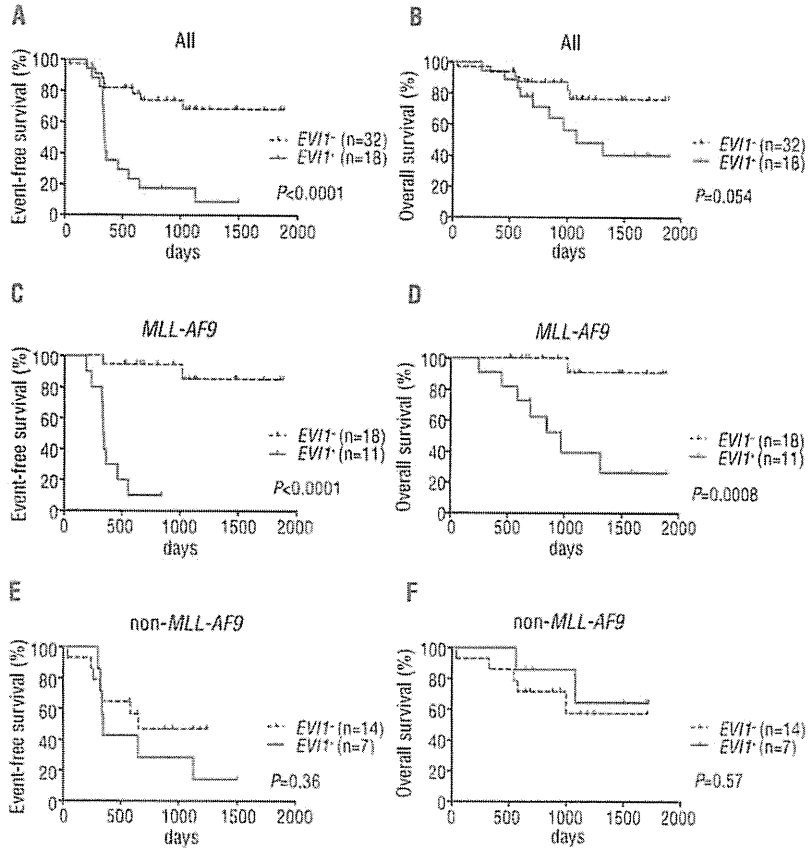

Figure 1. Kaplan-Meier survival curves of event-free survival (EFS) and overall survival (OS) from the time of diagnosis according to *EVI1* expression status. (A) Kaplan-Meier estimates of EFS in the cohort of *MLL*-rearranged AML in *EVI1*$^+$ and *EVI1*$^-$ patients. (B) Kaplan-Meier estimates of 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.

Hidemasa Matsuo,[1] Mio Kajihara,[1] Daisuke Tomizawa,[2] Tomoyuki Watanabe,[3] Akiko Moriya Saito,[4] Junichiro Fujimoto,[5] Keizo Horibe,[4] Kumi Kodama,[1] Mayu Tokunasu,[6] Hiroshi Itoh,[1] Hideki Nakayama,[7] Akitoshi Kinoshita,[8] Takashi Taga,[9] Akio Tawa,[10] Tomohiko Taki,[11] Norio Shiba,[12] Kentaro Ohki,[13] Yasuhide Hayashi,[13] Yuka Yamashita,[14] Akira Shimada,[15] Shiro Tanaka,[16] and Souichi Adachi[1]

[1]Department of Human Health Sciences, Kyoto University; [2]Department of Pediatrics, Tokyo Medical and Dental University (TMDU); [3]Department of Nutritional Science, Aichi Gakuin University, Nisshin; [4]Clinical Research Center, National Hospital Organization Nagoya Medical Center; [5]Epidemiology and Clinical Research Center for Children's Cancer, National Center for Child Health and Development, Tokyo; [6]Department of Pediatrics, Kyoto University; [7]Department of Pediatrics, National Hospital Organization Fukuoka-Higashi Medical Center, Koga; [8]Department of Pediatrics, St. Marianna University School of Medicine, Kawasaki; [9]Department of Pediatrics, Shiga University of Medical Science, Otsu; [10]Department of Pediatrics, National Hospital Organization Osaka National Hospital; [11]Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine; [12]Department of Pediatrics, Gunma University Graduate School of Medicine, Maebashi; [13]Department of Hematology and Oncology, Gunma Children's Medical Center, Shibukawa; [14]Department of Pediatrics, National Hospital Organization Nagoya Medical Center; [15]Department of Pediatrics, Okayama University Hospital; and [16]Department of Pharmacoepidemiology, Kyoto University, Japan

Trial registration: This trial is registered with the UMIN Clinical Trials Registry (UMIN-CTR, URL: http://www.umin.ac.jp/ctr/index.htm; number UMIN000000514).

Correspondence: adachiso@kuhp.kyoto-u.ac.jp

doi:10.3324/haematol.2014.107128

Acknowledgments: We thank all the clinicians participating in the JPLSG AML-05 study.

Funding: This work was supported by a Grant for Clinical Cancer Research and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

Key words: acute myeloid leukemia, pediatrics, MLL, EVI1, prognostic factor.

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Morishita K, Parker DS, Mucenski ML, Jenkins NA, Copeland NG, Ihle JN. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. Cell. 1988;54(6):831-40.
2. Kataoka K, Sato T, Yoshimi A, Goyama S, Tsuruta T, Kobayashi H et al. Evi1 is essential for hematopoietic stem cell self-renewal, and its expression marks hematopoietic cells with long-term multilineage repopulating activity. J Exp Med. 2011;208(12):2403-16.
3. Ho PA, Alonzo TA, Gerbing RB, Pollard JA, Hirsch B, Raimondi SC et al. High EVI1 expression is associated with MLL rearrangements and predicts decreased survival in paediatric acute myeloid leukaemia: a report from the children's oncology group. Br J Haematol. 2013;162(5):670-7.
4. Arai S, Yoshimi A, Shimabe M, Ichikawa M, Nakagawa M, Imai Y et al. Evi-1 is a transcriptional target of mixed-lineage leukemia oncoproteins in hematopoietic stem cells. Blood. 2011;117(23):6304-14.
5. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, van Putten WL, Valk PJ, van der Poel-van de Luytgaarde S, Hack R et al. High EVI1 expression predicts poor survival in acute myeloid leukemia: a study of 319 de novo AML patients. Blood. 2003; 101(5):837-45.
6. Lugthart S, van Drunen E, van Norden Y, van Hoven A, Erpelinck CA, Valk PJ et al. High EVI1 levels predict adverse outcome in acute myeloid leukemia: prevalence of EVI1 overexpression and chromosome 3q26 abnormalities underestimated. Blood. 2008;111(8):4329-37.
7. Gröschel S, Lugthart S, Schlenk RF, Valk PJ, Eiwen K, Goudswaard C et al. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. J Clin Oncol. 2010;28(12):2101-7.
8. Vázquez I, Maicas M, Cervera J, Agirre X, Marín-Béjar O, Marcotegui N, et al. Down-regulation of EVI1 is associated with epigenetic alterations and good prognosis in patients with acute myeloid leukemia. Haematologica. 2011;96(10):1448-56.
9. Gröschel S, Schlenk RF, Engelmann J, Rockova V, Teleanu V, Kühn MW et al. Deregulated expression of EVI1 defines a poor prognostic subset of MLL-rearranged acute myeloid leukemias: a study of the German-Austrian Acute Myeloid Leukemia Study Group and the Dutch-Belgian-Swiss HOVON/SAKK Cooperative Group. J Clin Oncol. 2013;31(1):95-103.
10. Balgobind BV, Lugthart S, Hollink IH, Arentsen-Peters ST, van Wering ER, de Graaf SS et al. EVI1 overexpression in distinct subtypes of pediatric acute myeloid leukemia. Leukemia. 2010; 24(5):942-9.
11. Balgobind BV, Raimondi SC, Harbott J, Zimmermann M, Alonzo TA, Auvrignon A et al. Novel prognostic subgroups in childhood 11q23/MLL-rearranged acute myeloid leukemia: results of an international retrospective study. Blood. 2009;114(12):2489-96.
12. Tomizawa D, Tawa A, Watanabe T, Saito AM, Kudo K, Taga T et al. Appropriate dose reduction in induction therapy is essential for the treatment of infants with acute myeloid leukemia: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group. Int J Hematol. 2013;98(5):578-88.
13. Bindels EM, Havermans M, Lugthart S, Erpelinck C, Wocjtowicz E, Krivtsov AV et al. EVI1 is critical for the pathogenesis of a subset of MLL-AF9-rearranged AMLs. Blood. 2012;119(24):5838-49.
14. Saito Y, Nakahata S, Yamakawa N, Kaneda K, Ichihara E, Suekane A et al. CD52 as a molecular target for immunotherapy to treat acute myeloid leukemia with high EVI1 expression. Leukemia. 2011; 25(6):921-31.

OPEN

Citation: Blood Cancer Journal (2014) 4, e226; doi:10.1038/bcj.2014.47

www.nature.com/bcj

ORIGINAL ARTICLE

Prognostic implications of *CEBPA* mutations in pediatric acute myeloid leukemia: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group

H Matsuo[1], M Kajihara[1], D Tomizawa[2], T Watanabe[3], AM Saito[4], J Fujimoto[5], K Horibe[4], K Kodama[1], M Tokumasu[6], H Itoh[1], H Nakayama[7], A Kinoshita[8], T Taga[9], A Tawa[10], T Taki[11], S Tanaka[12] and S Adachi[1]

CCAAT/enhancer-binding protein alpha (*CEBPA*) mutations are a favorable prognostic factor in adult acute myeloid leukemia (AML) patients; however, few studies have examined their significance in pediatric AML patients. Here we examined the *CEBPA* mutation status and clinical outcomes of pediatric AML patients treated in the AML-05 study. We found that 47 (14.9%) of the 315 evaluable patients harbored mutations in *CEBPA*; 26 cases (8.3%) harbored a single mutation (*CEBPA*-single) and 21 (6.7%) harbored double or triple mutations (*CEBPA*-double). After excluding core-binding factor-AML cases, patients harboring *CEBPA* mutations showed better overall survival (OS; $P=0.048$), but not event-free survival (EFS; $P=0.051$), than wild-type patients. Multivariate analysis identified *CEBPA*-single and *CEBPA*-double as independent favorable prognostic factors for EFS in the total cohort (hazard ratio (HR): 0.47 and 0.33; $P=0.02$ and 0.01, respectively). *CEBPA*-double was also an independent favorable prognostic factor for OS (HR: 0.30; $P=0.04$). *CEBPA*-double remained an independent favorable factor for EFS (HR: 0.28; $P=0.04$) in the normal karyotype cohort. These results suggest that *CEBPA* mutations, particularly *CEBPA*-double, are an independent favorable prognostic factor in pediatric AML patients, which will have important implications for risk-stratified therapy.

Blood Cancer Journal (2014) **4**, e226; doi:10.1038/bcj.2014.47; published online 11 July 2014

INTRODUCTION

CCAAT/enhancer-binding protein alpha (CEBPA) is a transcription factor that co-ordinates cellular differentiation. CEBPA is expressed in myeloid precursors during hematopoiesis, where it regulates the expression of several granulocyte-specific genes.[1] CEBPA inhibits E2F pathways, thereby downregulating c-Myc and allowing myeloid precursors to enter the granulocytic differentiation pathway.[2,3] The *CEBPA* gene is located on chromosome 19 band q13.1. Approximately 10% of acute myeloid leukemia (AML) patients harbor mutations in *CEBPA* genes, and these mutations can occur across the whole gene, but there are two main hotspots.[4,5] N-terminal out-of-frame mutations are located between the major translational start site and a second ATG further downstream. They abolish translation of the full-length p42 isoform of CEBPA, leading to overexpression of a shorter dominant-negative p30 isoform.[6] C-terminal mutations are generally in-frame insertions/deletions located in the basic leucine zipper (bZIP) domain; these mutations disrupt binding to DNA or dimerization.[7] Most AML patients with double *CEBPA* mutations harbor both N- and C-terminal mutations, which are typically present on different alleles; however, homozygous mutations have also been described.[8]

CEBPA mutations are a favorable prognostic factor for AML, particularly in patients harboring double *CEBPA* mutations and a normal karyotype.[8–13] However, the prognostic value of *CEBPA* mutations has been studied mostly in adult AML patients, with few studies examining mutations in pediatric AML patients. The first set of pediatric data was presented by the Taiwan Pediatric Oncology Group, but the report lacked data regarding clinical outcome.[14] The prognostic impact of *CEBPA* in pediatric AML was reported by two other groups, namely, the Children's Oncology Group and the Dutch Childhood Oncology Group/the Berlin–Frankfurt–Münster Study Group,[15,16] which both reported that, after excluding core-binding factor (CBF)-AML cases, patients harboring *CEBPA* mutations had a significantly better clinical outcome than those harboring the wild-type (WT) gene; however, the clinical implications of single vs double mutations were unclear. A more recent study conducted by the Nordic Society of Pediatric Hematology and Oncology suggests that *CEBPA* mutations in pediatric AML patients are not associated with improved survival;[17] thus the clinical significance of *CEBPA* mutations in pediatric AML patients is unclear. Although we previously reported the characteristics of *CEBPA* mutations in Japanese children with AML, the small sample size meant that further study was required.[18]

Here we examined the *CEBPA* mutation status and clinical outcomes of pediatric AML patients treated in the Japanese Pediatric Leukemia/Lymphoma Study Group (JPLSG) AML-05 study.

