Increased MCL-1 expression predicts poor prognosis and disease recurrence in acute myeloid leukemia

Xi-xi Li1,2,*
Jing-dong Zhou1,3,4,*
Xiang-mei Wen3,5,6,7
Ting-juan Zhang1,3,4
De-hong Wu6
Zhao-qun Deng3,5
Zhi-hui Zhang1,3,4
Xin-yue Lian1,3,4
Pin-fang He1,3,4
Xin-ru Yao7
Jiang Lin3,5
Jun Qian1,3,4

1Department of Hematology, Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu, People’s Republic of China; 2Department of Hematology, The Second Affiliated Hospital, Soochow University, Suzhou, Jiangsu, People’s Republic of China; 3Zhenjiang Clinical Research Center of Hematology, Zhenjiang, Jiangsu, People’s Republic of China; 4The Key Lab of Precision Diagnosis and Treatment in Hematologic Malignancies of Zhenjiang City, Zhenjiang, Jiangsu, People’s Republic of China; 5Laboratory Center, Affiliated People’s Hospital of Jiangsu University, Zhenjiang, Jiangsu, People’s Republic of China; 6Department of Hematology, The Third People’s Hospital of KunShan City, 215300 Kunshan, People’s Republic of China; 7School of Medicine, Jiangsu University, Zhenjiang, Jiangsu, People’s Republic of China

*These authors contributed equally to this work

Introduction

Acute myeloid leukemia (AML), a molecularly and clinically heterogeneous disease, is characterized by differentiation arrest and uncontrolled proliferation of myeloid progenitor cells, which ultimately interferes with the production of normal blood cells.1 Although recent treatment advances in hematopoietic stem cell transplantation and intensive chemotherapy, the clinical outcome for AML remained unsatisfactory.2 Since treatment with all-trans retinoic acid (ATRA) has dramatically improved the outcome of acute promyelocytic leukemia (APL) by binding to retinoic acid receptor α, the development of molecular risk-adapted treatment strategies and targeted therapies may improve the clinical outcome of AML.3 Therefore, the identifying biological markers correlated with prognosis, and recurrence is required for better management of AML patients and may provide potential...
targets for AML therapy. B-cell leukemia/lymphoma-2 (BCL-2) protein family consists of a pro-survival group and two pro-apoptotic subgroups. The pro-survival group comprise BCL-2, myeloid cell leukemia-1 (MCL-1), BCL-2 and BCL-2-like protein 1 isoform 1 (BCL-XL), which play important roles in regulating cell apoptosis, proliferation, differentiation and tumorigenesis. 4–8 Among them, MCL-1, isolated from a human myeloblastic leukemia cell line, can affect the development of tumor through the expression of its mRNA and protein in many cancers. 9–11 Allen et al indicated that overexpression of MCL-1 was related to poor overall survival (OS) in non-small cell lung cancer when MYC was up-regulated simultaneously. 6 In breast cancer, MCL-1 high-expressed was associated with later tumor stage and worse survival. 12 Glaser et al also found that knockdown of MCL-1, but not loss or pharmacological blockade of Bcl-xl, Bcl-2 or Bcl-w, caused the death of transformed AML and could cure disease in AML-affected mice. 13 Recently, MCL-1 protein inhibitor S63845 was reported to kill MCL-1-dependent cancer cells and has potential value in clinical application. 14 Herein, we aimed to investigate the MCL-1 expression pattern as well as its clinical implication in Chinese de novo patients with AML and may provide theoretical basis for AML patients using MCL-1 protein inhibitor in clinics.

Materials and methods

Patients

This study was approved by the Institutional Ethics Committee of the Affiliated People’s Hospital of Jiangsu University. Bone marrow (BM) aspirate specimens of 151 patients newly diagnosed AML, 26 healthy donors, 52 AML patients achieving complete remission and 23 relapsed AML patients were collected after written informed consent was signed. The diagnosis and classification of the patients were based on the 2008 WHO criteria. 15 All patients were treated at the Affiliated People’s Hospital of Jiangsu University from October 2005 to December 2016. Treatment protocol was described as reported previously. 16 The characteristics of all patients are summarized in Table 1.

