Early B-cell factor 3 (EBF3) is a novel tumor suppressor gene with promoter hypermethylation in pediatric acute myeloid leukemia

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Abstract

Background: Pediatric acute myeloid leukemia (AML) comprises up to 20% of all childhood leukemia. Recent research shows that aberrant DNA methylation patterning may play a role in leukemogenesis. The epigenetic silencing of the EBF3 locus is very frequent in glioblastoma. However, the expression profiles and molecular function of EBF3 in pediatric AML is still unclear.

Methods: Twelve human acute leukemia cell lines, 105 pediatric AML samples and 30 normal bone marrow/idiopathic thrombocytopenic purpura (NBM/ITP) control samples were analyzed. Transcriptional level of EBF3 was evaluated by semi-quantitative and real-time PCR. EBF3 methylation status was determined by methylation specific PCR (MSP) and bisulfite genomic sequencing (BGS). The molecular mechanism of EBF3 was investigated by apoptosis assays and PCR array analysis.

Results: EBF3 promoter was hypermethylated in 10/12 leukemia cell lines. Aberrant EBF3 methylation was observed in 42.9% (45/105) of the pediatric AML samples using MSP analysis, and the BGS results confirmed promoter methylation. EBF3 expression was decreased in the AML samples compared with control. Methylated samples revealed similar survival outcomes by Kaplan-Meier survival analysis. EBF3 overexpression significantly inhibited cell proliferation and increased apoptosis. Real-time PCR array analysis revealed 93 dysregulated genes possibly implicated in the apoptosis of EBF3-induced AML cells.

Conclusion: In this study, we firstly identified epigenetic inactivation of EBF3 in both AML cell lines and pediatric AML samples for the first time. Our findings also showed for the first time that transcriptional overexpression of EBF3 could inhibit proliferation and induce apoptosis in AML cells. We identified 93 dysregulated apoptosis-related genes in EBF3-overexpressing, including DCC, AIFM2 and DAPK1. Most of these genes have never been related with EBF3 over expression. These results may provide new insights into the molecular mechanism of EBF3-induced apoptosis; however, further research will be required to determine the underlying details. Our findings suggest that EBF3 may act as a putative tumor suppressor gene in pediatric AML.

Keywords: Early B-cell factor 3, Pediatric acute myeloid leukemia, Methylation, Tumor suppressor, Real-time PCR array

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Background

Acute myeloid leukemia (AML) is a type of cancer that arises from the myeloid cell. It is the most common form of acute leukemia in adults and the second most common form of leukemia in children after acute lymphoblastic leukemia (ALL) [1,2]. Pediatric AML comprises up to 20% of all childhood leukemia and the mechanism behind poor survival of acute myeloid leukemia (AML) patients remains unclear [3]. Several novel recurrent mutations have been found to involve epigenetically regulated genes in AML, including DNMT3A [4,5], TET2 [6,7], and IDH1/2 [8], which are involved in the regulation of DNA methylation, and EZH2 [9,10] and ASXL-1 [11], which are implicated in the regulation of histones [11]. Importantly, the presence of DNMT3A, IDH1, or IDH2 mutations may confer sensitivity to novel therapeutic approaches, including the use of demethylating agents. We propose that understanding the role of methylation in AML will lead to more rational therapeutic approaches targeting this disease [4,12].

One important role of epigenetic regulation is that it affects gene expression; recent research has shown that aberrant DNA methylation may play a role in leukemogenesis [13]. DNA methylation is an important regulator of gene transcription. DNA methylation is an epigenetic modification that typically occurs at CpG (cytosine-phosphate-guanine) sites in mammalian cells [14]. The prognostic impact of global DNA methylation and hydroxymethylation has been assessed and global DNA methylation predicted overall survival in myelodysplastic syndromes [15]. The importance of epigenetic aberrations in the pathogenesis of leukemias has been revealed by recurrent gene mutations that highlight epigenetic pathways as well as by the clinical success of therapies like 5-azacytidine and decitabine that work through epigenetic mechanisms. Azacitidine seems effective in WHO-AML, including patients with >30% BM blasts [16]. Multiple clinical trials have shown the promising activity of low-dose decitabine in AML, MDS, CML, and hemoglobinopathies, whereas its efficacy in solid tumors is rather limited. Recent clinical trials have investigated new dosing schedules, routes of administration, and combination of decitabine with other agents, including histone deacetylase (HDAC) inhibitors [17].

The early B-cell factors (EBF) are a family of four highly conserved DNA-binding transcription factors with an atypical zinc-finger and helix-loop-helix motif. EBF proteins have diverse functions in the development of multiple lineages, including neurons, B cells, and adipocytes. B lymphocytes are generated from hematopoietic stem cells in a series of steps controlled by transcription factors. One of the most important regulators of this process is early B cell factor (EBF). EBF and closely related proteins (EBF2, EBF3, EBF4, Collier/Knot and Unc-3) constitute a novel transcription factor family (here, termed the EBF family). All members of the EBF family possess a highly conserved DNA-binding domain (DBD) that is distinct from that of other known DNA-binding proteins. Multiple lines of evidence indicate that expression of EBF is a principle determinant of the B cell fate [18,19]. EBF activity is important for both stabilizing commitment and driving aspects of differentiation in Xenopus muscle cells [20]. Alterations in various developmental pathways are common themes in cancer. Accumulating evidence indicates that genomic deletion of the EBF1 gene contributes to the pathogenesis, drug resistance, and relapse of B-progenitor acute lymphoblastic leukemia (ALL) [21-23]. Epigenetic silencing and genomic deletion of the EBF3 locus on chromosome 10q are very frequent in glioblastoma (GBM). Strikingly, the frequency of EBF3 loss in GBM is similar to the loss of PTEN, a key suppressor of gliomagenesis. Cancer-specific somatic mutations were detected of EBF3 in GBM and both EBF1 and EBF3 in pancreatic ductal adenocarcinoma [24]. In a genome-wide screen for putative tumor suppressor genes, the EBF3 locus on the human chromosome 10q26.3 was found to be deleted or methylated in 73% of brain tumor cases. Silencing of the EBF3 locus has been observed in brain, colorectal, breast, liver, and bone tumor cell lines, and its reactivation was achieved with 5-aza-2′-deoxycytidine and trichostatin A treatment in a significant portion of these tumor cells [25]. In gastric carcinoma, inactivation of the EBF3 gene is frequently accompanied by promoter hypermethylation in several gastric cancer cell lines. Promoter methylation of EBF3 was detected in 42/104 (40.4%) gastric cancer tissues but not in normal gastric tissues. These results suggest that the EBF3 tumor suppressor is epigenetically silenced and that it serves as an independent prognostic marker in gastric carcinoma [26]. Therefore, EBF3 regulates a transcriptional program underlying a putative tumor suppression pathway [25]. Likewise, the expression of EBF3 results in cell cycle arrest and apoptosis. A previous study has shown that the expression of cyclin-dependent kinase inhibitors was profoundly affected upon early activation and then repression of p21 (cip1/waf1) and persistent activation of both p27 (kip1) and p57 (kip2), whereas genes involved in cell survival and proliferation were suppressed [25].

