High *EVI1* Expression Predicts Poor Outcomes in Adult Acute Myeloid Leukemia Patients with Intermediate Cytogenetic Risk Receiving Chemotherapy

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Background: Acute myeloid leukemia with intermediate cytogenetic risk (ICR-AML) needs to be stratified. The abnormal gene expression might be prognostic, and its cutoff value for patient grouping is pivotal.

Material/Methods: Ecotropic viral integration site 1 (*EVI1*) transcripts were assessed in 191 adult ICR-AML patients at diagnosis who received chemotherapy only. *MLL*-PTD, *WT1* transcript levels, *FLT3*-ITD, and *NPM1* mutations were simultaneously evaluated, and 27 normal bone marrow samples were tested to define normal threshold.

Results: The normal upper limit of *EVI1* transcript levels was 8.0%. Receiver operating characteristic curve analysis showed that 1.0% (a 0.9-log reduction from the normal limit) was the *EVI1* optimal cutoff value for significantly differentiating relapse (P=0.049). A total of 23 patients (12%) had *EVI1* levels ≥1.0%. *EVI1* ≥1.0% had no effect on CR achievement, whereas it was significantly associated with lower 2-year relapse-free survival (RFS), disease-free survival (DFS), and overall survival (OS) rates in the entire cohort (P=0.0003, 0.0017, and 0.0009, respectively), patients with normal karyotypes (P=0.0032, 0.0047, and 0.0007, respectively), and *FLT3*-ITD (–) patients (all P<0.0001). Multivariate analysis showed that *EVI1* ≥1.0% was an independent adverse prognostic factor for RFS, DFS, and OS in the entire cohort. In addition, patients with *EVI1* transcript levels between 1.0% and 8.0% had 2-year RFS rates similar to those with *EVI1* ≥8.0%, and they both had significantly lower RFS rates than those with *EVI1* <1.0% (P=0.0005 and 0.027).

Conclusions: High *EVI1* expression predicts poor outcome in ICR-AML patients receiving chemotherapy. The optimal cutoff value for patient stratification is different from the normal limit.

MeSH Keywords: Gene Expression • Leukemia, Myeloid, Acute • Patient Outcome Assessment • Real-Time Polymerase Chain Reaction

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Background

Acute myeloid leukemia (AML) is a heterogeneous disease, and cytogenetic analysis is the classical method and framework for stratification [1–3]. Nearly half of AML patients are defined as an intermediate cytogenetic risk group, but their outcomes greatly varied [1–3]. Therefore, further stratification is needed to guide appropriate treatment. Over the past 2 decades, dozens of gene mutations have been discovered in AML, and the characterization of their prognostic impact is ongoing [4–6].

Apart from gene mutation, abnormal gene expression might also be prognostic [7,8], and Ecotropic viral integration site 1 (*EVI1*), *WT1,* and *MLL* partial tandem duplications (*MLL*-PTD) are representatives [9–22]. The *EVI1* gene is located on human chromosome 3q26 and encodes a transcription factor essential for both normal and malignant hematopoiesis [23]. It was shown that *EVI1* was aberrantly highly expressed in AML patients, both with and without cytogenetic abnormalities 3q26 [24,25]. In the past decade, several studies have demonstrated an adverse prognostic role of *EVI1* in adult AML and AML patients with intermediate cytogenetic risk (ICR-AML) [9–14], but its effects on complete remission (CR) achievement were contradictory [10,11,13]. To date, almost all such studies have been undertaken in European populations and its prognostic significance in other populations needs to be evaluated. In addition, the *EVI1* cutoff value for patient grouping remains obscure.

The cutoff value is the key to defining abnormal expression and differentiating patients. An abnormally-expressed gene is typically expressed in normal hematopoietic cells but with different levels from those found with leukemia [26–28]. As for the determination of *EVI1* high expression, some used the upper limit in normal bone marrow (NBM) as the cutoff value [13], whereas others arbitrarily selected from several values [9–12]. Therefore, the optimal threshold for clinical practice remains a challenge.

In the present study, by measuring *EVI1* expression as well as additional molecular abnormalities in 191 consecutive adult ICR-AML patients receiving chemotherapy at our institute, we compared different cutoff values and evaluated their prognostic effects on outcomes.

Material and Methods

Patients and treatment

A total of 191 adult ICR-AML patients were enrolled in the present study. These patients were consecutively diagnosed from January 2009 to December 2015, had available cytogenetic results, received at least 2 cycles of chemotherapy, were followed up at our institute, and did not receive allogeneic hematopoietic stem cell transplantation (allo-HSCT). Furthermore, all patients had available RNA and DNA extracted from bone marrow samples at diagnosis. The definition of cytogenetic risk was based on NCCN guidelines [29]. Intermediate-risk cytogenetics included normal cytogenetics, +8 alone, t(9;11), and other abnormalities not classified as favorable or unfavorable. The analyzed patients included 148 (77.5%) patients with normal karyotypes and 1 (0.5%) patient with t(9;11)(p22;q23). The basic characteristics are shown in Table 1. In addition, a total of 27 NBM samples were aspirated from normal volunteers who were allo-HSCT donors and we extracted RNA.