[1]Department of Human Health Sciences, Kyoto University, Kyoto, Japan; [2]Department of Pediatrics, Tokyo Medical and Dental University (TMDU), Tokyo, Japan; [3]Department of Nutritional Science, Aichi Gakuin University, Aichi, Japan; [4]Clinical Research Center, National Hospital Organization Nagoya Medical Center, Aichi, Japan; [5]Clinical Research Center, National Center for Child Health and Development, Tokyo, Japan; [6]Department of Pediatrics, Kyoto University, Kyoto, Japan; [7]Department of Pediatrics, National Hospital Organization Fukuoka-Higashi Medical Center, Fukuoka, Japan; [8]Department of Pediatrics, St Marianna University School of Medicine, Kanagawa, Japan; [9]Department of Pediatrics, Shiga University of Medical Science, Shiga, Japan; [10]Department of Pediatrics, National Hospital Organization Osaka Medical Hospital, Osaka, Japan; [11]Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine, Kyoto, Japan and [12]Department of Pharmacoepidemiology, Kyoto University, Kyoto, Japan. Correspondence: Professor S Adachi, Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, 53 Kawahara-cho, Syogoin, Sakyoku, Kyoto 606-8507, Japan.
E-mail: adachiso@kuhp.kyoto-u.ac.jp
Received 4 May 2014; accepted 19 May 2014

SUBJECTS AND METHODS

Patients and study protocol

The AML-05 study is a Japanese nationwide multi-institutional study of children (age <18 years) with *de novo* AML, all of whom were enrolled between 1 November 2006 and 31 December 2010. The trial is registered with the UMIN Clinical Trials Registry (UMIN-CTR; http://www.umin.ac.jp/ctr/index.htm; number UMIN000000511).

In all, 485 patients with suspected AML (diagnosed at 118 centers and hospitals in Japan) were registered in the AML-05 study. Patients with acute promyelocytic leukemia, Down's syndrome, secondary AML, myeloid/natural killer cell leukemia and myeloid sarcoma, were not eligible. Overall, 38 patients were excluded, mainly because of misdiagnosis, while four additional patients were excluded for the following reasons: the patient's guardian refused permission to participate ($n=1$); there was a significant protocol violation during the initial induction course ($n=1$); the hospital withdrew from the JPLSG ($n=1$); and the patient was transferred to a non-JPLSG member hospital ($n=1$). Patients were stratified into three risk groups according to specific cytogenetic characteristics and morphological responses to treatment. CBF-AML patients were assigned to the low-risk group; those with unfavorable cytogenetics (−7, 5q-, t(16;21)(p11;q22), Ph1, Fms-like tyrosine kinase 3 internal tandem duplications (*FLT3*-ITD)) and poor induction responders were assigned to the high-risk group; and the rest were assigned to the intermediate-risk group. Details of the patient disposition, treatment schedules and risk stratification have been described previously.[19,20] In the present study, morphology was diagnosed prospectively using a central review system. Cytogenetic tests were performed in regional laboratories, but the reports were reviewed centrally. The 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.

Mutation analysis

cDNA was synthesized from RNA obtained from diagnostic bone marrow samples using the Omniscript Reverse Transcription Kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's recommendations. The entire coding region of the *CEBPA* gene was amplified using the overlapping PCR primer pairs followed by direct sequencing, as previously described.[6,16]

Statistical analysis

Patient characteristics were analyzed using Fisher's exact test (categorical variables) and the Kruskal–Wallis test (continuous variables). 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 death). Overall survival (OS) was defined as the time from the diagnosis of AML to any-cause death. The Kaplan–Meier method was used to estimate EFS and OS, and data were compared using the log-rank test. To determine the prognostic value of *CEBPA* mutation, Cox regression analysis was performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). All tests were two-tailed, and a *P*-value <0.05 was considered statistically significant.

RESULTS

Mutation analysis

Diagnostic samples from 315/443 (71.1%) eligible AML patients were analyzed for *CEBPA* mutations; *CEBPA* data were unavailable for 128 patients. There were no significant differences in the major characteristics or clinical outcomes of the 315 patients and the 128 patients for whom no data were available (EFS $P=0.78$, OS $P=0.30$). We found that 47/315 patients (14.9%) harbored a mutation in *CEBPA*, 26 (8.3%) harbored a single mutation and 21 (6.7%) harbored double or triple mutations. The location and combination pattern of all the detected mutations are shown in Figure 1 and Table 1. Single mutations were distributed across the entire gene, but most in-frame insertions/deletions were located in the bZIP domain. By contrast, double or triple mutations were clustered in the N- and C-terminal hotspots. Thirteen out of the 21 cases (61.9%) harbored both an N-terminal out-of-frame mutation and an in-frame mutation in the bZIP domain, which were predicted to result in a lack of WT CEBPA p42 expression. We identified five patients with triple mutations but could not exclude the possibility that these mutations occurred in different cells. Moreover, the method we used cannot identify whether mutations are located on different alleles. Further study is required to overcome these limitations.

Polymorphisms in the *CEBPA* mutations

Overall, 131 patients (41.6%) harbored an in-frame 6-bp insertion (ACCCGC) in the transactivation domain 2 (TAD2), resulting in a His-Pro duplication (HP196–197 insertion). This mutation is observed in approximately 10% of healthy controls and AML patients and is reported as a germline polymorphism.[21,22] We did not identify any differences in characteristics between the HP196–197 insertion-positive and -negative groups, and the clinical outcomes of both groups were similar (data not shown). Therefore, we ignored this mutation during our analysis of clinical outcome, along with other mutations that did not result in amino-acid changes.

Patient characteristics

Patient characteristics according to *CEBPA* mutation status are shown in Table 2. Patients harboring a single *CEBPA* mutation were described as '*CEBPA*-single' and those harboring double or triple *CEBPA* mutations were described as '*CEBPA*-double'. *CEBPA*-double patients showed a significantly higher percentage of M1 or M2 French–American–British subtypes ($P<0.001$). Compared with WT patients, patients with *CEBPA* mutations were older ($P=0.03$) at the time of diagnosis. *CEBPA* mutations were predominant in those with an intermediate risk ($P=0.002$) and a normal karyotype ($P<0.001$). There was a well-balanced gender distribution ($P=0.84$), and there were no significant differences in the number of patients with *FLT3*-ITD and *NPM1* mutations among the three *CEBPA* subgroups.

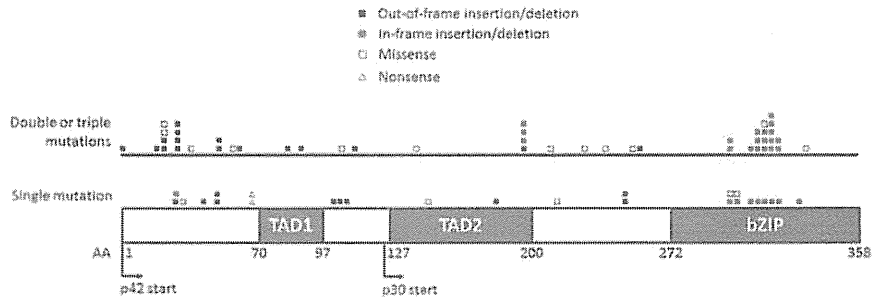

Figure 1. Location and type of the mutations detected in pediatric AML patients enrolled in the AML-05 study. AA, amino acid; BZIP, basic leucine zipper; TAD, transactivation domain.

Table 1. Summary of *CEBPA* mutations detected in 315 acute myeloid leukemia patients

Mutation status	*Mutation 1*			*Mutation 2 (Mutation 3)*			
	N-terminal AA 1-120	*Middle* AA 121-277	*C-terminal* AA 278-358	*N-terminal* AA 1-120	*Middle* AA 121-277	*C-terminal* AA 278-358	*No. of patients*
CEBPA-single	Out-of-frame ins/del						7
	In-frame ins/del						1
	Missense						1
	Nonsense						2
		Out-of-frame ins/del					3
		Missense					2
			In-frame ins/del				8
			Missense				2
Total							26
CEBPA-double	Out-of-frame ins/del					In-frame ins/del	11
	Missense			Missense			1
	Missense				Missense		1
	Missense					Missense	1
		Out-of-frame ins/del				In-frame ins/del	2
	Out-of-frame ins/del			Missense	(In-frame ins/del)		1
	Out-of-frame ins/del				In-frame ins/del	(In-frame ins/del)	2
		Missense			Missense (Missense)		1
		Missense				Missense (In-frame ins/del)	1
Total							21

Abbreviations: AA, amino acid; *CEBPA*, CCAAT/enhancer-binding protein alpha; del, deletion; ins, insertion. Note: Patients harboring a single *CEBPA* mutation were described as *CEBPA*-single and those harboring double or triple *CEBPA* mutations were described as *CEBPA*-double.

Prognostic impact of *CEBPA* in the total cohort

We first analyzed the clinical outcomes of patients harboring *CEBPA* mutations and then compared them with the outcomes of CBF-AML patients and patients without CBF or *CEBPA* mutations (denoted 'WT non-CBF') (Figure 2). The CBF-AML group included AML patients harboring t(8;21)(q22;q22) along with inv(16)(p13.1q22) or its variant t(16;16)(p13.1;q22). Seven CBF-AML patients harboring *CEBPA* mutations were categorized as '*CEBPA*-mutant'. Patients harboring *CEBPA* mutations showed better OS ($P=0.048$), but not EFS ($P=0.051$), than WT non-CBF patients (Figures 2a and b). However, patients with *CEBPA* mutations showed poorer OS ($P=0.0006$) than patients with CBF-AML. Furthermore, we examined whether the number of *CEBPA* mutations had an impact on prognosis (Figures 2c and d). *CEBPA*-double patients did not show significantly better EFS and OS than *CEBPA*-single patients ($P=0.33$ each). There was also no significant difference in EFS and OS between *CEBPA*-double patients and WT non-CBF patients ($P=0.055$ and $P=0.057$, respectively).

Prognostic impact of *CEBPA* in the normal karyotype cohort

We next examined prognosis in the normal karyotype cohort, because *CEBPA* mutations have been described as a favorable prognostic factor, particularly in cytogenetically normal AML (Figure 3). There was no significant difference in EFS and OS between *CEBPA*-double patients and WT or *CEBPA*-single patients (EFS: *CEBPA*-double vs WT, $P=0.15$; *CEBPA*-double vs *CEBPA*-single, $P=0.21$; OS: *CEBPA*-double vs WT, $P=0.28$; *CEBPA*-double vs *CEBPA*-single; $P=0.44$). Patients with *CEBPA*-single showed almost identical EFS ($P=0.97$) and OS ($P=0.77$) to those of WT patients.

Prognostic impact of *CEBPA* mutation type

We also examined the prognostic impact of the location of the *CEBPA* mutations, which has never been examined in pediatric AML patients. Only patients with hotspot mutations predicted to cause translation of the p30 isoform and/or disruption or loss of the C-terminal bZIP domain were included in the analysis. In the total cohort, patients with an N-terminal out-of-frame mutation and a C-terminal in-frame mutation ($n=13$, denoted as *CEBPA*-double N + C-term) had significantly better EFS ($P=0.01$), but not OS ($P=0.06$), than WT non-CBF patients (Figures 4a and b). This patient group also had significantly better EFS, but not OS, than other *CEBPA*-double patients ($n=8$), suggesting that a combination of N-terminal and C-terminal mutations results in a better prognosis for *CEBPA*-double patients (data not shown). We also investigated differences in outcome between *CEBPA*-single patients with an N-terminal mutation and those with a C-terminal mutation and found that the clinical outcomes were nearly identical. In the normal karyotype cohort, we found no significant difference in the outcome of four groups: patients with an N-terminal out-of-frame mutation, patients with a C-terminal in-frame mutation, patients with an N-terminal out-of-frame mutation and a C-terminal in-frame mutation, and WT patients, which may be due to the small sample size (Figures 4c and d).

Multivariate analysis

Multivariate Cox regression analysis, including age and white blood cell count at the time of diagnosis, was performed to examine whether *CEBPA* mutations were a favorable prognostic factor (Table 3). *FLT3*-ITD and *NPM1* mutations were not included as variables owing to the small number of positive cases and

Table 2. Characteristics of patients categorized according to *CEBPA* mutation status

	Total	*WT*	*CEBPA-single*	*CEBPA-double*	*P-value*
Number	315	268	26	21	
Age, years					0.03[b]
Median	7.9	7.6	9.9	9.6	
Range	0.0–17.5	0.0–17.5	0.3–16.2	1.3–15.9	
Sex, n *(%)*					0.84[a]
Male	167 (53)	144 (54)	13 (50)	10 (48)	
Female	148 (47)	124 (46)	13 (50)	11 (52)	
WBC ($\times 10^9$/l)					0.052[b]
Median	57.8	52.6	58.1	124	
Range	0.8–985	0.8–552	1.9–381	3.9–985	
Risk groups, n *(%)*					0.002[a]
Low	87 (28)	81 (30)	4 (15)	2 (10)	
Intermediate	132 (42)	101 (38)	15 (58)	16 (76)	
High	43 (14)	37 (14)	6 (23)	0 (0)	
Unclassified	53 (17)	49 (18)	1 (4)	3 (14)	
FAB, n *(%)*					<0.001[a]
M0	7 (2)	7 (3)	0 (0)	0 (0)	
M1	43 (14)	28 (10)	5 (19)	10 (48)	
M2	79 (25)	63 (24)	9 (35)	7 (33)	
M4	52 (17)	46 (17)	6 (23)	0 (0)	
M5	70 (22)	67 (25)	1 (4)	2 (10)	
M6	8 (3)	5 (2)	2 (8)	1 (5)	
M7	31 (10)	29 (11)	2 (8)	0 (0)	
RAEB-T	25 (8)	23 (9)	1 (4)	1 (5)	
Karyotype, n *(%)*					<0.001[a]
Normal	62 (20)	35 (13)	14 (54)	13 (62)	
t(8;21)	75 (24)	69 (26)	3 (12)	3 (14)	
inv(16)	25 (8)	24 (9)	1 (4)	0 (0)	
11q23	48 (15)	47 (18)	0 (0)	1 (5)	
other	105 (33)	93 (35)	8 (31)	4 (19)	
Molecular abnormalities, n *(%)*					
FLT3-ITD	42 (13)	35 (13)	6 (23)	1 (5)	0.21[a]
NPM1	12/167 (7)	10/128 (8)	2/22 (9)	0/17 (0)	0.59[a]

Abbreviations: *CEBPA*, CCAAT/enhancer-binding protein alpha; FAB, French–American–British; *FLT3*-ITD, Fms-like tyrosine kinase 3 internal tandem duplications; *NPM1*, nucleophosmin; WBC, white blood cell count; WT, wild type. [a]Fisher's exact test. [b]Kruskal–Wallis test.

because no statistically significant differences were detected by univariate analysis. For the total cohort ($n=315$), multivariate analysis identified both *CEBPA*-single and *CEBPA*-double as independent favorable prognostic factors for EFS (hazard ratio (HR): 0.47 and 0.33; $P=0.02$ and 0.01, respectively; upper column, Table 3). *CEBPA*-double was also identified as an independent favorable prognostic factor for OS (HR: 0.30; $P=0.04$). For the normal karyotype cohort ($n=62$), *CEBPA*-double was also identified as an independent prognostic factor for favorable EFS (HR: 0.28; $P=0.04$; lower column, Table 3). This may indicate that other factors, such as age and white blood cell count, had masked the benefit of *CEBPA* mutations in the univariate analysis.