However, reports on the methylation status of EBF3 in the blood system are rare, and its expression and role in pediatric AML remains unclear. The aim of this study was to analyze the methylation profile and molecular function of EBF3 in pediatric AML. Identifying aberrant methylated genes may provide better understanding of the pathogenesis of AML [27], thereby paving the way for the development of novel tumor markers and therapeutic targets.
Methods

Cell lines

Leukemia cell lines HL-60, MV4-11, U937, THP-1 and K562 were obtained from the American Type Culture Collection (ATCC). CCRF, Raji, Jurkat, 697, Daudi and SHI-1 cell lines (gifts from Professor Wang Jian-Rong, The Cyrus Tang Hematology center of Soochow University) [28], NALM-6 cell lines (gifts from Professor Tang Yong-Ming, Zhejiang University). HL-60, MV4-11, U937, THP-1 and SHI-1 are AML cell lines. All cell lines were maintained at 37°C in the RPMI 1640 (GibcoR, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Life Technologies, Carlsbad, CA).

Patients and samples

Bone marrow specimens were obtained at the time of diagnosis during routine clinical assessment of 105 pediatric patients with AML, who presented at the Department of Hematology and Oncology, Children's Hospital of Soochow University between 2000 and 2011. Ethical approval was provided by the Children's Hospital of Soochow University Ethics Committee (No.SUEC2000-021 & No.SUEC2011-037), and written informed consent was obtained from the parents or guardians. AML diagnosis was made in accordance with the revised French–American–British (FAB) classification. The main clinical and laboratory features of the patient cohort are summarized in Table 1. Additionally, bone marrow samples from 23 healthy donors and 7 patients with Idiopathic thrombocytopenic purpura (ITP) were analyzed as controls. Bone marrow mononuclear cells (BMNCs) were isolated using Ficoll solution within 2 h after bone marrow samples harvested and subjected for the extraction of total RNA and genomic DNA.

CD34⁺ cell purification

For CD34⁺ cell selection, the Miltenyi immunoaffinity device (VarioMACS 130-046-703) was used according to the manufacturer’s instructions (Miltenyi Biotech, Auburn, CA). Briefly, the CD34⁺ cells are magnetically labeled with CD34 MicroBeads. Then, the cell suspension is loaded onto a MACS R Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled CD34⁺ cells are retained within the column. The unlabeled cells run through; CD34⁺ cells were adsorbed on the magnetic poles. After removing the column from the magnetic field, the magnetically retained CD34⁺ cells can be eluted as the positively selected cell fraction.

Sodium bisulfite modification of genomic DNA

High-molecular-weight genomic DNA was extracted from cell lines and biopsies by a conventional phenol/chloroform method. The sodium bisulphite modification procedure was as described [29] with slight modification. In brief, 600 ng of genomic DNA was denatured in 3 M NaOH for 15 min at 37°C, then mixed with 2 volumes of 2% low-melting-point agarose. Agarose/DNA mixtures were then pipetted into chilled mineral oil to form agarose beads. Aliquots of 200 μl of 5 M bisulphite solution (2.5 M sodium metabisulphite, 100 mM hydroquinone, both Sigma, USA) were added into each tube containing a single bead. The bisulphite reaction was then carried out by incubating the reaction mixture for 4 h at 50°C in the dark. Treatments were stopped by equilibration against 1 ml of TE buffer, followed by desulphonation in 500 μl of 0.2 M NaOH. Finally, the beads were washed with 1 ml of TE buffer and directly used for PCR.

Methylation-specific PCR

The methylation status of the EBF3 promoter region was determined by methylation-specific PCR. Primers distinguishing unmethylated (U) and methylated (M) alleles were designed to amplify the sequence:

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EBF3 M-forward: 5'- TAGGAATTTTGTATGTGTG AGGTC-3;
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**EBF3** M-reverse: 5'- AAATACGTTATTAATTTTCT CGTT-3;
**EBF3** U-forward: 5'- TAGGAATTTTGTTATGTGTG AGGTT-3;
**EBF3** U-reverse: 5'- AATAAATACCATTATTAATTTT CTCATT-3.

Each PCR reaction contained 20 ng of sodium bisulphite-modified DNA, 250 pmol of each primer, 250 pmol deoxynucleoside triphosphate, 1 × PCR buffer, and one unit of ExTaq HS polymerase (Takara, Tokyo, Japan) in a final reaction volume of 20 μl. Cycling conditions were initial denaturation at 95°C for 3 min, 40 cycles of 94°C

![Figure 1](image)

**Figure 1** Analysis of promoter methylation in pediatric AML by NimbleGen Human DNA Methylation arrays. Analysis of the methylation status of genes in five pediatric AML samples (M1, M2, M3, M4 and M5) and three NBM samples (N1, N2, and N3) using NimbleGen Human DNA Methylation arrays shows that the **EBF3** promoter is significantly methylated in AML samples (5/5) and unmethylated in NBM samples (0/3). (A) Each red box represents the number of methylation peaks (PeakScore) overlapping the promoter region for the corresponding gene. The PeakScore is defined as the average -log10 (P value) from probes within the peak. (B) The scores reflect the probability of positive methylation enrichment.
Figure 2 (See legend on next page.)
for 30 s, 63°C (M) or 58°C (U) for 30 s, and 72°C for 30 s. For each set of methylation-specific PCR reactions, in vitro-methylated genomic DNA treated with sodium bisulphite served as a positive methylation control. PCR products were separated on 4% agarose gels, stained with ethidium bromide and visualized under UV illumination. For cases with borderline results, PCR analyses were repeated.

**Bisulfite genomic sequencing**

Bisulfite genomic sequencing (BGS) was performed as previously described [30]. BGS primers were from +709 to +1031 including 20 CpGs. EBF3 F: 5′-TTAGGAAATTTTGTTATGTGTGAGGT-3′ and EBF3 R: 5′-TTATATTTTATTTCCTTCTATACCATAAAA-3′. Amplified BGS products were TA-cloned; and five to six randomly chosen colonies were sequenced. DNA sequences were analyzed with QUMA Analyzer. (http://quma.cdb.riken.jp/).