All patients received idarubicin (8–10 mg/m^2) for 3 days in combination with cytarabine (100 mg/m^2) for 7 days as the first induction regimen. Patients who achieved partial remission repeated the first induction regimen, and those who had no response were treated with other regimens. The consolidation therapy included 4 cycles of high-dose cytarabine (2 g/m^2, q12h, 3 days) followed by 2 cycles of regimens containing cytarabine and anthracyclines. The cutoff date for follow-up was September 20, 2016. This study was approved by the Ethics Committee of Peking University People's Hospital. All patients and volunteers provided written informed consent in accordance with the Declaration of Helsinki to participate in the present study.

Polymerase chain reaction (PCR)

Nucleated cells were obtained by treating fresh bone marrow samples with 0.144 M NH4Cl, 0.01 M NH4HCO3 to lyse the red cells. Total RNA and genomic DNA were individually extracted from bone marrow nucleated cells using Trizol and DNAzol Reagent (Invitrogen, Carlsbad, CA, USA). RNA was used to test *EVI1*, *MLL*-PTD, and *WT1* transcript levels by TaqMan-based real-time quantitative PCR (RQ-PCR), and DNA was used to amplify *FLT3*-ITD (internal tandem duplication) by qualitative PCR and *NPM1* mutations (A, B, and D type) by TaqMan-based RQ-PCR [30]. RQ-PCR was performed with a ABI PRISM 7500 Sequence Detector (Applied Biosystems, Foster City, USA). The primers and probes for the *EVI1* transcript were designed using Primer Express Software (Applied Biosystems, Foster City, USA) to detect all subtypes (1a, 1b, 1c, 1d, and 3L), and the sequences were as follows:
Forward primer: 5'-CCCATGTGCCAGAGGAACTT-3'
(in exon 14)
Reverse primer: 5'-CAGTGACAGCATCATAGCATATGC-3'
(in exon 15)
Probe: 5'-FAM-CAGCCGTTACACAGAAAGTCCAAATCGC-TAMRA-3'
(in exon 14)

The *MLL* primers and probes annealed to locations in exons 8–10 and 3 of the *MLL* gene to detect fusion between exons 8–10 and exon 3. The *WT1* primers and probe have been previously reported [31]. ABL was used as a control gene, and the

Table 1. Relationship between *EVI1* expression and variables at diagnosis in ICR-AML.

Variables	All		*EVI1* <1.0%		*EVI1* ≥1.0%		*P*-value**
N	191		168		23		–
Age (y, median, range)	43	(17–65)	42	(17–65)	49	(21–59)	0.10
Males (%)*	106	(55.5%)	93	(55.4%)	13	(56.5%)	1.0
WBC (×10⁹/L; median; range)	18.6	(0.6–321)	19	(0.8–282.5)	16.2	(1.8–145.1)	0.84
Hb (g/L; median; range)	86	(40–155)	89	(40–155)	75	(39–135)	**0.022**
PLT (×10⁹/L; median; range)	45	(3–838)	45	(7–838)	67	(3–344)	0.34
Blasts in bone marrow (%, median, range)	66%	(20–95%)	68%	(20–95%)	50%	(22–94%)	**0.0090**
Normal karyotype (%)*	148	(77.5%)	131	(78.0%)	17	(73.9%)	0.61
FLT3-ITD (+) (%)*	40	(20.9%)	37	(22.0%)	3	(13.0%)	0.42
NPM1 mutation (+)(%)*	66	(34.6%)	61	(36.3%)	5	(21.7%)	0.24
NPM1 mutation (+)/FLT3-ITD (–) (%)*	49	(25.7%)	44	(26.2%)	5	(21.7%)	0.80
MLL-PTD transcript level ≥1.0% (%)*	16	(8.4%)	14	(8.3%)	2	(8.7%)	1.00
WT1 transcript level ≥10.0% (%)*	123	(64.4%)	102	(60.7%)	21	(91.3%)	**0.0043**
FAB type							0.092
M0	2		1	(0.006%)	1	(4.3%)	
M1	9		8	(4.8%)	1	(4.3%)	
M2	129		119	(70.8%)	10	(43.5%)	
M4	22		17	(10.1%)	5	(21.7%)	
M5	25		20	(11.9%)	5	(21.7%)	
M6	4		3	(1.8%)	1	(4.3%)	

* Values are presented as the number of patients followed by the percentage in parentheses; other values are presented as the median followed by a range in parentheses; ** The bold numbers represent *P* values <0.05.

corresponding primers and probe were based on a report from the Europe Against Cancer Program [32]. The *EVI1*, *MLL*-PTD, and *WT1* transcript levels were calculated as the percentage of target transcript copies/ABL copies.

**Statistical analysis and definitions**

Pairwise comparisons of the variables between groups were performed using the Mann–Whitney U test for continuous variables and Fisher's exact test for categorical variables. A receiver operating characteristic (ROC) curve was used to identify optimal cutoff levels that best discriminated patients with different responses (achieving CR) and outcomes (relapse). Survival functions were estimated using the Kaplan-Meier method and compared using the log-rank test. Relapse-free survival (RFS) and disease-free survival (DFS) were measured from the date when CR was achieved. The events were relapse for RFS and death during CR1 or relapse for DFS. The event for

overall survival (OS) was death (regardless of the cause), and patients were queried at the date of last follow-up to determine whether they were still alive, or were censored on the date they were last known to be alive. Variables associated with *P*<0.20 in the univariate analysis were entered in multi-variable analysis performed by the Cox models. The level for a statistically significant difference was set at *P*<0.05 for all univariate tests. The SPSS 13.0 software package (SPSS Inc., Chicago, IL), and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) were used for data analysis.

