phosphorylation of the receptor and ligand-independent cell growth (16,17,21).

FLT3 mutations are high risk factors in leukemia, and contribute to increased risk of treatment failure and poor prognosis (7,8). Mutations of *FLT3*-ITD have been reported to confer resistance to multiple tyrosine kinase inhibitors (22). The mutant allelic burden and ITD length are significantly associated with reduced overall survival and disease-free survival rates (23,24). The detection of ITD mutations at diagnosis is now a routine clinical practice to provide guidance for the optimal treatment of patients with AML.

Several previous studies have shown that the expression of *FLT3* is activated in acute promyelocytic leukemia (APL) and adult B lymphoblastic leukemia (25,26), and the upregulation of *FLT3* is a passive event in Hoxa9/Meis1-induced AML (27) indicating that the overexpression of *FLT3* may have a tumor-promotion effect. In addition, the overexpression of *FLT3* has been reported to activate the AKT and MAPK pathways, but not the STAT5 pathway (28), which suggests that the overexpression of *FLT3* has overlapping downstream pathways with *FLT3*-ITD.

In the present study, it was found that *FLT3* was upregulated in leukemia, and that the high expression of *FLT3* indicated a poor prognosis. By analyzing differentially-expressed genes (DEGs), certain leukemic oncogenes were identified, and the high expression of *FLT3* was found to have myeloid lymphoid leukemia (MLL)- and nucleophosmin 1-like (NPM1)-like signatures.

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Materials and methods

Expression profile analysis. *FLT3* expression data were collected from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), cBioportal (29,30), Oncomine (http://www.oncomine.org), Cancer Cell Line Encyclopedia (CCLE; https://portals.broadinstitute.org/ccle/home) (31) and the Human Protein Atlas (HPA; http://www.proteinatlas.org) (32). The expression levels of *FLT3* in various normal human tissues and cells were analyzed using three independent databases, which included the GEO GDS3834 dataset, and the HPA and Genotype-Tissue Expression (GTEx; https://www.gtexportal.org/home/) databases. The *FLT3* expression data were directly downloaded from the GEO and HPA databases; the tissue with the highest expression of *FLT3* was normalized to 1.

To compare the expression of *FLT3* in different types of cancer, data were directly downloaded from cBioportal, Oncomine and CCLE, and cancer cell lines with the same tissue origin were classified as the same group.

The expression levels of *FLT3* in different types of leukemia, including AML, B-cell ALL, B-cell childhood ALL, chronic lymphocytic leukemia, chronic myelogenous leukemia, myelodysplastic syndrome, pro-B ALL, and T-cell ALL, were analyzed using Oncomine. The peripheral blood mononuclear cells were considered a normal control.

The GDS4306 dataset was used to evaluate the effect of a DNA methyltransferase 1 (*DNMT1*) haploinsufficiency on the expression of *FLT3*. To compare the correlation between *FLT3* methylation and expression, data were downloaded from cBioportal to perform linear regression. The *FLT3* mutated samples and wild-type samples were analyzed separately.

Overall survival analysis. The GSE12417 GEO dataset, which contains data on survival rates and survival status, was selected to draw the overall survival curve, and the median expression of *FLT3* was used as the cut-off, according to a previous report (33), to classify patients into a high expression group and low expression group. The top 50% of patients were classified as the *FLT3* high expression group and the lowest 50% patients were defined as the *FLT3* low expression group, according to the expression of *FLT3* from high to low. The statistical difference between two curves was calculated using a log-rank test.

