GENES, CHROMOSOMES & CANCER 52:683–693 (2013)

NUP98-NSD1 Gene Fusion and Its Related Gene Expression Signature Are Strongly Associated with a Poor Prognosis in Pediatric Acute Myeloid Leukemia

Norio Shiba,[1,2] Hitoshi Ichikawa,[3] Tomohiko Taki,[4] Myoung-Ja Park,[1] Aoi Jo,[3] Sachiyo Mitani,[3] Tohru Kobayashi,[2] Akira Shimada,[5] Manabu Sotomatsu,[1] Hirokazu Arakawa,[2] Souichi Adachi,[6] Akio Tawa,[7] Keizo Horibe,[8] Masahiro Tsuchida,[9] Ryoji Hanada,[10] Ichiro Tsukimoto,[11] and Yasuhide Hayashi[1]*

[1]Department of Hematology/Oncology, Gunma Children's Medical Center, Shibukawa, Japan
[2]Department of Pediatrics, Gunma University Graduate School of Medicine, Maebashi, Japan
[3]Division of Genetics, National Cancer Center Research Institute, Tokyo, Japan
[4]Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan
[5]Department of Pediatrics, Okayama University Graduate School of Medicine, Okayama, Japan
[6]Department of Human Health Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan
[7]Department of Pediatrics, National Hospital Organization Osaka National Hospital, Osaka, Japan
[8]Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya, Japan
[9]Department of Pediatrics, Ibaraki Children's Hospital, Ibaraki, Japan
[10]Division of Hematology/Oncology, Saitama Children's Medical Center, Saitama, Japan
[11]Department of First Pediatrics, Toho University School of Medicine, Tokyo, Japan

The cryptic t(5;11)(q35;p15.5) creates a fusion gene between the *NUP98* and *NSD1* genes. To ascertain the significance of this gene fusion, we explored its frequency, clinical impact, and gene expression pattern using DNA microarray in pediatric acute myeloid leukemia (AML) patients. *NUP98-NSD1* fusion transcripts were detected in 6 (4.8%) of 124 pediatric AML patients. Supervised hierarchical clustering analyses using probe sets that were differentially expressed in these patients detected a characteristic gene expression pattern, including 18 *NUP98-NSD1*-negative patients (*NUP98-NSD1*-like patients). In total, a *NUP98-NSD1*-related gene expression signature (*NUP98-NSD1* signature) was found in 19% (24/124) and in 58% (15/26) of cytogenetically normal cases. Their 4-year overall survival (OS) and event-free survival (EFS) were poor (33.3% in *NUP98-NSD1*-positive and 38.9% in *NUP98-NSD1*-like patients) compared with 100 *NUP98-NSD1* signature-negative patients (4-year OS: 86.0%, 4-year EFS: 72.0%). Interestingly, t(7;11)(p15;p15)/*NUP98-HOXA13*, t(6;11)(q27;q23)/MLL-*MLLT4* and t(6;9)(p22;q34)/*DEK-NUP214*, which are known as poor prognostic markers, were found in *NUP98-NSD1*-like patients. Furthermore, another type of *NUP98-NSD1* fusion transcript was identified by additional RT-PCR analyses using other primers in a *NUP98-NSD1*-like patient, revealing the significance of this signature to detect *NUP98-NSD1* gene fusions and to identify a new poor prognostic subgroup in AML. © 2013 Wiley Periodicals, Inc.

INTRODUCTION

Acute myeloid leukemia (AML) is a complex disease caused by mutations, epigenetic modifications, and deregulated expression of genes, leading to increased proliferation and decreased differentiation of hematopoietic progenitor cells (Frohling et al., 2005; Marcucci et al., 2011; Pui et al., 2011). Several important molecular markers have been discovered in AML that have not only helped to characterize better patients, but also to improve risk stratification (Marcucci et al., 2011; Pui et al., 2011). However, in a subset of AML patients, no prognosis-associated cytogenetic aberrations or mutations are known (Frohling et al., 2005; Marcucci et al., 2011; Pui et al., 2011). In

Additional Supporting Information may be found in the online version of this article.

Supported by: a grant for Cancer Research, a grant for Research on Children and Families, and Research on Intractable Diseases, Health and Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare of Japan, Grants-in-Aid for Scientific Research (B, C) and Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio) of Japan, and a Research grant for Gunma Prefectural Hospitals.

*Correspondence to: Yasuhide Hayashi, Department of Hematology/Oncology, Gunma Children's Medical Center, 779, Shimohakoda, Hokkitsu, Shibukawa, 377-8577 Gunma, Japan. E-mail: hayashiy-tky@umin.ac.jp

Received 21 December 2012; Accepted 15 March 2013

DOI 10.1002/gcc.22064

Published online 30 April 2013 in Wiley Online Library (wileyonlinelibrary.com).

hematological malignancies, 11p15 translocations involving the nucleoporin 98-kDa (NUP98) protein gene are relatively rare. This notwithstanding, more than 20 different chromosomal rearrangements have been identified (Romana et al., 2006). These translocations fuse *NUP98* with respective partner genes, including many homeobox genes and nuclear non-homeobox genes (Arai et al., 1997; Taketani et al., 2002a,b,c). Although the *NUP98* fusion genes are rare, they have provided valuable information regarding the role of homeobox proteins in leukemogenesis (Nakamura et al., 1996; Nakamura, 2005).

The cryptic t(5;11)(q35;p15.5), which is frequently accompanied by deletion of the long arm of chromosome 5, del(5q), creates a fusion gene between *NUP98* and the nuclear receptor-binding SET-domain protein 1 (*NSD1*) gene (Jaju et al., 2001). This fusion gene has mainly been identified in pediatric AML, by the use of fluorescence in situ hybridization (FISH) with subtelomeric probes (Jaju et al., 2001; Brown et al., 2002; Panarello et al., 2002; Cerveira et al., 2003), it is rare in adult AML (Brown et al., 2002; Casas et al., 2003; Nebral et al., 2005; Walter et al., 2009). Recently, the *NUP98-NSD1* gene fusion, identified by high-resolution genome-wide copy number analysis and reverse transcription (RT)-PCR in pediatric and adult AML patients, was associated with poor prognosis (Hollink et al., 2011). To increase our understanding of this gene fusion, we explored the frequency, clinical significance, and gene expression pattern of *NUP98-NSD1* using DNA microarray in pediatric AML patients.

MATERIALS AND METHODS

Patients and Samples

From January 2000 to December 2002, 318 patients were diagnosed with de novo AML. The diagnosis of AML was based on the French-American-British (FAB) classification, and cytogenetic analysis was performed using conventional G-banding. Of these patients, samples from 124 patients with known mutation status and gene expression profiling data were available, including 10 patients with FAB-M3 and six patients with Down syndrome who were treated on different treatment protocols (Kudo et al., 2007; Tsukimoto et al., 2009; Imaizumi et al., 2011). Age and initial white blood cell (WBC) count were higher, patients with t(8;21) were more frequent, and M7 patients with Down syndrome were fewer in the present study cohort than in the non-analyzed patients (Supporting Information Table S1). There were no significant differences in survival between these two groups [4-year overall survival (OS): 76.6% versus 79.9% and 4-year event-free survival (EFS): 65.3% versus 71.1%]. Informed consent was obtained from the patients or the patients' parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review boards of Gunma Children's Medical Center and National Cancer Center approved this project.

Reverse Transcription-PCR and Sequence Analysis

Total RNA extracted from leukemic cells at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (GE Healthcare, Tokyo, Japan). PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ), using a DNA thermal cycler (Applied Biosystems). For the detection of *NUP98-NSD1* and the reciprocal fusion transcript, *NUP98*−5F and *NSD1*−1R, and *NSD1*−2F and *NUP98*−6R were used, respectively (Supporting Information Table S2) (Jaju et al., 2001; Brown et al., 2002). PCR conditions were as follows: initial denaturation at 94°C (9 min), 40 cycles of 96°C (45 sec), 58°C (45 sec), and 72°C (1 min), followed by final elongation at 72°C (7 min). For sequencing, PCR products were amplified using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under the following conditions: 95°C (2 min) followed by 25 cycles of 95°C (10 sec), 50°C (5 sec), and 60°C (4 min). Direct sequencing was performed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). To confirm and identify the gene fusions precisely, we used various primer sets for RT-PCR followed by direct sequencing (Supporting Information Table S2). Mutations of *NPM1* were also examined as previously reported (Döhner et al., 2005). Mutation analyses of the *DNMT3A*, *FLT3*, *MLL*, *KIT*, *NRAS*, *KRAS*, and *WT1* genes have been reported previously (Shimada et al., 2006, 2008; Sano et al., 2012; Shiba et al., 2012).

Microarray Analysis

Gene expression profiling data for the 124 patients (Gene Expression Omnibus accession number, GSE35784) were obtained and analyzed as follows. Total RNA was re-purified using the RNeasy MinElute cleanup kit (Qiagen, Hilden,

Germany), and the integrity of the purified RNA was confirmed using a 2100 Bioanalyzer and an RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA). The DNA microarray used was a Human Genome U133 plus 2.0 array (Affymetrix, Santa Clara, CA). Target cRNA was prepared from 20 ng purified RNA with a two-cycle cDNA synthesis kit and 3′-amplification reagents for IVT labeling (Affymetrix). Hybridization to the microarrays, washing, and staining with the antibody amplification procedure and scanning were performed according to the manufacturer's instructions. Using the GeneChip Operating Software version 1.4 (Affymetrix), the scanned image data were processed and the expression value (signal) of each probe set was calculated. The signal values were normalized so that the mean in each experiment was set at 100 to adjust for minor differences between the experiments. Statistical analyses and fold change calculations were performed using expression values that were log-transformed after addition of 10 to reduce adverse effects caused by noise at low expression levels (Ichikawa et al., 2006; Jo et al., 2009). To identify differentially expressed probe sets between *NUP98-NSD1*-positive and -negative patients, *P*-values in Student's *t*-test and fold change values were used. Hierarchical clustering analysis was performed using the Cluster and Tree View software (Eisen et al., 1998). For this analysis, log-transformed expression values were normalized for each probe set by subtracting the mean, and uncentered correlation metric and complete linkage clustering methods were used. Unsupervised clustering analyses was performed and visualized as previously described (Ichikawa et al., 2006).

Statistical Analysis

All analyses were carried out using the SPSS statistical package program (version 18.0J; SPSS Tokyo, Japan). Survival distributions were assessed using the Kaplan-Meier method and the differences were compared using the log-rank test. EFS and OS were defined as the times from diagnosis to event (relapse or death of any cause) and from diagnosis to death from any cause, respectively. Statistical analyses were performed using Fisher's exact test for categorical variables and Mann-Whitney's U test for continuous variables. For all analyses, the *P*-values were two-tailed, and $P < 0.05$ was considered significant. To identify independent predictors of poor prognosis, multivariate Cox regression analysis was performed using factors that were significant in frequencies between *NUP98-NSD1* signature positive and negative cases.