Results

Patient outcomes

The median follow-up time for the entire cohort was 13 (2–91) months. A total of 167 (87.4%) patients achieved CR after
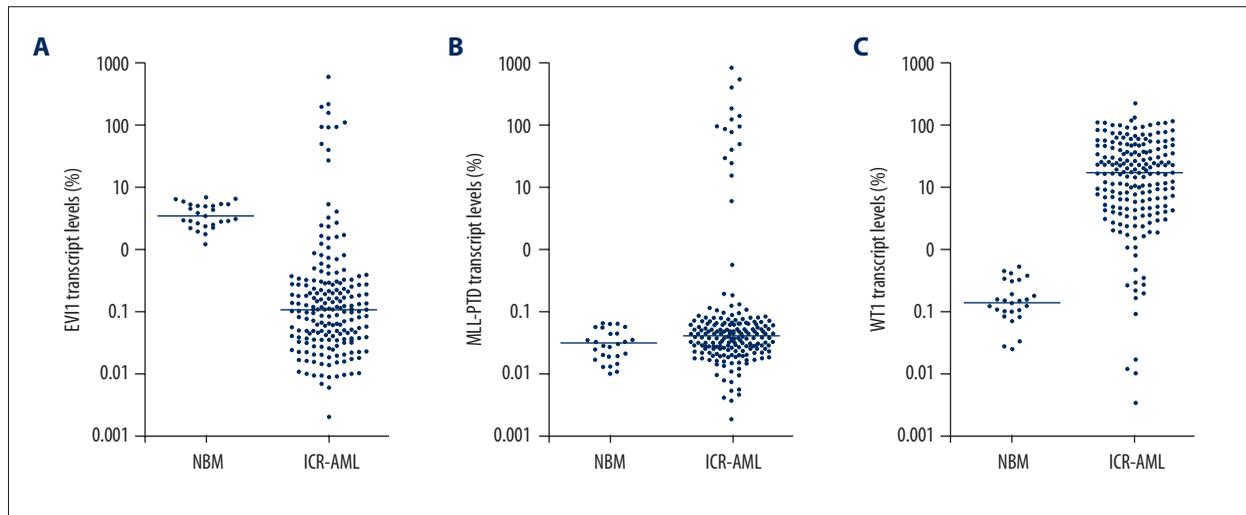
Figure 1. *EVI1*, *MLL*-PTD, and *WT1* expression patterns in 27 normal bone marrow (NBM) samples and BM samples collected from 191 newly diagnosed ICR-AML patients. (**A**) *EVI1*; (**B**) *MLL*-PTD; (**C**) *WT1*. Y-axis indicates the percentage of target transcript copies/ABL copies.
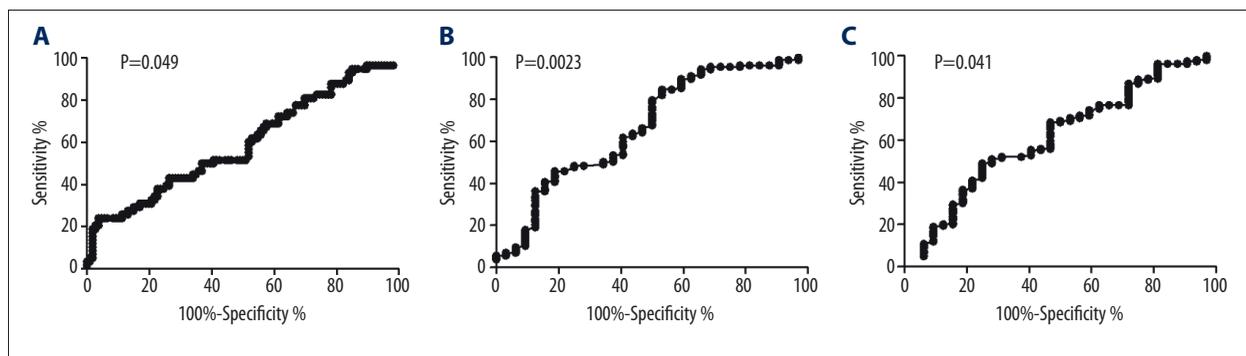
Figure 2. ROC curves. (**A**) Relationship between *EVI1* transcript levels and relapse. (**B**) Relationship between *MLL*-PTD transcript levels and 2-course induction of CR achievement. (**C**) Relationship between *WT1* transcript levels and 2-course induction CR achievement. The optimal cutoff value was determined according to maximal Youden index (sensitivity + specificity − 1).

induction during follow-up, and 117 (61.3%) patients were alive at last follow-up, with a median follow-up time of 16 (2–91) months. The 2-year RFS and DFS of the 167 patients who achieved CR were 54.2% (95% confidence interval (CI), 44.0–63.2%) and 49.4% (95% CI, 39.8–58.3%), respectively. The 2-year OS of the entire cohort was 58.4% (95% CI, 49.4–67.4%).