BMMNCs separation, RNA isolation and reverse transcription

BM mononuclear cells (BMMNCs) were separated by Lymphocyte Separation Medium (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed as reported previously. 17,18

Real-time quantitative PCR

Real-time quantitative PCR (RQ-PCR) was used to detect MCL-1 expression level on a 7,300 Thermo cycler (Applied Biosystems, CA, USA). AceQ qPCR SYBR Green Master Mix Kit (Vazyme Biotech Co., Piscataway, NJ, USA) and ROX Reference Dye 1 (Invitrogen, Carlsbad, CA, USA) were applied to RQ-PCR reactions. The primers (forward 5'-GATGATCCATGTTTTCAGCGAC-3' and reverse 5'-CTGGGATGGGTTTGTGGAG-3') were used for MCL-1 transcript detection. The housekeeping gene ABL detected by 2× SYBR Green PCR Mix (Multisciences, Hangzhou, China) was used to calculate the abundance of MCL-1 transcript. The primers of ABL expression were 5'-TCCTCCAGCTTGATTCTGGAAGA-3' (forward) and 5'-TCCAACGAGCGGCTTCAC-3' (reverse). The RQ-PCR was carried out at 95°C for 5 mins, followed by 40 cycles at 95°C for 10 s, 60°C (MCL-1) or 60°C (ABL) for 30 s, 72°C for 32 s and 80°C for 32 s to collect fluorescence, finally followed by 95°C for 15 s, 60°C for 60 s, 95°C for 15 s and 60°C for 15 s. Relative MCL-1 expression levels were calculated by 2^−△△CT method.

Gene mutation detection

NPM1 and C-KIT mutations were detected by high-resolution melting analysis using the LightScanner platform (Idaho Technology Inc., Salt Lake City, Utah, USA). 20 FLT3-ITD and CEBPA mutations were detected by direct DNA sequencing (BGI Tech Solutions Co., Shanghai, China). 19,20 Both positive (leukemic cell lines samples, cultured in RPMI-1640 medium containing 10% fetal calf serum [ExCell Bio, Shanghai, China]) and negative controls (ddH2O) were included in each assay.

Gene expression profiling data

Gene expression profiling (GEP) data (accession number GSE12417, http://www.ncbi.nlm.nih.gov/geo/) was used to validate the prognostic value of MCL-1 expression in two independent cohorts of 162 and 78 cytogenetically normal AML (CN-AML) patients by the online web tool Genomicscape (http://genomicscape.com/microarray/survival.php). 21
Statistical analysis

Statistical analyses were performed using the SPSS 20.0 software package. Mann–Whitney’s $U$ test was carried to compare the difference of continuous variables between two groups. Pearson Chi-square analysis or Fisher exact test was employed to compare the difference of categorical variables. Kaplan–Meier analysis and Cox regression analyses were performed to analyze the impact of MCL-1 expression on OS and leukemia-free survival (LFS) in AML. Receiver operating characteristic curve (ROC) and area under the ROC curve (AUC) were conducted to assess the value of MCL-1 expression in discriminating AML patients from normal controls. For all analyses, a two-tailed $P$-value of 0.05 or less was determined as statistically significant.

Table 1 Comparison of clinical manifestations and laboratory features between AML patients with low and high MCL1 expression

| Patient’s parameters          | High (n=75) | Low (n=76) | P-value |
|------------------------------|-------------|------------|---------|
| Sex, male/female             | 44/31       | 47/29      | 0.741   |
| Median age, years (range)    | 60 (10–93)  | 52 (15–85) | 0.029   |
| Median WBC, $\times 10^9$/L (range) | 19.5 (0.3–197.7) | 16.6 (0.9–528) | 0.248   |
| Median hemoglobin, g/L (range) | 77 (34–144)  | 77 (34–123) | 0.849   |
| Median platelets, $\times 10^9$/L (range) | 40 (8–399)   | 34 (3–415) | 0.355   |
| BM blasts, % (range)*        | 50 (1–94.5) | 43 (5.5–97.5) | 0.348   |

Karyotype classification
- Favorable: 18 (24%) vs. 25 (33%)
- Intermediate: 45 (60%) vs. 41 (54%)
- Poor: 10 (13%) vs. 8 (10%)
- No data: 2 (3%) vs. 2 (3%)