DISCUSSION

Here we examined *CEBPA* mutations in 315 pediatric AML patients enrolled in the AML-05 study. We detected *CEBPA* mutations in 47 patients (14.9%), which is comparable to the reported frequency in adult and pediatric AML patients (approximately 10%).[8–17] In all, 26 out of the 47 cases (55.3%) harbored a single *CEBPA* mutation; this percentage is higher than that reported in previous studies of pediatric AML patients.[15,16] We detected the HP196–197 insertion in 131/315 cases (41.6%). This well-known polymorphism was previously observed in approximately 10% of AML cases; thus the percentage identified in the present study was rather high.[21,22] Whether this polymorphism is also common in healthy Japanese populations remains to be seen. A recent study by a Korean group reported the incidence of this polymorphism as 30%; thus the frequency of this polymorphism may vary considerably according to geographical region.[23] The majority of *CEBPA*-double patients comprised M1 or M2 French–American–British subtypes, which is in agreement with the findings of previous studies.[15,16] *CEBPA* mutations were predominant in the intermediate risk and normal karyotype group, which is also consistent with previous findings.[15–17]

With regard to prognosis, the results presented herein suggest that *CEBPA* mutations, especially *CEBPA*-double, are an independent favorable prognostic factor in pediatric AML patients. Multivariate analysis of the normal karyotype cohort identified *CEBPA*-double as an independent favorable prognostic factor for EFS, but not OS; this finding may be due to the small sample size. As the majority of pediatric AML patients lack markers that indicate a favorable or poor prognosis, it is important to identify prognostic markers in intermediate-risk patients. *CEBPA* mutations show promise as markers of a favorable prognosis in pediatric AML patients, because they are strongly associated with intermediate risk.

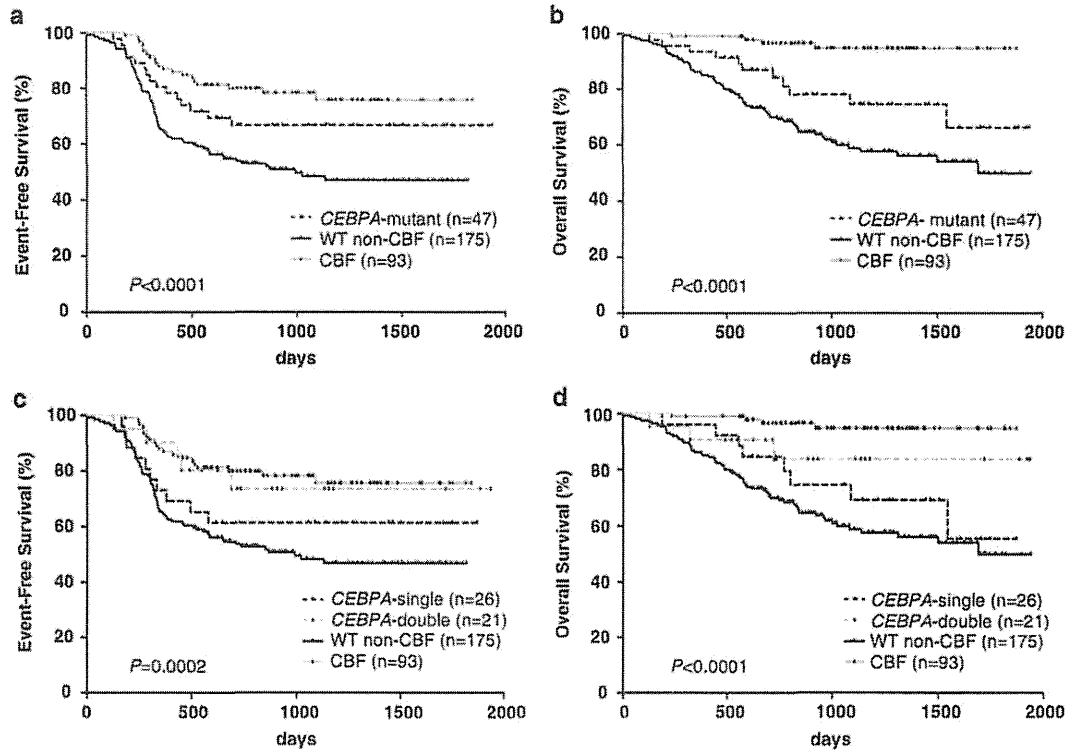

Figure 2. Kaplan–Meier survival curves showing EFS and OS from the time of diagnosis according to *CEBPA* mutation status. (**a**) EFS and (**b**) OS of patients harboring *CEBPA* mutations, patients harboring WT *CEBPA* (excluding core-binding factor-acute myeloid leukemia (CBF-AML) cases (WT non-CBF)) and patients with CBF-AML. (**c**) EFS and (**d**) OS of patients harboring a single *CEBPA* mutation (*CEBPA*-single), patients harboring double or triple *CEBPA* mutations (*CEBPA*-double), WT patients (excluding CBF-AML cases (WT non-CBF)) and patients with CBF-AML. *P*-values were determined using the log-rank test.

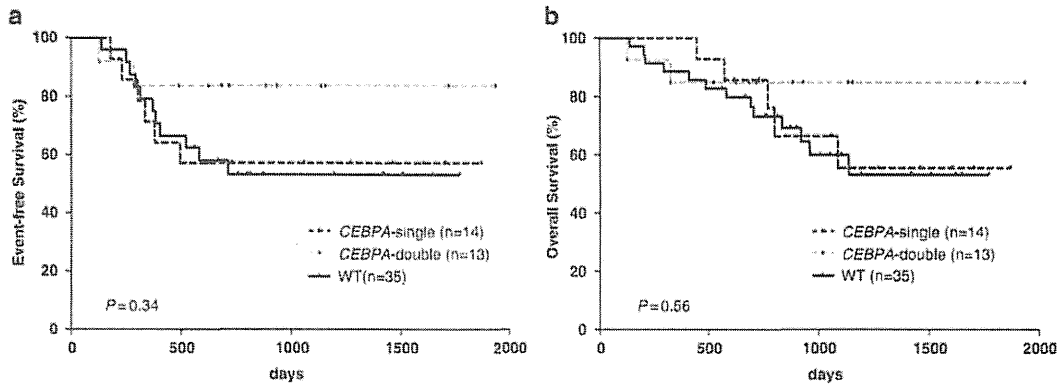

Figure 3. Kaplan–Meier survival curves showing EFS and OS in acute myeloid leukemia patients with a normal karyotype. (**a**) EFS and (**b**) OS of patients harboring a single *CEBPA* mutation (*CEBPA*-single), patients harboring double or triple *CEBPA* mutations (*CEBPA*-double) and WT patients. *P*-values were determined using the log-rank test.

Consistent with our results, several studies (including one pediatric study) postulated that AML patients harboring double *CEBPA* mutations have a favorable prognosis.[9–12] Two different *CEBPA* mutations have a synergistic effect on AML development, and the mechanism underlying leukemogenesis is likely to be different from that in AML patients harboring a single *CEBPA* mutation.[24,25] We found that a combination of N-terminal and C-terminal mutations is essential for a better prognosis in *CEBPA*-double patients (data not shown), indicating that a favorable prognosis is restricted in patients who lack WT CEBPA p42 expression among *CEBPA*-double patients. Moreover, a recent study of a large cohort of adult AML patients suggests that patients harboring double *CEBPA* mutations belong to a genetically distinct subtype and should be clearly distinguished from patients harboring a single mutation.[13] In this study, we could not examine the prognostic impact of concomitant molecular mutations because of their low incidence; therefore further analyses of pediatric AML patients is required.

In contrast to double *CEBPA* mutations, the prognostic value of single *CEBPA* mutation is currently under debate because of its small number. We detected a relatively large number of cases harboring a single *CEBPA* mutation in the total cohort, and

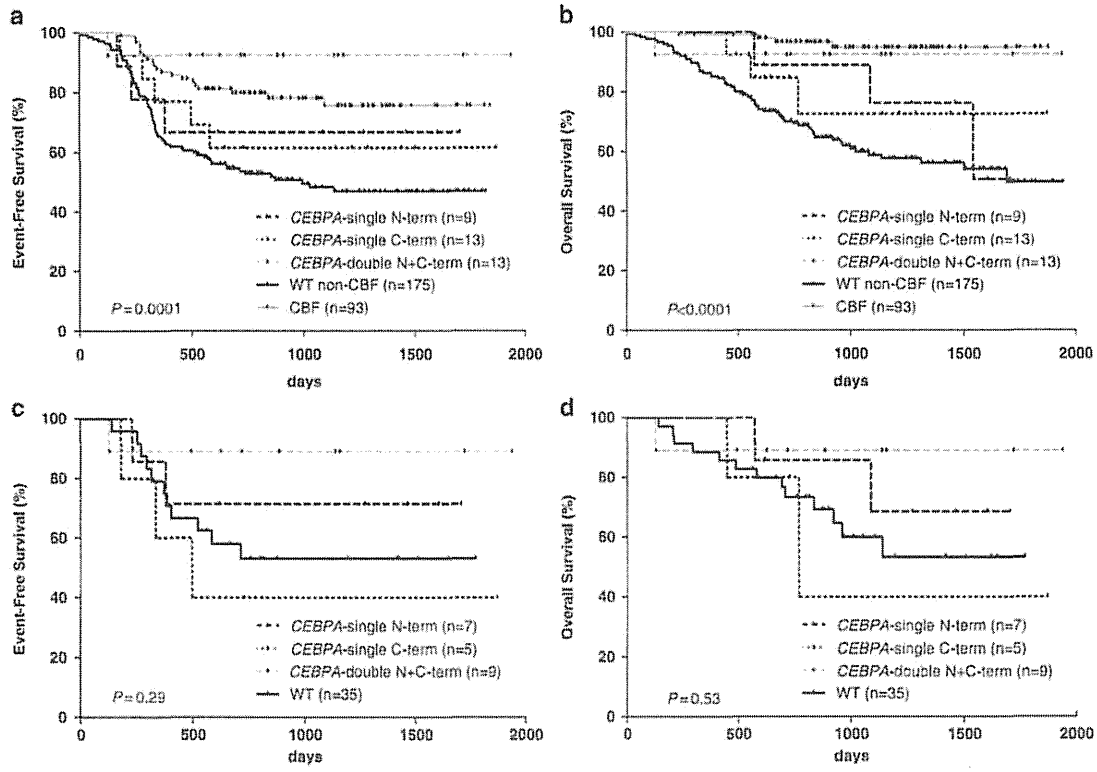

Figure 4. Kaplan–Meier survival curves showing EFS and OS according to the location and number of *CEBPA* mutations. Only patients with hotspot mutations predicted to cause p30 isoform translation and/or disruption or loss of the C-terminal bZIP domain were included in the analysis. (**a**) EFS and (**b**) OS in patients harboring a single N-terminal mutation (*CEBPA*-single N-term), patients harboring a single C-terminal mutation (*CEBPA*-single C-term), patients harboring both N and C-terminal mutations (*CEBPA*-double N + C-term), WT patients (excluding core-binding factor-acute myeloid leukemia (CBF-AML) cases (WT non-CBF)) and patients with CBF-AML. (**c**) EFS and (**d**) OS of AML patients with a normal karyotype. *P*-values were determined using the log-rank test.

Table 3. Multivariate Cox regression analysis for EFS and OS

	EFS			*OS*		
	HR	*95% CI*	*P-value*	*HR*	*95% CI*	*P-value*
Total cohort (n = *315*)						
Mutation status, vs WT non-CBF						
CBF	0.31	0.19–0.49	<0.01	0.09	0.03–0.25	<0.01
CEBPA-single	0.47	0.24–0.91	0.02	0.60	0.29–1.26	0.18
CEBPA-double	0.33	0.14–0.76	0.01	0.30	0.09–0.94	0.04
Age (+ 1 year)	1.00	0.97–1.03	0.86	1.03	0.99–1.07	0.17
WBC (⩾50 000)	1.81	1.29–2.55	<0.01	1.50	0.96–2.33	0.07
Normal karyotype cohort (n = *62*)						
Mutation status, vs WT						
CEBPA-single	0.54	0.22–1.33	0.18	0.88	0.31–2.47	0.81
CEBPA-double	0.28	0.08–0.95	0.04	0.49	0.11–2.17	0.35
Age (+ 1 year)	0.95	0.89–1.02	0.14	0.94	0.86–1.02	0.13
WBC (⩾50 000)	2.05	1.00–4.22	0.05	1.34	0.55–3.29	0.52

Abbreviations: CBF, core-binding factor; *CEBPA*, CCAAT/enhancer-binding protein alpha; CI, confidence interval; EFS, event-free survival; HR, hazard ratio; OS, overall survival; WBC, white blood cell count; WT, wild type.

multivariate analysis identified single mutation as an independent prognostic factor for favorable EFS (Table 3). Two adult AML studies (but no pediatric studies) showed that a single *CEBPA* mutation can be an independent favorable prognostic factor in patients harboring *NPM1* mutations.[26,27] Indeed, the two patients in the present study that harbored both a single *CEBPA* mutation and an *NPM1* mutation showed good long-term survival without any events. We also tried to examine the clinical significance of the location of the mutation in *CEBPA*-single patients but found no significant difference in outcomes for patients harboring N-terminal or C-terminal mutations. However, the *CEBPA*-single patients in the normal karyotype cohort who harbored a

C-terminal mutation may have slightly poorer EFS and OS than those who harbored an N-terminal mutation (Figures 4c and d), which is not consistent with previous adult AML studies.[12,13] Gene expression profiling suggests that *CEBPA*-single patients harboring a C-terminal mutation are more similar to *CEBPA*-double patients than to *CEBPA*-single patients harboring N-terminal mutations.[10] This latter study was performed in adult AML patients and needs to be validated in pediatric AML patients.