RESULTS

Detection of *NUP98-NSD1*-Positive Patients

We identified the *NUP98-NSD1* fusion transcript in 6 (4.8%) out of 124 Japanese pediatric AML patients. The reciprocal fusion transcript *NSD1-NUP98* was detected in five of the patients (Supporting Information Fig. S1A). Sequence analysis of the PCR products confirmed that *NUP98* and *NSD1* were fused in-frame, joining *NUP98* exon 12 with *NSD1* exon 6 (Supporting Information Fig. S1B) (Jaju et al., 2001; Brown et al., 2002; Panarello et al., 2002; Casas et al., 2003; Cerveira et al., 2003; Hollink et al., 2011). The reciprocal fusion products were also fused in-frame, joining *NSD1* exon 5 with *NUP98* exon 13 (Supporting Information Fig. S1C).

Detection of *NUP98-NSD1*-Like Patients by Gene Expression Analysis

To investigate the gene expression pattern characteristic of *NUP98-NSD1*-positive patients, we used gene expression profiling data on the 124 patients. In unsupervised hierarchical clustering analyses (Supporting Information Fig. S2), *NUP98-NSD1*-positive patients did not form a cluster, whereas patients with t(8;21)(q22;q22), t(15;17)(q22;q21), and inv(16)(p13;q22) formed unique clusters. Comparing the 6 *NUP98-NSD1*-positive patients with the other 118 patients resulted in the identification of 87 differentially expressed probe sets ($P < 0.001$, fold change ≥ 2) (Supporting Information Table S3). Fifty-one probe sets, including several *HOX* genes, were overexpressed, and 36 probe sets were underexpressed. Interestingly, in supervised hierarchical clustering analysis using those 87 probe sets, a relatively large cluster including 18 *NUP98-NSD1*-negative patients was found, not only a cluster of the 6 *NUP98-NSD1*-positive patients (Fig. 1). These 24 patients also formed a single cluster in similar supervised clustering analyses in which probe sets were selected by slightly different criteria (Supporting Information Fig. S3). The robustness in clustering suggests that these 24 patients have certain common features and that they constitute a distinct subtype. Thus, we termed this characteristic gene expression pattern the *NUP98-NSD1* signature. We also designated the 18 patients lacking the *NUP98-NSD1* gene fusion but displaying the *NUP98-NSD1* signature as *NUP98-NSD1*-like AML patients. Of the 24 patients displaying the *NUP98-NSD1* signature, a common overexpression of 3 *HOXB* genes (*HOXB3*,

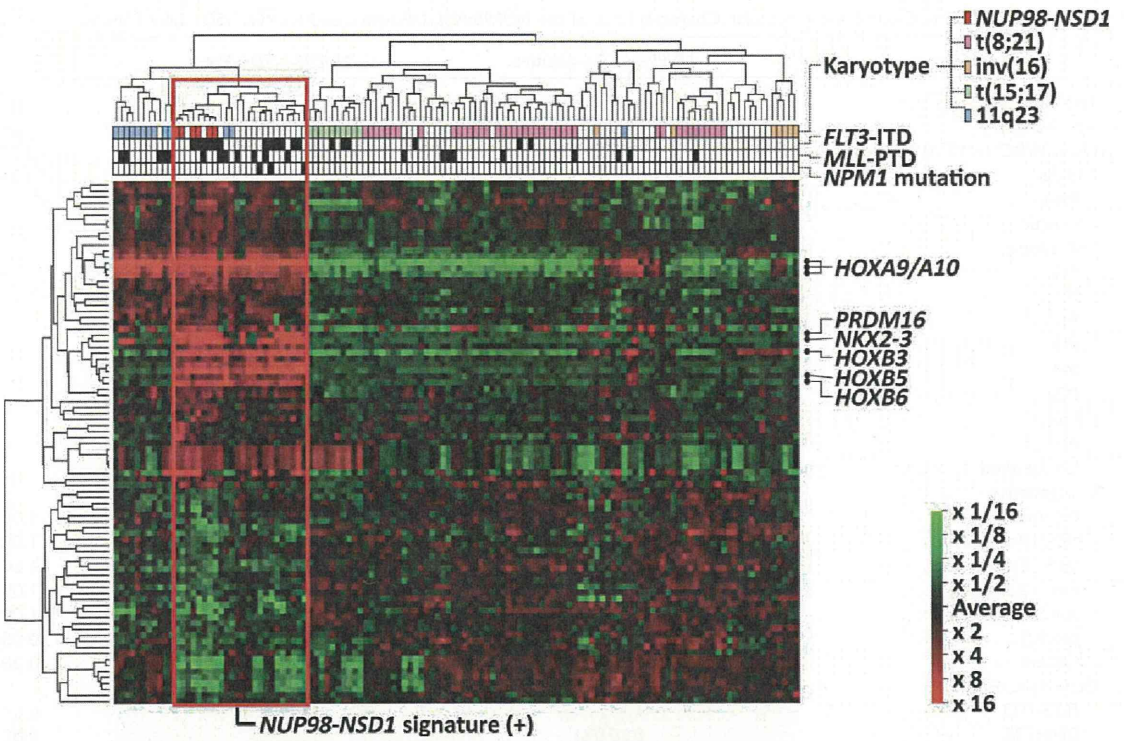

Figure 1. Supervised hierarchical clustering analysis of the 124 patients using differentially expressed genes between *NUP98-NSD1*-positive and -negative patients. Two-dimensional hierarchical clustering analysis of the 124 patients was performed using 87 probe sets that were differentially expressed between the *NUP98-NSD1*-positive and -negative patients (Table S3). Each column represents a patient and each row represents a probe set. The karyotype and the *FLT3*-ITD, *MLL*-PTD, and *NPM1* mutation status of each patient are indicated. Relative expression levels normalized to the average for each probe set are indicated by color, where red and green represent high and low expressions, respectively.

HOXB5, and *HOXB6*), another homeobox gene *NKX2–3*, a zinc finger transcription factor gene *PRDM16*, and a noncoding RNA gene *LOC404266* was particularly marked (Fig. 1). Although *HOXA9* and *HOXA10* were also overexpressed in these patients, their overexpression was shared by many patients with 11q23 abnormalities (Fig. 1).

Clinical and Molecular Characteristics of *NUP98-NSD1*-Positive and *NUP98-NSD1*-Like Patients

We compared the clinical and molecular features of the *NUP98-NSD1*-positive and *NUP98-NSD1*-like patients; there were no significant differences in terms of age, initial WBC count, gender, FAB subtypes, and cytogenetics (Table 1). Frequent gene mutations were also detected in both the *NUP98-NSD1*-positive and -like patients. All six *NUP98-NSD1*-positive patients and 16 *NUP98-NSD1*-like patients had one or several of the mutations analyzed (Table 2). *NUP98-NSD1*-positive patients frequently had class I aberrations, such as *FLT3*-ITD (4/6), and *NRAS* (1/6), *KRAS* (1/6), and *KIT* (1/6) mutations; however, no class II aberrations (*NPM1* or *MLL*-PTD) were detected in *NUP98-NSD1*-positive patients. On the other hand, *NUP98-NSD1*-like patients frequently had class II aberrations [*MLL*-PTD (7/18) and *NPM1* (3/18)] in addition to class I aberrations [*FLT3*-ITD (8/18), *KRAS* (3/18), and *KIT* (2/18)] (Table 2). Furthermore, we identified *DEK-NUP214*, *NUP98-HOXA13* and *MLL-MLLT4* fusion genes, which were generated by t(6;9)(p22;q34), t(1;7;11)(q32;p15;p15), and t(6;11)(q27;q23), respectively, in *NUP98-NSD1*-like patients (Table 2). In addition, we identified another type of *NUP98-NSD1* fusion transcript using another forward primer of *NUP98*-F', located on the 5′ side further from the known *NUP98* junction point (Supporting Information Table S2), in a *NUP98-NSD1*-like patient (Supporting Information Fig. S4).

Clinical and Molecular Characteristics of *NUP98-NSD1* Signature-Positive Patients

When compared with 100 *NUP98-NSD1* signature-negative patients, age (median 8.8 years vs.

TABLE 1. Clinical and Molecular Characteristics of the *NUP98-NSD1*-Positive and *NUP98-NSD1*-Like Patients

	NUP98-NSD1-positive	*NUP98-NSD1*-like	P-value
Total (n = 24)	6	18	
Age (range), y	7.2 (2–15)	9.3 (0–15)	0.28
Mean WBC ($\times 10^9$/l) (range)	115.9 (9.0–329.0)	60.5 (2.3–255.0)	0.31
Gender			0.34
Male	5 (83.3%)	9 (50.0%)	
Female	1 (16.7%)	9 (50.0%)	
FAB subtype			
M0	0 (0.0%)	1 (5.6%)	
M1	1 (16.7%)	4 (22.2%)	
M2	0 (0.0%)	2 (11.1%)	
M3	0 (0.0%)	0 (0.0%)	
M4	2 (33.3%)	6 (33.3%)	
M5	2 (33.3%)	4 (22.2%)	
M6	0 (0.0%)	0 (0.0%)	
M7	0 (0.0%)	1 (5.6%)	
Unclassified	1 (16.7%)	0 (0.0%)	
Cytogenetics			
Normal	4 (66.7%)	11 (61.1%)	1.00
t(8;21)(q22;q22)	0 (0.0%)	0 (0.0%)	1.00
t(15;17)(q22;q12)	0 (0.0%)	0 (0.0%)	1.00
inv(16)(p13q22)	0 (0.0%)	0 (0.0%)	1.00
abnormal 11q23	0 (0.0%)	2 (11.1%)	1.00
del(9q)	2 (33.3%)	0 (0.0%)	0.054
Others	0 (0.0%)	5 (27.8%)	0.28
Gene mutations			
FLT3-ITD	4 (66.7%)	8 (44.4%)	0.64
DNMT3A	0 (0.0%)	0 (0.0%)	1.00
NPM1	0 (0.0%)	3 (16.7%)	0.55
KIT	1 (16.7%)	2 (11.1%)	1.00
NRAS	1 (16.7%)	0 (0.0%)	0.25
KRAS	1 (16.7%)	3 (16.7%)	1.00
WT1	2 (33.3%)	3 (16.7%)	0.57
MLL-PTD	0 (0.0%)	7 (38.9%)	0.13

Abbreviations: y, years; WBC, white blood cell count; FAB,French-American-British subtype.