Analysis of genetic alterations. In order to summarize the genetic alterations of *FLT3* in different types of cancer, The Cancer Genome Atlas (TCGA) data were downloaded through cBioportal. A total of 30 types of cancer, including AML, skin cutaneous melanoma, colorectal adenocarcinoma, esophageal carcinoma, lung adenocarcinoma, stomach adenocarcinoma, lung squamous cell cancer, lymphoid neoplasm diffuse large B-cell lymphoma, bladder urothelial carcinoma, uterine corpus endometrial carcinoma, sarcoma, cholangiocarcinoma, prostate adenocarcinoma, breast invasive carcinoma, glioblastoma multiforme, liver hepatocellular carcinoma, ovarian serous cystadenocarcinoma, cervical squamous cell carcinoma and endocervical adenocarcinoma, head and neck squamous cell carcinoma, kidney renal clear cell carcinoma, uterine carcinosarcoma, brain lower grade glioma, pancreatic adenocarcinoma, pheochromocytoma and paraganglioma, adrenocortical carcinoma, thyroid carcinoma, testicular germ cell cancer, kidney renal papillary cell carcinoma, thymoma, kidney chromophobe and mesothelioma, were selected to analyze *FLT3* mutations and copy number alterations.

Screening of DEGs. Three GEO datasets of AML (GSE10358, GSE14468 and GSE34860) were selected to analyze the DEGs between the *FLT3* high expression group and *FLT3* low expression group. Initially, the raw data in the CEL file were downloaded from the GEO database (34), and the robust multiarray average algorithm in the affy R-3.3.1 package was used to perform background correction, normalization and expression calculation (35-37). The Limma package in R (38) was used to identify the DEGs at the probe level between these two groups. $P<0.05$ and $|\log2 \text{ fold change (FC)}|>0.585$ were used as the cut-off criteria. Finally, these probes were annotated into gene names.

Chemical and genetic perturbations enrichment of DEGs. In order to identify the association between the overexpression of *FLT3* and other risk factors for leukemia, the enrichment of DEGs in the chemical and genetic perturbations gene set were determined using Gene Set Enrichment Analysis (http://software.broadinstitute.org/gsea) (39,40). Input of the upregulated and downregulated genes was performed on the website separately, and chemical and genetic perturbations was selected to calculate the enrichment. $P<0.05$ and FDR q-value <0.05 were used as the cut-off criteria.

Statistical analysis. Student's t-test was used to calculate statistically significant differences between quantitative variables. The log-rank test was used to compare the overall survival curve. $P<0.05$ was considered to indicate a statistically significant difference. GraphPad Prism 5.01 was used for statistical analysis (GraphPad Software, Inc., La Jolla, CA, USA).

Results

FLT3 is upregulated in leukemia and a high expression of FLT3 is a prognostic biomarker. It has been reported that the *FLT3*-ITD mutation is correlated with prognosis and drug response in leukemia. To investigate the role of the expression of FLT3 in leukemia, the present study firstly evaluated the expression of *FLT3* in different types of cancer and normal tissues. In normal tissues, *FLT3* was expressed at high levels in bone marrow, lymph nodes, the thymus and spleen in three independent databases (Fig. 1A), which indicated that *FLT3* was involved in hematopoiesis. In cancer, the expression of *FLT3* was significantly higher in leukemia, compared with other types of tumor (Fig. 1B and C), and *FLT3* was specifically upregulated in AML and ALL (Fig. 1D), suggesting that *FLT3* contributed to the progression of AML and ALL. In order to analyze the association between the expression of FLT3 and survival rates in AML, the GSE12417 dataset, in which patients with AML exhibited a normal karyotype, was selected for analysis. Kaplan-Meier survival analysis showed that patients with AML with high expression levels of *FLT3* (n=121) had reduced survival rates, compared with those with low expression levels of *FLT3* (n=121; $P<0.05$; Fig. 1E). These

