EVI1 overexpression is a poor prognostic factor in pediatric patients with *mixed lineage leukemia-AF9* rearranged acute myeloid leukemia

The ecotropic viral integration site-1 gene (*EVI1*) encodes a zinc finger protein that functions as a transcriptional regulator of hematopoietic stem cell self-renewal and long-term multilineage repopulating activity.[1,2] The mixed lineage leukemia gene *(MLL)* rearrangements [i.e. t(11q23)] occur at high frequency in pediatric acute myeloid leukemia (AML) patients with *EVI1* overexpression,[3] and *EVI1* is a transcriptional target of MLL oncoproteins.[4] *EVI1* overexpression has been reported in up to 10% of patients with AML and is associated with an adverse prognosis. However, the prognostic value of *EVI1* overexpression has been studied mostly in adult AML.[5-9] Only two studies have examined *EVI1* overexpression in pediatric AML, but a detailed analysis according to the type of leukemia was not performed because of the small sample size.[3,10]

Recent data from an international consortium, including those from our group, suggest that pediatric *MLL*-rearranged AML can be divided into certain risk groups on the basis of different translocation partners.[11] However, clinical outcome data leading to risk stratification of the *MLL*-rearranged subgroups are still scarce and further investigation is necessary to identify new prognostic factors. Here, we retrospectively examined *EVI1* expression levels and clinical outcomes of pediatric *MLL*-rearranged AML patients treated in the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) AML-05 study.

After excluding patients with acute promyelocytic leukemia, Down syndrome, secondary AML, myeloid/natural killer cell leukemia and myeloid sarcoma, 485 AML patients were enrolled in the AML-05 study. Overall, 42 patients were excluded, mainly because of misdiagnosis. Details of the treatment schedules and risk stratification were described in previous publication.[12] This study was conducted in accordance with the principles set down in the Declaration of Helsinki and was approved by the Ethics Committees of all participating institutions. All patients, or the patients' parents/guardians, provided written informed consent.

RNA obtained from diagnostic bone marrow samples was used to analyze the expression of *EVI1* using a previously established *EVI1* quantitative real-time polymerase chain reaction assay that covers the various *EVI1* splice variants.[7] Event-free survival (EFS) was defined as the time from the diagnosis of AML to the last follow up or the first event (failure to achieve remission, relapse, secondary malignancy, or any cause of death). In this study, most of the events were relapses (n=23) and the rest were deaths with sepsis (n=1) and acute respiratory distress syndrome (n=1). Overall survival (OS) was defined as the time from the diagnosis of AML to any cause of death. All tests were two-tailed and $P<0.05$ was considered statistically significant.

Among 443 eligible AML patients, 69 were diagnosed as *MLL*-rearranged AML and diagnostic samples from 50 patients were analyzed for *EVI1* mRNA expression. No significant differences in the characteristics and clinical outcomes were observed between these 50 patients and the 19 patients who did not have *EVI1* data [EFS (P=0.20), OS (P=0.45)]. *EVI1* expression levels were dichotomized based on a cut off of 0.1 relative to SKOV3, an ovarian carcinoma cell line over-expressing *EVI1*: values higher than 0.1 were defined as *EVI1*$^+$ and those lower than 0.1 or undetectable

Table 1. Characteristics of patients categorized according to *EVI1* expression status.

	All (n=50)				
	EVI1$^-$ (n=32)		*EVI1*$^+$ (n=18)		*P*
Age (years)					0.03#
median	4.5		6.6		
range	0.1-14.7		0.8-15.1		
Sex, n(%)					0.77*
male	16	(50)	8	(44)	
female	16	(50)	10	(56)	
WBC(x10^9/L)					0.01#
median	48.4		88.7		
range	0.8-459		4.1-322		
Types of *MLL* rearrangement, n(%)					0.96*
MLL-AF6	2	(6)	1	(6)	
MLL-AF9	18	(56)	11	(61)	
MLL-AF10	5	(16)	2	(11)	
MLL-ELL	3	(9)	3	(17)	
MLL-ENL	3	(9)	1	(6)	
MLL-AF17	1	(3)	0	(0)	
FAB, n(%)					<0.0001*
M1	1	(3)	3	(17)	
M2	0	(0)	1	(6)	
M4	2	(6)	6	(33)	
M5	27	(84)	4	(22)	
RAEB-T	0	(0)	3	(17)	
Unclassified	2	(6)	1	(6)	
FLT3-ITD, n(%)	0	(0)	3	(17)	0.04*