EVI1 expression and other molecular abnormality patterns in NBM and ICR-AML patients at diagnosis

EVI1, *MLL*-PTD, and *WT1* expression patterns in NBM and newly diagnosed ICR-AML patients are shown in Figure 1. The upper limits of *EVI1*, *MLL*-PTD, and *WT1* transcript levels of 27 NBM samples were 8.0%, 0.08%, and 0.6%, respectively (Figure 1). For the entire patient cohort, the median *EVI1*, *MLL*-PTD, and *WT1* transcript levels at diagnosis were 0.11% (range, 0.003–643.5%), 0.04% (range, 0.003–859.6%), and 19.4% (range, 0.004–251.2%), respectively (Figure 1). Compared with

the upper limit in NBM, 5.8% (11/191), 18.3% (35/191), and 93.2% (178/191) of patients individually overexpressed *EVI1*, *MLL*-PTD, and *WT1,* respectively. Furthermore, the frequencies of *FLT3*-ITD and *NPM1* mutations were 20.9% (40/191) and 34.6% (66/191), respectively.

Determination of optimal cutoff values of *EVI1*, *MLL*-**PTD,** and *WT1*

The ROC curves showed that *EVI1* transcript levels significantly differentiated patients in relapse (area under curve 0.59, *P*=0.049, Figure 2A). A value of 1.0% (a 0.9-log reduction from the upper limit in NBM) was identified as the optimal cutoff value based on its maximal Youden index (0.20) among all values. Therefore, *EVI1* ≥1.0% and <1.0% were defined as high expression and low expression, respectively. In the entire cohort, 23 (12.0%) patients had high *EVI1* expression (≥1.0%). In addition, EVI1 transcript levels did not significantly differentiate

Table 2. Impacts of molecular abnormalities on CR achievement.

Variables	After 1 course of induction		After 2 courses of induction	
	CR rate	*P* value*	CR rate	*P* value*
EVI1 transcript levels				
<1.0%	67.9%	0.16	83.9%	0.55
≥1.0%	52.2%		78.3%	
MLL-PTD transcript levels				
<1.0%	68.6%	**0.0047**	86.9%	**0.0002**
≥1.0%	31.3%		43.8%	
WT1 transcript levels				
<10.0%	69.1%	0.53	89.7%	0.10
≥10.0%	63.4%		79.7%	
NPM1 mutation				
(+)	80.3%	**0.0022**	93.9%	**0.0065**
(–)	58.1%		78.2%	
FLT3-ITD				
(+)	57.5%	0.26	70.0%	**0.017**
(–)	67.5%		86.8%	

* The bold numbers represent *P* values <0.05.

patients in CR achievement after 1 and 2 courses of induction (*P*=0.16 and 0.42).

Similarly, both *MLL*-PTD and *WT1* significantly differentiated patients achieving CR after 2 courses of induction (Figure 2B, 2C, *P*=0.023 and 0.041) but not in relapse (*P*=0.58 and 0.16), and the optimal cutoff value was 1.0% and 10.0%, respectively. In the entire cohort, 16 (8.4%) and 123 (64.4%) patients individually had high *MLL*-PTD and *WT1* expression, respectively (≥1.0% and 10.0%).

Relationship between *EVI1* expression and other patient characteristics and molecular abnormalities at diagnosis

As shown in Table 1, high *EVI1* expression (≥1.0%) was significantly related to low hemoglobin levels, low blast percentage in bone marrow, and high *WT1* expression (all *P*<0.05) but not age, sex, white blood cell (WBC) or platelet counts, *FLT3*-ITD frequency, *NPM1* mutation frequency, and FAB subtype. Furthermore, *EVI1* expression was not relevant to the distribution of *FLT3*-ITD (–)/*NPM1* mutation (+) (*P*=0.80).

Effects of *EVI1* expression and other molecular abnormalities on CR achievement

The CR rates after 1 and 2 courses of induction in the entire cohort were 65.4% (125/191) and 83.2% (159/191), respectively. As shown in Table 2, *EVI1* grouped by 1.0% had no impact

on CR achievement (all *P*>0.05). Similarly, *WT1* expression did not affect CR achievement. Differing from them, both high *MLL*-PTD expression and *NPM1* mutation (+) were significantly related to lower 1-course and 2-course induction CR rate (Table 2, *EVI1*: *P*=0.0047 and 0.0002; *MLL*-PTD: *P*=0.0022 and 0.0065), and *FLT3*-ITD (+) was significantly related to a lower 2-course induction CR rate (Table 2, *P*=0.017).