**Leukemia cells treated with 5-aza-2′-deoxycytidine**

De-methylation was induced with 5-aza-dC (5-Aza, Sigma-Aldrich, St Louis, MO, USA) treatment at a concentration that induced de-methylation of the DNA without killing the cells. Culture media for HL-60 and MV4-11 cells contained 5 µM or 10µM 5-Aza. DNA and RNA were extracted after 72 hours of 5-Aza treatment for the following analysis.

**Quantitative reverse-transcription PCR for EBF3**

Quantitative real-time PCR was performed to determine the expression levels of EBF3 genes. Total RNA was reverse transcribed using the Reverse Transcription Kit, according to the manufacturer's protocol (Applied Biosystems Inc., Foster City, CA). The real time PCR primers used to quantify GAPDH expression were: F: 5′-AGAAGGCTGGGGCTCATTGTG-3′ and R: 5′-AGGGGCCATCACCAGTCTTCTTCC-3′ and for EBF3 were: F: 5′-ATGGCTCTCCCGCTAACTCCT-3′ and R: 5′-TCCGTTCTTTGTGCTGGGTT-3′. Expression of EBF3 was normalized to endogenous GAPDH expression.

**EBF3 lentiviral expression constructs and lentivirus production**

Briefly, an approximately 1650 bp fragment containing the human EBF3 gene was directly cloned into the pMD18-T vector. Positive clones were confirmed by sequencing and subcloned into the pLVX-IRES-ZsGreen vector (Clontech Laboratories, Inc. Tokyo, Japan). The vector plasmids, pLVX-IRES-ZsGreen1, pLP1, pLP2 and pLP/VSVG were amplified in E.Coli and purified using the Endofree Maxiprep Kit (QIAGEN, Inc. Duesseldorf, Germany). 270 µg of transfer vector, 176 µg of pLP1, 95 µg of pLP/VSVG and 68 µg of pLP2 was mixed with 0.25 M CaCl2 (Sigma-Aldrich, St Louis, MO, USA) and added to same volume of 2 × HEPES (Sigma-Aldrich, St Louis, MO, USA) and mixed while bubbling for 20 min to allow a precipitate to form. This was then added to a 175 cm2 flask of approximately 60% confluent 293 T cells containing 20 mL DMEM supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mM glutamine and incubated for 48 h at 37°C in 5% CO2. The supernatant was centrifuged at 1,700 g for 10 min to pellet cell debris, and ultracentrifuged at 121,603 g for 2 h. The pellet containing concentrated virus was resuspended in DMEM without supplements and stored at −80°C.

**Cell proliferation analysis**

Acute myeloid leukemia cells were seeded in 96-well plates at 2 × 10^4 cells per well. 20 µl CCK-8 (Dojindo Molecular Technologies, Tokyo, Japan) was added to each well and incubated at 37°C for a further 4 hours. The optical density (OD) values were measured at 450 nm on a scanning multi-well spectrophotometer (BioRad Model 550, USA). Compared with the control group, cell proliferation was calculated as proliferation values. All experiments were performed in triplicate and repeated twice. The results were analyzed using ANOVA and the Student-Newman-Keuls tests, p < 0.05 were considered significant.

**Apoptosis assay**

Apoptosis assay was according to the manual operation of BD Annexin V Staining Kit (Cat: 556420, BD Biosciences, Franklin Lakes and NJ USA). Briefly, wash cells twice with cold PBS and then resuspend cells in 1 x Binding Buffer at a concentration of ~1 × 10^6 cells/ml. Transfer 100 µl of the solution (~1 × 10^5 cells) to a 5 ml culture tube. Add Annexin V and PI 5 µl/test. Gently mix the cells and incubate for 15 min at RT in the dark. Add 400 µl of 1 x Binding Buffer to each tube. Analyzed by flow cytometry as soon as possible (within 1 hour).

**Western blot analysis**

For western blot analysis, cellular proteins were extracted in 40 mM Tris–HCl (pH 7.4) containing 150 mM NaCl and 1% (v/v) Triton X-100, supplemented with a cocktail of protease inhibitors. Equal amounts of protein were
resolved on 12% SDS-PAGE gels, and then transferred to a PVDF membrane (Millipore, Bedford, MA). Blots were blocked and then probed with antibodies against PARP (1:1000, 9532 s, Cell Signaling Technology, Inc. Danvers, MA), Caspase3 (1:1000, 9665 s, Cell Signaling Technology, Inc. Danvers, MA), Caspase9 (1:1000, 9505 s, Cell Signaling Technology, Inc. Danvers, MA), EBF3 (1:1000, ab122917, Abcam, Cambridge, MA Office, USA), GAPDH (1:5000, G8795, Sigma, St. Louis, MO), AIFM2 (1:1000, sc-377120, Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), BIRC8.

Figure 3 MSP analysis showing EBF3 promoter hypermethylation in AML samples. (A) Western blot analysis depicting the expression of EBF3 in eight NBM samples and nine leukemia cells. (B) MSP analysis the promoter methylation of EBF3 and aberrant EBF3 methylation was observed in 42.9% (45/105) of the pediatric AML samples compared with 13.3% (4/30) of the NBM control samples. M and U represent MSP results using primer sets for methylated and unmethylated EBF3 genes, respectively.
Figure 4 BGS analysis depicts $EBF3$ promoter hypermethylation in AML samples. Eight NBM samples and eight AML samples were selected for further analysis by BGS. The $EBF3$ promoter was methylated in the AML samples (67.0% - 77.0%); whereas the $EBF3$ promoter was methylated in only 41.0% - 50.0% in the NBM samples. ● methylated cytosines; ○ unmethylated cytosines.
(1:1000, sc-130107, Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), BCL2L11 (1:1000, sc-8267, Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), CDKN1A (1:1000, 2947 s, Cell Signaling Technology, Inc. Danvers, MA). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence kit (Pierce, Rockford, IL). Protein bands were visualized after exposure of the membrane to Kodak X-ray film.