Karyotype
- normal: 31 (41%) vs. 34 (45%)
- t(8:21): 8 (11%) vs. 6 (8%)
- t(16:16): 2 (3%) vs. 3 (4%)
- t(15;17): 2 (3%) vs. 3 (4%)
- +8: 3 (4%) vs. 0 (0%)
- −5/5q−: 0 (0%) vs. 1 (1%)
- −7/7q−: 1 (1%) vs. 3 (4%)
- t(9;22): 12 (16%) vs. 17 (9%)
- complex: 7 (9%) vs. 7 (9%)
- No data: 2 (3%) vs. 2 (3%)

Gene mutation
- CEBPA (±): 4/60 vs. 8/53
- NPM1 (±): 10/54 vs. 2/59
- FLT3-ITD (±): 8/56 vs. 7/54
- c-KIT (±): 2/62 vs. 4/57

CR, all AML (±)** | 49/24 vs. 30/38 | 0.007
CR, non-APL AML (±) | 48/17 vs. 27/24 | 0.031
CR, CN-AML (±) | 24/7 vs. 12/18 | 0.004

Notes: *AML patients less than 20% BM blasts often with typical cytogenetics such as t(15;17) (q22;q12). **For CR analysis, a total of 141 patients with available follow-up data were included.

Abbreviations: AML, acute myeloid leukemia; WBC, white blood cells; BM, bone marrow; CR, complete remission; CN-AML, cytogenetically normal AML.
Results

MCL-1 expression in controls and AML patients

As is shown in Figure 1, MCL-1 transcript level in 151 AML patients (range 0.0000–311.0063, median 6.6821) was significantly higher as compared with 26 controls (range 0.0002–22.4120, median 3.7865), which indicated that MCL-1 expression was up-regulated in AML (P=0.042). In addition, increased MCL-1 expression was also presented in both 123 non-APL (range 0.0000–311.0063, median 10.6716) and 65 CN-AML (range 0.0023–108.2933, median 5.1723) patients (P=0.005 and 0.044, respectively, Figure 1).

Discriminative capacity of MCL-1 expression

ROC was used to evaluate the discriminative capacity of MCL-1 expression. It revealed that MCL-1 expression could act as a potential biomarker for distinguishing whole-cohort AML (AUC=0.625, 95% CI: 0.526–0.723, P=0.043, Figure 2A), non-APL AML (AUC=0.676, 95% CI: 0.577–0.774, P=0.005, Figure 2B) and CN-AML (AUC=0.636, 95% CI: 0.515–0.756, P=0.044, Figure 2C) from normal controls.

Clinical and laboratory characteristics of AML patients

In order to investigate the clinical implication of MCL-1 expression in AML, we further divided the patients into two groups (MCL-1^{high} and MCL-1^{low}) based on the median level of 6.6821. The comparison of clinical characteristics of two groups is presented in Table 1. MCL-1^{high} patients showed older age as compared with MCL-1^{low} patients (P=0.029). Moreover, there was a significant difference in the distribution of karyotypes between two groups (P=0.042). However, no significant differences were found between the two groups in the sex, white blood cell (WBC), hemoglobin, platelets, percentage of BM blasts and karyotype classification (P>0.05). MCL-1^{high} patients had a significantly higher frequency of NPM1 mutation than MCL-1^{low} patients (Table 1). No significant differences were observed between MCL-1^{high} and MCL-1^{low} groups among other gene mutations.
Effect of MCL-1 expression on chemotherapy response in AML

The follow-up data is available in 141 patients after induction chemotherapy. $MCL-1^{\text{high}}$ patients presented significantly lower CR rate than $MCL-1^{\text{low}}$ patients (33% vs 57%, $P=0.007$, Table 1). In addition, we further showed $MCL-1$ transcript level between the patients with and without CR ($P=0.002$, Figure 3). Apart from APL patients, $MCL-1^{\text{high}}$ cases also had markedly lower CR rate than $MCL-1^{\text{low}}$ cases (26% vs 47%, $P=0.031$, Table 1). There was also a significant difference in CR rate between the two groups in CN-AML (23% vs 60%, $P=0.004$, Table 1).