6.7 years; $P = 0.069$) and initial WBC count (median 74.4 $\times$ 10^9/ L vs. 49.7 $\times$ 10^9/ L; $P = 0.025$) were higher in the 24 *NUP98-NSD1* signature-positive patients. They frequently had a normal karyotype (62.5%) or del(9q) (8.3%), but did not have the favorable chromosomal translocations t(8;21), t(15;17), and inv(16) (Tables 2 and 3). The frequencies of the M4 and M5 subtypes (14/24; $P < 0.001$) were higher than in *NUP98-NSD1* signature-negative patients.

Prognosis of *NUP98-NSD1*-Positive and *NUP98-NSD1*-Like Patients

Both the 4-year OS and EFS were 33.3% in *NUP98-NSD1*-positive patients and 38.9% in *NUP98-NSD1*-like patients, which is significantly worse than for those with *NUP98-NSD1* signature-negative patients (86.0% in OS and 72.0% in EFS; Figs. 2A and 2B). Five of the six *NUP98-NSD1*-positive and 8 of the 18 *NUP98-NSD1*-like patients received allogeneic-stem cell transplantation (allo-SCT) (Table 2). Of the *NUP98-NSD1*-positive patients, only two of the three who underwent allo-SCT in first CR are still alive and, in the *NUP98-NSD1*-like patients, all 4 who received SCT in first CR and 3 of 10 who were treated with only chemotherapy are still alive without relapse. All 12 relapsed patients and two who did not achieve CR died (Table 2). Multivariate Cox regression analysis of OS was used to construct a model including the *NUP98-NSD1* signature, *FLT3*-ITD, *WT1*, *NPM1*, t(8;21), del(9q), and initial WBC, which were statistically significant in univariate analysis. In this model, the *NUP98-NSD1* signature and *FLT3*-ITD were independent poor prognostic factors (Table 4). Among the 24 patients displaying the *NUP98-NSD1* signature, the outcome of the 12 *FLT3*-ITD-positive patients was worse than that of the 12 *FLT3*-ITD-negative

TABLE 2. Individual Characteristics of the *NUP98-NSD1* Signature-Positive Patients

ID	Age (y)	Sex	FAB	Cytogenetic aberrations	*NUP98-NSD1*	Risk	CR	Relapse	SCT	Class I mutations	Class II mutations	Other mutations	Survival (m)
A106	5	F	M1	50,XX,+6,+8,del (9q?),+21,+22	+	Off study	+	–	CR 1	*FLT3*-ITD	–	–	63+
A188	10	M	U/C	Normal	+	High	+	+	CR 2	*FLT3*-ITD	–	*WT1*	21
A282	2	M	M5	46,XY,del(9)(q13q22)	+	Intermediate	+	–	CR 1	*FLT3*-ITD	–	–	43+
A325	5	M	M5	Normal	+	High	+	+	CR 2	*FLT3*-ITD	–	–	15
A333	15	M	M4	Normal	+	Intermediate	+	+	–	*NRAS*	–	–	31
A335	6	M	M4	Normal	+	Intermediate	+	–	CR 1	*KRAS*+*KIT*	–	*WT1*	7[a]
A044	6	M	M2	Normal	–	Intermediate	+	+	–	–	–	–	30
A059	12	F	M7	Normal	–	Intermediate	+	+	CR 2	*FLT3*-ITD	–	*WT1*	16
A089	9	F	M4	Normal	–	Off study	–	–	Non-CR	*FLT3*-ITD	*MLL*-PTD	–	25
A154	8	F	M0	Normal	–	High	+	+	CR 2	*FLT3*-ITD	–	–	20
A167	2	M	M5	Normal	–	High	+	–	CR 1	–	*MLL*-PTD	–	55+
A171	12	M	M1	46,XY,t(1;7;11) (q32;p15;p15)	–	Intermediate	+	–	CR 1	–	*MLL*-PTD	*WT1*	55+
A173	13	M	M1	Normal	–	Intermediate	+	–	CR 1	*FLT3*-ITD +*KIT*	*MLL*-PTD	*WT1*	54+
A199	10	F	M5	46,XX,add(10)(p11.2), del(11)(q13q23)	–	Intermediate	+	+	–	*KIT*+*KRAS*	–	–	41
A202	15	F	M4	Normal	–	Intermediate	+	–	–	–	*NPM1*	–	50+
A211	14	F	M1	47,XX,+8	+[b]	Intermediate	+	+	CR 2	*FLT3*-ITD	*MLL*-PTD	–	14
A234	13	M	M4	46,XY, t(6;11) (q27;q23)	–	Intermediate	+	+	–	*KRAS*	*MLL*-PTD	–	29
A243	5	F	M1	Normal	–	Intermediate	+	+	–	*FLT3*-ITD	–	–	8
A245	11	F	M4	Normal	–	Intermediate	+	–	–	*FLT3*-TK (D835)	*NPM1*	–	46+
A249	0	M	M4	46,XY,t(5;6) (q33;q22)	–	Low	+	–	–	–	–	–	46+
A259	5	M	M5	46,XY,t(6;9) (p23;q34)	–	Intermediate	+	–	CR 1	*FLT3*-TK (D835) + *KRAS*	–	–	48+
A297	6	M	M5	Normal	–	Off study	–	–	–	*FLT3*-ITD	*NPM1*	–	6
A299	13	F	M4	Normal	–	Intermediate	+	+	–	–	*MLL*-PTD	–	27
A355	13	M	M2	46,XY,t(15;17) (q13;q11)	–	Intermediate	+	+	–	*FLT3*-ITD	–	–	15

Abbreviations: y, years; F, female; M, male; FAB, French-American-British subtype; U/C, unclassified; CR, complete remission; SCT, stem cell transplantation; CR 1, first CR; CR 2, second CR; m, month; +, alive. The *NUP98-NSD1*-positive cases are indicated in bold type.

[a]A335 died of severe GVHD and acute pneumonia.

[b]Another type of *NUP98-NSD1* fusion transcript was identified by additional RT-PCR using another forward primer.

TABLE 3. Clinical and Molecular Characteristics of the *NUP98-NSD1* Signature Positive and Negative Cases

	NUP98-NSD1 signature (+)	*NUP98-NSD1* signature (−)	*P*-value
Total ($n = 124$)	24	100	
Age (range), y	8.8 (0–15)	6.7 (0–15)	0.069
Mean WBC (-10^9/l) (range)	74.4 (2.3–329.0)	49.7 (1.0–440.0)	0.025
Gender			1.00
Male	14 (58.3%)	57 (57.0%)	
Female	10 (41.7%)	43 (43.0%)	
FAB subtype[a]			
M0	1 (4.2%)	3 (3.0%)	
M1	5 (20.8%)	14 (14.0%)	
M2	2 (8.3%)	39 (39.0%)	
M3	0 (0.0%)	10 (10.0%)	
M4	8 (33.3%)	9 (9.0%)	
M5	6 (25.0%)	11 (11.0%)	
M6	0 (0.0%)	1 (1.0%)	
M7	1(4.2%)	12 (12.0%)	
Unclassified	1 (4.2%)	1 (1.0%)	
Cytogenetics			
Normal	15 (62.5%)	11 (11.0%)	<0.001
t(8;21)(q22;q22)	0 (0.0%)	41 (41.0%)	<0.001
t(15;17)(q22;q12)	0 (0.0%)	10 (10.0%)	0.21
inv(16)(p13q22)	0 (0.0%)	6 (6.0%)	0.60
abnormal 11q23	2 (8.3%)	9 (9.0%)	1.00
del(9q)	2 (8.3%)	0 (0.0%)	0.036
Others	5 (20.8%)	23 (23.0%)	1.00
Gene mutations			
FLT3-ITD	12 (50.0%)	6 (6.0%)	<0.001
DNMT3A	0 (0.0%)	0 (0.0%)	1.00
NPM1	3 (12.5%)	0 (0.0%)	0.007
KIT	3 (12.5%)	12 (12.0%)	1.00
NRAS	1 (4.2%)	9 (9.0%)	0.69
KRAS	4 (16.7%)	9 (9.0%)	0.28
WT1	5 (20.8%)	6 (6.0%)	0.037
MLL-PTD	7 (29.2%)	14 (14.0%)	0.12

[a]*NUP98-NSD1* signature-positive patients were significantly associated with the M4 and M5 subtypes (14/24; $P < 0.001$) when compared with *NUP98-NSD1* signature-negative patients.

patients (4-year OS: 25% vs. 50%), although the difference was not significant ($P = 0.400$).

We further analyzed the prognostic significance of *NUP98-NSD1*-positive patients and *NUP98-NSD1*-like patients other than those with t(15;17) and Down syndrome because they represent distinct AML entities. All 10 patients with t(15;17) and all six patients with Down syndrome were *NUP98-NSD1* signature negative. The outcome of the six patients with *NUP98-NSD1* gene fusion was significantly worse than that of the *NUP98-NSD1* signature-negative patients in OS ($P < 0.001$; 4-year OS: 33.3% vs. 85.7%; Fig. 2C) and in EFS ($P = 0.022$; 4-year EFS: 33.3% versus 70.2%; Fig. 2D). Furthermore, the outcome of the 18 *NUP98-NSD1*-like AML patients was significantly worse than that of the *NUP98-NSD1* signature-negative patients in OS ($P < 0.001$; 4-year OS: 38.9% versus 85.7%; Fig. 2C) and in EFS ($P = 0.002$; 4-year EFS: 38.9% versus 70.2%; Fig. 2D).

DISCUSSION

In this study, we found 24 patients with *NUP98-NSD1*-related gene expression signature, including six with the *NUP98-NSD1* gene fusion (*NUP98-NSD1*-positive) and 18 without (*NUP98-NSD1*-like). This signature represented 19% (24/124) of all pediatric AML patients and 58% (15/26) of all cytogenetically normal cases (Fig. 3). Our results also revealed that the *NUP98-NSD1* signature, irrespective of the presence of the *NUP98-NSD1* fusion, is a novel poor prognostic factor in AML.