So far, the biological mechanisms underlying a favorable clinical outcome for AML patients harboring *CEBPA* mutations (including relative drug sensitivity) are not clear. Further studies of single and double *CEBPA* mutations and the underlying biology are required to enable better risk assessment and therapeutic approaches in pediatric AML.

CONCLUSION

This is the first nationwide study to examine the clinical significance of *CEBPA* mutations in Japanese pediatric AML patients. The results suggest that *CEBPA* mutations, especially double or triple *CEBPA* mutations, are an independent favorable prognostic factor for pediatric AML patients. *CEBPA*-double patients should be stratified into the favorable risk group, and the prognostic significance of these mutations should be validated prospectively.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by a Grant for Clinical Cancer Research and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan. We also thank to H Miyachi, N Kiyokawa, T Deguchi, Y Hayashi and all doctors participating in JPLSG AML-05 protocol.

REFERENCES

1 Tenen DG, Hromas R, Licht JD, Zhang DE. Transcription factors, normal myeloid development, and leukemia. *Blood* 1997; **90**: 489–519.
2 Porse BT, Pedersen TA, Xu X, Lindberg B, Wewer UM, Friis-Hansen L *et al.* E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis *in vivo*. *Cell* 2001; **107**: 247–258.
3 Johansen LM, Iwama A, Lodie TA, Sasaki K, Felsher DW, Golub TR *et al.* c-Myc is a critical target for c/EBPalpha in granulopoiesis. *Mol Cell Biol* 2001; **21**: 3789–3806.
4 Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. CEBPA point mutations in hematological malignancies. *Leukemia* 2005; **19**: 329–334.
5 Nerlov C. C/EBPalpha mutations in acute myeloid leukaemias. *Nat Rev Cancer* 2004; **4**: 394–400.
6 Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S *et al.* Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet* 2001; **27**: 263–270.
7 Asou H, Gombart AF, Takeuchi S, Tanaka H, Tanioka M, Matsui H *et al.* Establishment of the acute myeloid leukemia cell line Kasumi-6 from a patient with a dominant-negative mutation in the DNA-binding region of the C/EBPalpha gene. *Genes Chromosomes Cancer* 2003; **36**: 167–174.
8 Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S *et al.* Favorable prognostic significance of CEBPA mutations in patients with *de novo* acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood* 2002; **100**: 2717–2723.
9 Pabst T, Eyholzer M, Fos J, Mueller BU. Heterogeneity within AML with *CEBPA* mutations; only *CEBPA* double mutations, but not single *CEBPA* mutations are associated with favourable prognosis. *Br J Cancer* 2009; **100**: 1343–1346.
10 Wouters BJ, Löwenberg B, Erpelinck-Verschueren CA, van Putten WL, Valk PJ, Delwel R. Double *CEBPA* mutations, but not single *CEBPA* mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome. *Blood* 2009; **113**: 3088–3091.
11 Dufour A, Schneider F, Metzeler KH, Hoster E, Schneider S, Zellmeier E *et al.* Acute myeloid leukemia with biallelic *CEBPA* gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. *J Clin Oncol* 2010; **28**: 570–577.
12 Green CL, Koo KK, Hills RK, Burnett AK, Linch DC, Gale RE. Prognostic significance of *CEBPA* mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double *CEBPA* mutations and the interaction with *FLT3* and *NPM1* mutations. *J Clin Oncol* 2010; **28**: 2739–2747.
13 Fasan A, Haferlach C, Alpermann T, Jeromin S, Grossmann V, Eder C *et al.* The role of different genetic subtypes of *CEBPA* mutated AML. *Leukemia* 2013; **28**: 794–803.
14 Liang DC, Shih LY, Huang CF, Hung IJ, Yang CP, Liu HC *et al.* CEBPalpha mutations in childhood acute myeloid leukemia. *Leukemia* 2005; **19**: 410–414.
15 Ho PA, Alonzo TA, Gerbing RB, Pollard J, Stirewalt DL, Hurwitz C *et al.* Prevalence and prognostic implications of *CEBPA* mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. *Blood* 2009; **113**: 6558–6566.
16 Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Zimmermann M, Peeters JK, Valk PJ *et al.* Characterization of *CEBPA* mutations and promoter hypermethylation in pediatric acute myeloid leukemia. *Haematologica* 2011; **96**: 384–392.
17 Staffas A, Kanduri M, Hovland R, Rosenquist R, Ommen HB, Abrahamsson J *et al.* Presence of *FLT3*-ITD and high *BAALC* expression are independent prognostic markers in childhood acute myeloid leukemia. *Blood* 2011; **118**: 5905–5913.
18 Mizushima Y, Taki T, Shimada A, Yui Y, Hiraumi Y, Matsubara H *et al.* Prognostic significance of the *BAALC* isoform pattern and *CEBPA* mutations in pediatric acute myeloid leukemia with normal karyotype: a study by the Japanese Childhood AML Cooperative Study Group. *Int J Hematol* 2010; **91**: 831–837.
19 Tomizawa D, Tawa A, Watanabe T, Saito AM, Kudo K, Taga T *et al.* Excess treatment reduction including anthracyclines results in higher incidence of relapse in core binding factor acute myeloid leukemia in children. *Leukemia* 2013; **27**: 2413–2416.
20 Tomizawa D, Tawa A, Watanabe T, Saito AM, Kudo K, Taga T *et al.* Appropriate dose reduction in induction therapy is essential for the treatment of infants with acute myeloid leukemia: a report from the Japanese Pediatric Leukemia/Lymphoma Study Group. *Int J Hematol* 2013; **98**: 578–588.
21 Wouters BJ, Louwers I, Valk PJ, Löwenberg B, Delwel R. A recurrent in-frame insertion in a CEBPA transactivation domain is a polymorphism rather than a mutation that does not affect gene expression profiling-based clustering of AML. *Blood* 2007; **109**: 389–390.
22 Biggio V, Renneville A, Nibourel O, Philippe N, Terriou L, Roumier C *et al.* Recurrent in-frame insertion in C/EBPalpha TAD2 region is a polymorphism without prognostic value in AML. *Leukemia* 2008; **22**: 655–657.
23 Kim S, Kim DH, Jang JH, Jung CW, Jang MA, Ki CS *et al.* Novel mutations in CEBPA in Korean Patients with acute myeloid leukemia with a normal karyotype. *Ann Lab Med* 2012; **32**: 153–157.
24 Bereshchenko O, Mancini E, Moore S, Bilbao D, Månsson R, Luc S *et al.* Hematopoietic stem cell expansion precedes the generation of committed myeloid leukemia-initiating cells in C/EBPalpha mutant AML. *Cancer Cell* 2009; **16**: 390–400.
25 Somervaille TC, Cleary ML. Preview. Mutant CEBPA: priming stem cells for myeloid leukemogenesis. *Cell Stem Cell* 2009; **5**: 453–454.
26 Dufour A, Schneider F, Hoster E, Benthaus T, Ksienzyk B, Schneider S *et al.* Monoallelic CEBPA mutations in normal karyotype acute myeloid leukemia: independent favorable prognostic factor within NPM1 mutated patients. *Ann Hematol* 2012; **91**: 1051–1063.
27 Park SH, Chi HS, Cho YU, Jang S, Park CJ. CEBPA single mutation can be a possible favorable prognostic indicator in NPM1 and FLT3-ITD wild-type acute myeloid leukemia patients with intermediate cytogenetic risk. *Leuk Res* 2013; **37**: 1488–1494.

t(6;9)(p22;q34)/*DEK-NUP214*-rearranged pediatric myeloid leukemia: an international study of 62 patients

Julie Damgaard Sandahl,[1] Eva A. Coenen,[2] Erik Forestier,[3] Jochen Harbott,[4] Bertil Johansson,[5] Gitte Kerndrup,[6] Souichi Adachi,[7] Anne Auvrignon,[8] H. Berna Beverloo,[9,10] Jean-Michel Cayuela,[11,12] Lucy Chilton,[13] Maarten Fornerod,[2,14] Valérie de Haas,[15] Christine J. Harrison,[13] Hiroto Inaba,[16] Gertjan J.L. Kaspers,[15,17] Der-Cherng Liang,[18] Franco Locatelli,[19] Riccardo Masetti,[20] Christine Perot,[21] Susana C. Raimondi,[16] Katarina Reinhardt,[22] Daisuke Tomizawa,[23] Nils von Neuhoff,[24] Marco Zecca,[25] C. Michel Zwaan,[2] Marry M. van den Heuvel-Eibrink,[2] and Henrik Hasle[1]

[1]Department of Pediatrics, Aarhus University Hospital Skejby, Denmark; [2]Department of Pediatric Oncology/Hematology, Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands; [3]Department of Medical Biosciences, Genetics, Umeå University Hospital, Sweden; [4]Department of Pediatric Hematology/Oncology, Justus-Liebig-University, Giessen, Germany; [5]Department of Clinical Genetics, University and Regional Laboratories Region Skåne, Lund, and Division of Clinical Genetics, Department of Laboratory Medicine, Lund University, Sweden; [6]Department of Pathology, Vejle Hospital, Denmark; [7]Human Health Sciences, Kyoto University, Japan; [8]French Leucémie Aigue Myeloide Enfant, Hôpital Trousseau, Paris, France; [9]Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, the Netherlands; [10]Dutch Childhood Oncology Group, Dutch Working Group on Hemato-Oncologic Genome Diagnostics, The Hague, the Netherlands; [11]Laboratoire d'hématologie, Hôpital Saint-Louis, Paris, France; [12]Saint Louis Institute Paris Diderot, France; [13]Northern Institute for Cancer Research, Newcastle University, Newcastle upon Tyne, UK; [14]Department of Biochemistry Medical Genetics Cluster, Erasmus University Medical Center, Rotterdam, the Netherlands; [15]Dutch Childhood Oncology Group, The Hague, the Netherlands; [16]Department of Oncology and Pathology, St. Jude Children's Research Hospital, Memphis, TN, USA; [17]Pediatric Oncology/Hematology, VU University Medical Center, Amsterdam, the Netherlands; [18]Department of Pediatrics, Mackay Memorial Hospital, Taipei, Taiwan; [19]Department of Pediatric Hematology and Oncology, IRCCS, Ospedale Bambino Gesù, Rome, and University of Pavia, Italy; [20]Oncologia ed Ematologia Pediatrica, Ospedale Sant'Orsola Malpighi, Università di Bologna, Italy; [21]Unité de Cytogénétique Onco-hématologique, Hôpital Saint-Antoine, Paris, France; [22]Pediatric Hematology/Oncology, AML-BFM Group, Germany; [23]Department of Pediatrics, Tokyo Medical and Dental University, Japan; [24]Institute of Cell and Molecular Pathology, Hannover Medical School, Germany; and [25]Pediatric Hematology/Oncology, Fondazione IRCCS Policlinico "San Matteo", Pavia, Italy

ABSTRACT

Acute myeloid leukemia with t(6;9)(p22;q34) is listed as a distinct entity in the 2008 World Health Organization classification, but little is known about the clinical implications of t(6;9)-positive myeloid leukemia in children. This international multicenter study presents the clinical and genetic characteristics of 62 pediatric patients with t(6;9)/*DEK-NUP214*-rearranged myeloid leukemia; 54 diagnosed as having acute myeloid leukemia, representing <1% of all childhood acute myeloid leukemia, and eight as having myelodysplastic syndrome. The t(6;9)/*DEK-NUP214* was associated with relatively late onset (median age 10.4 years), male predominance (sex ratio 1.7), French-American-British M2 classification (54%), myelodysplasia (100%), and *FLT3*-ITD (42%). Outcome was substantially better than previously reported with a 5-year event-free survival of 32%, 5-year overall survival of 53%, and a 5-year cumulative incidence of relapse of 57%. Hematopoietic stem cell transplantation in first complete remission improved the 5-year event-free survival compared with chemotherapy alone (68% *versus* 18%; $P<0.01$) but not the overall survival (68% *versus* 54%; $P=0.48$). The presence of *FLT3*-ITD had a non-significant negative effect on 5-year overall survival compared with non-mutated cases (22% *versus* 62%; $P=0.13$). Gene expression profiling showed a unique signature characterized by significantly higher expression of *EYA3, SESN4, PRDM2/RIZ,* and *HIST2H4* genes. In conclusion, t(6;9)/*DEK-NUP214* represents a unique subtype of acute myeloid leukemia with a high risk of relapse, high frequency of *FLT3*-ITD, and a specific gene expression signature.