**Real-time PCR array analysis**

For RNA extraction, cells were immediately submerged in 2 ml Trizol (Invitrogen Co., NY, USA), stored at –80°C until further processed. A volume of 1 ml of each sample was spun at 4°C for 15 min at 12,000 g to remove debris and DNA, 1 ml of supernatant was mixed with 200 ul chloroform, shaken for 15 seconds, incubated at Room Temperature for 2–3 minutes and spun for 10 minutes at 12,000 g at 4°C. RNA was precipitated by adding 500 µl of the aqueous phase to an equal volume of isopropanol and spun at 14,000 g at 4°C for 10 minutes. RNA was washed with 75% ethanol, spun at 14,000 g at 4°C for 10 minutes, dried and resuspended in 40 µl DEPC-treated H2O. The final RNA concentration was determined using a spectrophotometer (Nanodrop 2000) and the purity was assessed by agarose gel electrophoresis. cDNA synthesis was performed on 4 µg of RNA in a 10 µl sample volume using SuperScript II reverse transcriptase (Invitrogen Co., NY, USA) as recommended by the manufacturer. The RNA was incubated with 0.5 µg of oligo(dT)12–18mers primers (Invitrogen Co., NY, USA) for 7 minutes at 70°C and then transferred onto ice. Then, 9 µl of a master mix containing 4 µl of SuperScript II buffer, 2 µl of 0.1 M DTT, and 1 µl each of dNTPs stock (10 mM), Rnasin (40 UI) and SuperScript II were added to the RNA sample, spun and incubated at 42°C for 60 min followed by 5 min at 70°C to inactivate the enzyme. cDNA was stored at –20°C. Real-time PCR array (SA Bioscience Human Apoptosis PCR Array PAHS-3012) analysis was performed in a total volume of 20 µl including 2 µl of cDNA, primers (0.2 mM each) and 10 µl of SYBR Green mix (Roche Co., Basel, Switzerland.). Reactions were run on an Light cycler 480 using the universal thermal cycling parameters (95°C 5 min, 45 cycles of 10 sec at 95°C,20 sec at 60°C and 15 sec at 72°C; melting curve: 10 sec at 95°C, 60 sec at 60°C and continues melting). Results were obtained using the sequence detection software Light cycler 480 and analyzed using Microsoft Excel. For all samples melting curves were acquired for quality control purposes. For gene expression quantification, we used the comparative Ct method. First, gene expression levels for each sample were normalized to the expression level of the housekeeping gene encoding Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) within a given sample (~ΔCt); the relative expression of each gene was calculated with10*Log2(ΔCt). The difference between the EBF3 over-expression samples compared to the control samples was used to determine the10*Log2(ΔCt). Statistical significance of the gene expression difference between the EBF3 over-expression and the control samples was calculated with the T-test using SPSS 11.5 software.

**Ingenuity pathway analysis (IPA)**

Datasets representing genes with altered expression profile derived from Real-time PCR array analyses were imported into the Ingenuity Pathway Analysis Tool (IPA Tool; Ingenuity H Systems, Redwood City, CA, USA; http://www.ingenuity.com). In IPA, differentially expressed genes are mapped to genetic networks available in the Ingenuity database and then ranked by score. The basis of the IPA program consists of the Ingenuity Pathway Knowledge Base (IPKB) which is derived from known functions and interactions of genes published in the literature. Thus, the IPA Tool allows the identification of biological networks, global functions and functional pathways of a particular dataset. The program also gives the significance value of the genes, the other genes with which it interacts, and how the products of the genes directly or indirectly act on each other, including those not involved in the

| Table 2 Association of EBF3 promoter methylation with clinico-pathological characteristics in 105 pediatric AML samples |
|-----------------|-----------------|-----------------|-----------|
| Gender                       | No. of patients | EBF3 methylation (n) | P        |
| Male                         | 42              | 28               | 14        | 0.107 |
| Female                       | 63              | 32               | 31        |       |
| Age (years)                  |                 |                  |           |       |
| <6                           | 60              | 35               | 25        | 0.776 |
| ≥6                           | 45              | 25               | 20        |       |
| Leukocyte (/µl)              |                 |                  |           |       |
| >10000                       | 61              | 35               | 26        | 0.954 |
| ≤10000                       | 44              | 25               | 19        | 0.250 |
| FAB                         |                 |                  |           |       |
| M1-M6                        | 93              | 55               | 38        | 0.250 |
| M7                           | 12              | 5                | 7         |       |
| Cytogenetics                 |                 |                  |           |       |
| Favorable                    | 50              | 27               | 23        | 0.060 |
| Intermediate                 | 27              | 12               | 15        |       |
| Unfavorable                  | 28              | 21               | 7         |       |
| MRD                          |                 |                  |           |       |
| <0.25%                       | 49              | 28               | 21        | 1.000 |
| ≥0.25%                       | 56              | 32               | 24        |       |
microarray analysis. The networks created are ranked depending on the number of significantly expressed genes they contain and also list diseases that were most significant. A network is a graphical representation of the molecular relationships between molecules. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least 1 reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base.

Statistical analysis
SPSS v11.5 (SPSS Inc., Chicago, IL) was used for statistical analysis. Data are presented as means ± standard deviation. Group t-test was used to compare the expression of EBF3 between DMSO group and 5-Aza group. Statistical significance between methylated sample data and clinical pathological features of AML patients were analyzed by Pearson chi-square test or Fisher’s exact test. Statistical significance of EBF3 expression among NBM and pediatric AML groups was determined using one-way ANOVA. A p <0.05 was considered statistically significant.

Results and Discussion
The EBF3 promoter is hypermethylated in AML cells
Our long-term research is focused on epigenetic modifications in pediatric AML. In previous studies, we have found a series of abnormally methylated genes in AML [31,32]. We conducted CpG island array analysis to explore promoter methylation in pediatric AML. Our results implied that the EBF3 promoter is hypermethylated in AML (Figure 1). Subsequent analysis identified two CpG islands in the EBF3 promoter region (Figure 2A). Therefore, we conducted methylation sensitive PCR (MSP) in 12 leukemia cell lines using a primer that encompassed the CpG islands within the EBF3 promoter. Our results showed that the EBF3 promoter was hypermethylated in 10/12 leukemia cell lines, with the highest methylation levels observed in HL-60, NB4, SHI-1, U937 (four AML cell lines) and K562 cells; whereas EBF3 was unmethylated in