The relationship between *NUP98-NSD1*-positive AML and *NUP98-NSD1*-like AML resembles that of *BCR-ABL*-positive acute lymphoblastic

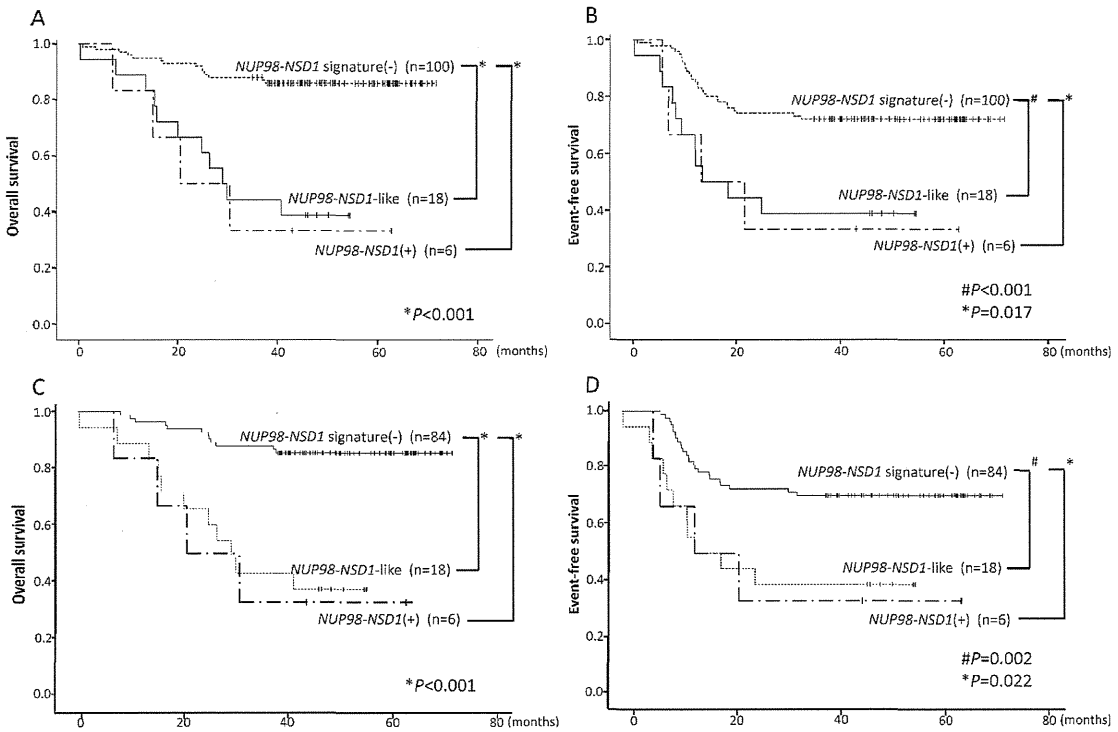

Figure 2. Survival based on *NUP98-NSD1* status by Kaplan-Meier method. Comparison of *NUP98-NSD1*-positive, *NUP98-NSD1*-like, and *NUP98-NSD1* signature-negative patients as regards OS (A) and EFS (B). Comparison of *NUP98-NSD1*-positive, *NUP98-NSD1*-like patients, and *NUP98-NSD1* signature-negative patients other than t(15;17) and Down syndrome, as regards OS (C) and EFS (D).

TABLE 4. Multivariate Analysis of OS of the Pediatric AML Patients

	P-value	SE	HR	Lower CI	Upper CI
NUP98-NSD1 signature	0.005	0.38	2.89	1.38	6.02
FLT3-ITD	0.005	0.40	3.06	1.41	6.63
WT1	0.20	0.54	0.50	0.18	1.43
NPM1	0.49	1.05	0.48	0.06	3.77
t(8;21)	0.18	0.46	0.54	0.22	1.32
del(9q)	0.97	371.69	<0.001	<0.001	–
Initial WBC	0.10	0.002	1.00	1.00	1.01

Abbreviations: SE, standard error; HR, hazard ratio; CI, confidence interval.

leukemia (ALL) and *BCR-ABL*-like ALL (Den Boer et al., 2009; Mullighan et al., 2009). In their gene expression analyses, a significant number of genetically unclassified B-cell precursor ALL patients clustered together with *BCR-ABL*-positive patients, and these *BCR-ABL*-like patients had a poor prognosis, similar to *BCR-ABL*-positive patients. Recently, it was reported that some *BCR-ABL*-like patients express tyrosine kinase/cytokine receptor gene-related fusion genes, such as *NUP214-ABL1*, *EBF1-PDGFRB*, *BCR-JAK2*, and *STRN3-JAK2* (Roberts et al., 2012). Thus, as potentially important fusions might be detected in *NUP98-NSD1*-like patients, we performed RT-PCR using various primer sets for detecting *NUP98-HOXA9*, *NUP98-HOXA11*, *NUP98-HOXA13*, *NUP98-TOP1*, *NUP98-PRRX1*, *NUP98-DDX10*, *NUP98-MLL*, *NUP98-NSD3*, *DEK-NUP214*, *MLL-MLLT4*, and other junction points of *NUP98-NSD1* (Supporting Information Table S2). As a result, t(6;9)/*DEK-NUP214*, t(1;7;11)/*NUP98-HOXA13*, and t(6;11)/*MLL-MLLT4*, which are well-known poor prognostic markers, were found in some *NUP98-NSD1*-like patients in our study (Table 2). Furthermore, another type of *NUP98-NSD1* fusion transcript was identified in a *NUP98-NSD1*-like patient (Supporting Information Fig. S4). This fusion transcript has previously been reported in an adult patient with refractory anemia with excess blasts (La Starza et al., 2004). It is likely that other *NUP98-NSD1*-like patients also have unknown fusion genes with the same functions as *NUP98-NSD1* gene fusion.

It has been reported that hematological malignancies with *NUP98*-fusion genes are strongly

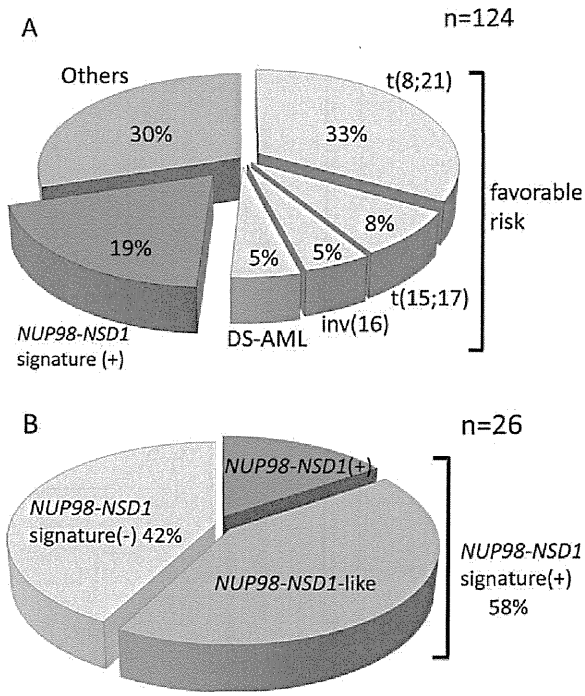

Figure 3. The *NUP98-NSD1* signature is frequent in pediatric AML. Frequencies of *NUP98-NSD1* signature-positive patients in the total pediatric AML cohort (A) and in cytogenetically normal AML (B).

associated with class I mutations (Chou et al., 2009; Taketani et al., 2010). Interestingly, *NUP98-NSD1*-positive patients had only class I aberrations, except for *WT1* mutations, although *NUP98-NSD1*-like patients frequently had Class II aberrations (*NPM1* mutations, *MLL*-PTD, and the *NUP98-HOXA13*, *MLL-MLLT4*, and *DEK-NUP214* fusion genes) in addition to class I aberrations. Because the *NUP98-NSD1* fusion is considered to act as a Class II aberration, no additional Class II aberrations might be necessary for leukemogenesis in *NUP98-NSD1*-positive patients.

The frequency of *FLT3*-ITD was higher than other mutations in both *NUP98-NSD1*-positive and *NUP98-NSD1*-like patients. Many studies have shown that *FLT3*-ITD has a negative impact on outcome in both adult and pediatric AML patients. In fact, in this study, *FLT3*-ITD was an independent poor prognostic factor in addition to the *NUP98-NSD1* signature (Table 4). This suggests that the *NUP98-NSD1* signature and *FLT3*-ITD play a key potential role in AML patients with poor prognosis.

Among the 100 *NUP98-NSD1* signature-negative patients, 23 patients relapsed and of those patients, nine died. On the other hand, of the 24 *NUP98-NSD1* signature-positive patients, 13 patients relapsed and all of those died. Except for 2 patients who did not achieve CR, 12 of the 22 *NUP98-NSD1* signature-positive patients received allo-SCT. Six of the seven patients (86%) who received allo-SCT in first CR were still alive without relapse; however, all five patients who received allo-SCT in second CR died. Only three patients were alive among the 10 patients who were treated with chemotherapy alone. Thus, allo-SCT is recommended in first CR of *NUP98-NSD1* signature-positive patients.

In the *NUP98-NSD1* signature-positive patients, the *HOXA9*, *HOXA10*, *HOXB3*, *HOXB5*, and *HOXB6* genes were up-regulated (Fig. 1). *NUP98* is frequently fused to homeobox genes, and some *NUP98*-homeobox and *NUP98*-non-homeobox fusion genes were revealed to activate *HOXA* cluster genes in hematopoietic cells (Gough et al., 2011). Overexpression of some *HOX* genes is known to enhance the self-renewal of hematopoietic stem and progenitor cells and to perturb differentiation (Grier et al., 2005). It is expected that the aberrant expression of *HOX* genes plays an important role in the leukemogenesis of AML displaying the *NUP98-NSD1* signature. In addition,

two transcription factor genes, *PRDM16* and *NKX2–3*, were markedly up-regulated (Fig. 1). *PRDM16* (also known as *MEL1*) was originally isolated as a translocated gene in t(1;3)(p36;q21) AML (Mochizuki et al., 2000) and encodes a zinc finger protein with a PR domain, which is critical for the establishment and maintenance of the hematopoietic stem cell pool (Agulio et al., 2011). *NKX2–3* is an NKX family homeobox gene (Pabst et al., 1999), whose involvement in leukemogenesis has not been reported; however, its highly homologous paralog *NKX2–5* is rearranged and ectopically expressed in T-cell ALL with t(5;14)(q35;q32) and t(5;14)(q35;q11.2) (Nagel et al., 2003; Przybylski et al., 2006). Taken together, these data suggest that both *PRDM16* and *NKX2–3* play an important role in leukemogenesis.

Our results indicate that the *NUP98-NSD1*-related gene expression signature is associated with a poor outcome in addition to the *NUP98-NSD1* gene fusion in pediatric AML. Most of the patients displaying the *NUP98-NSD1* signature were classified into an intermediate risk group, but their unfavorable outcome suggests that a high-risk group is a more suitable stratification. Although further investigations are necessary, we believe that our work contributes to improving the risk stratification of pediatric AML.