Introduction

The t(6;9)(p22;q34), frequently reported with a breakpoint in 6p23 but now known to involve the *DEK* gene mapping to 6p22.3, is a rare translocation, estimated to occur in 1-2% of cases of childhood acute myeloid leukemia (AML).[1] The translocation was first identified in 1976, and the first pediatric patient was described in 1982.[2,3] The World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia from 2008 listed the t(6;9)(p22;q34) as a distinct entity.[4] However, our current knowledge of t(6;9)(p22;q34) in AML is drawn from relatively small series of patients, predominantly adults, associating t(6;9) with young age at onset and a poor outcome.[1,5-7] Typically, the t(6;9) presents as *de novo* AML, morphologically associated with French-American-British (FAB) type M2, bone marrow basophilia, Auer rods, and dysplasia.[1,5,7,8] The translocation is primarily the sole cytogenetic abnormality (80%); among the 20% of

 doi:10.3324/haematol.2013.098517
The online version of this article has a Supplementary Appendix.
Manuscript received on September 24, 2013. Manuscript accepted on January 13, 2014.
Correspondence: hasle@dadlnet.dk

patients with additional secondary changes, gains of chromosomes 8 and 13 are most frequent.[1,8,9] Internal tandem duplication (ITD) in the FMS-related tyrosine kinase 3 (*FLT3*) gene occurs in 20-30% of *de novo* AML in adults and in approximately 10% of childhood AML but in up to 70% of t(6;9)-positive cases.[1,8,10,11] The clinical outcome is poor, with 5-year overall survival rates of 28% reported in children and 9% in adults.[1,8,9] Recent smaller studies, including both adult and pediatric patients, have shown that treatment with early allogeneic hematopoietic stem cell transplantation (HSCT) in first complete remission may improve the outcome.[1,11,12]

The t(6;9) results in a fusion of the 5' part of the *DEK* gene at 6p22.3 and the 3' part of the *NUP214* gene, formerly known as *CAN*, located at 9q34, forming the chimeric *DEK-NUP214* gene.[13] The DEK-NUP214 protein has been reported to enhance protein synthesis in cells of the myeloid lineage but is unable to block differentiation.[15,16] The leukemogenic potential of the fusion protein is restricted to a very small subpopulation of hematopoietic stem cells.[17]

The characteristics of t(6;9)/*DEK-NUP214* AML have so far not been separately described in pediatric patients and the prognostic impact in pediatric AML is unclear.[1,12,14,18] The aims of this study were to characterize the clinical, genetic and morphological features of t(6;9)-positive childhood myeloid neoplasms in the largest series so far; to evaluate outcome and to identify genes potentially involved in leukemogenesis of t(6;9) using gene expression profiling.

Methods

Patients

The inclusion criteria comprised age between 0-18 years and a diagnosis of *de novo* AML or myelodysplastic syndrome (MDS) with t(6;9)/*DEK-NUP214* made between 1 January, 1998 and 31 December, 2011.

The study was conducted within the International Berlin-Frankfurt-Munster study group cooperation and 24 study groups and treatment centers participated, providing background data for frequency analysis and submitting clinical and genetic data on 62 patients. In addition, the study groups contributed smears, bone marrow biopsies, and material for gene expression analysis. The nomenclature was reviewed following the International System for Human Cytogenetic Nomenclature 2009[19] by three of the co-authors (JH, BJ, and EF). Available diagnostic smears and bone marrow biopsies were reviewed by co-author GK.

Patients were treated according to national AML trials, and the treatment protocols were approved by local ethical committees in compliance with national regulations.

Gene expression profiling and quantitative real time polymerase chain reaction analysis

Gene expression profiling was performed on 297 pediatric AML patients' samples of which eight were t(6;9)-positive. The gene expression profiling data on the full cohort have already been published but an individual analysis of t(6;9)/*DEK-NUP214* was not performed.[20] Original data are available in the Gene Expression Omnibus repository (*http://www.ncbi.nlm.nih.gov/geo*; accession GSE17855). The gene expression profiles of t(6;9)-positive cases were compared with those of other representative pediatric AML samples.[21] Four top scoring, differentially expressed genes were selected for mRNA expression validation by quantitative real time polymerase chain reaction (RT-qPCR) analysis based on statistical significance, occurrence of multiple probes in the top-list, and log-fold change, combined with potential biological relevance; *EYA5* (eyes absent homolog 3, Drosophila), *SESN1* (sestrin 1), *PRDM2/RIZ* (PR domain containing 2, with ZNF domain), and *HIST2H4* (histone cluster 2, H4). RT-qPCR was performed on t(6;9)/*DEK-NUP214*-positive samples from 17 patients and one cell line (FKH-1) and compared with AML without t(6;9)/*DEK-NUP214* (31 samples from patients and 13 cell lines) using the ABI PRISM 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA). Primer sequences are listed in *Online Supplementary Table S1*.

Statistical analyses

The Kaplan-Meier method was used to estimate the 5-year probabilities of overall survival and event-free survival. The 5-year cumulative incidence of relapse was calculated by the method of Kalbfleisch and Prentice.[22] The median time to HSCT in first complete remission was 150 days, with 98 days as minimum. Patients with an event within 150 days of treatment were excluded from the analysis comparing the effect of HSCT in first complete remission with that of conventional chemotherapy alone. Statistical analyses were conducted using SPSS for Mac, version 20 (SPSS Science, Chicago, IL, USA).

Gene expression profiling data were acquired using Expresso (Bioconductor package Affy). Probe-set intensities were normalized using variance stabilization normalization (Bioconductor package VSN) in the statistical data analysis environment R, version 2.11.[23] An empirical Bayes linear regression model was used to compare the signatures for the t(6;9)-positive cases with those of the other AML cases.[24] Moderated T-statistics *P*-values were corrected for multiple testing using the false discovery rate method, as defined by Benjamini and Hochberg.[25]

Details of the methods and primer sequences are provided in the *Online Supplementary Appendix*.

Results

Of the 24 study groups/centers that participated in the study, 15 provided clinical and cytogenetic data and samples for morphologic and gene expression analyses. The remaining nine study groups reported no patients with t(6;9)/*DEK-NUP214*. Data on 70 children were submitted. Eight patients were excluded; four were diagnosed before 1998 and four had incomplete karyotype with unknown t(6;9) breakpoints and no proof of the *DEK-NUP214* gene fusion. Accordingly 62 patients fulfilled the inclusion criteria. One study group did not report frequency background data and was not included in the frequency analysis, and since this was an AML estimate, EWOG-MDS was not included either. Thus 22 of the 24 study groups/centers reported altogether 7368 childhood AML cases with complete cytogenetic data, 45 of which had t(6;9)/*DEK-NUP214*, corresponding to a frequency of 0.6% of pediatric AML.

Of the 62 t(6;9)/*DEK-NUP214* myeloid malignancies, 54 were diagnosed as *de novo* AML and eight as MDS. The clinical, morphological, and genetic characteristics of the cohort are listed in Table 1. The AML and MDS groups were comparable except for age; children diagnosed with MDS were younger (median age 7.4 years *versus* 11.4 years; $P<0.05$). This notwithstanding, considering all other similarities, we combined t(6;9)/*DEK-NUP214* AML and MDS cases into one entity.

Morphology

The majority of t(6;9) cases were categorized as FAB type M2 (54%) or FAB type M4 (26%) (Table 1). Peripheral blood and bone marrow smears from 11 and 15 cases, respectively, of which two were MDS cases, were evaluable for review along with 7 bone marrow biopsies. Dysplasia was defined according to EWOG-MDS guidelines.[25] All bone marrow biopsies displayed mild to moderate bilinear dysplasia. Basophils were present in the bone marrow from five (83%) of the reviewed cases, but did not exceed 2% in any case. Data on basophils were available for 16 cases without central review; five had 0.5-2% basophils in the bone marrow while no basophils were reported in the remaining 11 patients. No Auer rods were identified in the material reviewed. Among the cases not centrally reviewed, Auer rods were reported in ten of 28 (36%) cases. The morphological characteristics found by the central review are shown in *Online Supplementary Figure S1*.

Table 1. Characteristics of patients with t(6;9)(p22;q34)/*DEK-NUP214*-rearranged myeloid leukemia.

	AML N=54	MDS N=8	Total N=62
Boys	34 (63%)	5 (63%)	39 (63%)
Median age in years (range)	11.4* (3.4-17.6)	7.4 * (4.1-13.2)	10.4 (3.4-17.6)
0-1 year	0	0	0
2-9 years	21 (39%)	5 (63 %)	26 (42%)
10-18 years	33 (61%)	3 (37%)	36 (58%)
Hematologic parameters median (range)			
WBC (x10⁹/L) (N=58)	16.4 (0.2-191.0)	11.8 (1.9-29.0)	16.0 (0.2-191.0)
Hb (g/dL) (N=56)	8.2 (1.9-12.2)	7.3 (4.0-12.3)	8.0 (1.9-12.3)
Platelets (x10⁹/L) (N=48)	63.0 (6.0-235.0)	76.5 (15.0-92.0)	70.5 (6.0-235.0)
PB blasts (%) (N=50)	36 (0-99)**	9 (0-16)**	31 (0-99)
BM blasts (%) (N=53)	60 (14-95)**	10 (5-19)**	55 (5-95)
FAB			
M0	1 (2%)		1
M1	4 (7%)		4
M2	29 (54%)		29
M4	14 (26%)		14
M5	1 (2%)		1
Unclassifiable	5 (9%)		5
MDS		8	8
Cytogenetics			
t(6;9) sole abnormality	40 (74%)	7 (88%)	47 (81%)
Additional aberrations	10 (19%)	1 (13%)	11 (19%)
FISH or PCR only	4 (7%)	0	4 (6%)
HSCT	32 (59%)	7 (88%)	39 (63%)
First complete remission	18 (55%)	3 (43%)	21 (54%)
Residual disease	0	3 (43%)	3 (8%)
After relapse	14 (45%)	1 (14%)	15 (38%)
Complete remission	50 (93%)	5 (63%)	55 (89%)
No event	20 (37%)	5 (63%)	25 (40%)
Relapse	25 (46%)	3 (38%)	28 (45%)
Survival (SE)			
5-year EFS	32% (7)	55% (20)	35% (7)
5-year OS	53% (7)	86% (13)	57% (7)
5-year CIR	57 % (7)	45% (20)	56% (7)

* $P<0.05$, ** $P<0.01$

AML: acute myeloid leukemia; MDS: myelodysplastic syndrome; WBC: white blood cell count; Hb: hemoglobin; PB: peripheral blood; BM: bone marrow; FAB: French-American-British subtype; FISH: fluorescence in situ hybridization, PCR: polymerase chain reaction; HSCT: hematopoietic stem-cell transplantation; EFS: event-free survival; OS: overall survival; CIR: cumulative incidence of relapse

Cytogenetics

Successful cytogenetic results were available in 58 of the cases; in the remaining four, the *DEK-NUP214* gene fusion was detected by RT-PCR or fluorescence *in situ* hybridization. Among the 58 cytogenetically informative cases, t(6;9)(p22;q34) was the sole cytogenetic abnormality in 47 (81%) while 11 (19%) had additional aberrations, including loss of chromosome Y in three boys and trisomy 8 and trisomy 13 each present in three cases, either alone or combined. The karyotypes with additional aberrations are listed in *Online Supplementary Table S2*.

Outcome and prognostic factors

Outcome data were available for all 62 children. There were no differences in overall and event-free survival between children diagnosed with AML or with MDS; however, due to the heterogeneity of the treatment strategies for AML and MDS, the following survival estimates are based on AML cases only (Table 2). The median follow-up for the survivors was 4.7 years (range, 0.2 – 17.1). Fifty of the children (93%) achieved a complete remission and 25 (46%) experienced relapse. The latest event occurred 41 months after diagnosis. The 5-year event-free survival rate was 32% (±14%), the 5-year overall survival rate was 53% (±14%), and the 5-year cumulative incidence of relapse was 57% (±14%).

Sex, age, and white blood cell count were not prognostic factors for event-free survival, overall survival or cumulative incidence of relapse (Table 2). Patients with FAB M4 had a worse outcome than patients with other FAB subtypes, with a 5-year event-free survival of 9% *versus* 40% (P=0.04) and 5-year cumulative incidence of relapse of 91% *versus* 49% (P<0.01) respectively, but they were over-represented among the *FLT3*-ITD-positive cases (P=0.02).

In total, *FLT3*-ITD status was known in 33 (53%) cases: 14 (42%) were *FLT3*-ITD-positive and 19 (58%) were *FLT3*-ITD-negative (wild-type and one tyrosine kinase domain point mutation). The allelic ratio of *FLT3*-ITD was not available. Among the 29 *FLT3* informative AML cases included in the survival analysis, presence of *FLT3*-ITD had a non-significant negative effect on outcome compared with absence of *FLT3*-ITD (Table 2 and *Online Supplementary Figure S2C,D*).

In the evaluation of HSCT, MDS patients were excluded along with cases with events earlier than 150 days to correct for time to transplantation. Eighteen AML patients were transplanted in first complete remission and 14 after relapse. The characteristics of the HSCT are presented in *Online Supplementary Table S3*. HSCT in first complete remission significantly improved the 5-year event-free survival compared with treatment with chemotherapy alone in first complete remission: 68% *versus* 18% (P<0.01), but did not improve the overall survival rate (68% *versus* 54%; P=0.48; *Online Supplementary Figure S2A,B*). A total of five patients died after HSCT; one from progressive disease following relapse and four from procedure-related toxicity (two infections and two cases of multiorgan failure).

FLT3-ITD status was known for 24 of the 48 cases included in the HSCT analysis. Twelve were *FLT3*-ITD-positive, of which eight received chemotherapy only and four had HSCT in first complete remission. None of the eight patients treated with conventional chemotherapy survived without an event, whereas there were no events among the four ITD-positive patients treated with HSCT in first complete remission. Twelve patients were *FLT3*-

ITD-negative; three were transplanted in first complete remission, of whom one relapsed and nine received chemotherapy alone in first complete remission, of whom six relapsed.

Multivariate analysis was used to assess survival parameters of patients with *FLT3*-ITD treated with HSCT in first complete remission. The limited number of patients and information on *FLT3*-ITD status necessitated a strict selection of variables that could be included in the Cox model. We therefore performed stepwise exclusion and the best possible model was adjusted for HSCT and *FLT3*-ITD. FAB type M4 was overrepresented among *FLT3*-ITD-positive cases and was not an independent prognostic factor; thus, FAB type M4 was excluded from further analysis. Treatment with HSCT in first complete remission was independently associated with higher event-free survival (P=0.03) whereas *FLT3*-ITD mutation was not an independent prognostic factor (*Online Supplementary Table S4*).