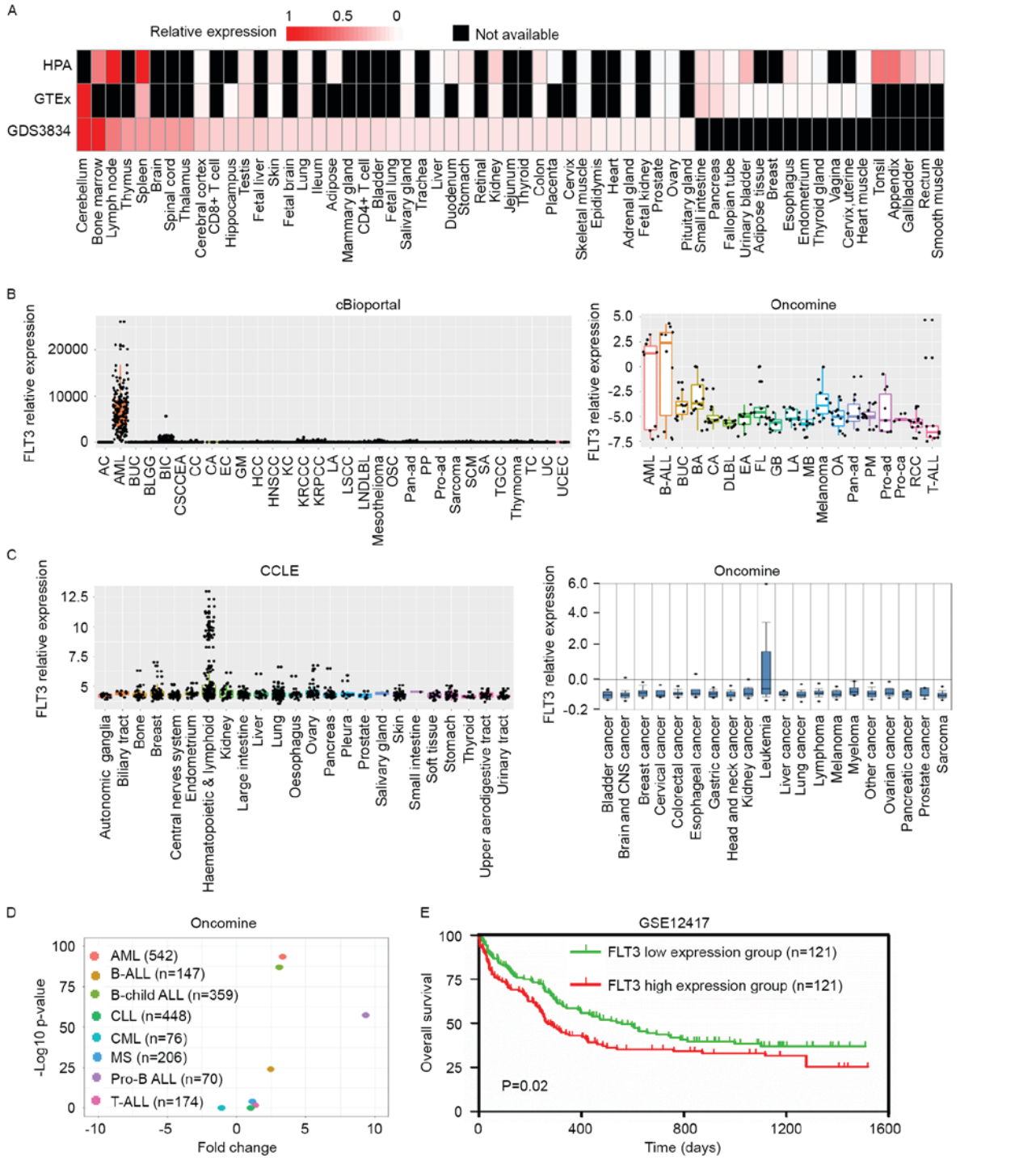

Figure 1. Tissue expression profile identifying the overexpression of *FLT3* in leukemia. (A) RNA expression of *FLT3* in various normal human tissues and cells, analyzed using three independent databases (HPA, GTEx and GDS3834). Red color indicates relative expression of *FLT3*, black indicates expression data are not available. (B) TCGA and Oncomine databases were used to evaluate expression levels of *FLT3* in different types of tumor. (C) Expression of *FLT3* in different cancer cell lines was analyzed using CCLE and Oncomine databases. Cell lines of the same tissue origin were classified into the same group. (D) *FLT3* was significantly upregulated in several types of leukemia. The X-axis shows the fold change between different types of leukemia and peripheral blood mononuclear cells. The Y-axis shows the log 10 transformed P-value. Different colors of points indicate different types of leukemia. (E) Results of Kaplan-Meier survival analysis showed that patients with AML and a high expression of *FLT3* (n=121) had shorter overall survival rates, compared with those with a low expression of *FLT3* (n=121). FLT3, FMS-like tyrosine kinase; HPA, Human Protein Atlas; GTEx, Genotype-Tissue Expression; CCLE, Cancer Cell Line