Figure 5 The expression of EBF3 was downregulated in patients with pediatric AML. (A) Real-time PCR analysis of the transcript levels of EBF3 in 105 pediatric AML samples and 30 NBM control samples. (B) Quantification shows that EBF3 expression was found to be robustly decreased in the AML samples compared with the control samples (26.91 ± 50.86 vs. 121.14 ± 95.11, respectively; P <0.001). Those with methylated EBF3 showed significantly lower levels of EBF3 expression compared with unmethylated EBF3 (16.32 ± 12.93 vs. 34.86 ± 65.46, respectively; P = 0.043). (C) The prognostic significance of EBF3 expression was assessed in 105 Chinese pediatric AML patients with clinical follow-up records. Kaplan-Meier survival analysis revealed similar survival outcomes in tumors with high or low EBF3 expression among 105 pediatric AML patients (P = 0.091). (D) Samples with EBF3 promoter methylation revealed similar survival outcomes through Kaplan-Meier survival analysis (P = 0.190).
2/11 cell lines, including 697 and Jurkat cells (Figure 2B). To confirm methylation of the EBF3 promoter, we treated the leukemia cell lines with 5-Aza. This demethylation reagent is an epigenetic modifier that inhibits DNA methyltransferase activity resulting in hypomethylation and gene activation. MSP analysis showed a decrease in EBF3 methylation in leukemia cells following 5-Aza treatment compared with control cells treated with DMSO. In addition, we showed that EBF3 expression was significantly upregulated in leukemia cells following 5-Aza treatment compared with control cells treated with DMSO (Figure 2C). EBF3 expression was upregulated 50.71 fold in HL-60 cells (30.93 vs. 0.61, respectively; \( P = 0.019 \)), and 19.00 fold in MV4-11 cells (24.27 vs. 1.28, respectively; \( P = 0.012 \)). Western blot analysis showed significantly higher EBF3 expression in NBM samples (n = 8) compared with leukemia cells (n = 9), which is consistent with the MSP results (Figure 3A).

The EBF3 promoter is methylated in patients with pediatric AML

To examine the methylation status of the EBF3 promoter in pediatric AML, we obtained samples from 105 patients with pediatric AML and 30 control patients with NBM/ITP. Aberrant EBF3 methylation was observed in 42.9% (45/105) of the pediatric AML samples compared with 13.3% (4/30) of the NBM control samples (Figure 3B). Subsequently, eight NBM samples and eight AML samples were selected for further analysis by bisulfite genomic sequencing (BGS; Figure 4). Consistent with the MSP results, BGS confirmed that the CpG islands in the EBF3 promoter were methylated in the AML samples (67.0% - 77.0%), whereas the CpG sites were methylated in 41.0% - 50.0% in the NBM samples. No significant differences in clinical features, such as sex, age, initial hemoglobin level, white blood cell counts, platelet counts, and chromosomal abnormalities, were observed between methylated EBF3 samples and unmethylated samples by examination of the clinicopathologic characteristics (Table 2). Real-time qPCR was employed to examine the transcriptional levels of EBF3 in 105 pediatric AML samples and 30 NBM/ITP control samples (Figure 5A and 5B; Table 1). EBF3 expression was found to be robustly decreased in the AML samples compared with the control samples (26.91 ± 50.86 vs. 121.14 ± 95.11; \( P < 0.001 \)). Further analysis of the AML samples showed that 45/105 pediatric AML patients displayed EBF3 methylation compared with 60/105 patients that exhibited unmethylated EBF3 (Table 1). Furthermore, patients with methylated EBF3 showed significantly lower levels of EBF3 expression compared with patients exhibiting unmethylated EBF3 (16.32 ± 12.93 vs. 34.86 ± 65.46; \( P = 0.043 \); Figure 5B). In summary, the hypermethylation status of the EBF3 promoter in pediatric AML patient tissue was consistent with results in human myeloid leukemia cell lines. The prognostic significance of EBF3 expression was assessed in 105 Chinese pediatric AML patients with clinical follow-up records. No significant association was found between EBF3 expression and patient age, sex, FAB, or cytogenetics (Table 1). Kaplan-Meier survival analysis

### Table 3 Association of EBF3 expression/promoter methylation with Kaplan-Meier survival in 105 pediatric AML samples

| Variable                  | No. of patients | Over survival | P       |
|---------------------------|-----------------|---------------|---------|
|                           |                 | Median ± SE   |         |
| Cytogenetics              |                 |               |         |
| Favorable                 | 50              | 46.664 ± 3.717| <0.001  |
| Intermediate              | 27              | 29.220 ± 3.188|         |
| Unfavorable               | 28              | 11.161 ± 1.827|         |
| FAB                       |                 |               |         |
| M1-M6                     | 93              | 36.113 ± 2.885| <0.001  |
| M7                        | 12              | 8.542 ± 1.820 |         |
| Leukocyte (/µl)           |                 |               |         |
| >10000                    | 61              | 30.220 ± 2.974| 0.803   |
| ≤10000                    | 44              | 33.631 ± 4.063|         |
| MRD                       |                 |               |         |
| <0.25%                    | 49              | 53.627 ± 3.151| <0.001  |
| ≥0.25%                    | 56              | 18.893 ± 2.425|         |
| EBF3 expression           |                 |               |         |
| Low < 12.11               | 52              | 38.971 ± 4.402| 0.091   |
| High ≥ 12.11              | 53              | 32.864 ± 3.654|         |
| EBF3 methylation          |                 |               |         |
| Negative                  | 60              | 35.143 ± 3.509| 0.190   |
| Positive                  | 45              | 29.458 ± 2.607|         |

### Table 4 Cox multivariate analysis of EBF3 expression/promoter methylation and clinico-pathological features in pediatric AML

| Variable                  | Odds ratio | EXP(B) 95% CI   | P     |
|---------------------------|------------|----------------|-------|
| Cytogenetics              | 8.132      | 3.246(1.445-7.290)| 0.004 |
| <0.25% vs. ≥0.25%         | 12.951     | 5.496(2.173-13.901)| 0.000 |
| Leukocyte (/µl)           | 1.371      | 1.433(0.785-2.618)| 0.242 |
| >10000 vs. ≤10000         | 1.371      | 1.433(0.785-2.618)| 0.242 |
| FAB classification        | 4.301      | 2.379(1.049-5.397)| 0.038 |
| M7 vs. M1-M6              | 0.882      | 0.732(0.382-1.403)| 0.348 |
| EBF3 Expression           | 0.317      | 1.201(0.635-2.272)| 0.574 |
| Negative vs. Positive     | 0.317      | 1.201(0.635-2.272)| 0.574 |
Figure 6 (See legend on next page.)
Figure 6 Overexpression of EBF3 inhibited proliferation and induced apoptosis in leukemia cells. (A) Western blot analysis of EBF3 expression in EBF3 transfected leukemia cells. Transfection with EBF3 lentivirus PLVX-EBF3 significantly upregulated expression of EBF3 in AML cells compared with mock-transfected cells. An expression level of cleaved PARP, a marker of apoptosis, was analyzed by Western blotting. (B) CCK-8 assays show that transfection with EBF3 lentivirus inhibits proliferation in HL-60 and MV4-11 cells compared with mock-transfected cells. (C) The number of cells displaying apoptotic features is higher in the HL-60 and MV4-11 cells transfected with PLVX-EBF3 compared with the mock-transfected cells. (D) Quantification shows that the proportion of apoptotic cells in the EBF3-overexpressing cells (PLVX-EBF3) was significantly greater than the vector control group (PLVX-Ve). **P < 0.01.

