Low EVI1 expression at diagnosis predicted poor outcomes in pediatric Ph-negative B cell precursor acute lymphoblastic leukemia patients

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March 30, 2022

Abstract

Background: Abnormal high ecotropic viral integration site 1 (EVI1) expression was recognized as a poor prognostic factor in acute myeloid leukemia (AML) patients. However, its prognostic impact in B cell precursor acute lymphoblastic leukemia (BCP-ALL) remains unknown. Procedures: A total of 176 pediatric Ph-negative BCP-ALL patients who received at least 1 course of chemotherapy and received chemotherapy only during follow-up were retrospectively tested EVI1 transcript levels by real-time quantitative PCR at diagnosis and survival analysis was performed. Clinical and EVI1 expression data of 129 pediatric BCP-ALL patients were downloaded from the therapeutically applicable research to generate effective treatments (TARGET) database for validation. Results: In our cohort, the median EVI1 transcript levels were 0.33% (range, 0.0068-136.2%), and 0.10% was determined as the optimal cutoff value for patient grouping by receiver operating characteristic curve. Low EVI1 expression (<0.10%) was significantly related to lower 5-year relapse-free survival (RFS) and overall survival (OS) rates (P=0.017 and 0.018), respectively. Multivariate analysis showed that EVI1<0.10% was an independent adverse prognostic factor for RFS and OS. TARGET data showed that low EVI1 expression tended to be related to a lower 5-year OS rate (P=0.066). Conclusions: Low EVI1 expression at diagnosis may predict poor outcome in pediatric Ph-negative BCP-ALL patients receiving chemotherapy.

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Abstract word count: 204

Main text word count: 2668

Figures: 2

Tables: 3

Supporting information files: 1

Running title : Low EVI1 predicted poor outcomes in BCP-ALL

Keywords: Pediatric B cell precursor acute lymphoblastic leukemia; Ph-negative; EVI1 expression; At diagnosis; Prognosis

Abbreviations

EVI1	ecotropic viral integration site 1
AML	acute myeloid leukemia
BCP-ALL	B cell precursor acute lymphoblastic leukemia
TARGET	the therapeutically applicable research to generate effective treatments
RFS	relapse-free survival
OS	overall survival
HSC	hematopoietic stem cell
MDS	myelodysplastic syndrome
CML	chronic myeloid leukemia
ICR-AML	intermediate cytogenetic risk receiving chemotherapy
ALL	acute lymphoblastic leukemia
CLL	chronic lymphocytic leukemia
RQ-PCR	real-time quantitative PCR
CR	Complete remission
ROC	receiver operating characteristic
NBM	normal bone marrow

Abstract

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Procedures: A total of 176 pediatric Ph-negative BCP-ALL patients who received at least 1 course of chemotherapy and received chemotherapy only during follow-up were retrospectively tested EVI1 transcript levels by real-time quantitative PCR at diagnosis and survival analysis was performed. Clinical and EVI1 expression data of 129 pediatric BCP-ALL patients were downloaded from the therapeutically applicable research to generate effective treatments (TARGET) database for validation.

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Conclusions: Low EVI1 expression at diagnosis may predict poor outcome in pediatric Ph-negative BCP-ALL patients receiving chemotherapy.

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1. Introduction

The ecotropic viral integration site 1 (EVI1) gene was first recognized as a common locus of retroviral integration in murine myeloid leukemia models and found to be critical in transformation of murine hematopoietic cells.[1,2]Subsequent studies demonstrated that EVI1 is a hematopoietic stemness and transcription factor, which plays a pivotal role in the regulation of hematopoietic stem cell (HSC) self-renewal and myeloid leukemogenesis.[3-5] EVI1 is located on chromosome 3q26, and rearrangements on chromosome 3q26 often activate EVI1 expression which could be seen in acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and chronic myeloid leukemia (CML).[6]In addition, overexpression of EVI1 also occurs in patients without 3q26 abnormalities.[7,8] Importantly, several studies have uniformly demonstrated that high EVI1 expression confers a poor prognosis to AML.[9-12] We once evaluated adult AML patients with intermediate cytogenetic risk receiving chemotherapy (ICR-AML) and found that high EVI1 expression was an independent negative prognostic indicator.[13]

Compared to myeloid malignancies, the expression pattern and the role of EVI1 in lymphoid malignancies are far less investigated. EVI1 mRNA expression was confirmed in the majority of analyzed lymphoid cell lines and primary samples from patients with pediatric and adult acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL) by real-time quantitative PCR (RQ-PCR).[14-17] The microarray data showed that the range of EVI1 expression levels in pediatric ALL is much smaller than that in AML.[18] The in vitro and mice transplantation study demonstrated that EVI1 contributes to the leukemogenic potential and apoptosis resistance of ALL cells, which implied its link to poor prognosis.[14]Whereas in CLL, EVI1 was assigned tumor suppressor-like functions because of a negative interaction with the TCL1A oncogene to impact cell survival and clinical outcomes.[17] The effect of EVI1 expression on outcomes of patients with ALL could only be obtained by clinical cohort studies. However, it remains absent to date.