Encyclopedia; AC, adrenocortical carcinoma; AML, acute myeloid leukemia; BUC, bladder urothelial carcinoma; BLGG, brain lower grade glioma; BIC, breast invasive carcinoma, CSCCEA, cervical squamous cell carcinoma and endocervical adenocarcinoma; CC, cholangiocarcinoma; CA, colorectal adenocarcinoma; EC esophageal carcinoma; GM, glioblastoma multiforme; HCC hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; KC, kidney chromophobe; KRCCC, kidney renal clear cell carcinoma; KRPCC, kidney renal papillary cell carcinoma; LA, lung adenocarcinoma; LSCC, lung squamous cell carcinoma; LNDLBL, lymphoid neoplasm diffuse large B-cell lymphoma; OSC, ovarian serous cystadenocarcinoma; Pan-ad, pancreatic adenocarcinoma; PP, pheochromocytoma and paraganglioma; Pro-ad, prostate adenocarcinoma; SCM, skin cutaneous melanoma; SA, stomach adenocarcinoma; TGCC, testicular germ cell cancer; TC, thyroid carcinoma; UC, uterine carcinosarcoma; UCEC, uterine corpus endometrial carcinoma; B-ALL, B-cell acute lymphoblastic leukemia; BA, breast adenocarcinoma; DLBL, diffuse large B-cell lymphoma; EA, endometrial adenocarcinoma; FL, follicular lymphoma GB, glioblastoma; MB, medulloblastoma; OA, ovarian adenocarcinoma; PM, pleural mesothelioma; Pro-ca, prostate carcinoma; RCC, renal cell carcinoma.