High *EVI1* expression (≥1.0%) predicted poor outcomes

In the entire cohort, the *EVI1* ≥1.0% group had significantly lower 2-year RFS, DFS, and OS rates than the *EVI1* <1.0% group (RFS: 6.7% [95% CI 0.4–26.2%] *vs.* 62.0% [95% CI 51.2–71.1%], *P*<0.0001; DFS: 11.9% [95% CI 2.0–31.5%] *vs.* 56.0% [95% CI 45.6–65.2%], *P*=0.0017; OS: 43.0% [95% CI 21.6–64.4%] *vs.* 64.4% [95% CI 61.9–76.5%], *P*=0.0009; Table 3 and Figure 3A–3C).
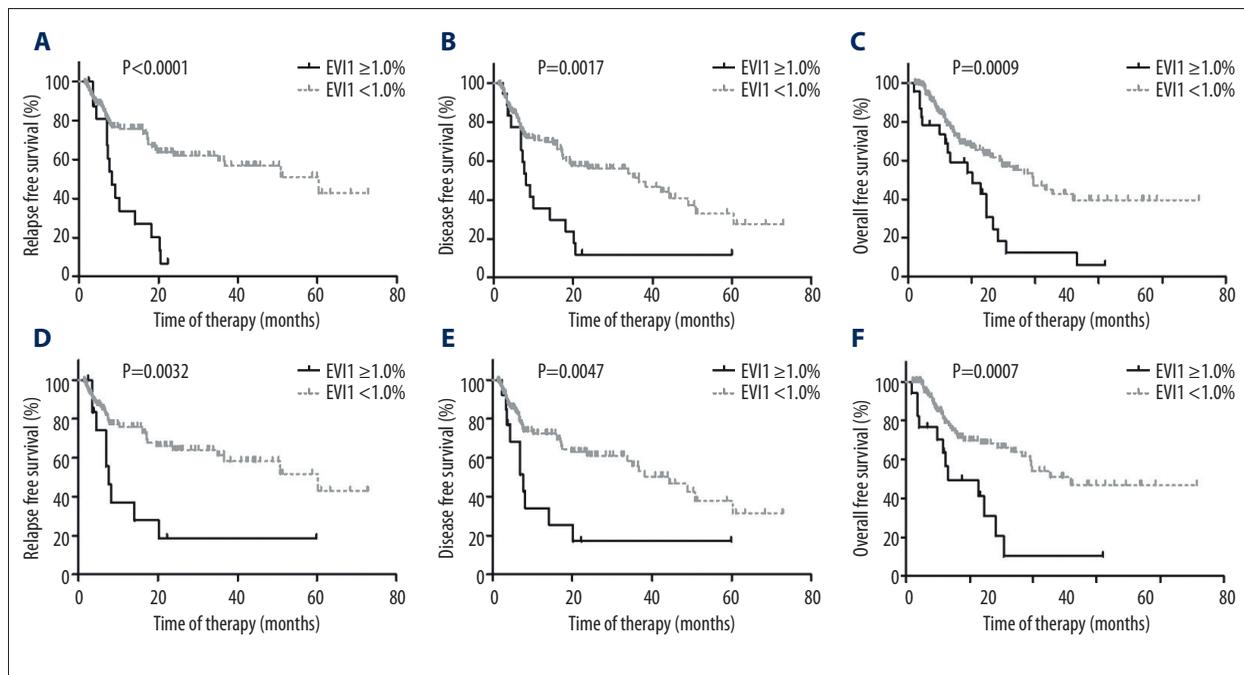
Patients with normal karyotypes (n=148) were further analyzed. Similarly, *EVI1* ≥1.0% (n=17) was significantly associated with lower 2-year RFS, DFS, and OS rates than *EVI1* <1.0% (RFS: 18.5% [95% CI 2.9–44.7%] *vs.* 64.0% [95% CI 52.1–73.7%], *P*=0.0032; DFS: 17.1% [95% CI 2.7–42.1%] *vs.* 61.1% [95% CI 49.5–70.8%], *P*=0.0047; OS: 41.3% [95% CI 16.8–64.5%] *vs.* 68.1% [95% CI 57.1–76.9%], *P*=0.0007; Figure 3D–3F).

FLT3-ITD (–) patients (n=150) were analyzed. *EVI1* ≥1.0% was significantly associated with lower 2-year RFS, DFS, and

Table 3. Univariate analysis of relapse and survival in the entire cohort.

| Variable	RFS		DFS		OS	
	HR (95%CI)	*P* value*	HR (95%CI)	*P* value*	HR (95%CI)	*P* value*
EVI1 ≥1.0%	7.7 (3.0–19.4)	**<0.0001**	3.5 (1.6–7.6)	**0.0017**	3.3 (1.6–6.7)	**0.0009**
MLL-PTD ≥1.0%	2.0 (0.70–5.9)	0.19	1.7 (0.67–4.4)	0.26	1.4 (0.64–3.1)	0.39
WT1 ≥10.0%	1.6 (0.91–2.7)	0.10	1.4 (0.87–2.3)	0.17	1.2 (0.73–2.0)	0.51
FLT3-ITD (+)	5.7 (2.6–12.6)	**<0.0001**	3.4 (1.7–6.8)	**0.0005**	2.6 (1.4–4.9)	**0.0022**
NPM1 mutation (+)	1.0 (0.62–1.8)	0.87	0.98 (0.61–1.6)	0.93	1.0 (0.63–1.6)	0.98
Age >40 y	1.1 (0.64–1.9)	0.75	1.2 (0.73–1.9)	0.51	1.1 (0.68–1.7)	0.72
Female	1.1 (0.64–1.8)	0.79	1.2 (0.78–2.0)	0.37	0.96 (0.61–1.5)	0.86
WBC >10×10⁹/L	1.2 (0.72–2.1)	0.47	1.1 (0.67–1.1)	0.74	0.99 (0.61–1.6)	0.96
Hb <90 g/L	1.2 (0.68–2.0)	0.59	1.0 (0.65–1.7)	0.89	1.0 (0.64–1.6)	0.93
PLT <100×10⁹/L	1.6 (0.91–2.9)	0.10	1.7 (1.0–2.8)	**0.051**	1.3 (0.76–2.3)	0.33
BM blast >65%	1.7 (0.99–2.8)	**0.056**	1.4 (0.87–2.2)	0.17	1.1 (0.73–1.8)	0.55
Normal karyotype	0.73 (0.38–1.4)	0.35	0.54 (0.30–1.0)	**0.047**	0.51 (0.29–0.88)	**0.017**

* The bold numbers represent *P* values <0.05.