In the present study, the bone marrow samples collected from 176 newly diagnosed pediatric Ph-negative B cell precursor ALL (BCP-ALL) patients were performed RQ-PCR to detect EVI1 transcript levels, and the survival analysis was performed to investigate its prognostic role. We further validated the result by the RNA-seq data of ALL Expansion Phase 2 Project which downloaded from the Therapeutically Available Research to Generate Effective Treatments (TARGET, http://ocg.cancer.gov/programs/target) database.

2. Patients and methods

2.1 Patients and treatment

A total of 176 pediatric patients with Ph-negative BCP-ALL were enrolled in the current study. They were diagnosed at our hospital from December 2008 to January 2016, received at least 1 course of chemotherapy and received chemotherapy only as post-remission treatment. The median age at diagnosis was 4 (range, 0-16) years. A total of 94 (53.4%) patients were male. The diagnosis was based on bone marrow morphology, immunophenotyping, karyotyping and molecular testing. The cutoff date for follow-up was December 2019.

As we reported previously,[19,20] all the patients received treatments according to an improved ALL-Berlin-Frankfurt-Munster (BFM) protocol or the Chinese Children's Protocol for ALL 2008 (CCLG-ALL-2008). Briefly, the CODPL (cyclophosphamide and prednisone or dexamethasone, vincristine, and daunorubicin or idarubicin and L-asparaginase) regimen was used during induction therapy; 15 courses of high-dose methotrexate with or without pegaspargase, 3 courses of high-dose cytarabine, and a round of ifosfamide were used during consolidation therapy. Re-induction therapy was administered at half-year intervals during consolidation therapy. The cumulative doses of L-asparaginase and daunorubicin (or idarubicin) were 300,000 units/m^2 and 400(or 100) mg/m^2, respectively. 6-mercaptopurine and methotrexate were used for maintenance therapy. Besides, 23-25 doses of intrathecal methotrexate, cytarabine, and dexamethasone administration was used for central nervous system leukemia (CNSL) prevention. The whole treatment course lasted 3-3.5 years.

The study was approved by the Ethics Committee of Peking University People's Hospital, and all of the patients' parents/guardians provided written informed consent to participate in the study in accordance with the Declaration of Helsinki.

2.2 Detection of EVI1 and fusion transcript levels

All patients were collected bone marrow samples at diagnosis. Total RNA was extracted from nucleated cells. Reverse transcription of RNA and TaqMan-based RQ-PCR to measure the EVI1 transcript as well as BCR-ABL1, E2A-PBX1, TEL-AML1 and MLL rearrangement (MLL-AF4, MLL-AF9, MLL-AF1p and MLL-AF1q) fusion transcripts were performed as described in our previous studies.[13,21] ABL was selected as the control gene.[22] The amplification was performed in duplicate, and the EVI1 transcript level was calculated as EVI1 transcript copies/ABL copies in percentage.

2.3 Validation by TARGET database

The clinical and RNA-seq EVI1 expression data of 129 pediatric BCP-ALL patients were downloaded from the TARGET database and used for validation. The patients included met the following criteria: newly diagnosed, <18 years, Ph-negative and receiving chemotherapy only. EVI1 transcript level was represented as Log_2transcript per million.

2.4 Definitions

Complete remission (CR) was defined as no circulating lymphoblasts or extramedullary disease, bone marrow with trilineage hematopoiesis and less than 5% blasts, neutrophil counts of more than 1×10^9/L, platelet counts of more than 100×10^9/L, and no recurrence for 4 weeks.[23] Relapse was defined as the recurrence of higher than 5% BM blasts, the reappearance of blasts in the blood, or the development of extramedullary disease infiltrates at any site. Relapse-free survival (RFS) was measured from the date when CR was achieved to relapse. Overall survival (OS) was measured from diagnosis to death (regardless of cause) or last follow-up.