ACKNOWLEDGMENTS

The authors would like to thank the following Japanese childhood AML Cooperative Study Group institutions and investigators that participated in the study: Akira Morimoto, Department of Pediatrics, Kyoto Prefectural University of Medicine; Ryoji Kobayashi, Department of Pediatrics, Hokkaido University School of Medicine; Hiromasa Yabe, Department of Pediatrics, Tokai University School of Medicine; Kazuko Hamamoto, Department of Pediatrics, Hiroshima Red Cross Hospital; Shigeru Tsuchiya, Department of Pediatric Oncology, Institute of Development, Aging and Cancer, Tohoku University; Yuichi Akiyama, Department of Pediatrics, National Hospital Organization Kyoto Medical Center; Hisato Kigasawa, Department of Hematology, Kanagawa Children's Medical Center; Akira Ohara, Department of First Pediatrics, Toho University School of Medicine; Hideki Nakayama, Department of Pediatrics, Hamanomachi Hospital; Kazuko Kudo, Department of Pediatrics, Nagoya University Graduate School of Medicine; and Masue Imaizumi, Department of Hematology/Oncology, Miyagi Prefectural Children's Hospital.

REFERENCES

Agulio F, Avagyan S, Labar A, Sevilla A, Lee DF, Kumar P, Lemischka IR, Zhou BY, Snoeck HW. 2011. Prdm16 is a physiologic regulator of hematopoietic stem cells. Blood 117:5057–5066.

Arai Y, Hosoda F, Kobayashi H, Kawakami K, Takahara J. 1997. The inv(11)(p15q22) chromosome translocation of de novo and therapy-related myeloid malignancies results in fusion of the nucleoporin gene, NUP98, with the putative RNA helicase case, DDX10. Blood 89:3936–3944.

Brown J, Jawad M, Twigg SR, Saracoglu K, Sauerbrey A, Thomas AE, Eils R, Harbott J, Kearney L. 2002. A cryptic t(5;11)(q35;p15.5) in 2 children with acute myeloid leukemaia with apparently normal karyotypes, identified by a multiplex fluorescence in situ hybridization telomere assay. Blood 99:2526–2531.

Casas S, Aventín A, Nomdedéu J, Sierra J. 2003. Cryptic t(5;11)(q35;p15.5) in adult de novo acute myelocytic leukemia with normal karyotype. Cancer Genet Cytogenet 145:183.

Cerveira N, Correia C, Dória S, Bizarro S, Rocha P, Gomes P, Torres L, Norton L, Borges BS, Castedo S, Teixeira MR. 2003. Frequency of NUP98-NSD1 fusion transcript in childhood acute myeloid leukaemia. Leukemia 17:2244–2247.

Chou WC, Chen CY, Hou HA, Lin LI, Tang JL, Yao M, Tsay W, Ko BS, Wu SJ, Huang SY, Hsu SC, Chen YC, Huang YN, Tseng MH, Huang CF, Tien HF. 2009. Acute myeloid leukemia bearing t(7;11)(p15;p15) is a distinct cytogenetic entity with poor outcome and a distinct mutation profile: comparative analysis of 493 adult patients. Leukemia 23:1303–1310.

Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JG, Peters ST, Van Zutven LJ, Beverloo HB, Van der Spek PJ, Escherich G, Horstmann MA, Janka-Schaub GE, Kamps WA, Evans WE, Pieters R. 2009. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. Lancet Oncol 10:125–134.

Döhner K, Schlenk RF, Habdank M, Scholl C, Rücker FG, Corbacioglu A, Bullinger L, Fröhling S, Döhner H. 2005. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood 106:3740–3746.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 95:14863–14868.

Frohling S, Scholl C, Gilliland DG, Levine RL. 2005. Genetics of myeloid malignancies: Pathogenetic and clinical implications. J Clin Oncol 23:6285–6295.

Gough SM, Slape CI, Aplan PD. 2011. NUP98 gene fusions and hematopoietic malignancies: Common themes and new biological insights. Blood 118:6247–6257.

Grier DG, Thompson A, Kwasniewska A, McGonigle GJ, Halliday HL, Lappin TR. 2005. The pathophysiology of HOX genes and their role in cancer. J Pathol 205:154–171.

Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, Pratcorona M, Abbas S, Kuipers JE, van Galen JF, Beverloo HB, Sonneveld E, Kaspers GJ, Trka J, Baruchel A, Zimmermann M, Creutzig U, Reinhardt D, Pieters R, Valk PJ, Zwaan CM. 2011. NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern. Blood 118:3645–3656.

Ichikawa H, Tanabe K, Mizushima H, Hayashi Y, Mizutani S, Ishii E, Hongo T, Kikuchi A, Satake M. 2006. Common gene expression signatures in t(8;21)- and inv(16)-acute myeloid leukemia. Br J Haematol 135:336–347.

Imaizumi M, Tawa A, Hanada R, Tsuchida M, Tabuchi K, Kigasawa H, Kobayashi R, Morimoto A, Nakayama H, Hamamoto K, Kudo K, Yabe H, Horibe K, Tsuchiya S, Tsukimoto I. 2011. Prospective study of a therapeutic regimen with all-trans retinoic acid and anthracyclines in combination of cytarabine in children with acute promyelocytic leukaemia: the Japanese childhood acute myeloid leukaemia cooperative study. Br J Haematol 152:89–98.

Jaju RJ, Fidler C, Haas OA, Strickson AJ, Watkins F, Clark K, Cross NC, Cheng JF, Aplan PD, Kearney L, Boultwood J,

Wainscoat JS. 2001. A novel gene, NSD1, is fused to NUP98 in the t(5;11)(q35;p15.5) in de novo childhood acute myeloid leukemia. Blood 98:1264–1267.

Jo A, Tsukimoto I, Ishii E, Asou N, Mitani S, Shimada A, Igarashi T, Hayashi Y, Ichikawa H. 2009. Age-associated difference in gene expression of paediatric acute myelomonocytic lineage leukaemia (FAB M4 and M5 subtypes) and its correlation with prognosis. Br J Haematol 144:917–929.

Kudo K, Kojima S, Tabuchi K, Yabe H, Tawa A, Imaizumi M, Hanada R, Hamamoto K, Kobayashi R, Morimoto A, Nakayama H, Tsuchida M, Horibe K, Kigasawa H, Tsukimoto I. 2007. Prospective study of a pirarubicin, intermediate-dose cytarabine, and etoposide regimen in children with Down syndrome and acute myeloid leukemia: The Japanese Childhood AML Cooperative Study Group. J Clin Oncol 25:5442–5447.

La Starza R, Gorello P, Rosati R, Riezzo A, Veronese A, Ferrazzi E, Martelli MF, Negrini M, Mecucci C. 2004. Cryptic insertion producing two NUP98/NSD1 chimeric transcripts in adult refractory anemia with an excess of blasts. Genes Chromosomes Cancer 41:395–399.

Marcucci G, Haferlach T, Döhner H. 2011. Molecular genetics of adult acute myeloid leukemia: Prognostic and therapeutic implications. J Clin Oncol 29:475–486.

Mochizuki N, Shimizu S, Nagasawa T, Tanaka H, Taniwaki M, Yokota J, Morishita K. 2000. A novel gene, MEL1, mapped to 1p36.3 is highly homologous to the MDS1/EVI1 gene and is transcriptionally activated in t(1;3)(p36;q21)-positive leukemia cells. Blood 96:3209–3214.

Mullighan CG, Su X, Zhang J, Radtke I, Phillips LA, Miller CB, Ma J, Liu W, Cheng C, Schulman BA, Harvey RC, Chen IM, Clifford RJ, Carroll WL, Reaman G, Bowman WP, Devidas M, Gerhard DS, Yang W, Relling MV, Shurtleff SA, Campana D, Borowitz MJ, Pui CH, Smith M, Hunger SP, Willman CL, Downing JR. 2009. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. N Engl J Med 360:470–480.

Nagel S, Kaufmann M, Drexler HG, MacLeod RA. 2003. The cardiac homeobox gene NKX2-5 is deregulated by juxtaposition with BCL11B in pediatric T-ALL cell lines via a novel t(5;14)(q35.1;q32.2). Cancer Res 63:5329–5334.

Nakamura T. 2005. NUP98 fusion in human leukemia: Dysregulation of the nuclear pore and homeodomain proteins. Int J Hematol 82:21–27.

Nakamura T, Largaespada DA, Lee MP, Johnson LA, Ohyashiki K, Toyama K, Chen SJ, Willman CL, Chen IM, Feinberg AP, Jenkins NA, Copeland NG, Shaughnessy JD Jr. 1996. Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. Nature Genet 12:154–158.

Nebral K, König M, Schmidt HH, Lutz D, Sperr WR, Kalwak K, Brugger S, Dworzak MN, Haas OA, Strehl S. 2005. Screening for NUP98 rearrangements in hematopoietic malignancies by fluorescence in situ hybridization. Haematologica 90:746–752.

Pabst O, Zweigerdt R, Arnold HH. 1999. Targeted disruption of the homeobox transcription factor Nkx2-3 in mice results in postnatal lethality and abnormal development of small intestine and spleen. Development 126:2215–2225.

Panarello C, Rosanda C, Morerio C. 2002. Cryptic translocation t(5;11)(q35;p15.5) with involvement of the NSD1 and NUP98 genes without 5q deletion in childhood acute myeloid leukemia. Genes Chromosomes Cancer 35:277–281.

Przybylski GK, Dik WA, Grabarczyk P, Wanzeck J, Chudobska P, Jankowski K, von Bergh A, van Dongen JJ, Schmidt CA, Langerak AW. 2006. The effect of a novel recombination between the homeobox gene NKX2-5 and the TRD locus in T-cell acute lymphoblastic leukemia on activation of the NKX2-5 gene. Haematologica 91:317–321.

Pui CH, Carroll WL, Meshinchi S, Arceci RJ. 2011. Biology, risk stratification, and therapy of pediatric acute leukemias: An update. J Clin Oncol 29:551–565.

Roberts KG, Morin RD, Zhang J, Hirst M, Zhao Y, Su X, Chen SC, Payne-Turner D, Churchman ML, Harvey RC, Chen X, Kasap C, Yan C, Becksfort J, Finney RP, Teachey DT, Maude SL, Tse K, Moore R, Jones S, Mungall K, Birol I, Edmonson MN, Hu Y, Buetow KE, Chen IM, Carroll WL, Wei L, Ma J, Kleppe M, Levine RL, Garcia-Manero G, Larsen E, Shah NP, Devidas M, Reaman G, Smith M, Paugh SW, Evans WE, Grupp SA, Jeha S, Pui CH, Gerhard DS, Downing JR, Willman CL, Loh M, Hunger SP, Marra MA, Mullighan CG. 2012. Genetic alterations activating kinase and cytokine receptor signaling in high-risk acute lymphoblastic leukemia. Cancer Cell 22:153–166.