Figure 3. The impacts of *EVI1* expression on relapse-free survival (**A, D**), disease-free survival (**B, E**), and overall survival (**C, F**). **A–C** showed the impacts in the entire cohort (n=191), and **D–F** showed the impacts in patients with normal karyotypes (n=148).

OS rates (RFS: 13.5% [95% CI 2.2–34.9%] *vs.* 71.0% [95% CI 58.9–80.1%], Figure 4A; DFS: 12.7% [95% CI 2.1–33.2%] *vs.* 64.2% [95% CI 52.5–73.7%]; OS: 41.1% [95% CI 19.1–62.0%] *vs.* 73.4% [95% CI 62.8–81.4%]. All *P*<0.0001). If *NPM1* mutation status was simultaneously considered, 5 *NPM1* mutation (+) and 15 *NPM1* mutation (–) patients had *EVI1* expression

≥1.0% (5/49 *vs.* 15/101, 10.2% *vs.* 14.9%), respectively. The *NPM1* mutation had no impact on the RFS rate in *FLT3*-ITD (–) patients (*P*=0.53), whereas *EVI1* ≥1.0% was significantly related to a higher 2-year RFS rate in both the *NPM1* mutation (–) and (+) patients (*P*<0.0001 and *P*=0.0079, Figure 4B).
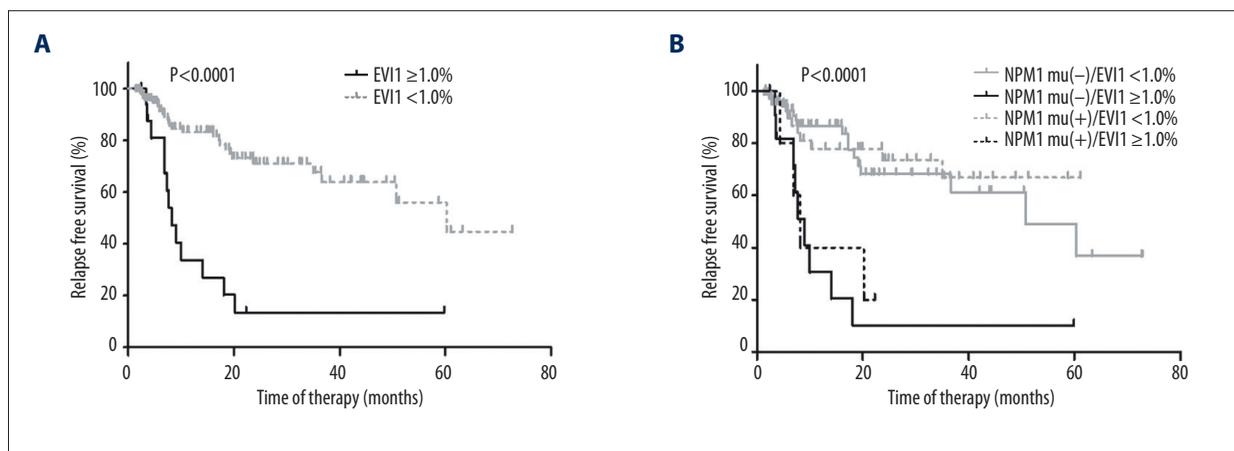
Figure 4. The impact of *EVI1* expression and *NPM1* mutation status on RFS in *FLT3*-ITD (–) patients. (**A**) Patients were grouped according to *EVI1* expression. (**B**) Patients were grouped according to both *EVI1* expression and *NPM1* mutation status.

High *EVI1* expression (≥1.0%) independently predicts poor outcomes in ICR-AML patients

Univariate analysis was performed in the entire cohort and is shown in Table 3. In addition to high *EVI1* expression, *FLT3*-ITD was significantly related to lower 2-year RFS, DFS, and OS rates (RFS: 19.8% [95% CI 5.3–40.9%] *vs.* 61.7% [95% CI 50.4–71.1%]; DFS: 18.9% [95% CI 5.1–39.3%] *vs.* 56.0% [95% CI 45.3–65.4%]; OS: 29.3% [95% CI 13.2–47.5%] *vs.* 65.2% [95% CI 55.0–73.7%]). However, *MLL*-PTD expression, *WT1* expression, and *NPM1* mutation all had no effects on relapse and survival.

The effects of variables associated with $P<0.20$ in univariate analysis were analyzed by multivariable analysis. As shown in Table 4, both high *EVI1* expression (≥1.0%) and *FLT3*-ITD (+) were independent adverse prognostic factors for RFS, DFS, and OS in the entire cohort. Furthermore, PLT count <100×10^9/L was an independent adverse prognostic factor for RFS and DFS, and BM blast >65% was an independent adverse prognostic factor for RFS.