2.5 Statistical analysis

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 the optimal cutoff levels that best discriminated patients in relapse. Survival functions were estimated using the Kaplan–Meier method and were compared using the log-rank test. The variables with P <0.20 by the univariate analysis were entered into a multivariate model using a Cox proportional hazards model to identify the most statistically significant parameters associated with RFS and OS. The level for a statistically significant difference was set at P <0.05 for all univariate tests. The SPSS 19.0 software package (SPSS Inc., Chicago, IL) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA) were used for the data analysis.

3. Results

3.1 Patient characteristics and outcomes

The characteristics of all 176 patients at diagnosis were shown in Table 1. The median follow-up period was 52.5 months (range, 3-128 months). All patients (100%) achieved CR after the induction therapy. 27 (15.3%) patients relapsed during follow-up, and 12 (6.8%) of them died after relapse. The 5-year RFS and OS rates of the entire cohort were 84.5% (95% confidence interval (CI), 78.0-89.2%) and 93.3% (95% CI, 88.2-96.3%), respectively.

3.2 EVI1 expression patterns of the entire cohort

All 176 patients had detectable EVI1 transcript and the median levels were 0.33% (range, 0.0068-136.2%, Fig. 1A). We previously reported that the median EVI1 transcript levels in 27 normal bone marrow (NBM) were 4.5% (range, 2.1-8.0%, Fig. 1A).[13] Compared to EVI1 transcript levels in NBM, pediatric Ph-negative BCP-ALL patients had significantly lower EVI1 transcript levels (P<0.0001).

3.3 Determination of optimal cutoff values of EVI1 transcript for patient grouping

Firstly, we used the normal threshold 8.0% as the cutoff value. As shown in Fig. 1B, the grouped patients had similar 5-year RFS rates (P =0.48). Next, patients were grouped into quartiles according to EVI1 transcript

levels (1st quartile to 4th quartile, from low to high levels). Although the 5-year RFS rates were similar among the four groups (P =0.37), there was a trend that 1st quartile patients had lower 5-year RFS rate compared to the other 3 groups (P =0.17, 0.40, and 0.15, Fig. 1C), and the other 3 groups had the similar RFS rate (P =0.75, Fig. 1C).

Then, the ROC curve analysis was performed to determine the optimal cutoff value of EVI1 transcript levels. Although the EVI1 transcript levels could not significantly differentiate patients in relapse (area under the curve 0.57, P =0.24), 0.10% had the maximal Youden index and was determined as the optimal cutoff values. As a result, 32 (18.2%) and 144 (81.8%) patients had EVI1 transcript levels <0.10% and [?]0.10% and were defined as low and high EVI1 expression in our cohort, respectively.

3.4 Low EVI1 expression predicted poor outcomes

In the whole cohort, patients with low EVI1 expression had significantly lower 5-year RFS and OS rates than those with high EVI1 expression, respectively (RFS: 70.7% [95% CI 51.0-83.6%] vs. 87.6% [95% CI 80.8-92.1%], P =0.017, Fig. 2A; OS: 83.4% [95% CI 64.6-92.8%] vs. 95.5% [95% CI 90.3-98.0%], P =0.018, Fig. 2B).

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

As shown in Table 1, low platelet count, high blast percentage in bone marrow and MLL rearrangement were significantly related to low EVI1 expression (all P <0.05), and TEL-AML1 fusion gene was significantly related to high EVI1 expression (P<0.05). Other parameters including age, sex, WBC count, hemoglobin, high-hyperdiploidy, E2A-PBX1 fusion gene, immunophenotypic subtype at diagnosis and adverse cytogenetic risk feature (include MLL rearrangement, E2A-PBX1 fusion gene, or hypodiploidy) had no association with EVI1 expression (all P >0.05).

3.6 Low EVI1 expression independently predicted poor outcomes

Univariate analysis of RFS and OS in the whole cohort were shown in Table 2. In addition to EVI1 expression at diagnosis, age <1 or >10 and E2A-PBX1 fusion gene were significantly related to lower RFS rate. Furthermore, WBC count [?]30x10^9/L and E2A-PBX1 fusion gene were significantly related to lower OS rate (all P <0.05).

Variables with P <0.20 in univariate analysis were entered into multivariate analysis. As shown in Table 3, low EVI1 expression, age <1 or >10 and E2A-PBX1 fusion gene were independent adverse prognostic factors for RFS, and low EVI1 expression was the only independent adverse prognostic factor for OS.