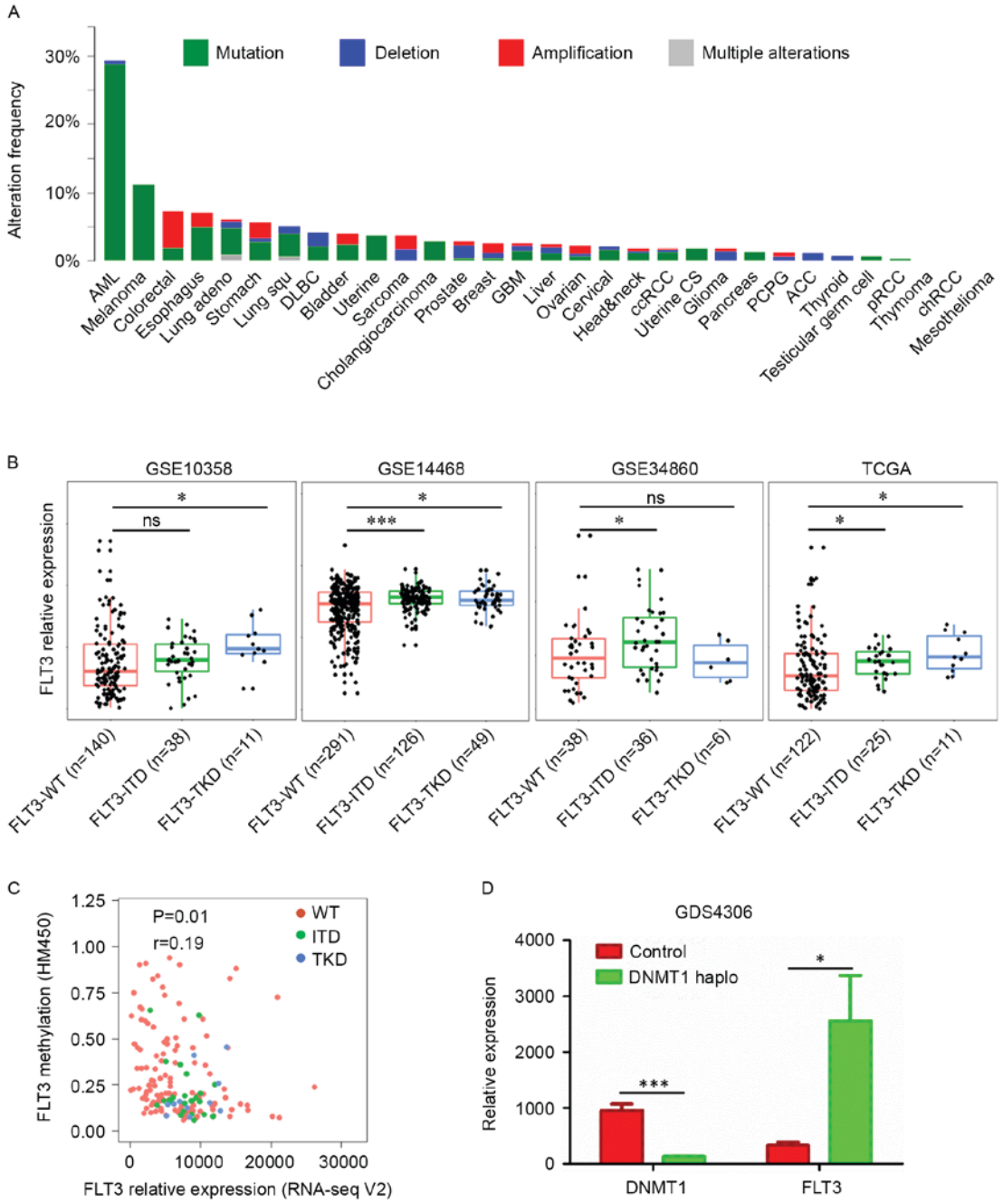

Figure 2. Genetic and epigenetic alterations increase the expression of *FLT3* in AML. (A) cBioportal database was used to analyze mutations and copy number alterations of *FLT3*. Genetic alterations of *FLT3* in different types of cancer showed the most mutations of *FLT3* in leukemia. (B) Four databases (GSE10358, GSE14468, GSE34860 and TCGA) were utilized to examine the effect of *FLT3* mutations on the expression of *FLT3*. (C) cBioportal result showed the expression of *FLT3* was negatively correlated with *FLT3* methylation in leukemia. (D) Gene Expression Omnibus dataset (GDS4306) showed that *DNMT1* haploinsufficiency (haplo) increased expression of *Flt3* in mouse leukemia cells. FLT3, FMS-like tyrosine kinase; AML, acute myeloid leukemia; DLBC, diffuse large B-cell; GBM, glioblastoma multiforme; ccRCC, clear cell renal cell carcinoma; UC, uterine carcinosarcoma; PCPG, pheochromocytoma and paraganglioma; ACC, adenoid cystic carcinoma; pRCC, papillary renal cell carcinoma; chRCC, chromophobe renal cell carcinoma; WT, wild-type; ITD, internal tandem duplication; TKD, tyrosine kinase domain. DNMT1, DNA methyltransferase 1; *P<0.05, **P<0.01 and ***P<0.001; ns, not significant.

collective data indicated that *FLT3* was overexpressed in leukemia and was a prognostic biomarker.