Comparison between ROC curve and the upper limit of NBM-determined cutoff value

To further evaluate the impacts of *EVI1* expression on relapse and *MLL*-PTD expression on CR achievement, the patients were individually classified into 3 groups according to the ROC curve, and the upper limit of NBM-determined cutoff values. Patients with *EVI1* levels between 1.0% and 8.0% had 2-year RFS rates similar to those with *EVI1* ≥8.0% ($P=0.16$, Figure 5A), and both patient groups had significantly lower 2-year RFS rates than those with *EVI1* <1.0% ($P=0.0005$ and 0.027, Figure 5A). Furthermore, the 1- and 2-course induction CR rates of patients with MLL-PTD levels between 0.08% and 1.0% were similar to those with MLL-PTD <0.08% ($P=0.80$ and 0.28, Figure 5B), and they were all significantly higher than those of patients with MLL-PTD ≥1.0% (All $P<0.05$, Figure 5B).

Discussion

AML patients with intermediate cytogenetic risk need to be differentiated [1–4]. In the present study, *EVI1* expression was evaluated in combination with other molecular abnormalities in adult ICR-AML patients who received chemotherapy only. We found that the ROC curve-determined high *EVI1* expression was an independent poor prognostic factor for relapse and survival in ICR-AML patients, patients with normal karyotypes, and *FLT3*-ITD(–) patients.

Many genes involved in leukemogenesis are expressed in both leukemic cells and normal hematopoietic stem cells/progenitors (e.g., EVI1, *WT1,* and *MLL*-PTD) [26–28]. The abnormal expression of some genes (e.g., *WT1*) has been widely used to monitor minimal residual disease (MRD), in which the upper limit of NBM expression was usually used to define overexpression [33]. Another role of gene overexpression is prognosis. To best differentiate patients, determining the optimal cutoff value is important. Notably, the optimal cutoff value for prognosis may not always be the same as the upper limit in NBM. For example, we recently reported that *WT1* ≤5.0% (approximately 1-log increase compared with the upper limit in NBM) at diagnosis was significantly related to poor outcomes in t(8;21) AML patients [17]. In the present study, we demonstrated that a value less than the normal upper limit was the optimal cutoff value for *EVI1* with the largest Youden index. Comparisons revealed that the ROC curve analysis-determined cutoff value, but not the upper limit in NBM, significantly differentiated patients with respect to relapse. It was the same for *MLL*-PTD levels to differentiate patients in CR achievement. Therefore, the optimal prognostic cutoff value for abnormally-expressed genes needs to be identified by patient outcome data.

Contradictory results existed for the impact of *EVI1* expression on CR achievement. Lugthart et al. and Groschel et al.

Table 4. Independent prognostic factors for outcomes in the entire cohort.

	HR (95%CI)	*P* value
RFS		
EVI1 expression		
<1.0%	1.0	<0.0001
≥1.0%	4.0 (2.1–7.7)	
FLT3-ITD		
(–)	1.0	<0.0001
(+)	3.4 (1.9–6.0)	
PLT count		
≥100×10⁹/L	1.0	0.030
<100×10⁹/L	2.1 (1.1–4.3)	
Blast percentage in BM		
≤65%	1.0	0.017
>65%	2.1 (1.1–3.6)	
DFS		
EVI1 expression		
<1.0%	1.0	0.001
≥1.0%	2.6 (1.5–4.7)	
FLT3-ITD		
(–)	1.0	<0.0001
(+)	2.8 (1.6–4.7)	
PLT count		
≥100×10⁹/L	1.0	0.015
<100×10⁹/L	2.2 (1.2–4.1)	
OS		
EVI1 expression		
<1.0%	1.0	0.001
≥1.0%	2.4 (1.4–4.1)	
FLT3-ITD		
(–)	1.0	0.002
(+)	2.2 (1.3–3.6)	

individually showed that *EVI1* (+) AML patients had significantly lower induction CR rates than *EVI1* (–) AML patients [10,11], but Haas et al. did not observe this association [13]. With respect to ICR-AML, Groschel et al. reported that the CR rate was not related to *EVI1* expression [11]. Similarly, our results showed that *EVI1* transcript levels had no effect on CR achievement.