3.7 Validation of the prognostic impact of EVI1 expression by TARGET data

Of 129 pediatric Ph-negative BCP-ALL patients from TARGET database for validation, 63 (48.8%) were male, and the median age was 6 (range 1-18) years. 11.3% (12/106), 14.7% (14/95) and 1.8% (2/111) of patients individually had TEL-AML and E2A-PBX1 fusion gene and MLL rearrangement, and 15.6% (15/96) of patients had high-hyperdiploidy karyotype. Totally, 92 (71.3%) patients relapsed and 72 (55.8%) patients died, and the median follow-up time was 50 (range, 3-122) months.

By performing ROC curve, the EVI1 transcript levels with the maximal Youden index was identified as the cutoff value for grouping. As a result, 94 (72.9%) and 35 (27.1%) patients individually belonged to low EVI1 expression and high EVI1 expression. As shown in Fig. 2C, patients with low EVI1 expression tended to have lower 5-year OS rate compared with the patients with high EVI1 expression (47.2% [95% CI 36.7-56.9%] vs 63.3% [95% CI 44.4-77.4%], P =0.066).

We further analyzed the relationship between EVI1 expression and variables at diagnosis (Table S1). TEL-AML1 fusion gene was significantly related to high EVI1 expression (P =0.0006) and E2A-PBX1 was not related to EVI1 expression, which is similar to our cohort. Whereas, no relationship existed between MLL

arrangement and EVI1 expression, which was different from our cohort. Furthermore, platelet count and blast percentage in bone marrow were absent in TARGET data.

4. Discussion

So far survival analysis has not been performed to evaluate the impact of EVI1 mRNA expression in ALL. In the current study, basing on the EVI1 transcript detected by RQ-PCR in our cohort and further validating by RNA-seq data from TARGET database, we demonstrated that low EVI1 expression at diagnosis predicted poor outcomes in pediatric Ph-negative BCP-ALL patients receiving chemotherapy only.

EVI1 gene encodes an oncogenic transcription factor and aberrant EVI1 expression has been found in various human myeloid malignancies, including AML, MDS, and CML.[6] High EVI1 expression predicted poor outcomes in adult and pediatric AML patients as well as ICR-AML patients.[9-13] EVI1 could induce MDS in the mouse model, and it was a poor prognostic marker for MDS patients.[24,25] In CML patients, high EVI1 expression was related to blast crisis.[26] Compared to AML, the prognostic role of EVI1 in ALL remains obscure.

ALL is the most common cancer and the most frequent cause of death from cancer among children. It is a heterogeneous disease with various genetic abnormalities.[27] Although the outcomes of pediatric BCP-ALL have improved greatly in the past decades, relapse remains occur in the minority leading to death.[28]Therefore, searching for new prognostic markers is still necessary in order to guide appropriate treatment selection and improve the overall outcomes. Therefore, the prognostic impact of EVI1 expression in pediatric BCP-ALL needs to be evaluated.

EVI1 expression patterns were evaluated firstly. Several studies have showed EVI1 transcript levels in ALL which tested by PCR or microarray.[14,15,18] T-ALL patients and cell lines were found to have no detectable EVI1 transcript by RT-PCR.[16] Whereas, Su G et al performed RQ-PCR and found that T-ALL had significantly higher EVI1 levels than BCP-ALL. Furthermore, they showed that the overall EVI1 transcript levels were significantly higher in ALL than AML.[15] We also found the similar result in pediatric Ph-negative BCP-ALL patients. That is, they generally had significantly higher levels than adult ICR-AML patients which we reported previously,[13] and great variation existed among patients.

The cutoff value for patient grouping usually affected the final comparison result greatly. As for the cutoff value of EVI1 transcript, both the upper limit and the mean+-standard deviation values of NBM were ever used in previous studies.[13,15] Furthermore, some studies arbitrarily determined.[9,14] In the current study, quartile grouping implied that low EVI1 expression was related to low RFS rate, and ROC curve was used to determine the optimal cutoff value.

The survival analysis in our cohort showed that low EVI1 expression independently predicted low RFS and OS rate. The TARGET data for validation showed the similarly negative impact of low EVI1 expression on OS rate though the P value is no less than 0.05. However, a previous in vitro and mice transplantation study demonstrated that EVI1 contributes to the leukemogenic potential and apoptosis resistance of ALL cells, which implied the poor prognostic role of EVI1.[14] One reason for this contradictory result is that protein function is usually cell context-specific. Therefore, the in vitro result might be different from that in vivo and cell line could not represent primary leukemic cells completely. Another reason might be the EVI1 level. Just as illustrated by our quartile grouping result, the 2nd to 4th quartile group had similar RFS. Therefore, so-called low or high EVI1 expression must be clearly defined and the in vitro study should correspond to clinical meaningful value in order to clarify the function of EVI1 in ALL.