Gene expression profiling

The supervised analysis of gene expression levels of *DEK-NUP214*-positive samples *versus* other pediatric AML samples resulted in a top-table of 180 distinctive probe-sets with a false discovery rate-adjusted P value <0.05. The t(6;9)/*DEK-NUP214* cases were characterized by high expression of four genes in particular: *HIST2H4* (log fold-change 2.75; adjusted P value of 8.17×10^{-15}); *PRDM2/RIZ* (log fold-change 1.5, adjusted P value 2.86×10^{-9}), represented by four probe-sets in the top-table, *SESN1* (log fold-change 1.24; adjusted P value 6.30×10^{-8}), and *EYA-3*, represented twice with log fold-changes 0.46 and 0.33 (adjusted P value 1.75×10^{-11}). mRNA expression levels determined by RT-qPCR correlated well with the expression profiles derived from gene expression profiling. The expression of these four genes among cytogenetic subgroups of pediatric AML, determined by both gene expression profiling and RT-qPCR, and their correlations are shown in Figure 1. The unsupervised analysis is presented in *Online Supplementary Figure S3*, revealing that the t(6;9)/*DEK-NUP214* cases did not cluster in an unsupervised manner.

In the top-table, 158 probe sets represented up-regulated genes, including several *HOXA* and *HOXB* genes; (*HOXB2*, *B3*, *B4*, *B5*, *B6*, *B8*, and *B9*). The high expression levels of the *HOXA* and the *HOXB* genes were validated in a previous study.[25]

Table 2. Overall survival, event-free survival, and cumulative incidence of relapse for children with t(6;9)(p22;q34)/*DEK-NUP214*-rearranged AML (N=54).

	5-year OS % (SE)	P-log rank	5-year EFS % (SE)	P-log rank	5-year CIR % (SE)	P-Gray
Year of diagnosis						
1993-1999 (N=18)	44 (12)	0.75	17 (9)	0.72	66 (12)	0.48
2000-2006 (N=18)	61 (12)		44 (12)		52 (12)	
2007-2012 (N=18)	55 (14)		34 (14)		53 (15)	
Sex						
Female (N=20)	51 (13)	0.84	33 (12)	0.90	62 (12)	0.35
Male (N=34)	53 (9)		31 (8)		54 (9)	
Age groups						
2-9 (N=21)	64 (11)	0.29	32 (10)	0.83	50 (11)	0.34
>10 (N=33)	44 (10)		33 (9)		62 (10)	
WBC count						
WBC <20x10⁹/L (N=29)	56 (10)	0.19	35 (10)	0.66	58 (10)	0.51
WBC 20-99x10⁹/L (N=20)	58 (11)		32 (11)		61 (12)	
WBC ≥100x10⁹/L (N=4)	0		0		no relapse	
FAB classification*						
M0 (N=1)	no events	0.47	no events	0.36	no relapse	0.55
M1 (N=4)	67 (27)		50 (25)		25 (22)	
M2 (N=29)	56 (10)		35 (10)		54 (10)	
M4 (N=14)	39 (14)	0.17	9 (8)	0.04	91 (8)	<0.01
M5 (N=1)	no events		no events		no relapse	
Unclassifiable (N=5)	40 (22)		40 (22)		no relapse	
Cytogenetics						
t(6;9)(p22;q34), sole abnormality (N=40)	50 (8)	0.81	28 (8)	0.57	60 (8)	0.32
Additional aberrations (N=10)	55 (17)		37 (19)		48 (21)	
FLT3-ITD**						
Positive (N=14)	22 (14)	0.13	17 (14)	0.29	75 (16)	0.10
Negative (N=15)	62 (13)		31 (14)		46 (16)	
HSCT in first complete remission***						
Yes (N=18)	68 (12)	0.48	68 (12)	<0.01	13 (9)	<0.01
No (N=30)	54 (10)		18 (7)		81 (8)	

*The log-rank values are based on a comparison between all FAB subtypes. Only FAB M4 differed from the others, hence the additional analysis of FAB M4 vs. all others. **Four FLT3-ITD-positive patients were treated with HSCT in first complete remission with no events; all eight FLT3-ITD-positive patients not treated with HSCT relapsed. *** The analysis of survival is based on patients with EFS >150 days, no patients were censored after HSCT. WBC: white blood cell count; FAB: French-American-British subtype; HSCT: hematopoietic stem-cell transplantation; EFS: event-free survival; OS: overall survival; CIR: cumulative incidence of relapse.*

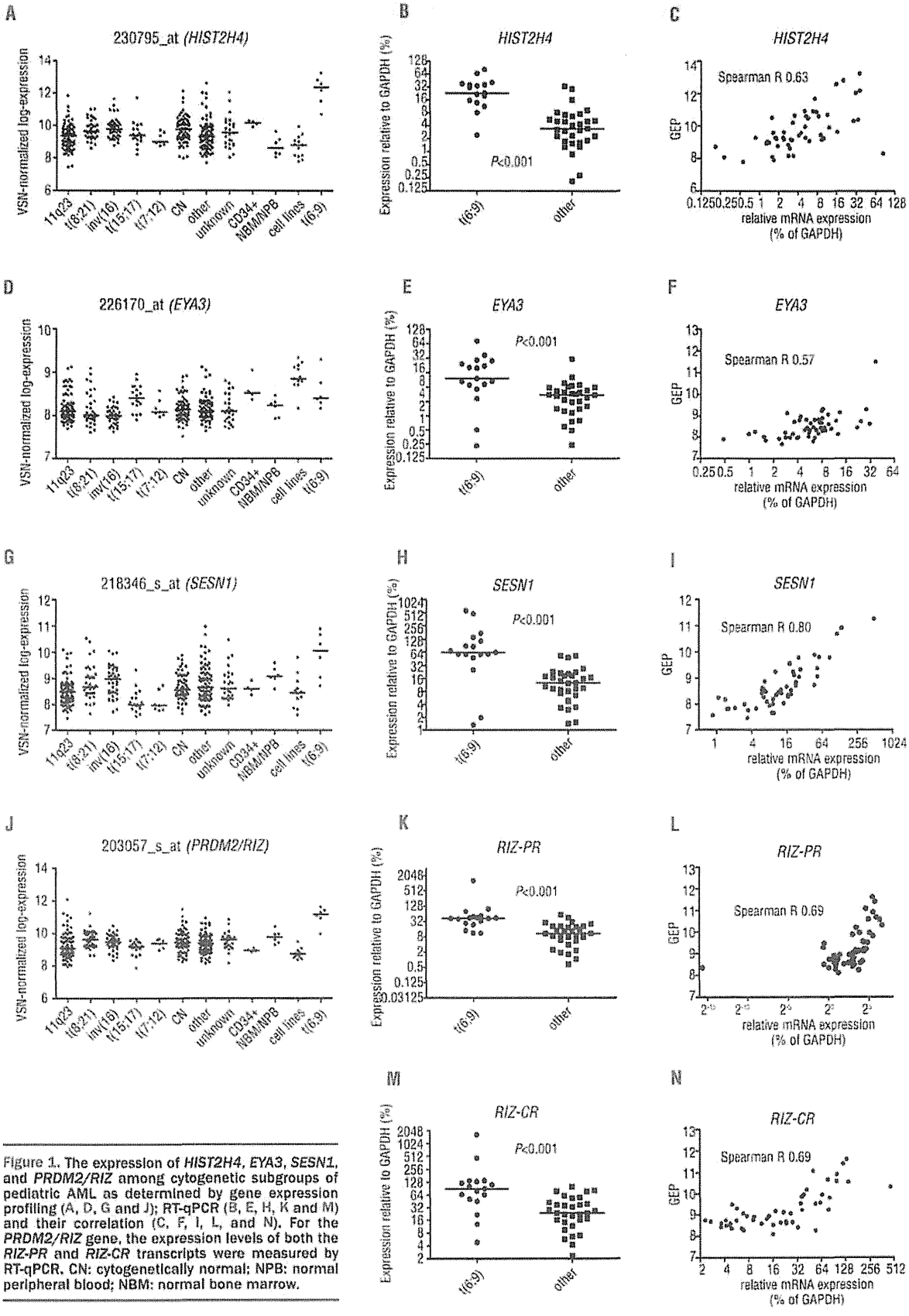

Figure 1. The expression of *HIST2H4*, *EYA3*, *SESN1*, and *PRDM2/RIZ* among cytogenetic subgroups of pediatric AML as determined by gene expression profiling (A, D, G and J); RT-qPCR (B, E, H, K and M) and their correlation (C, F, I, L, and N). For the *PRDM2/RIZ* gene, the expression levels of both the *RIZ-PR* and *RIZ-CR* transcripts were measured by RT-qPCR. CN: cytogenetically normal; NPB: normal peripheral blood; NBM: normal bone marrow.

Discussion

The t(6;9)/*DEK-NUP214*-rearranged cases in this cohort represented less than 1% of childhood AML with available cytogenetics based on the data collected from 22 major study groups/centers - a frequency lower than previously reported.[15] The translocation is easily detected by conventional karyotyping and the risk of undetected cases is considered minimal. An explanation for the low frequency could be the inclusion in the denominator of AML children from the study groups who did not report t(6;9) cases in the frequency analysis.

The median age was 10.4 years and no patient was diagnosed before 3 years of age. The age distribution is very different from the general age distribution in AML in which approximately 30% of patients are less than 3 years of age at diagnosis.[27] To our knowledge, no t(6;9)-positive cases have been reported in children below 3 years of age[*], which strengthens our finding. The male dominance in this study has also been observed in adult series of t(6;9).[3,5,7]

As in most previous adult studies on t(6;9) AML, the FAB subtypes FAB M2 and FAB M4 were most common,[3,5,6,28] together constituting 80% of all cases in the present cohort (Table 1). The present study suggests that there are differences in the morphology among pediatric and adult t(6;9) myeloid malignancies. Basophilia seems less common in children than in adults along with a significant but milder degree of dysplasia in childhood myeloid malignancies.[3,5,6] Pseudo-Pelger Huët cells were found in all 15 patients reviewed. This observation is in contrast to that in pediatric MDS, in which such cells are only rarely found (GK, *personal communication 2012*). The similar clinical and morphological characteristics between t(6;9)-positive AML and MDS found in this large pediatric series suggest that t(6;9)/*DEK-NUP214*, like t(8;21)/*RUNX1-RUNX1T1* and inv(16)(p13q22) or t(16;16)(p13;q22)/*CBFB-MYH11*, should be classified as AML regardless of the blast count.

FLT3-ITD mutations were present in 42% of the present cohort, which is less than reported in the literature,[3,5] and had a negative impact on outcome (Table 2), though this did not reach statistical significance. The COG study on t(6;9) AML reported no significant impact of *FLT3*-ITD on outcome (overall survival rates for *FLT3*-ITD-positive and -negative cases were 39% *versus* 20%, respectively). However, the ITD-positive patients in the COG study were allocated to a high-risk treatment protocol including HSCT while ITD-negative cases were assigned to an intermediate-risk protocol without transplantation,[3] which might have influenced the outcome. In the present study, all eight *FLT3*-ITD-positive patients treated without HSCT in first complete remission experienced a relapse and subsequently only two survived (with follow-ups of 12 and 13 months), whereas the four *FLT3*-ITD-positive patients treated with HSCT were alive without disease in first complete remission at follow-up. Six of the nine ITD-negative patients, treated with chemotherapy in first complete remission, relapsed. We found a significant improvement of event-free survival resulting from treatment with HSCT in first complete remission compared with treatment with chemotherapy only, but this did not translate into superior overall survival (Table 2 and *Online Supplementary Table S4*), suggesting a high salvage rate. Only St. Jude reported t(6;9) as a treatment-stratifying aberration in the AML02 and 08 protocols relevant for two patients included in this study and allocated to the high-risk arm including HSCT. It should be emphasized that retrospectively collected data have serious limitations since the factors for allocating patients to HSCT, such as co-morbidity, individual assessment of the treating physician, and availability of donor, remain unknown and this weakness must be taken into account when evaluating the value of HSCT. We have statistically tried to overcome some of the obstacles, such as disease stage, by only evaluating HSCT in first complete remission and corrected for time to transplantation. Parameters important for the outcome of HSCT, such as donor characteristics, and HLA match are given in *Online Supplementary Table S3*. The numbers are small and considering the above-mentioned limitations, our results are in accordance with those of other small series.[12,13] Notably, 14 children were transplanted after relapse and it is possible that a potential beneficial effect of HSCT after relapse is reflected in the high salvage rate. HSCT seems beneficial in patients with t(6;9)/*DEK-NUP214* and in particular for patients with *FLT3*-ITD.

We identified a unique gene expression signature with, among others, high expression of *HIST2H4*, *PRDM2/RIZ*, *EYA3* and *SESN1* in addition to *HOXA* and *HOXB* gene overexpression being characteristic of *DEK-NUP214*-positive cases, as it is for AML with *NPM1*-mutations, *NUP98-NSD1*-rearrangements and partial tandem duplications of the *MLL* gene, suggesting a common pathway of leukemogenesis in these cases. The overexpression of *HOXA* and *HOXB* genes in *DEK-NUP214*-positive cases was previously validated.[26] It is noteworthy that both *DEK-NUP214* and *NUP98-NSD1* are characterized by high *HOXA* and *HOXB* gene expression since these two cytogenetic subgroups share other genetic and clinical characteristics: a high frequency of *FLT3*-ITD (40-70% and >90%, respectively)[3,26] and absence of patients below 2 years of age.[26] Furthermore, the NUP214 and NUP98 oncogenic fusion proteins are similar in many respects. First, both fusions include a nucleoporin-specific FG region (phenylalanine-glycine repeats), which is associated with various histone-modifying complexes.[29,30] Second, both fuse to a nuclear factor, mostly but not uniquely a direct transcription factor.[14,31,32] Third, both fusion products are localized to the nucleus, as opposed to the wild-type nucleoporins, which are mainly present in the nuclear pore complex.[31,33] It is, therefore, likely that these nucleoporin-containing fusion proteins are functionally similar, acting as aberrant transcriptional modulators.