Expression of FLT3 is regulated by genetic and epigenetic alterations. In order to determine the mechanism increasing the expression of *FLT3* in leukemia, the present study evaluated genetic and epigenetic alterations of *FLT3* in AML. By mining TCGA data, it was found that *FLT3* was significantly mutated in AML, compared with other types of cancer, which was similar to the results obtained on the expression of *FLT3*. The results revealed ~28% of patients with AML had somatic mutations in *FLT3* (Fig. 2A). Therefore, it was hypothesized that *FLT3* mutations may increase the expression of *FLT3* in AML. To confirm this, the expression of *FLT3* was compared between wild-type and mutated groups in four independent databases, (GSE10358, GSE14468, GSE34860 and TCGA). It was found that ITD and TKD mutations significantly increased the expression of *FLT3* (Fig. 2B). In addition, methylation data

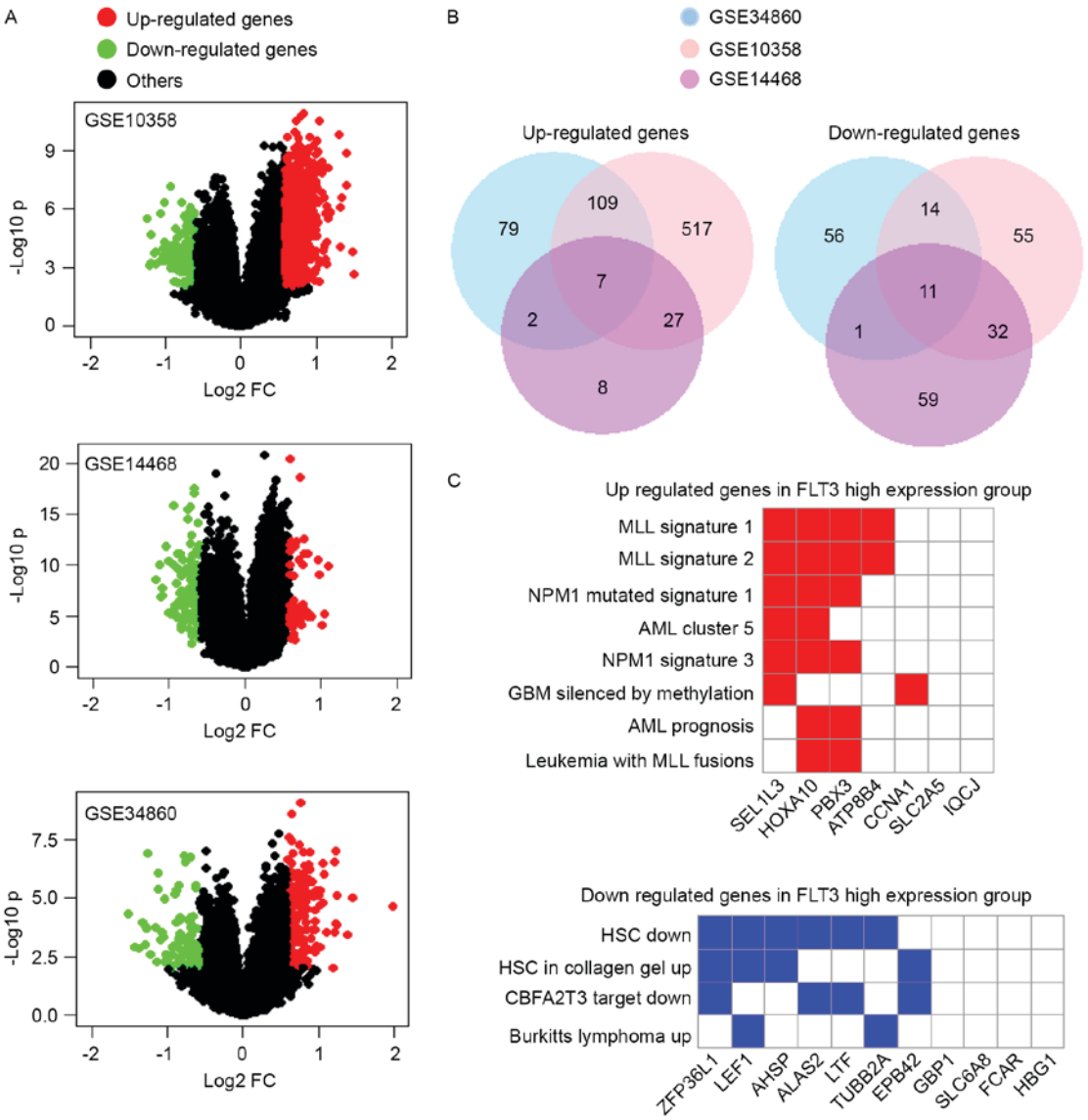

Figure 3. DEGs between the *FLT3* high expression group and *FLT3* low expression group. Three databases (GSE10358, GSE14468 and GSE34860) were used to analyze DEGs. (A) Genes with P<0.05 and FC >1.5 are indicated in red and green colors in the volcano plot. Red indicates genes upregulated in the *FLT3* high expression group, and green indicates genes downregulated in the *FLT3* high expression group. The X-axis is the log2-transformed fold change, and the Y-axis is the log10-transformed P-value. (B) Pie charts shows number of DEGs in the three databases. A total of seven genes were upregulated in the *FLT3* high expression group (left) and 11 genes were downregulated (right). (C) Gene Set Enrichment Analysis was used to assess the association between DEGs and chemical and genetic perturbations. The seven upregulated genes were mainly associated with the MLL signature and NPM1 signature (above). The majority of the downregulated genes were also reduced in HSCs (below). DEGs, differentially-expressed genes; FC, fold change; HCSs, hematopoietic stem cells; MLL, myeloid lymphoid leukemia; NPM1, nucleophosmin 1-like; AML, acute myeloid leukemia; GBM, glioblastoma multiforme.