Romana SP, Radford-Weiss I, Ben Abdelali R, Schluth C, Petit A, Dastugue N, Talmant P, Bilhou-Nabera C, Mugneret F, Lafage-Pochitaloff M, Mozziconacci MJ, Andrieu J, Lai JL, Terre C, Rack K, Cornillet-Lefebvre P, Luquet I, Nadal N, Nguyen-Khac F, Perot C, Van den Akker J, Fert-Ferrer S, Cabrol C, Charrin C, Tigaud I, Poirel H, Vekemans M, Bernard OA, Berger R. 2006. NUP98 rearrangements in hematopoietic malignancies: a study of the Groupe Francophone de Cytogénétique Hématologique. Leukemia 20:696–706.

Sano H, Shimada A, Taki T, Murata C, Park MJ, Sotomatsu M, Tabuchi K, Tawa A, Kobayashi R, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Hayashi Y. 2012. RAS mutations are frequent in FAB type M4 and M5 of acute myeloid leukemia, and related to late relapse: A study of the Japanese Childhood AML Cooperative Study Group. Int J Hematol 95:509–515.

Shiba N, Taki T, Park MJ, Shimada A, Sotomatsu M, Adachi S, Tawa A, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Arakawa H, Hayashi Y. 2012. DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia. Br J Haematol 156:413–414.

Shimada A, Taki T, Tabuchi K, Tawa A, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Hayashi Y. 2006. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood 107:1806–1809.

Shimada A, Taki T, Tabuchi K, Taketani T, Hanada R, Tawa A, Tsuchida M, Horibe K, Tsukimoto I, Hayashi Y. 2008. Tandem duplications of MLL and FLT3 are correlated with poor prognoses in pediatric acute myeloid leukemia: a study of the Japanese childhood AML Cooperative Study Group. Pediatr Blood Cancer 50:264–269.

Taketani T, Taki T, Shibuya N, Ito E, Kitazawa J, Terui K, Hayashi Y. 2002a The HOXD11 gene is fused to the NUP98 gene in acute myeloid leukemia with t(2;11)(q31;p15). Cancer Res 62:33–37.

Taketani T, Taki T, Ono R, Kobayashi Y, Ida K, Hayashi Y. 2002b. The chromosome translocation t(7;11)(p15;p15) in acute myeloid leukemia results in fusion of the NUP98 gene with a HOXA cluster gene, HOXA13, but not HOXA9. Genes Chromosomes Cancer 34:437–443.

Taketani T, Taki T, Shibuya N, Kikuchi A, Hanada R, Hayashi Y. 2002c Novel NUP98-HOXC11 fusion gene resulted from a chromosomal break within exon 1 of HOXC11 in acute myeloid leukemia with t(11;12)(p15;q13). Cancer Res 62: 4571–4574.

Taketani T, Taki T, Nakamura T, Kobayashi Y, Ito E, Fukuda S, Yamaguchi S, Hayashi Y. 2010. High frequencies of simultaneous FLT3-ITD, WT1 and KIT mutations in hematological malignancies with NUP98-fusion genes. Leukemia 24: 1975–1977.

Tsukimoto I, Tawa A, Horibe K, Tabuchi K, Kigasawa H, Tsuchida M, Yabe H, Nakayama H, Kudo K, Kobayashi R, Hamamoto K, Imaizumi M, Morimoto A, Tsuchiya S, Hanada R. 2009. Risk-stratified therapy and the intensive use of cytarabine improves the outcome in childhood acute myeloid leukemia: The AML99 trial from the Japanese Childhood AML Cooperative Study Group. J Clin Oncol 27:4007–4013.

Walter MJ, Payton JE, Ries RE, Shannon WD, Deshmukh H, Zhao Y, Baty J, Heath S, Westervelt P, Watson MA, Tomasson MH, Nagarajan R, O'Gara BP, Bloomfield CD, Mrózek K, Selzer RR, Richmond TA, Kitzman J, Geoghegan J, Eis PS, Maupin R, Fulton RS, McLellan M, Wilson RK, Mardis ER, Link DC, Graubert TA, DiPersio JF, Ley TJ. 2009. Acquired copy number alterations in adult acute myeloid leukemia genomes. Proc Natl Acad Sci USA 106:12950–12955.

Mutations of the *GATA2* and *CEBPA* genes in paediatric acute myeloid leukaemia

Hereditary *GATA2* mutations show predisposition to acute myeloid leukaemia (AML) and myelodysplastic syndrome (MDS) (Hahn *et al*, 2011). These mutations have also been reported in chronic myeloid leukaemia (Zhang *et al*, 2008) and monocytopenia and mycobacterial infection (MonoMAC) syndrome (Hsu *et al*, 2011). More recently, *GATA2* mutations have been identified in *de novo* AML, especially in adult patients with biallelic *CEBPA* mutations (Greif *et al*, 2012; Green *et al*, 2013). GATA2 and CEBPA are transcription factors that are crucial for haematopoietic development. These findings prompted us to identify possible *GATA2* and *CEBPA* mutations in patients with various paediatric leukaemias.

Direct Sequencing of *GATA2* was performed in 157 *de novo* AML patients, including 13 patients with acute promyelocytic leukaemia (APL; French–American–British type-M3) and 10 with Down syndrome (DS; Table S1), 22 secondary AML patients, 40 juvenile myelomonocytic leukaemia (JMML) patients, 50 acute lymphoblastic leukaemia (ALL) patients, 70 cell lines (25 B-cell precursor-ALL, 15 T-cell-ALL, 22 AML, and 8 neuroblastomas), and 60 healthy subjects. *GATA2* mutation analysis was performed by direct sequencing for all coding exons (exons 2–6) using an ABI PRISM 3130 Genetic Analyser (Applied Biosystems, Branchburg, NJ, USA) (Table S2). For AML patients, *CEBPA* and *NPM1* mutations were also examined. Mutational analyses of *FLT3*, *KIT*, *WT1* and *RAS* genes in our AML patients was performed as described previously (Shimada *et al*, 2006). Informed consent was obtained from the patients or the patients' parents according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review boards of Gunma Children's Medical Centre approved this project.

GATA2 mutations were found in eight out of 157 AML patients (5·1%), including three APL patients (Fig 1A,B), but were absent in 18 patients with acute megakaryocytic leukaemia (FAB-M7; Table S3). Furthermore, there were no *GATA2* mutations in patients with other leukaemias, in the cell lines, or in the 60 healthy subjects, suggesting that *GATA2* mutations were indeed associated with leukaemogenesis in a subset of patients with *de novo* AML.

Germline *GATA2* mutations were also examined in five AML patients whose complete remission (CR) samples were available, and a germline mutation was identified in one patient. Furthermore, we performed *GATA2* mutation analyses of the patient's parents and two siblings, and identified the same *GATA2* mutations in her father (II-4) and brother (III-1) but not in her mother (II-5) or sister (III-2) (Fig 1C). Her father and brother lacked abnormalities in their full blood cell counts, lymphocyte subsets, or episodes of opportunistic infections. The proband experienced severe mycotic pneumonia during induction chemotherapy. Remarkably, she has been in CR for more than 11 years, despite discontinuation of chemotherapy. Three patients, for whom CR samples were not available, had no history of MonoMAC syndrome.

In addition, 16 *CEBPA* mutations (10·2%) and three *NPM1* mutations (1·9%) were found in 157 paediatric AML patients. Thirteen (81·3%) of 16 patients with *CEBPA* mutations had been in CR for more than 4 years, suggesting that *CEBPA* mutations may be associated with favourable outcomes. Although most *GATA2* mutations were found in patients with biallelic *CEBPA* mutations in adult AML (Greif *et al*, 2012; Green *et al*, 2013), only two of eight *GATA2* mutation-positive patients had monoallelic *CEBPA* mutations in this study (Table I).

We compared the clinical and molecular features between patients with and without *GATA2* mutations. However, there were no significant differences in terms of age, initial white blood cell count, gender, and cytogenetics (Table S3). Of the eight patients with *GATA2* mutations, one had a *WT1* mutation, one had a *KIT* mutation, and two patients had *RAS* mutations (Table I). *FLT3*-internal tandem duplication, *MLL*-partial tandem duplication, and *NPM1* mutations were not found in any patients with *GATA2* mutations (Table S3). All of the *GATA2* mutations were found in the intermediate risk subgroup or APL patients with t(15;17), whereas none were found in those with core-binding factor AML [i.e. t(8;21) and inv(16)]. *GATA2* mutations were found in two patients with 11q23 translocations, including t(11;19) and t(7;11), and three patients with complex chromosomal abnormalities, whereas most *GATA2* mutations were found in cytogenetically normal AML patients in previous reports (Table I) (Greif *et al*, 2012; Luesink *et al*, 2012).

GATA2 mutations were previously reported in patients with M1, M2, and M4 subtypes of AML (Greif *et al*, 2012; Luesink *et al*, 2012), which is in accordance with our results. *GATA2* mutations have not been previously reported in APL, but our study found these mutations in three APL patients. Of note, promyelocytic leukaemia protein has been shown to interact with GATA2 and potentiate its transactivation capacity (Tsuzuki *et al*, 2000).