Figure 7 Real-time PCR array analysis shows dysregulated genes implicated in EBF3 over-expression. (A) Cluster of apoptosis genes related with EBF3 over-expression. We analyzed and clustered the expression of 370 key genes involved in apoptosis using the SABioscience Human Apoptosis PCR Array PAHS-3012 kit. (B) Relative expression of the genes most up-regulated in EBF3-overexpressing AML cells compared with mock-transfected cells. (C) Relative expression of the genes most down-regulated in EBF3-overexpressing cells compared with mock transfected cells.
| Gene         | Description                                      | Vector | EBF3   | FC     | P       |
|-------------|--------------------------------------------------|--------|--------|--------|---------|
| ANXA4       | annexin A4                                       | 60.7992| 65484.55| 1077.063| 0.000000|
| MTL5        | metallothionein-like 5,                         | 3327.433| 78707.44| 23.6541 | 0.004317|
| CDKN1A      | cyclin-dependent kinase inhibitor 1A (p21, Cip1) | 477.772| 9830.082| 20.57462| 0.005702|
| PRLR        | prolactin receptor                               | 166.5942| 3355.185| 20.13987| 0.005959|
| DCC         | DCC netrin 1 receptor                            | 60.97703| 1106.082| 18.13932| 0.007335|
| AIFM2       | apoptosis-inducing factor, mitochondrion-associated, 2 | 5221.303| 76648.86| 14.68003| 0.011117|
| BIRC7       | baculoviral IAP repeat containing 7             | 10810.86| 134973.2| 12.48496| 0.015411|
| APOE        | apolipoprotein E                                 | 1234.913| 15389.71| 12.46218| 0.015466|
| FAIM        | Fas apoptotic inhibitory molecule                | 59.72215| 723.2259| 12.10985| 0.016371|
| BCL6        | B-cell CLL/lymphoma 6                           | 300.2861| 3465.696| 11.54131| 0.018009|
| EEF1A2      | eukaryotic translation elongation factor 1 alpha 2 | 3430991| 34629902| 10.09326| 0.023482|
| DAPK1       | death-associated protein kinase 1               | 36.00672| 341.4206| 9.482138| 0.026565|
| DAPK2       | death-associated protein kinase 2               | 106.9056| 926.7119| 8.668504| 0.031703|
| CUL5        | cullin 5                                         | 1607.044| 10876.05| 6.767734| 0.021489|
| CARD10      | caspase recruitment domain family, member 10    | 73643.27| 4740088| 6.436553| 0.036773|
| HTATIP2     | HIV-1 Tat interactive protein 2                 | 21030.48| 125463.2| 5.965781| 0.006577|
| HSPA1B      | heat shock 70 kDa protein 1B                    | 9411.403| 51996.48| 5.524838| 0.046279|
| EIF2AK3     | eukaryotic translation initiation factor 2-alpha kinase 3 | 1296.309| 6741.561| 5.200581| 0.035667|
| PTH         | parathyroid hormone                              | 3.364038| 14.63482| 4.350374| 0.020214|
| FAS         | Fas cell surface death receptor                  | 278.2416| 1168.472| 4.199489| 0.028443|
| PPP2CA      | protein phosphatase 2, catalytic subunit         | 19486.59| 77413.57| 3.972658| 0.012455|
| GFRAL       | GDNF family receptor alpha like                  | 2.948935| 11.44733| 3.881851| 0.018966|
| CBX4        | chromobox homolog 4                              | 12247.45| 47277.53| 3.860193| 0.010244|
| Bd2-A1      | BCL2-related protein A1                          | 191.3664| 733.6087| 3.833528| 0.012181|
| CASP7       | caspase 7, apoptosis-related cysteine peptidase  | 3214.089| 11901.59| 3.702944| 0.012218|
| AIFM3       | apoptosis-inducing factor, mitochondrion-associated, 3 | 2320.458| 8123.495| 3.500815| 0.019759|
| TNFAIP3     | tumor necrosis factor, alpha-induced protein 3   | 396.2299| 1312.111| 3.311488| 0.018799|
| PRODH       | proline dehydrogenase (oxidase) 1                | 1017.064| 3162.695| 3.106934| 0.022482|
| BIK         | BCL2-interacting killer (apoptosis-inducing)     | 3304.449| 10077.62| 3.049712| 0.020294|
| CFL1        | cofilin 1                                        | 96630.71| 284065.3| 2.9397 | 0.024696|
| PIK3CA      | phosphatidylinositol-4,5-bisphosphate 3-kinase   | 119.848| 3307.876| 2.773091| 0.027971|
| SERPINB2    | serpin peptidase inhibitor, clade B              | 324.0773| 898.5624| 2.77268| 0.020241|
| DAP3        | death associated protein 3                       | 59483.18| 156832.7| 2.636589| 0.029971|
| MSH6        | mutS homolog 6                                   | 10961.78| 26493.5| 2.416898| 0.033269|
| XIAP        | X-linked inhibitor of apoptosis                  | 680.3787| 1616.734| 2.376227| 0.033028|
| PRKRA       | protein kinase, interferon-inducible double stranded RNA dependent activator | 6339.679| 15064.52| 2.376227| 0.035028|
| CD28        | CD28 molecule                                    | 60.97703| 140.9465| 2.311469| 0.039382|
| DEDD        | death effector domain containing                 | 1058.38| 24135.46| 2.27943| 0.037863|
| CUL4A       | cullin 4A                                        | 43243.45| 98570.4| 2.27943| 0.037863|
| ERN1        | endoplasmic reticulum to nucleus signaling 1     | 786.986| 1766.819| 2.245046| 0.038268|
| PMAIP1      | phorbol-12-myristate-13-acetate-induced protein 1 | 3492.862| 7820.367| 2.238957| 0.038967|
| API5        | apoptosis inhibitor 5                            | 1207.84| 26298.57| 2.177243| 0.040781|

Table 5 Up-regulated genes in HL-60 cells treated with PLVX-EBF3 compared with control group
revealed similar survival outcomes in tumors with high or low EBF3 expression among 105 pediatric AML patients ($P = 0.091$, Table 3, and Figure 5C). Multivariate analysis also suggested that expression of EBF3 failed to be an independent prognostic factor in pediatric AML ($P = 0.348$, Table 4). The prognostic significance of EBF3 promoter methylation was assessed by clinical follow-up records of the 105 cases of Chinese pediatric AML patients. Table 2 shows no significant differences in clinical features, such as sex, age, FAB, cytogenetics, or MRD between patients with and without methylated EBF3 (Table 2). Samples exhibiting EBF3 promoter methylation revealed similar survival outcomes by Kaplan-Meier survival analysis ($P = 0.190$, Table 3, and Figure 5D). Furthermore, multivariate analysis revealed that EBF3 promoter methylation is not an independent prognostic factor in pediatric AML ($P = 0.574$, Table 4).