Correlation between MCL-1 expression and clinical outcome

To investigate the prognostic significance of $MCL-1$ expression in AML, 141 AML patients with follow-up data (median 8 months, range 1–115 months) were included in survival analysis. Due to independent disease entity, APL was excluded from the analysis. Kaplan–Meier analyses revealed that there was a significant difference in OS time between $MCL-1^{\text{high}}$ and $MCL-1^{\text{low}}$ groups in non-APL patients (median 4 vs 9 months, respectively, $P=0.008$, Figure 4A). $MCL-1^{\text{high}}$ patients also had a shorter OS time than $MCL-1^{\text{low}}$ patients in CN-AML patients (median 3 vs 16.5 months, respectively, $P=0.004$, Figure 4B). In addition, multivariate analysis, including variables (age ($>60/\leq60$y), WBC ($\geq30/<30\times10^9$/L), $MCL-1$ expression (high/low) and gene mutations) with $P<0.200$ in univariate analysis, demonstrated that increased expression of $MCL-1$ was an independent adverse prognostic factor for OS among both non-APL and CN-AML patients (Tables 2 and 3). However, there was no significant difference in LFS between two groups both in non-APL ($P=0.157$) and CN-AML patients ($P=0.228$).

To further validate the prognostic impact of $MCL-1$ expression in CN-AML patients, we focused on the GEP

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**Figure 3** The comparison of $MCL-1$ expression among AML patients with and without CR. $MCL-1$ expression among AML patients ($n=62$, range 0.0000–105.5293, median 3.9643) who could achieve CR was significantly lower than those who could not achieve CR ($n=79$, range 0.0000–311.0063, median 15.5237) after induction therapy.

**Abbreviations:** AML, acute myeloid leukemia; CR, complete remission.

**Figure 4** The impact of $MCL-1$ expression on prognosis of AML patients. Overall survival between $MCL-1^{\text{high}}$ and $MCL-1^{\text{low}}$ group. (A) non-APL patients; (B) CN-AML patients.

**Abbreviations:** AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; CN-AML, cytogenetically normal AML.
Table 2 Univariate and multivariate analyses of prognostic factors for overall survival in non-APL patients

| Prognostic factors | Univariate analysis | Multivariate analysis |
|--------------------|---------------------|-----------------------|
|                    | Hazard ratio (95% CI) | P-value | Hazard ratio (95% CI) | P-value |
| Age                | 2.433 (1.599–3.702)  | <0.001   | 2.088 (1.359–3.207)  | 0.001   |
| WBC                | 2.118 (1.405–3.193)  | <0.001   | 1.000 (0.997–1.002)  | 0.804   |
| Karyotype          | 1.744 (1.404–2.165)  | <0.001   | 1.751 (1.266–2.421)  | 0.001   |
| MCL-1 expression   | 1.710 (1.125–2.600)  | 0.012    | 1.777 (1.164–2.714)  | 0.008   |
| FLT3-ITD mutation  | 1.403 (0.698–2.822)  | 0.342    | -                    | -       |
| NPM1 mutation      | 1.484 (0.738–2.982)  | 0.268    | -                    | -       |
| CEBPA mutation     | 0.818 (0.375–1.783)  | 0.613    | -                    | -       |
| c-KIT mutation     | 0.613 (0.150–2.503)  | 0.496    | -                    | -       |

Notes: Multivariate analyses, including variables [age (>60/≤ 60 y), WBC (≥30/<30*10^9/L), MCL-1 expression (high/low), and gene mutations] with P<0.200 in univariate analysis.
Abbreviations: APL, acute promyelocytic leukemia; WBC, white blood cells.

Table 3 Univariate and multivariate analyses of prognostic factors for overall survival in CN-AML patients

| Prognostic factors | Univariate analysis | Multivariate analysis |
|--------------------|---------------------|-----------------------|
|                    | Hazard ratio (95% CI) | P-value | Hazard ratio (95% CI) | P-value |
| age                | 2.437 (1.336–4.447)  | 0.004   | 2.126 (1.155–3.914)  | 0.015   |
| WBC                | 2.348 (1.312–4.202)  | 0.004   | 1.755 (0.958–3.215)  | 0.069   |
| MCL-1 expression   | 2.235 (1.123–4.035)  | 0.008   | 1.876 (1.013–3.473)  | 0.045   |
| FLT3-ITD mutation  | 0.944 (0.361–2.452)  | 0.901   | -                    | -       |
| NPM1 mutation      | 1.311 (0.545–3.149)  | 0.545   | -                    | -       |
| CEBPA mutation     | 1.055 (0.409–2.718)  | 0.912   | -                    | -       |
| c-KIT mutation     | 0.631 (0.086–4.643)  | 0.651   | -                    | -       |

Notes: Multivariate analyses, including variables [age (>60/≤ 60 y), WBC (≥30/<30*10^9/L), MCL-1 expression (high/low), and gene mutations] with P<0.200 in univariate analysis.
Abbreviations: AML, acute myeloid leukemia; CN-AML: cytogenetically normal AML; WBC: white blood cells.