Almost all relevant studies have shown an adverse impact of high *EVI1* expression on outcomes in AML, despite considering different end points. High *EVI1* expression was demonstrated to independently predict event-free survival (EFS), DFS, RFS, and OS [9–11,13]. As for ICR-AML, *EVI1* high expression was shown to predict EFS, RFS, and OS by univariable or multivariable survival analysis [9–14]. In the present study, we confirmed the strong poor prognostic impact of *EVI1* overexpression in the Chinese cohort. Therefore, high *EVI1* expression strongly predicts poor outcomes for both AML and ICR-AML, which suggests its possible role in patient stratification in clinical routine.
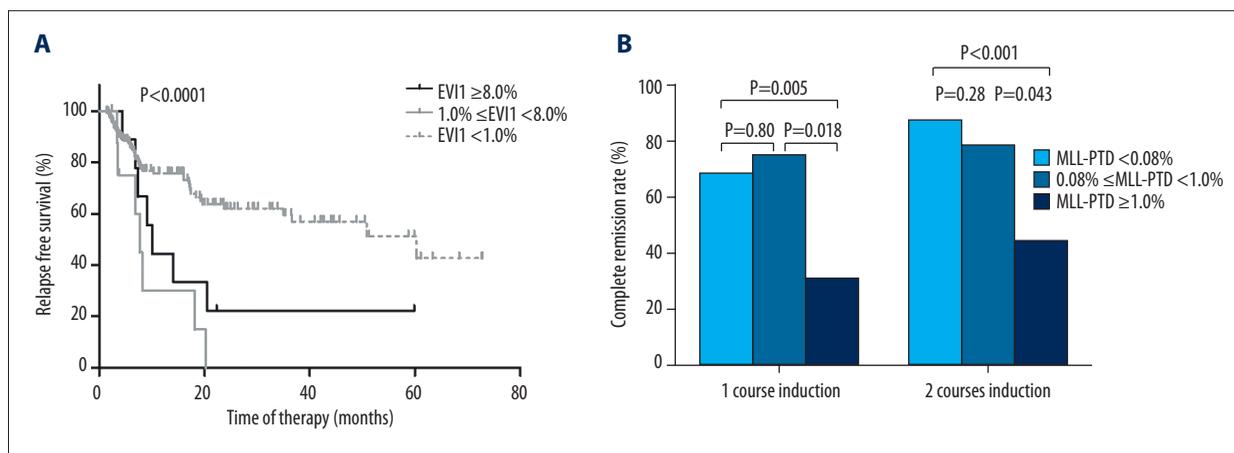
Figure 5. Comparisons among patients grouped according to the ROC curve and the upper limit of NBM-determined cutoff values. (**A**) Comparison of RFS rates among patients grouped according to *EVI1* expression. (**B**) Comparison of CR rates among patients grouped according to *MLL*-PTD expression.

In the AML cohort, Groschel et al. reported that *EVI1* (+) was inversely correlated with both *FLT3*-ITD (+) and *NPM1* mutation (+) [11]. However, in the present study, no significant correlations were observed. Furthermore, *FLT3*-ITD (–)/*NPM1* mutation (+) patients were demonstrated to have better outcomes and were defined as favorable risk [29,34]. In the present study, *NPM1* mutation was not found to be prognostic in *FLT3*-ITD (–) patients. These findings might be caused by the exclusion of patients receiving allo-HSCT. In this study, we found that high *EVI1* expression was associated with a lower RFS rate in both *NPM1* mutation (–) and (+) patients without *FLT3*-ITD. Therefore, *EVI1* expression may further stratify *FLT3*-ITD (–) patients receiving chemotherapy, regardless of *NPM1* mutation.

Although almost all relevant studies have shown an adverse impact of *EVI1* high expression on outcome in AML, it was difficult to make direct comparisons among them, which hinders the direct application of *EVI1* expression testing in clinical practice. In addition to the cutoff value selection method, differences also existed in the detection method and control gene for normalization. In the early studies, each subtype of *EVI1* was individually tested and analyzed. Subsequently, the common site of all subtypes was amplified and quantitated. Both absolute and relative real-time quantitative PCR methods were used [9–13]. Gene expression profiling (GEP) was also used [14]. The control gene used included PBGD, cyclophilin, ubiquitin C, G6PD, and GUSB [9–13]. Thus, standardization of the *EVI1* transcript testing and reporting is required. In the present study, we selected ABL as a control gene and the *EVI1* transcript level was expressed as a percentage of *EVI1* to ABL copies, a widely used quantitation method for other fusion genes in hematologic malignancies [32].

MLL-PTD and *WT1* expression were simultaneously assessed in the present study. In contrast to previous reports [18–22,35], high

MLL-PTD expression was shown to be significantly associated with a low CR achievement rate. For outcomes, the reported impact of *MLL*-PTD was discordant [18–22,35]. In the present study, *MLL*-PTD (+) was not found to be relevant to relapse and survival in ICR-AML. The therapy composition, drug dose, and race all might affect CR achievement. In addition, differences in the cutoff values for defining *MLL*-PTD (+) might also affect its prognostic role. The impact of *WT1* expression on outcomes in AML remains controversial [15–17]. *WT1* expression was not prognostic in ICR-AML in the present study. This inconsistency indicates that the prognostic value of *WT1* expression is weak and is affected by other factors, such as AML subtype and treatment modality.

The included variables affected the multivariate analysis results. Great progress has been made in the discovery of prognostic gene mutations in AML in the past 2 decades [6]. The limitation of this study is that it was a retrospective study. Although we investigated the widely used overexpression markers *WT1*, *MLL*-PTD, and *FLT3*-ITD, we did not screen *CEBPA*, *DNMT3A*, and other newly identified mutations.

Conclusions

EVI1 expression at diagnosis could further stratify ICR-AML, and high *EVI1* expression predicted poor outcomes in patients receiving chemotherapy. *EVI1* transcript levels should be routinely assessed at diagnosis for stratification once a standard laboratory protocol is established and the cutoff value is determined. Furthermore, the impact of *EVI1* expression should be fully investigated in the context of all newly identified gene mutations in AML.

Conflict of interest

None.

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