The percentage of patients belonging to low EVI1 expression were different in our cohort and TARGET cohort for validation, 18.2% and 72.9%, respectively. It might be caused by different case composition. The TARGET ALL Pilot project included comprehensive genomic profiles of nearly 200 high-risk, clinically annotated, BCP-ALL patient cases from Children's Oncology Group (COG), most of which experienced an early bone marrow relapse (within 4 years of initial diagnosis).

A significant correlation between high EVI1 expression and MLL rearrangement has been demonstrated in

both adult and pediatric AML patients.[9-12,29] However, we found an association between low EVI1 expression and MLL rearrangement in pediatric BCP-ALL patients. Furthermore, TARGET data and previous reports did not show this relationship in BCP-ALL patients.[15,18] These inconsistent results reflected that the association between MLL rearrangement and EVI1 expression were related to leukemia type. Difference in the partner of MLL fusion and all enrolled patients received chemotherapy only in our cohort might also be the reasons. In addition, both our cohort and TARGET data showed that patients with TEL-AML1 fusion gene had high EVI1 expression, and its mechanism remains to be studied.

5. Conclusion

We demonstrated in our cohort that low EVI1 expression at diagnosis predicted poor outcomes in pediatric Ph-negative BCP-ALL patients receiving chemotherapy only, which was validated by TARGET ALL Expansion (Phase 2) project cohort. It implied the necessity to test EVI1 transcript at diagnosis for stratification. The current result needs to be confirmed in other cohort and the prognostic impact of EVI1 expression in BCP-ALL patients receiving allogeneic stem cell transplantation remains to be evaluated. Furthermore, prospective multicenter trial is warranted, and the mechanism of the effect need be investigated.

Conflict of interest statement

We confirm that no conflicts of interest exist.

Funding information

This study was supported by Scientific Research Foundation for Capital's Funds for Health Improvement and Research (2020-2Z-40811) and the National Nature Science Foundation of China (81870125).

Acknowledgement

We thank the TARGET databases for the availability of the data.

Author contributions

YZQ designed the study; LY, FTD, WMC, LDL and LYL performed the PCR analysis; ADL, YRL, KYL and LPZ collected clinical data; LY wrote manuscript; YZQ revised the manuscript. All authors gave final approval.

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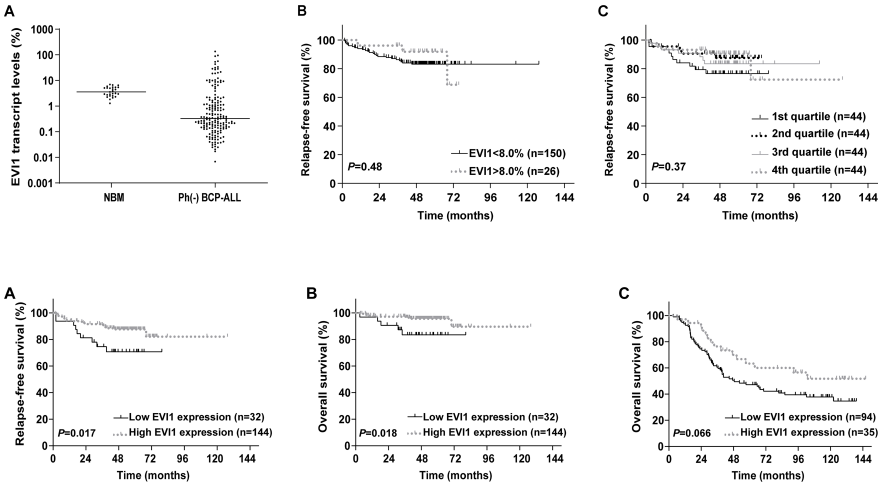
Figure legends:

FIGURE 1 Comparison of EVI1 expression patterns(A) and RFS analysis by different patient grouping ways (B, C). (A) Data of 27 NBM was quoted from Ref13. The horizontal lines represent the median levels; (B) Patients were grouped by the upper limits of NBM of EVI1 transcript levels; (C) Patients were grouped into quartiles according to EVI1 transcript levels.

FIGURE 2 The impact of EVI1 expression at diagnosis on survival. A: RFS, our cohort; B: OS, our cohort; C: OS, TARGET data.



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