Figure 5 Prognostic value of MCL-1 expression validated by public database. Gene expression profiling (GEP) data (accession number GSE12417, http://www.ncbi.nlm.nih.gov/geo/) was used to validate the prognostic value of MCL-1 expression in two independent cohorts of 162 and 78 cytogenetically normal AML (CN-AML) patients by the online web tool Genomicscape (http://genomicscape.com/microarray/survival.php).

Surveillance of MCL-1 expression in follow-up AML patients

To investigate whether the expression of MCL-1 could monitor disease recurrence in AML, we assessed MCL-1 expression in follow-up AML patients.
expression in 52 AML patients in CR and 23 cases in relapse. The median MCL-1 levels in patients at CR and relapse were 3.194 (range 0.000 to 76.368) and 12.076 (range 0.216–151.220), respectively. Obviously, MCL-1 expression was significantly decreased in CR time and was significantly increased in relapsed time compared with initial diagnosis time ($P=0.020$ and $P=0.004$, respectively, Figure 6). In addition, the dynamic change of MCL-1 expression in the five paired AML patients of different stages (Figure 7).

**Discussion**

Accumulating studies stated that MCL-1 expression was associated with the progression and outcome of various human malignant tumors.\(^4\)\(^–\)\(^6\) In multiple myeloma, MCL-1 high-expression was reported to be associated with relapse and shorter event-free survival.\(^11\) Mylin et al demonstrated that MCL-1 was up-regulated in multiple myeloma compared with monoclonal gammopathy of undetermined significance, but had no impact on OS and event-free survival.\(^22\) Moreover, the activated vascular endothelial growth factor induced the overexpression of MCL-1 and predicted a poor outcome and aggressive disease progression in non-Hodgkin’s lymphoma.\(^23\) More interestingly, Kempkensteffen et al elucidated that the antiapoptotic full-length splicing variant of MCL-1, down-regulation in clear-cell renal cancer, was related with poor tumor differentiation and predicted a higher risk for relapse; they also revealed that low MCL-1L was associated with short recurrence-free and disease-specific survival.\(^24\) Glaser et al used RNAi for knockdown of MCL-1 to determine that MCL-1 was essential for development and sustained growth of this hematological malignancy and it could be targeted for therapeutic benefit.\(^13\)

In this study, we observed that up-regulation of MCL-1 expression was a frequent event in Chinese de novo AML patients. Survival analyses revealed that high MCL-1 expression was associated with shorter OS both in non-APL and in CN-AML patients. Notably, our data showed that high-expression of MCL-1 had lower CR rate. Meanwhile, expression of MCL-1 in CR and relapsed patients illustrated that MCL-1 were significantly decreased from the initial diagnosis to CR and increased after relapsed. These results together
indicated that MCL-1 expression was a valuable predictor in assessing treatment outcome as well as status, and might serve as a criterion for the therapeutic evaluation in AML.