Within this study, we validated the selective up-regulation of four genes in t(6;9)/*DEK-NUP214*–positive pediatric cases: *HIST2H4*, *PRDM2/RIZ*, *EYA3* and *SESN1*. The *HIST2H4*, mapping at 1q21.2, encodes a member of the histone H4 family but the function of *HIST2H4* in leukemogenesis is unknown. The *PRDM2* (1p36.21) gene, also known as *RIZ*, encodes two proteins: RIZ-PR (RIZ1) and RIZ-CR (RIZ2). The proteins are identical except that RIZ-PR has an N-terminal PR domain with methyltransferase activity that is lacking in RIZ-CR. *RIZ*-PR has tumor suppressor activity whereas *RIZ-CR* has been described to acts as an oncogene.[34,35] The probe used in our gene expression profiling analyses did not distinguish between the two transcripts. However, in the RT-qPCR validation of the expression levels, we analyzed the gene expression of each transcript and found that both the tumor-suppressive *RIZ-PR* and the oncogenic *RIZ-CR* were up-regulated. The

EYA3 gene (1p35.3) encodes a member of the 'eyes absent' protein family and is involved in repair and cell survival as a response to DNA damage in organogenesis.[35,37] The fourth gene *SESN1* (6q21) codes for a member of the sestrin family and is known to be a TP53 target.[38,39] Considering their known tumor suppressor function, it is surprising that the *SESN1* gene and the *RIZ-PR* transcript were both up-regulated in t(6;9)-positive cases.

It is striking that several of the genes found to be significantly up-regulated in *DEK-NUP214*-rearranged cases are known to influence the modeling and function of histones. Depletion of both *DEK* and *EYA3* causes phosphorylation of H2Ax (γH2Ax), a subunit of histone H2A,[37,40] DEK protein is able to bind to histones and reduce the levels of histone H3 and H4 acetylation, thus playing an important role in chromatin modification, histone acetylation and transcription.[41,42] In addition, the most significantly up-regulated gene was *HIST2H4* and the tumor suppressor activity of RIZ-PR is related to the histone methyl-transferase activity of the PR domain.[43] We hypothesize that specific histone modifications are key events during leukemogenesis, possibly through modulating the epigenetic state of the cell.

In conclusion, t(6;9)/*DEK-NUP214*-rearranged cases represent less than 1% of all childhood AML and are characterized by a late onset, male predominance, *FLT3*-ITD mutations, and a high risk of relapse. Nevertheless, a large proportion of the patients can be cured and HSCT potentially benefits patients with t(6;9) and especially those with *FLT3*-ITD. In addition, we identified a unique gene expression signature including several up-regulated genes involved in histone modification, and a typical *HOXA/B* profile, which may be a target for future therapy.

Acknowledgments

The authors would like to thank all groups involved in retrieving and providing data and material (a list of additional participants involved in the study is given in the online supplement). We especially thank Saint-Louis Hospital's Tumor Biobank in Paris (France), the NOPHO Biobank in Uppsala (Sweden), the St. Jude Children's Research Hospital Tissue Resources Laboratory in Memphis (USA), and the Giuseppe Basso Laboratory of Pediatric Oncology, Pediatric Department, University of Padova, Padova (Italy) for managing patients' samples, and for valuable samples and smears provided by regional treatment centers. We thank Susan Arentsen-Peters at the Laboratory of Pediatric Oncology/Hematology, Erasmus MC, Rotterdam, NL for technical assistance. This work was supported by the Danish Childhood Cancer Foundation, the Swedish Childhood Cancer Foundation, the Dutch Childhood Oncology Group, a Grant from the Clinical Cancer Research and Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Slovak ML, Gundacker H, Bloomfield CD, Dewald G, Appelbaum FR, Larson RA, et al. A retrospective study of 69 patients with t(6;9)(p23;q34) AML emphasizes the need for a prospective, multicenter initiative for rare 'poor prognosis' myeloid malignancies. Leukemia. 2006;20(7):1295-7.
2. Rowley JD, Potter D. Chromosomal banding patterns in acute nonlymphocytic leukemia. Blood. 1976;47(5):705-21.
3. Kaneko Y, Rowley JD, Maurer HS. Chromosome pattern in childhood acute nonlymphocytic leukemia (ANLL). Blood. 1982;60(2):589-99.
4. Vardiman JW, Thiele J, Arber DA, Brunning RD, Borowitz MJ, Porwit A, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114(5):937-51.
5. Gupta M, Ashok Kumar J, Sitaram U, Neeraj S, Nancy A, Balasubramanian P, et al. The t(6;9)(p22;q34) in myeloid neoplasms: a retrospective study of 16 cases. Cancer Genet Cytogenet. 2010;203(2):297-302.
6. Garcon L, Libura M, Delabesse E, Valensi F, Asnafi V, Berger C, et al. DEK-CAN molecular monitoring of myeloid malignancies could aid therapeutic stratification. Leukemia. 2005;19(8):1338-44.
7. Alsabeh R, Brynes RK, Slovak ML, Arber DA. Acute myeloid leukemia with t(6;9)(p23;q34): association with myelodysplasia, basophilia, and initial CD34 negative immunophenotype. Am J Clin Pathol. 1997;107(4):430-7.
8. Oyarzo MP, Lin P, Glassman A, Bueso-Ramos CE, Luthra R, Medeiros LJ. Acute myeloid leukemia with t(6;9)(p23;q34) is associated with dysplasia and a high frequency of flt3 gene mutations. Am J Clin Pathol. 2004;122(3):348-58.
9. Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer (2013). Mitelman F, Johansson B and Mertens F (Eds.), http://cgap.nci.nih.gov/Chromosomes/Mitelman.
10. Chi Y, Lindgren V, Quigley S, Gaitonde S. Acute myelogenous leukemia with t(6;9)(p23;q34) and marrow basophilia: an overview. Arch Pathol Lab Med. 2008;132(11):1835-7.
11. Zwaan CM, Meshinchi S, Radich JP, Veerman AJP, Huismans DR, Munske L, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. Blood. 2003;102(7):2387-94.
12. Ishiyama K, Takami A, Kanda Y, Nakao S, Hidaka M, Maeda T, et al. Allogeneic hematopoietic stem cell transplantation for acute myeloid leukemia with t(6;9)(p23;q34) dramatically improves the patient prognosis: a matched-pair analysis. Leukemia. 2013;26(3):461-4.
13. Ishiyama K, Takami A, Kanda Y, Nakao S, Hidaka M, Maeda T, et al. Prognostic factors for acute myeloid leukemia patients with t(6;9)(p23;q34) who underwent an allogeneic hematopoietic stem cell transplant. Leukemia. 2012;26(6):1416-9.
14. Von Lindern M, Fornerod M, Van Baal S, Jaegle M, De Wit T, Buijs A, et al. The translocation (6;9), associated with a specific subtype of acute myeloid leukemia, results in the fusion of two genes, dek and can, and the expression of a chimeric, leukemia-specific dek-can mRNA. Mol Cell Biol. 1992;12(4):1687-97.
15. Ageberg M, Drott K, Olofsson T, Gullberg U, Lindmark A. Identification of a novel and myeloid specific role of the leukemia-associated fusion protein DEK-NUP214 leading to increased protein synthesis. Genes Chromosomes and Cancer. 2008;47(4):276-87.
16. Boer J, Bonten-Surtel J, Grosveld G. Overexpression of the nucleoporin CAN/NUP214 induces growth arrest, nucleocytoplasmic transport defects, and apoptosis. Mol Cell Biol. 1998;18(3):1236-47.
17. Oancea C, Rüster B, Henschler R, Puccetti E, Ruthardt M. The t(6;9) associated DEK/CAN fusion protein targets a population of long-term repopulating hematopoietic stem cells for leukemogenic transformation. Leukemia. 2010;24(11):1910-9.
18. Harrison CJ, Hills RK, Moorman AV, Grimwade DJ, Hann I, Webb DK, et al. Cytogenetics of childhood acute myeloid leukemia: United Kingdom Medical Research Council Treatment trials AML 10 and 12. J Clin Oncol. 2010;28(16):2674-81.
19. Shaffer LG, Slovak ML, Campbell LJ. ISCN (2009): International System of Human Cytogenetic Nomenclature. Basel S Karger AG; 2009.
20. Balgobind BV, van den Heuvel-Eibrink MM, De Menezes RX, Reinhardt D, Hollink IH, Arentsen-Peters STJCM, et al. Evaluation of gene expression signatures predictive of cytogenetic and molecular subtypes of pediatric acute myeloid leukemia. Haematologica. 2011;96(2):221-30.
21. Kalbfleisch JD, Prentice RL. The Statistical Analysis of Failure Time Data (2nd ed).

Hoboken NJ: J. Wiley; 2002.
22. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004;20 (3):307-15.
23. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3.
24. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289-300.
25. Cantu Rajnoldi A, Fenu S, Kerndrup G, van Wering ER, Niemeyer CM, Baumann I. Evaluation of dysplastic features in myelodysplastic syndromes: experience from the morphology group of the European Working Group of MDS in Childhood (EWOG-MDS). Ann Hematol. 2005;84(7): 429-33.
26. Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Pratcorona M, Abbas S, Kuipers JE, et al. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood. 2011;118(13): 3645-56.
27. Forestier E, Schmiegelow K. The incidence peaks of the childhood acute leukemias reflect specific cytogenetic aberrations. J Pediatr Hematol Oncol. 2006;28(8):486-95.
28. Pearson MG, Vardiman JW, Le Beau MM. Increased numbers of marrow basophils may be associated with a t(6;9) in ANLL. Am J Hematol. 1985;18(4):393-408.
29. Moraleda PP, Alonzo TA, Gerbing RB, Raimondi SC, Hirsch BA, Ravindranath Y, et al. Acute myeloid leukemia with t(6;9)(p23;q34) is associated with poor outcome in childhood AML regardless of FLT3/ITD status, a report from Children's Oncology Group. ASH Annual Meeting Abstracts. 2012;120(21):2541.
30. Bai XT, Gu BW, Yin T, Niu C, Xi XD, Zhang J, et al. Trans-repressive effect of NUP98-PMX1 on PMX1-regulated c-FOS gene through recruitment of histone deacetylase 1 by FG repeats. Cancer Res. 2006;66(9): 4584-90.
31. Kasper LH, Brindle PK, Schnabel CA, Pritchard CE, Cleary ML, van Deursen JM. CREB binding protein interacts with nucleoporin-specific FG repeats that activate transcription and mediate NUP98-HOXA9 oncogenicity. Mol Cell Biol. 1999;19(1):764-76.
32. Romana SP, Radford-Weiss I, Ben Abdelali R, Schluth C, Petit A, Dastugue N, et al. NUP98 rearrangements in hematopoietic malignancies: a study of the Groupe Francophone de Cytogenetique Hematologique. Leukemia. 2006;20(4):696-706.
33. Fornerod M, Boer J, van Baal S, Jaegle M, von Lindern M, Murti KG, et al. Relocation of the carboxyterminal part of CAN from the nuclear envelope to the nucleus as a result of leukemia-specific chromosome rearrangements. Oncogene. 1995;10(9): 1739-48.
34. Sun W, Qiao L, Liu Q, Chen L, Ling B, Sammynaiken R, et al. Anticancer activity of the PR domain of tumor suppressor RIZ1. Int J Med Sci. 2011;8(2):161-7.
35. Sasaki O, Meguro K, Tohmiya Y, Funato T, Shibahara S, Sasaki T. Altered expression of retinoblastoma protein-interacting zinc finger gene, RIZ, in human leukaemia. Br J Haematol. 2002;119(4):940-8.
36. Jeme J, Rebay I. The eyes absent family of phosphotyrosine phosphatases: properties and roles in developmental regulation of transcription. Annu Rev Biochem. 2007; 76:513-38.
37. Cook PJ, Ju BG, Telese F, Wang X, Glass CK, Rosenfeld MG. Tyrosine dephosphorylation of H2AX modulates apoptosis and survival decisions. Nature. 2009;458(7238):591-6.
38. Velasco-Miguel S, Buckbinder L, Jean P, Gelbert L, Talbott R, Laidlaw J, et al. PA26, a novel target of the p53 tumor suppressor and member of the GADD family of DNA damage and growth arrest inducible genes. Oncogene. 1999;18(1):127-37.
39. Budanov AV, Karin M. p53 target genes Sestrin1 and Sestrin2 connect genotoxic stress and mTOR signaling. Cell. 2008;134(3):451-60. Erratum in Cell. 2009; 136(2):378.
40. Ko SI, Lee IS, Kim JY, Kim SM, Kim DW, Lee KS, et al. Regulation of histone acetyltransferase activity of p300 and PCAF by proto-oncogene protein DEK. FEBS Lett. 2006;580(18):3217-22.
41. Waldmann T, Eckerich C, Baack M, Gruss C. The ubiquitous chromatin protein DEK alters the structure of DNA by introducing positive supercoils. J Biol Chem. 2002; 277(28):24988-94.
42. Derunes C, Briknarova K, Geng L, Li S, Gessner CR, Hewitt K, et al. Characterization of the PR domain of RIZ1 histone methyltransferase. Biochem Biophys Res Commun. 2005;333(3):925-34.