*WBC: white blood cell count; FAB: French-American-British. *Fisher's exact test. #Mann-Whitney U test.*

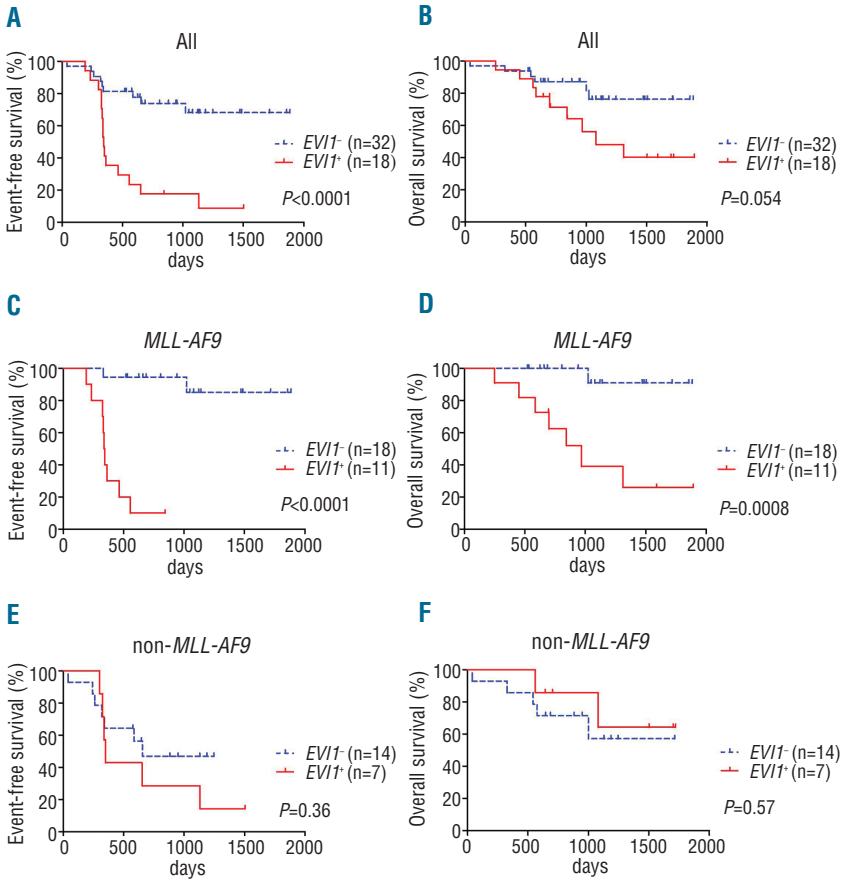

Figure 1. Kaplan-Meier survival curves of event-free survival (EFS) and overall survival (OS) from the time of diagnosis according to *EVI1* expression status. (A) Kaplan-Meier estimates of EFS in the cohort of *MLL*-rearranged AML in *EVI1*$^+$ and *EVI1*$^-$ patients. (B) Kaplan-Meier estimates of OS in the cohort of *MLL*-rearranged AML in *EVI1*$^+$ and *EVI1*$^-$ patients. (C) Kaplan-Meier estimates of EFS in the cohort of *MLL-AF9* in *EVI1*$^+$ and *EVI1*$^-$ patients. (D) Kaplan-Meier estimates of OS in the cohort of *MLL-AF9* in *EVI1*$^+$ and *EVI1*$^-$ patients. (E) Kaplan-Meier estimates of EFS in the cohort of *MLL*-rearranged AML without *MLL-AF9* in *EVI1*$^+$ and *EVI1*$^-$ patients. (F) Kaplan-Meier estimates of OS in the cohort of *MLL*-rearranged AML without *MLL-AF9* in *EVI1*$^+$ and *EVI1*$^-$ patients. *P* values determined using the log rank test.