**Overexpression of EBF3 inhibited proliferation and induced apoptosis in leukemia cells**

To determine whether EBF3 is as an important player in leukemia cells, HL-60 or MV4-11 cells were stably transfected with EBF3. Expression of EBF3 was significantly upregulated after transfection of the PLVX-EBF3 lentivirus into HL-60 and MV4-11 leukemia cells (Figure 6A), and EBF3 overexpression significantly inhibit cell proliferation (Figure 6B). A CCK-8 assay in HL-60 and MV4-11 cells showed that the inhibition rate at 5 days post-transfection was 35.4 ± 19.8% and 39.3 ± 17.6% in EBF3-overexpressing cells compared with the mock transfection group ($P < 0.05$). To determine whether EBF3-induced apoptosis in leukemia cells, we performed an Annexin V assay in HL-60 and MV4-11 leukemia cells following transfection (Figure 6C and 6D). Our results showed that the proportion of apoptotic cells in the EBF3-overexpressing cells (PLVX-EBF3) was significantly greater than the vector control group (PLVX-Ve) [HL-60 (7.63% ± 1.11% vs. 1.23% ± 0.38%, respectively; $P = 0.006$) and MV4-11 (8.30% ± 1.08% vs. 1.67 ± 0.78%, respectively; $P = 0.002$)]. To further confirm the apoptotic effect of EBF3 in HL-60 and MV4-11 cells, we investigated the expression levels of cleaved PARP, a marker of apoptosis, by Western blotting. The results were consistent with the Annexin V data, confirming that EBF3 induced apoptosis in leukemia cells (Figure 6A).

**RT-PCR array analysis showed the dysregulation of apoptosis-related genes in HL-60 cells overexpressing EBF3**

Because we observed that overexpression of EBF3 induced apoptosis in leukemia cells, we examined the apoptosis-related genes by real-time PCR array that are implicated in EBF3 overexpressing HL-60 cells, cells that harbor an empty vector, or a vector overexpressing EBF3. The real-time PCR array was composed of 370 key genes that have been associated with apoptosis (Figure 7A). Examination of the
array data revealed that 62 genes were significantly upregulated and 31 genes were significantly downregulated in the EBF3-overexpression group compared with the control group (Table 5 and Table 6, respectively). Genes that were most significantly upregulated and downregulated in response to EBF3 overexpression are shown in Figure 7B and 7C, respectively. The up-regulated genes included cyclin-dependent kinase inhibitor 1A (p21, Cip1), DCC netrin 1 receptor, apoptosis-inducing factor, mitochondrion-associated 2, death-associated protein kinase 1 and 2, and caspase recruitment domain family, member 10. The down-regulated genes included zinc finger protein 443, baculoviral IAP repeat containing 8, and BCL2-like 11 (apoptosis facilitator). To validate the results of the real-time PCR array, we examined some of the dysregulated molecules at the protein level. The up-regulation of CDKN1A, DCC, and AIFM2 and the down-regulation of ZNF443, BIRC8, and BCL2L11 in EBF3-overexpressing group was verified by western-blot analysis (Figure 8).

**Ingenuity pathway analysis tool displays a pathway regulated by EBF3 overexpression in HL-60 cells**

To investigate the possible biological interactions of differentially regulated genes, datasets representing genes with altered expression profiles derived from our real-time PCR array analysis were imported into the Ingenuity Pathway Analysis (IPA) Tool. The list of differentially

| Gene   | Description                               | Vector | EBF3 | FC   | P      |
|--------|-------------------------------------------|--------|------|------|--------|
| ZNF443 | zinc finger protein 443                    | 199.493| 3.80756 | 0.019086 | 0.000602 |
| BIRC8  | baculoviral IAP repeat containing 8       | 433.5919 | 29.62754 | 0.06833 | 0.007686 |
| BCL2L11| BCL2-like 11 (apoptosis facilitator)      | 8365.257 | 663.1 | 0.079268 | 0.01033 |
| CARD17 | caspase recruitment domain family, member 17 | 28.84389 | 2.494701 | 0.08649 | 0.012285 |
| NLRP2  | NLR family, pyrin domain containing 2     | 16.79773 | 1.462936 | 0.087091 | 0.012456 |
| NIF3   | neurotrophin 3                            | 910.2974 | 122.6894 | 0.134779 | 0.025974 |
| INHA   | inhibin, alpha                            | 2435.824 | 404.1836 | 0.165933 | 0.04486 |
| IL10   | interleukin 10                            | 115.3756 | 24.23243 | 0.210031 | 0.007331 |
| FADD   | Fas (TNFRSF6)-associated via death domain | 3416.141 | 8072.876 | 0.236297 | 0.008898 |
| CD5    | CD5 molecule                              | 233.9723 | 60.92097 | 0.260377 | 0.010608 |
| SNCA   | synuclein, alpha                          | 38703.97 | 10360.94 | 0.267697 | 0.011798 |
| SST    | somatostatin                              | 5495.558 | 15.65842 | 0.284929 | 0.015721 |
| SERPINB9| serpin peptidase inhibitor, clade B       | 4153.737 | 1191.751 | 0.286911 | 0.017364 |
| PIDD   | p53-induced death domain protein          | 25712.71 | 7480.23 | 0.290916 | 0.013071 |
| TP52L1 | tumor protein DS2-like 1                  | 621.7514 | 187.2558 | 0.301175 | 0.013438 |
| RARG   | retinoic acid receptor, gamma             | 2628.81 | 808.3672 | 0.307303 | 0.01493 |
| ALOX15B| arachidonate 15-lipoxygenase, type B      | 8365.257 | 2700.231 | 0.322791 | 0.015529 |
| PAK7   | p21 protein (Cdc42/Rac)-activated kinase 7| 657.2025 | 219.6202 | 0.334174 | 0.016946 |
| LCK    | LCK proto-oncogene, Src family tyrosine kinase | 17082.06 | 5788.071 | 0.338839 | 0.017284 |
| ADRA1D | adrenoreceptor alpha 1D                   | 247.313 | 91.70109 | 0.37079 | 0.024013 |
| PCBP4  | poly(C) binding protein 4                 | 9675.993 | 3587.758 | 0.37079 | 0.024013 |
| TNFRSF18| tumor necrosis factor receptor superfamily, member 18 | 3420.98 | 1340.789 | 0.391931 | 0.022524 |
| GRM4   | glutamate receptor, metabotropic 4        | 576.1074 | 233.7572 | 0.405753 | 0.023978 |
| CRYAA  | crystallin, alpha A                       | 1184.608 | 490.7576 | 0.414278 | 0.024834 |
| FASLG  | Fas ligand (TNF superfamily, member 6)    | 233.9723 | 99.65477 | 0.425925 | 0.026786 |
| ALOX12 | arachidonate 12-lipoxygenase             | 153.2979 | 66.31 | 0.432556 | 0.026786 |
| PEA15  | phosphoprotein enriched in astrocytes 15  | 3083.16 | 1350.115 | 0.4379 | 0.027532 |
| S100B  | S100 calcium binding protein B            | 4211.721 | 1896.163 | 0.450211 | 0.028891 |
| NLRP3  | NLR family, pyrin domain containing 3    | 1661.495 | 7797.884 | 0.469329 | 0.035794 |
| TNFRSF18| tumor necrosis factor receptor superfamily, member 18 | 3984.331 | 1936.005 | 0.48588 | 0.036438 |
| TNFRSF8| tumor necrosis factor receptor superfamily, member 8 | 7487.114 | 3740.115 | 0.49954 | 0.038469 |
expressed genes analyzed by IPA revealed significant networks. Figure 9A depicts the list of the top 5 networks identified by IPA. Of these networks, cell death was the highest rated network with 77 focus molecules and a significance score of 54 (Figure 9D). The score is the probability that a collection of genes equal to or greater than the number in a network could be achieved by chance alone. A score of three indicates a 1/1000 chance that the focus genes are in a network not due to random chance. Figure 9D indicated firstly that ERK1/2, DAPK1, and caspase might be related to the EBF3 pathway. The IPA analysis also groups the differentially expressed genes into biological mechanisms that are related to cell death and survival, cellular growth and proliferation, DNA replication, cell morphology and cellular function, and maintenance (Figure 9B). The top five most significant pathways were presented in Figure 9C. These results may provide new clues for the molecular mechanisms of apoptosis induced by EBF3 overexpression.