from TCGA was used to compare the association between the expression of *FLT3* and methylation status. In AML, the expression of *FLT3* was negatively correlated with its methylation (Fig. 2C), indicating that the hypomethylation of *FLT3* may be a potential mechanism resulting in the upregulation of *FLT3*. Of note, mining of the GEO dataset (GDS4306) revealed that the haploinsufficiency of *DNMT1* significantly increased the expression of *Flt3* in mouse leukemia cells (Fig. 2D), suggesting that DNMT1 modified the methylation of *FLT3*. Collectively, these data showed that genetic and epigenetic alterations may be potential mechanisms by which the expression of *FLT3* is increased in AML.

High expression of FLT3 has MLL- and NPM1-like signatures. In order to identify the DEGs associated with a high expression of *FLT3*, three independent AML databases (GSE10358, GSE14468 and GSE34860) were examined (Fig. 3A). The expression of seven genes were significantly upregulated in the *FLT3* high expression group, comprising *ATP8B4*, *CCNA1*, *HOXA10*, *IQCJ*, *PBX3*, *SEL1L3* and *SLC2A5*. *CCNA1*, *HOXA10*, *PBX3* and *SLC2A5* have been reported to function as oncogenes in leukemia (41-44). A total of 11 genes were downregulated, comprising *AHSP*, *ALAS2*, *EPB42*, *FCAR*, *GBP1*, *HBG1*, *LEF1*, *LTF*, *SLC6A8*, *TUBB2A* and *ZFP36L1*, as shown in Fig. 3B. The deletion of RNA-binding protein *ZFP36L1* has been reported to lead to perturbed thymic development and T lymphoblastic leukemia (45). Subsequently, the present study analyzed the enrichment of these DEGs in the chemical and genetic perturbations dataset. Of note, the seven overexpressed genes were enriched in MLL and NPM1 signatures,

indicating that the majority were also upregulated in MLL- or NPM1-mutated AML (Fig. 3C). The 11 downregulated genes were reduced in hematopoietic stem cells (Fig. 3C). Taken together, these results showed that a high expression of *FLT3* was associated with MLL- and NMP1-like signatures.