were defined as *EVI1*$^-$, as described in a previous study.[7] *EVI1*$^+$ was present in 18 patients (36%). *EVI1* expression levels in different *MLL* translocation partners relative to that in SKOV3 cells are shown in *Online Supplementary Figure S1*. The clinical features of *EVI1*$^+$ and *EVI1*$^-$ patients are summarized in Table 1. *EVI1*$^+$ patients were significantly older (P=0.03) and had a higher WBC count (P=0.01) at the time of diagnosis than *EVI1*$^-$ patients. Most of the *MLL*-rearranged AML cases were classified as FAB-M5 or FAB-M4. Specifically, most *EVI1*$^-$ patients (84%) presented with FAB-M5 morphology, which was less frequent in *EVI1*$^+$ patients (22%), consistent with the findings of a previous study.[8] *EVI1*$^+$ was not correlated with sex or *MLL* translocation partners. The frequency of *FLT3*-ITD was significantly higher in *EVI1*$^+$ patients (P=0.04). We also analyzed *CEBPA* and *NPM1* mutations, which are established favorable prognostic factors; however, none of the patients harbored these mutations, except for one *EVI1*$^-$ patient harboring double *CEBPA* mutations.

Next, clinical outcomes were compared between *EVI1*$^+$ patients and *EVI1*$^-$ patients (Figure 1). In the *MLL*-rearranged AML cohort (n=50), *EVI1*$^+$ patients had a significantly worse EFS than *EVI1*$^-$ patients (P<0.0001) (Figure 1A). However, OS did not differ significantly between the two groups (P=0.054) (Figure 1B). Among several types of *MLL*-rearrangements, *MLL-AF9* was the most common translocation (n=29, 58%) (Table 1). Therefore, clinical outcomes in the cohort of *MLL-AF9* positive patients were compared between *EVI1*$^+$ patients (n=11) and *EVI1*$^-$ patients (n=18). The results showed significant differences in EFS (P<0.0001) and OS (P=0.0008) (Figure 1C and D). By contrast, no differences in EFS (P=0.36) or OS (P=0.57) were observed among patients with *MLL*-rearranged AML after excluding *MLL-AF9* positive patients (Figure 1E and F). The clinical outcomes associated with each type of *MLL*-rearrangement could not be analyzed because of the small sample size. Multivariate Cox regression analysis, including *FLT3*-ITD, WBC count, and age identified *EVI1*$^+$ as the only prognostic factor predicting poor EFS in the total cohort of *MLL*-rearranged AML (hazard ratio (HR), 4.94; P<0.01) and in the *MLL-AF9* positive cohort (HR, 33.81; P<0.01), but not OS (*Online Supplementary Table S1*).

These results suggest that *EVI1* overexpression is an independent adverse prognostic factor because of its association with reduced remission duration in pediatric patients with *MLL*-rearranged AML, especially in patients harboring *MLL-AF9*. A recent large study identified several novel prognostic *MLL*-rearranged subgroups, including a favorable-risk *MLL-AF1q* positive subgroup and a poor-risk *MLL-AF6* positive subgroup.[11] However, *MLL-AF9* positive patients are categorized as an intermediate risk group, and this subgroup may be dichotomized as a favorable and poor-risk subgroup based on *EVI1* expression levels. Pretreatment screening for *EVI1* expression should be considered in patients with *MLL*-rearranged AML to enable better risk assessment and alternative consolidation therapies to be considered. Our results need to be confirmed in larger studies because of the limited case numbers.

From a biological viewpoint, the 'evil'-like adverse effects of *EVI1* in patients with *MLL-AF9*-positive AML were partially elucidated in a recent study in which *EVI1* positive cells harboring *MLL-AF9* showed distinct morphological,

molecular, and mechanistic differences from *EVI1* negative cells.[13] Moreover, *EVI1* overexpression has been linked to CD52 overexpression, which could be a therapeutic target for monoclonal antibody treatment.[14] Further investigation is required to identify novel prognostic factors in the various subgroups of *MLL*-rearranged AML and to develop therapeutic strategies effective for patients with *EVI1* overexpression.

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