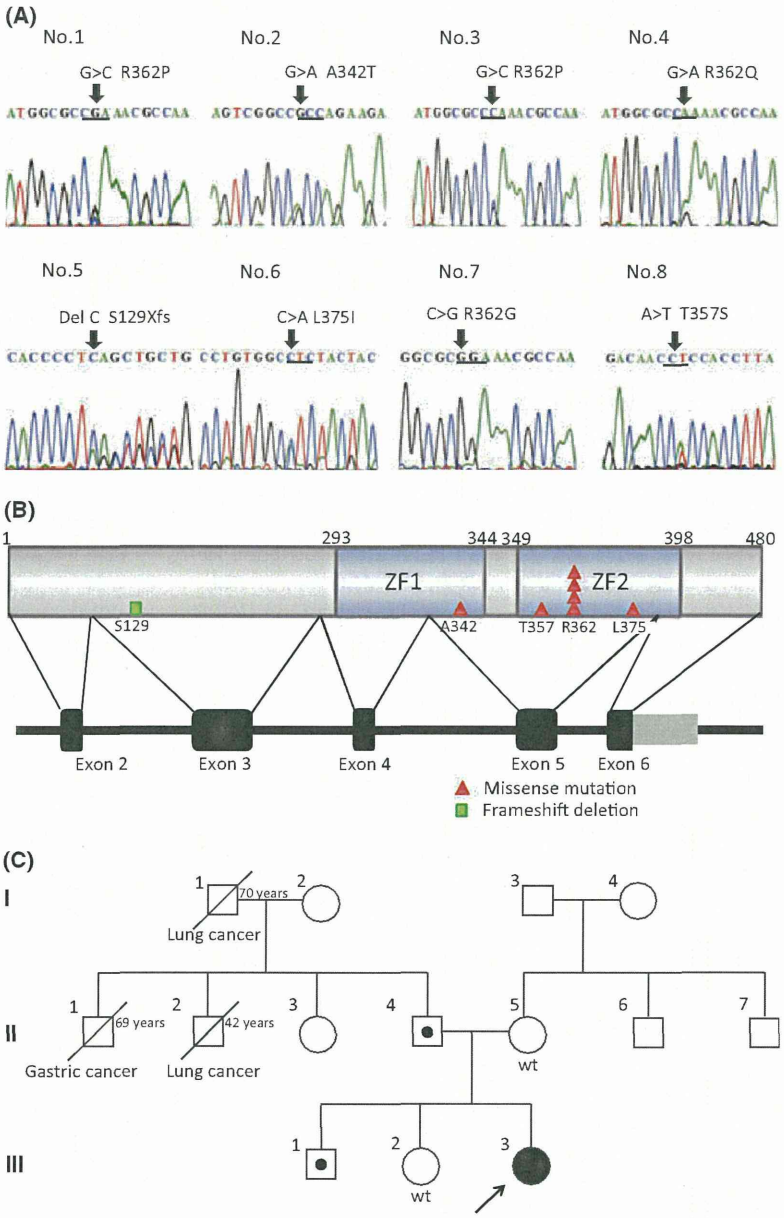

Fig 1. Identification of *GATA2* mutations by direct sequencing. (A) Eight *GATA2* mutations were identified in 157 Japanese paediatric *de novo* acute myeloid leukaemia (AML) patients (5·1%). Major missense mutations were R362 (R362P, R362Q, and R362G). Small vertical arrows indicate the mutated nucleotides. (B) Of the eight mutations, six mutations were identified in the ZF2 domain, one mutation was identified in the ZF1, and a mutation was identified on the outside of the ZF domain. (C) The family pedigree is shown. Squares indicate males and circles indicate females. The proband (III-3) is indicated by an arrow. The proband, her father (II-4), and her brother (III-1) harboured *GATA2* mutations (shown by squares containing dots). Her uncles and grandfather died of lung cancer (I-1 and II-2) and gastric cancer (II-1). wt, wild-type.

The outcomes of our patients with *GATA2* mutations was not poor (3-year overall survival and event free survival: 87·5%), which is in agreement with previous reports on *de novo* AML (Greif *et al*, 2012; Luesink *et al*, 2012): two of eight patients received autologous-stem cell transplantation (Auto-SCT), and one died of gastrointestinal haemorrhage after Auto-SCT. The remaining six patients who did not receive Auto-SCT were still alive (Table I).

In this study, one patient with a germline *GATA2* mutation developed AML. Her paternal grandfather (I-1) and second uncle (II-2) died of lung cancer at the age of 70 and 42 years, respectively, while her first uncle (II-1) died of gastric cancer at 69 years of age (Fig 1C). Increased GATA2 protein expression has been associated with biochemical recurrence and distant metastatic progression in prostate cancer (Böhm *et al*, 2009), as loss of GATA2 reduced the viability of Non-small cell lung cancer cells with RAS-pathway mutations, whereas wild-type cells were unaffected (Kumar *et al*, 2012). These facts indicate that GATA2 upregulation is strongly associated with maintenance of cancer cells. The association between *GATA2* mutations and solid tumours remains to be elucidated.

Our results indicate that *GATA2* mutations are associated with a favourable outcome in paediatric AML. Therefore, less aggressive treatment strategies without SCT may be

Table I. Clinical and molecular characteristics of patients with *GATA2* mutations.

Pt	Sex	Age (years)	FAB	WBC ($\times 10^9$/l)	Chromosome	Risk	Tx	Relapse	Prognosis (months)	*GATA2* mutation	Germline	Additional mutations
1	M	3	M4	23·8	46, XY, t(11;19) (q23; p13·1)	IR	Auto	Yes	16	R362P	N/A	–
2	F	7	M0	3·7	45,XX,add(3)(p13),del(6)(q?), der(8) t(3;8)(p21;q24), −13	IR	Chemo	No	+141	A342T	Yes	*NRAS*
3	F	8	M1	1·8	46, XX	IR	Chemo	No	+56	R362P	No	*KRAS*
4	M	14	M1	440·0	46XY [2/8], 46, XY, del(6) (q15 q21), −7, −9, −10, +3mar[1/8], 46, XY, ?de(3) (p25)[1/8], 47, XY, −5, −8, −10, add(12)(q24·1), −16, −18, +6mar [1/8], 46, XY, −2, −6, −8, +3mer [1/8], 46, XY, −8, +mar [1/8], 46, Y, ?add(X)(p11·2) [1/8]	IR	Auto	No	+51	R362Q	No	*WT1*, *CEBPA*-SM
5	M	11	M3	16·1	46,XX,inv(9)(p11q13),t(15;17)(q22;q11·21)	M3	Chemo	No	+50	S129X	N/A	–
6	M	3	M3	11·6	46,XY,t(15;17)(q22;q11?21)	M3	Chemo	No	+45	L375I	No	*CEBPA*-SM
7	M	10	M3	13·6	47,XY, +8, t(15;17)(q22;q11·21)	M3	Chemo	No	+41	R362G	N/A	*KIT*
8	F	2	M4	12·7	48, XX, +6, +10, t(11?; ?) (q23;q25)	IR	Chemo	No	+38	T357S	No	–

Pt, Patient; FAB, French–American–British classification; WBC, white blood cell count; Tx, Treatment; M, Male; F, Female; IR, Intermediate risk; Auto, Autologous stem cell transplantation; Chemo, Chemotherapy; N/A, not available; +, alive; SM, single mutation.

appropriate for paediatric AML patients with *GATA2* mutations, although most patients with *GATA2* mutations were classified into an intermediate risk group. Furthermore, the association between germline *GATA2* mutations and solid tumours remains to be elucidated.

Acknowledgements

We are grateful to all members of the Japanese Childhood AML Cooperative Study Group. Members of the Japanese Childhood AML Cooperative Study Group who contributed data to the study include Ryoji Hanada, Department of Haematology/Oncology, Saitama Children's Medical Centre; Masahiro Tsuchida, Department of Haematology/Oncology, Ibaraki Children's Medical Centre; Akira Morimoto, Department of Paediatrics, Kyoto Prefectural University of Medicine; Ryoji Kobayashi, Department of Paediatrics, Hokkaido University School of Medicine; Hiromasa Yabe, Department of Paediatrics, Tokai University School of Medicine; Kazuko Hamamoto, Department of Paediatrics, Hiroshima Red Cross Hospital; Shigeru Tsuchiya, Department of Paediatric Oncology, Institute of Development, Aging and Cancer, Tohoku University; Yuichi Akiyama, Department of Paediatrics, National Hospital Organization Kyoto Medical Centre; Hisato Kigasawa, Department of Haematology, Kanagawa Children's Medical Centre; Akira Ohara, Department of First Paediatrics, Toho University School of Medicine; Hideki Nakayama, Department of Paediatrics, Hamanomachi Hospital; Kazuko Kudo, Department of Paediatrics, Nagoya University Graduate School of Medicine; and Masue Imaizumi, Department of Haematology/Oncology, Miyagi Prefectural Children's Hospital. The authors would like to thank Enago (www.enago.jp) for the English language review. This work was supported by a grant for Cancer Research, a grant for Research on Children and Families, and Research on Intractable Diseases, Health and Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, by Grants-in-Aid for Scientific Research (B, C) and Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Programme for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio) of Japan, and by a Research grant for Gunma Prefectural Hospitals.

Author contributions

Y.H. designed the study; M.F., S.A., M.K., A.K., M.S., A.T., K.H. and I.T. collected patient samples and clinical data; N.S., K.O., M.-J.P., Y.M. and S.M. performed the laboratory research; N.S., M.-J.P. and Y.H. analysed and interpreted the data; N.S. performed the statistical analysis; N.S. and Y.H. wrote the manuscript; H.A. and Y.H. supervised the work; and all authors critically reviewed the manuscript and gave their final approval.

Conflicts of interest

The authors declare no competing financial interests.

Norio Shiba[1,2]
Michinori Funato[3]
Kentaro Ohki[1]
Myoung-ja Park[1]
Yasuhiro Mizushima[4]
Souichi Adachi[5]
Masao Kobayashi[6]
Akitoshi Kinoshita[7]
Manabu Sotomatsu[1]
Hirokazu Arakawa[2]
Akio Tawa[8]
Keizo Horibe[9]
Ichiro Tsukimoto[10]
Yasuhide Hayashi[1]

[1]Department of Haematology/Oncology, Gunma Children's Medical Centre, Shibukawa, [2]Department of Paediatrics, Gunma University Graduate School of Medicine, Maebashi, [3]Department of Paediatrics, Graduate School of Medicine, Gifu University, Gifu, [4]Department of Paediatrics, Kyoto-Katsura Hospital, [5]Department of Human Health Sciences, Kyoto University Graduate School of Medicine, Kyoto, [6]Department of Paediatrics, Hiroshima University Graduate School of Biomedical and Health Sciences, Hiroshima, [7]Department of Paediatrics, St. Marianna University School of Medicine, Kawasaki, [8]Department of Paediatrics, National Hospital Organization Osaka National Hospital, Osaka, [9]Clinical Research Centre, National Hospital Organization Nagoya Medical Centre, Nagoya, and [10]First Department of Paediatrics, Toho University School of Medicine, Tokyo, Japan
E-mail: hayashiy-tky@umin.ac.jp

Keywords: *GATA2, CEBPA*, paediatric acute myeloid leukaemia, acute promyelocytic leukaemia, germline mutation

First published online 14 September 2013
doi: 10.1111/bjh.12559

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical and cytogenetically characteristics of 157 AML patients.

Table S2. PCR primers used for mutation screening.

Table S3. Clinical and molecular characteristics of *GATA2* mutation positive patients.

References

Böhm, M., Locke, W.J., Sutherland, R.L., Kench, J.G. & Henshall, S.M. (2009) A role for GATA-2 in transition to an aggressive phenotype in prostate cancer through modulation of key androgen-regulated genes. *Oncogene*, **28**, 3847–3856.

Green, C.L., Tawana, K., Hills, R.K., Bödör, C., Fitzgibbon, J., Inglott, S., Ancliff, P., Burnett, A.K., Linch, D.C. & Gale, R.E. (2013) GATA2 mutations in sporadic and familial acute myeloid leukaemia patients with CEBPA mutations. *British Journal of Haematology*, **161**, 701–705.

Greif, P.A., Dufour, A., Konstandin, N.P., Ksienzyk, B., Zellmeier, E., Tizazu, B., Sturm, J., Benthaus, T., Herold, T., Yaghmaie, M., Dörge, P., Hopfner, K.P., Hauser, A., Graf, A., Krebs, S., Blum, H., Kakadia, P.M., Schneider, S., Hoster, E., Schneider, F., Stanulla, M., Braess, J., Sauerland, M.C., Berdel, W.E., Büchner, T., Woermann, B.J., Hiddemann, W., Spiekermann, K. & Bohlander, S.K. (2012) GATA2 zinc finger 1 mutations associated with biallelic CEBPA mutations define a unique genetic entity of acute myeloid leukemia. *Blood*, **120**, 395–403.