Discussion

In the present study, it was found that the expression of *FLT3* was high in normal hemopoietic tissues (Fig. 1A) and, in leukemia, *FLT3* was specifically upregulated in AML and ALL (Fig. 1D), indicating that a high expression of *FLT3* may contribute to the progression of leukemia. It has been reported that the overexpression of *FLT3* can induce autophosphorylation (46), and activate the AKT and MAPK pathways in AML (47). ITD mutations clustered in the juxtamembrane domain of *FLT3* are the most frequent forms in AML, and *FLT3*-ITD mutations are associated with a poor prognosis (47-49). In the present study, it was also found that a high expression of *FLT3* was a prognostic factor for poor prognosis in AML using the GEO database (Fig. 1E).

FLT3-related pathways were activated in AML by ITD mutations or the overexpression of *FLT3*. Several interacting proteins are also reported to interact with FLT3 to negatively or positively regulate FLT3 pathways. Spleen tyrosine kinase (SYK) and the mucin 1-C-terminal subunit (MUC1-C) oncoprotein are reported to directly bind to and activate FLT3-related pathways (50,51), whereas suppressor of cytokine signaling 2 (SOCS2) and src-like adaptor protein 2 (SLAP2) interact with FLT3 protein to inhibit its signaling (52,53). In addition, the transcription of *FLT3* can be regulated in AML. Certain AML-related transcription factors, including CCAAT/enhancer binding protein α and the proto-oncogene MYB, can bind to the *FLT3* promoter to activate the transcription of *FLT3* (54). In the present study, another two mechanism were found to increase the expression of *FLT3* in AML. ITD and TKD mutations, and the methylation of *FLT3* increased its expression in AML (Fig. 2B and C). In addition, DNMT1 was identified as is a potential regulator of *FLT3* (Fig. 2D).

By analyzing DEGs in the *FLT3* high expression group, a total of 18 genes were identified using three independent AML datasets, seven of which were upregulated and 11 of which were downregulated (Fig. 3B). These upregulated genes were enriched in MLL and NPM1 signatures (Fig. 3C), and *CCNA1*, *HOXA10*, *PBX3* and *SLC2A5* were reported to function as oncogenes in leukemia. *CCNA1* was overexpressed in ALL, and patients with high levels of *CCNA1* exhibit poor event-free survival rates (55). *CCNA1* transgenic mice have also been shown to exhibit abnormal myelopoiesis and progressed to overt AML (40). Similarly, the overexpression of HOXA10 can cooperate with active SHP2 to induce AML (41), and *HOXA10* was usually fused with the *NUP98*, collaborating with overexpressed FLT3 receptor tyrosine kinase to induce aggressive AML (56). PBX3 is an important cofactor of HOXA9 in leukemogenesis (57) and the coexpression of PBX3 and MEIS1 (PBX3/MEIS1) can cause AML *in vivo* (58). *SLC2A5* is also overexpressed in AML and can increase the fructose utilization of leukemic cells, with a high expression of *SLC2A5* being associated with poor outcomes (44). Among these 11 downregulated genes, *ZFP36L1* has a tumor suppressor role in leukemia. ZFP36L1 is an RNA-binding protein and leads to mRNA degradation; deletion of *ZFP36L1* in mice can induce T cell ALL (45).

Collectively the findings obtained in the present study showed that *FLT3* is overexpressed in leukemia and is prognostic factor for poor prognosis in AML. Patients with a high expression of *FLT3* simultaneously express high levels of leukemic oncogenes. Therefore, a high expression of *FLT3* is a risk factor in leukemia.

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