Comparison of a Fludarabine and Melphalan Combination-Based Reduced Toxicity Conditioning With Myeloablative Conditioning by Radiation and/or Busulfan in Acute Myeloid Leukemia in Japanese Children and Adolescents

Hiroyuki Ishida, MD, PhD,[1,2]* Souichi Adachi, MD, PhD,[3] Daiichiro Hasegawa, MD, PhD,[4] Yasuhiro Okamoto, MD, PhD,[5] Hiroaki Goto, MD, PhD,[6] Jiro Inagaki, MD, PhD,[7] Masami Inoue, MD,[8] Katsuyoshi Koh, MD,[9] Hiromasa Yabe, MD, PhD,[10] Keisei Kawa, MD, PhD,[11] Koji Kato, MD, PhD,[12] Yoshiko Atsuta, MD, PhD,[13,14] and Kazuko Kudo, MD, PhD[15]

Background. The relative efficacy of allogeneic hematopoietic cell transplantation (allo-HCT) after reduced toxicity conditioning (RTC) compared with standard myeloablative conditioning (MAC) in pediatric patients with acute myeloid leukemia (AML) has not been studied extensively. To address whether RTC is a feasible approach for pediatric patients with AML in remission, we performed a retrospective investigation of the outcomes of the first transplant in patients who had received an allo-HCT after RTC or standard MAC, using nationwide registration data collected between 2000 and 2011 in Japan. ***Procedure.*** We compared a fludarabine (Flu) and melphalan (Mel)-based regimen (RTC; n = 34) with total body irradiation (TBI) and/or busulfan (Bu)-based conditioning (MAC; n = 102) in demographic- and disease-criteria-matched childhood and adolescent patients with AML in first or second complete remission (CR1/CR2). ***Results.*** The incidence of engraftment, early complications, grade II–IV acute graft-versus-host disease (GVHD), and chronic GVHD were similar in each conditioning group. The risk of relapse (25% *vs.* 26%) and non-relapse mortality (13% *vs.* 11%) after 3 years did not differ between these groups, and univariate and multivariate analyses demonstrated that the 3-year overall survival (OS) rates after Flu/Mel-RTC and MAC were comparable (mean, 72% [range, 51–85%] and 68% [range, 58–77%], respectively). ***Conclusions.*** The results suggest that the Flu/Mel-RTC regimen is a clinically acceptable conditioning strategy for childhood and adolescent patients with AML in remission. Although this retrospective, registry-based analysis has several limitations, RTC deserves to be further investigated in prospective trials. Pediatr Blood Cancer

Key words: acute myeloid leukemia; childhood; hematopoietic stem cell transplantation; melphalan; reduced toxicity conditioning; reduced intensity conditioning

INTRODUCTION

Intensive combination chemotherapy is associated with a 52–75% probability of survival for childhood and adolescent patients with acute myeloid leukemia (AML); however, more than 30% of patients eventually relapse [1–4]. While allogeneic hematopoietic stem cell transplantation (allo-HCT) is reportedly the most promising therapy for intractable disease, the intensity of the conditioning regimen as well as the control of graft-versus-leukemia effects are paramount in reducing the incidence of relapse after allo-HCT [5,6]. Although conventionally, total body irradiation (TBI) and cyclophosphamide (Cy) (TBI/Cy) have been the primary conditioning regimen, the optimal regimen for childhood and adolescent AML has not yet been identified. In addition to the

Abbreviations: Allo-HCT, allogeneic hematopoietic cell transplantation; AML, acute myeloid leukemia; Bu, busulfan; CIR, cumulative incidence of relapse; CR, complete remission; Cy, cyclophosphamide; FLTT3, fms-like tyrosine kinase receptor 3; Flu, fludarabine; GVHD, graft-versus-host disease; HiR, high-risk cytogenetics/gene mutation; JSHCT, Japanese Society for Hematopoietic Cell Transplantation; ITD, internal tandem duplication; LFS, leukemia-free survival; LoR, low-risk cytogenetics; MAC, myeloablative conditioning; Mel, melphalan; MDS, myelodysplastic syndrome; NRM, non-relapse mortality; OS, overall survival; PS, performance status; RTC, reduced toxicity conditioning; TBI, total body irradiation

[1]Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto, Japan; [2]Division of Pediatrics, Matsushita Memorial Hospital, Moriguchi, Japan; [3]Human Health Sciences, Graduate School of Medicine, Kyoto University, Kyoto, Japan; [4]Department of Hematology and Oncology, Kobe Children's Hospital, Kobe, Japan; [5]Department of Pediatrics, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan; [6]Department of Hematology, Kanagawa Children's Medical Center, Yokohama, Japan; [7]Department of Pediatrics, National Kyushu Cancer Center, Fukuoka, Japan; [8]Department of Hematology and Oncology, Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Japan; [9]Department of Hematology/Oncology, Saitama Children's Medical Center, Saitama, Japan; [10]Department of Cell Transplantation, Tokai University Hospital, Isehara, Japan; [11]Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Japan; [12]Department of Hematology and Oncology, Children's Medical Center, Japanese Red Cross Nagoya First Hospital, Nagoya, Japan; [13]Department of HSCT Data Management/Biostatistics, Nagoya University Graduate School of Medicine, Nagoya, Japan; [14]Japanese Data Center for Hematopoietic Cell Transplantation, Nagoya, Japan; [15]Division of Hematology and Oncology, Shizuoka Children's Hospital, Shizuoka, Japan

Conflict of interest: Nothing to declare.

H.I., K. Kudo, and S.A. designed the research, analyzed the data, and wrote the manuscript. D.H., Y.O., H.K., J.I., M.I., K. Koh, M.Y., K. Kawa, K. Kato, and Y.A. collected and managed the clinical data and discussed the results. Y.A. supervised the data analyses.

*Correspondence to: Hiroyuki Ishida, Division of Pediatrics, Matsushita Memorial Hospital, 5-55 Sotojimacho, Moriguchi, Osaka 570-8540, Japan. E-mail: ishidah@koto.kpu-m.ac.jp

Received 4 July 2014; Accepted 11 November 2014

DOI 10.1002/pbc.25389
Published online in Wiley Online Library (wileyonlinelibrary.com).

common late effects after high-dose TBI–growth retardation, neurocognitive defects, gonadal dysfunction, hypothyroidism, cataracts, and infertility–there is an elevated risk of secondary malignancy [7,8]. Some alternative strategies used to reduce the incidence of these late complications of TBI/Cy include busulfan (Bu)-based myeloablative conditioning (MAC) and reduced intensity conditioning using fludarabine (Flu) combined with an alkylator, with or without low-dose TBI. A retrospective non-randomized study showed that reduced intensity conditioning was associated with lower non-relapse mortality (NRM); however, there was a higher incidence of relapse in recipients with adult AML and myelodysplastic syndrome (MDS), leading to survival rates comparable to those receiving MAC [5,6,9]. Although reduced intensity conditioning relies on the graft-versus-leukemia effect of alloantigen-reactive cells, the conditioning regimen itself also plays a significant role in disease control. Gradually, reduced intensity conditioning has been replaced with reduced toxicity conditioning (RTC) regimens, and currently, RTC is the main conditioning regimen used worldwide. Thus, the incorporation into the RTC regimen of drugs with high anti-tumor activity and acceptable toxicity merits further investigation [10]. RTC is defined as a regimen associated with various degrees of myeloablation, but with decreased toxicity associated with the conditioning compared with that of traditional MAC [11]. Consequently, RTC, without the full dose of TBI and Bu but with the potential for myeloablative intensity comparable to MAC, is preferable.

Although several reports indicate the utility of RTC, little is known about the efficacy and adverse events associated with its use for childhood and adolescent patients with leukemia. The Pediatric Blood and Marrow Transplant Consortium Study ONC0313 showed that favorable outcomes could be achieved with an RTC approach in pediatric patients in remission who are otherwise ineligible for myeloablative transplantation [12]. Results from the Center for International Blood and Marrow Transplant Research indicated that the 3-year disease-free survival rates after RTC and allo-HCT were comparable to those after dose-intensive MAC regimens and allo-HCT, when children with acute lymphoblastic leukemia underwent first transplantation [13]. Another recent study reported to the Center for International Blood and Marrow Transplant Research that the 5-year survival rate in patients younger than 18 years with AML who received RTC regimens was similar to those who received MAC regimens. Intriguingly, this retrospective case-controlled study showed no increase in relapse rates and no change in rates of NRM in recipients of RTC, suggesting that RTC strategies are acceptable for children [14]. However, the patients in this analysis had received different first-line chemotherapies, different combinations of anti-cancer drugs as RTC, and represented various ethnic groups. The present study aimed to investigate the efficacy and toxicity of Flu/melphalan (Mel)-based RTC in remission in Japanese childhood and adolescent patients with AML who had been treated with a well-defined first-line chemotherapy.

PATIENTS AND METHODS

Patients and Transplantation

According to results of the nationwide AML99 trial in Japan, overall survival (OS) of children with HLA-matched and related donors in the intermediate-risk group defined by cytogenetics and response to the induction therapies, and then selected to receive the transplantion in CR1 is similar to that of non-transplanted children in CR1, indicating that in this group at least, transplantation from a matched and related donor should be reserved for those in CR2 [3]. In the Japanese population, transplantation is now indicated in pediatric patients with AML in the CR1 group only in cases of induction delay/failure or with high-risk cytogenetics/gene mutation (HiR), according to the AML-05 study [15]. Patients in the CR2 group receive a transplant whether or not they have additional risks. Patients registered to the Japanese Society for Hematopoietic Cell Transplantation (JSHCT) under 20 years of age with de novo AML (excluding AML-M3) who underwent a first allo-HCT in CR1 or CR2 between January 2000 and December 2011 were selected for present study (n = 693); 91 patients with Down syndrome, Fanconi anemia or neurofibromatosis type 1, or who received a graft with ex vivo T cell depletion, CD34-positive selection, or from an HLA-haploidentical donor were excluded. Patients with low-risk cytogenetics (LoR) who underwent transplantation in CR1 were excluded (n = 42) because the reason for transplantation in CR1 was unknown. In addition, patients who recieved miscellaneous combinations (excluding RTC regimens consisting of Flu and Mel) which did not meet the criteia for MAC were also excluded (n = 78) [16]. The remaining 482 patients had received the following conditioning regimens: TBI (>8 Gy fractionated) combined with either standard-dose Cy or Mel in the absence or presence of a cytotoxic drug (TBI-MAC, n = 336); Bu (>8 mg/kg, oral or intravenous equivalent) [16] combined with either standard-dose Cy or Mel in the absence or presence of a cytotoxic drug (Bu-MAC, n = 112); or Flu combined with Mel in the absence or presence of cytarabine, etoposide, and/or low-dose TBI (≤4 Gy) (Flu/Mel-RTC, n = 34). LoR was defined as either t(8;21) or inv(16), HiR was defined as either t(6;11), t(9:22), t(16;21), 7-/7q-, 5q-, or fms-like tyrosine kinase receptor 3 (FLT3)-internal tandem duplication (ITD). Intermediate-risk cytogenetics/gene mutation (IntR) was defined as neither LoR nor HiR. Graft-versus-host disease (GVHD) was graded based on the previously published and accepted criteria [17]. NRM was defined as death occurring during continuous remission, and leukemia-free survival (LFS) was defined as survival without any relapse of the underlying hematological malignant disease or death from any cause.

Pair-match Analysis

Patients who received Flu/Mel-RTC were matched to those who received TBI-MAC (at a ratio of 1:2) or Bu-MAC (1:1) using an optimal matching strategy (EZR software [Version 1.10]) with

TABLE I. Conditioning Regimens

	n
Reduced toxicity conditioning (RTC)	
Fludarabine + melphalan ± low-dose TBI	23
Fludarabine + melphalan + cytarabine ± low-dose TBI	7
Fludarabine + melphalan + etoposide ± low-dose TBI	4
Myeloablative conditioning (MAC)	
TBI + cyclophosphamide ± another cytotoxic drug	49
TBI + melphalan ± another cytotoxic drug	19
Busulfan + cyclophosphamide ± another cytotoxic drug	11
Busulfan + melphalan ± another cytotoxic drug	23

TBI, total body irradiation.

TABLE II. Characteristics of Patients in the RTC and MAC Groups

	RTC		MAC		*P* value	Total
	34	(%)	102	(%)		136
Year of transplant					0.32	
2000–2005	11	(32)	44	(43)		55
2006–2011	23	(68)	58	(57)		81
Gender					0.69	
Female	22	(65)	61	(60)		83
Male	12	(35)	41	(40)		53
Age at transplant					1.00	
<10 years	23	(68)	67	(66)		90
≥10 years	11	(32)	35	(34)		46
FAB classification						
M0	3	(9)	4	(4)		7
M1	5	(15)	10	(10)		15
M2	5	(15)	27	(26)		32
M4	8	(24)	13	(13)		21
M5	7	(21)	21	(21)		28
M6	3	(9)	7	(7)		10
M7	3	(9)	10	(10)		13
Others[a]/missing data	0		10	(10)		10
					0.17	
M0–M4	21	(62)	54	(53)		75
M5–M7	13	(38)	38	(37)		51
Others/missing data	0		10	(10)		10
Status at transplant					1.00	
CR1	22	(65)	67	(65)		89
CR2	12	(35)	35	(35)		47
Cytogenetics/gene mutation					0.78	
LoR	7	(21)	14	(14)		21
IntR	24	(71)	78	(75)		102
HiR	2	(6)	7	(7)		9
UE/missing data	1	(3)	3	(3)		4
Graft type					1.00	
Related BM	8	(24)	24	(24)		32
Related PBSC	2	(6)	6	(6)		8
Unrelated BM	13	(38)	39	(38)		52
Unrelated CB	11	(32)	33	(32)		44
GVHD prophylaxis					0.21	
Cyclosporine A-based	10	(29)	37	(36)		47
Tacrolimus-based	24	(71)	58	(57)		82
Others	0		7	(7)		7
Performance status at transplant					1.00	
0	16	(47)	47	(46)		63
1–4	12	(35)	37	(36)		49
Missing data	6	(18)	18	(18)		24
HCTCI score					0.29	
0	14	(41)	46	(45)		60
≥1	5	(15)	6	(6)		11
Missing data	15	(44)	50	(55)		65
Median observation period						
Months	35		35			

RTC, reduced-toxicity conditioning; MAC, myeloablative conditioning; FAB, French-American-British classification; LoR, IntR, HiR: low-, intermediate- or high-risk cytogenetics/gene mutation; UE, unevaluable; BM, bone marrow; PBSC, peripheral blood stem cell; CB, cord blood; GVHD, graft-versus-host disease; HCTCI, hematopoietic cell transplantation comorbidity index. [a]AML with multi-lineage dysplasia or acute undifferentiated leukemia.