Pediatric AML is a heterogeneous disease, which currently can be cured in approximately 70% of children. Five-year survival of pediatric AML varies from 15% – 70%, relapse rate varies from 33% – 78%, and its incidence is expected to increase. Hypermethylation of EBF3 has been reported in patients with rheumatoid arthritis [33], gastric carcinoma [26], head and neck squamous cell carcinoma [34], and neoplasms of the pancreas [35]. In this study, we found that the EBF3 promoter was hypermethylated in pediatric AML. Our results showed that the EBF3 promoter was hypermethylated in 10/12 leukemia cell lines. PCR analysis showed that EBF3 expression was significantly upregulated in leukemia cells following 5-Aza treatment compared with control cells treated with DMSO. In addition, western blot analysis showed that expression of EBF3 in NBM samples (n = 8) was significantly higher than leukemia cell lines (n = 9); these results are consistent with the MSP assay in leukemia cell lines. Aberrant methylation of EBF3 was observed in 39.0% (16/41) of pediatric AML samples compared with 6.7% (2/30) of NBM control samples. Consistent with the MSP results, the BGS results confirmed that the CpG islands in the EBF3 promoter were methylated in the AML samples (67.0% - 77.0%), whereas they were unmethylated in the NBM samples (41.0% - 50.0%). Taken together, these results imply that methylation may be involved in the downregulation of EBF3 in pediatric AML.

Although the EBF3 locus on chromosome 10q26.3 is epigenetically silenced or deleted in several types of cancers, the prognostic value of EBF3 has only been reported in gastric carcinoma. Promoter methylation of EBF3 was found to be significantly correlated with lymphatic invasion (p = 0.013) and poor survival (p = 0.038) in gastric carcinoma. These results suggest that the EBF3 tumor suppressor is epigenetically silenced and that it serves as an independent prognostic marker in gastric carcinoma [26]. Our results showed no significant differences in clinical features, such as sex, age, initial hemoglobin level, white blood cell counts, platelet counts and chromosomal abnormalities, between patients with methylated EBF3 and those with unmethylated EBF3 by examination of the clinicopathologic characteristics. Samples with EBF3 promoter methylation revealed similar survival outcomes through Kaplan-Meier survival analysis. Multivariate analysis revealed that EBF3 promoter methylation is not an independent prognostic factor in pediatric AML. This study represents the first report showing that the methylation of EBF3 is not an independent predictor of poor survival in AML.

A previous study in brain tumors revealed that expression of EBF3 resulted in cell cycle arrest and apoptosis. The expression of cyclin-dependent kinase inhibitors was
Figure 9 (See legend on next page.)
The basis of the IPA program consists of the Ingenuity Pathway Knowledge Base (IPKB), which is derived from known functions and interactions of genes published in the literature. The IPA Tool allows the identification of biological networks, global functions, and functional pathways of a particular dataset. This work shows that cell death was the highest rated network with 77 focus molecules and a significance score of 54. In addition, this study was the first to indicate that ERK1/2, DAPK1, and caspase may be related in the EBF3 pathway. However, the mechanism and the role of these genes in EBF3-induced apoptosis in AML remain to be elucidated further.

Conclusions
In this study, we identified epigenetic inactivation of EBF3 in both AML cell lines and pediatric AML samples for the first time. The expression of EBF3 was significantly lower in pediatric AML compared with control samples. In addition, our findings showed for the first time that transcriptional overexpression of EBF3 could inhibit proliferation and induce apoptosis in AML cells. We identified 93 dysregulated apoptosis-related genes in EBF3-overexpressing cells, including DCC, AIFM2, and DAPK1. Most of these genes have never been related to EBF3 overexpression. These results may provide new insights into the molecular mechanism of EBF3-induced apoptosis; however, further research will be required to determine the underlying details. Taken together, our findings suggest that EBF3 may act as a putative tumor suppressor gene in pediatric AML.

Abbreviations
ALL: Acute lymphoblastic leukemia; AML: Acute myeloid leukemia; MSP: Methylation specific PCR; BGS: Bisulfite genomic sequencing; NBM: Normal bone marrow; ITP: Idiopathic thrombocytopenic purpura.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
PJ and ZWL designed and directed the study. TYF, XLX and LJ finished the most of experiments. LZZ and WNN finished the real-time PCR array. LYP, XXY, ZHT, WY, JMF, LL and CL collected the leukemia sample. XPF, HSY and SGH collected the clinical information of samples. DXJ and SLC supported the design of primer for BGS and MSP analysis. FF, LG, ZH and LYH drafted this manuscript. ZXM, WI, FX and NJ participated in study design and coordination, data analysis and interpretation and drafted the manuscript. All authors read and approved the final manuscript.
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