It has been well demonstrated that BCL-2 family proteins act as positive or negative regulators on cellular apoptosis and druggable targets.\textsuperscript{4-11,25} Kaufmann et al found that cells overexpressing MCL-1 were resistant to a variety of chemotherapeutic agents and raised the possibility that some chemotherapeutic regimens might select for leukemia cells with elevated levels of this particular apoptosis inhibitor.\textsuperscript{26} Recently, ABT-199, a highly potent and selective inhibitor of BCL-2, has been granted breakthrough designation by FDA for relapsed or refractory chronic lymphocytic leukemia with 17p deletion.\textsuperscript{27} Moreover, Pan et al found that ABT-199 also potently killed a diverse array of AML cell lines by establishing an aggressive mouse xenograft model of MOLM-13.\textsuperscript{27} Notably, some scholars stated that MCL-1 induced the resistance to ABT-737 (the first molecule studied extensively preclinically) and its analogs ABT-263 (the first of this series to enter the clinic) and ABT-199,\textsuperscript{28-30} while MCL-1 suppression could restore sensitivity to these compounds in leukemia cells,\textsuperscript{31,32} suggesting that drug resistance was attributed to high level of MCL-1. Kotschy et al found that the selective MCL-1 inhibitor S63845 killed MCL-1-dependent cancer cells by activating the BAX/BAK-dependent mitochondrial apoptotic pathway in diverse cancer models.\textsuperscript{14} Doi et al showed that maritoclax, a novel kind of BCL-2 family inhibitors, induced the selective degradation of MCL-1 through the proteasome to kill AML cells that express elevated levels of MCL-1.\textsuperscript{33} Recently, Wang et al revealed that inhibition of CDK9, a key component of the positive transcriptional regulator complex pTEFb which activates transcription via phosphorylation of RNA polymerase II, blocked transcription resulting in the repression of short-lived proteins such as MCL-1.\textsuperscript{34} Dey J et al found that voruciclib, a novel CDK9 inhibitor which addressed a well-characterized key-mediator of resistance to venetoclax by repressing MCL-1, and the BCL-2 specific inhibitor, venetoclax, with promise for treating high-risk subtype of diffuse large B-cell lymphoma by establishing specific models.\textsuperscript{35} Previous researches proved that higher levels of MCL-1 have been reported to result in the failure to achieve CR after fludarabine and chlorambucil treatment,\textsuperscript{36,37} while higher MCL-1/BAX ratio was related to poor response to rituximab in some patients.\textsuperscript{38} Taken together, these results suggested that MCL-1 combined with other targets have great potential in AML targeted therapies.

In addition, our study further observed that MCL-1 overexpression was associated with NPM1 mutation, which was a favorable prognostic indicator in AML and frequently occurred in patients with normal karyotype.\textsuperscript{39} In some studies, AML patients with NPM1 mutation appeared to benefit from ATRA as an adjunct to conventional chemotherapy.\textsuperscript{40,41} Moreover, it was reported that AML cells carrying NPM1 mutation were more sensitive to ATO, because of the expression of NPM1 mutant protein and its acquired C-terminus cysteine 288.\textsuperscript{42} Martelli et al also showed that ATO/ATRA induced proteasome-dependent degradation of NPM1 leukemic protein leading to cell growth inhibition and apoptosis in NPM1-mutated AML.\textsuperscript{43} Although a large number of findings suggest that the NPM1 mutation has a favorable effect on the outcome for AML,\textsuperscript{44-47} Falini et al found that NPM1 exon 12 mutations which caused cytoplasmic dislocation of NPM were related to the malignant clone and leukemogenesis.\textsuperscript{48} Moreover, a study conducted by Konoplev et al suggested that NPM1 mutations did not impact OS and event-free survival (EFS) in AML patients.\textsuperscript{49} However, Verhaak et al demonstrated that patients with intermediate cytogenetic risk AML with NPM1 mutations had a significantly better OS and EFS than those without NPM1 mutations.\textsuperscript{44} Further studies are needed to confirm and reveal the underlying association between MCL-1 expression and NPM1 mutation.

Collectively, these results demonstrated that MCL-1 is overexpressed and confers a poor prognosis in Chinese de novo AML patients and could be also used as a potential therapeutic target in the treatment of newly diagnosed and relapsed AML.

**Abbreviation list**

AML, acute myeloid leukemia; APL, acute promyelocytic leukemia; ATRA, all-trans retinoic acid; AUC, area under the ROC curve; BCL-2, B-cell leukemia/lymephma-2; BCL-XL, BCL-2 and BCL-2-like protein 1 isoform 1; BM, Bone marrow; BMMNCs, BM mononuclear cells; CN-AML, cytogenetically normal AML; CR, complete remission; GEP, melting analysis; LFS, leukemia-free survival; MCL-1, myeloid cell leukemia-1; OS, overall survival; PLT, platelets; RARα, retinoic acid receptor α; ROC, Receiver operating characteristic curve; RQ-PCR, Real-time quantitative PCR; WBC, white blood cell; WHO, World Health Organization.

**Ethics Statements**

We confirm in the revised manuscript that a parent or legal guardian provided written informed consent for any patient under 18 years of age, and that this study was conducted in accordance with the Declaration of Helsinki.
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Disclosure

The authors report no conflicts of interest in this work.

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