Hahn, C.N., Chong, C.E., Carmichael, C.L., Wilkins, E.J., Brautigan, P.J., Li, X.C., Babic, M., Lin, M., Carmagnac, A., Lee, Y.K., Kok, C.H., Gagliardi, L., Friend, K.L., Ekert, P.G., Butcher, C.M., Brown, A.L., Lewis, I.D., To, L.B., Timms, A.E., Storek, J., Moore, S., Altree, M., Escher, R., Bardy, P.G., Suthers, G.K., D'Andrea, R.J., Horwitz, M.S. & Scott, H.S. (2011) Heritable GATA2 mutations associated with familial myelodysplastic syndrome and acute myeloid leukemia. *Nature Genetics*, **43**, 1012–1017.

Hsu, A.P., Sampaio, E.P., Khan, J., Calvo, K.R., Lemieux, J.E., Patel, S.Y., Frucht, D.M., Vinh, D.C., Auth, R.D., Freeman, A.F., Olivier, K.N., Uzel, G., Zerbe, C.S., Spalding, C., Pittaluga, S., Raffeld, M., Kuhns, D.B., Ding, L., Paulson, M.L., Marciano, B.E., Gea-Banacloche, J.C., Orange, J.S., Cuellar-Rodriguez, J., Hickstein, D.D. & Holland, S.M. (2011) Mutations in GATA2 are associated with the autosomal dominant and sporadic monocytopenia and mycobacterial infection (MonoMAC) syndrome. *Blood*, **118**, 2653–2655.

Kumar, M.S., Hancock, D.C., Molina-Arcas, M., Steckel, M., East, P., Diefenbacher, M., Armenteros-Monterroso, E., Lassailly, F., Matthews, N., Nye, E., Stamp, G., Behrens, A. & Downward, J. (2012) The GATA2 transcriptional network is requisite for RAS oncogene-driven non-small cell lung cancer. *Cell*, **149**, 642–655.

Luesink, M., Hollink, I.H., van der Velden, V.H., Knops, R.H., Boezeman, J.B., de Haas, V., Trka, J., Baruchel, A., Reinhardt, D., van der Reijden, B.A., van den Heuvel-Eibrink, M.M., Zwaan, C.M. & Jansen, J.H. (2012) High GATA2 expression is a poor prognostic marker in pediatric acute myeloid leukemia. *Blood*, **120**, 2064–2075.

Shimada, A., Taki, T., Tabuchi, K., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I. & Hayashi, Y. (2006) KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. *Blood*, **107**, 1806–1809.

Tsuzuki, S., Towatari, M., Saito, H. & Enver, T. (2000) Potentiation of GATA-2 activity through interactions with the promyelocytic leukemia protein (PML) and the t(15;17)-generated PML-retinoic acid receptor alpha oncoprotein. *Molecular and Cellular Biology*, **20**, 6276–6286.

Zhang, S.J., Ma, L.Y., Huang, Q.H., Li, G., Gu, B.W., Gao, X.D., Shi, J.Y., Wang, Y.Y., Gao, L., Cai, X., Ren, R.B., Zhu, J., Chen, Z. & Chen, S.J. (2008) Gain-of-function mutation of GATA-2 in acute myeloid transformation of chronic myeloid leukemia. *Proceedings of the National Academy of Sciences*, **105**, 2076–2081.

OPEN

Assessment of Corticosteroid-induced Osteonecrosis in Children Undergoing Chemotherapy for Acute Lymphoblastic Leukemia: A Report From the Japanese Childhood Cancer and Leukemia Study Group

Nobuyuki Hyakuna, MD, Yasuto Shimomura, MD, Arata Watanabe, MD, Takashi Taga, MD, Atsushi Kikuta, MD, Takeji Matsushita, MD, Kazuhiro Kogawa, MD, Chihiro Kawakami, MD, Yasuo Horikoshi, MD, Tsuyako Iwai, MD, Yasuhiro Okamoto, MD, Masahito Tsurusawa, MD, Keiko Asami, MD, and for The Japanese Childhood Cancer and Leukemia Study Group (JCCLSG)

Summary: Steroid-induced osteonecrosis (ON) is a challenging complication encountered during modern chemotherapy for childhood acute lymphoblastic leukemia (ALL). We retrospectively assessed the incidence of ON and its risk factors in a total of 1095 patients enrolled in 3 consecutive Japanese Children's Cancer and Leukemia Study Group ALL studies (ALL941 [1994 to 2000], n = 464; ALL2000 [2000 to 2004], n = 305; and ALL2004 [2004 to 2010], n = 326). ON was diagnosed in 16 patients, of whom 15 were symptomatic. The cumulative incidence of ON was 0.76% in ALL941, 0.35% in ALL2000, and 3.6% in ALL2004. The incidence of ON in ALL941/2000, in which only prednisolone was administered as a steroid, was significantly lower than that in ALL2004, in which dexamethasone was used as a partial substitute for prednisolone ($P < 0.01$). In ALL2004, sex and age were significantly correlated with the incidence of ON (1.3% in boys vs. 6.7% in girls, $P = 0.0132$; 0.42% for age < 10 y vs. 15.6% for age ≥ 10 y, $P < 0.0001$), suggesting that girls aged 10 years and above are at a greater risk of ON onset. These results indicate that the risk of ON should be considered when administering dexamethasone as part of ALL protocol treatment in girls aged 10 years and above.

Key Words: acute lymphoblastic leukemia, osteonecrosis, corticosteroid, dexamethasone

(*J Pediatr Hematol Oncol* 2014;36:22–29)

Recent advances in treatment strategies for childhood acute lymphoblastic leukemia (ALL) have improved the overall survival rate by 80% to 90%.[1–3] Enhanced chemotherapeutic agents, refined risk classification criteria, and improved supportive care have contributed to these high cure rates, but significant toxicity remains a major risk factor that causes long-term morbidity and decreased quality of life. Osteonecrosis (ON) has been increasingly documented in pediatric ALL and presents a challenging complication during modern chemotherapy.[4,7] ON can result in joint dysfunction and subsequent impairments in activities of daily living among long-term survivors.[8,9] Well-known risk factors for ON include age above 10 years, female sex, and use of dexamethasone (DEX).[5,7] Although the precise pathophysiology of ON remains unknown, corticosteroid administration has been shown to induce ischemia, upregulate apoptosis of osteoblasts and osteocytes, and prolong osteoclast lifespans.[10]

Most previous studies regarding ON in children with ALL have been limited to European and North American study groups, as there is little data concerning Japanese or Asian patients. Therefore, the aim of the present study was to assess the incidence, risk factors, and morbidity of corticosteroid-induced ON in ALL studies conducted by the Japanese Childhood Cancer and Leukemia Study Group (JCCLSG). We retrospectively analyzed the data of 1095 patients enrolled in 3 consecutive ALL studies (ALL941, ALL2000, and ALL2004) conducted by the JCCLSG. Prednisolone (PSL) was used as the primary corticosteroid in all studies, with DEX acting as a partial substitute for PSL in ALL2004. ON patients were practically identified by symptoms and ON was confirmed with imaging studies in all patients.

MATERIALS AND METHODS

Patients and Treatment

ALL941, ALL2000, and ALL2004 were conducted between 1994 and 2000, 2000 and 2004, and 2004 and 2010, respectively. The therapies on these studies were risk adjusted but not randomized. Patients enrolled in ALL941 and ALL2000 were aged 1 to 15 years, whereas those enrolled in ALL2004 were aged 1 to 18 years. All participants were newly diagnosed with B-precursor ALL or T-cell ALL, and those with a mature B-cell phenotype and Philadelphia chromosome-positive ALL were excluded. All studies were conducted across 18 hospitals that were members of the JCCLSG. The study protocol was approved by the institutional review board of each study,

Received for publication March 27, 2013; accepted August 26, 2013.
From the Center of Bone Marrow Transplantation, Hospital of University of the Ryukyus, Okinawa, Japan.
The authors declare no conflict of interest.
Reprints: Nobuyuki Hyakuna, MD, Center of Bone Marrow Transplantation, Hospital of University of the Ryukyus, 207 Uehara, Nishihara-cho, Okinawa 903-0215, Japan (e-mail: hyakunan@med.u-ryukyu.ac.jp).
Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Website, www.jpho-online.com.

and written informed consent was provided by the patients or their legal guardians before treatment.

The treatment protocols adopted in ALL941 and ALL2000 were reported previously.[11,12] The patients were stratified according to leukocyte count and age at the time of diagnosis into standard-risk (SR), high-risk (HR), and high-high-risk (HHR) groups. The ALL941 and ALL2000 study protocols were almost identical except for the addition of doxorubicin administration to patients with a leukocyte count $<10{,}000/\mu L$ and age below 5 years in ALL2000. Treatment schedules and adopted drugs are briefly described in the supplement (see Supplemental Digital Content 1, Table 1,http://links.lww.com/JPHO/A55).

The ALL2004 treatment protocols are described in Figure 1 and Table 1. Previous risk classification criteria were modified according to the National Cancer Institute criteria,[13] resulting in a shift from HR to SR in 6- to 9-year-old patients with leukocyte counts of 5000 to 10,000 cells/μL. After a 7-day PSL regimen, induction therapy in the SR and HR groups was almost identical to that in previous studies.[11,12] In the HHR group, cyclophosphamide was added on day 8. After achieving complete remission, all risk groups received the same intensification therapy (Int-1). At week 15, SR patients with MRD levels $<10^{-3}$ received further intensification therapy (Int-2) that was followed by maintenance therapy (M-1 and M-2) until week 110. In the HR and HHR groups, patients with MRD levels $<10^{-3}$ at week 15 received 2 cycles of reinduction/intensification therapy (Rc1/Int-2 and Rc1/Int-3 in HR, Rc2/Int-4 and Rc2/Int-5 in HHR group) that was followed by the same maintenance therapy (M-3 and M-4) until week 165. Patients with MRD levels $\geq 10^{-3}$ at week 12 in the SR and HR/HHR groups were assigned to salvage arms 1 and 2, respectively. In the salvage regimen, patients received intensification therapy comprising 2 cycles of Rc-2/etoposide + cytarabine + L-asparaginase. For CNS prophylaxis, SR and HR patients received extended TIT injections beginning from day 1. When IT was combined with a high dose of methotrexate, only cytarabine and hydrocortisone were injected (double intrathecal [DIT] injection). TIT injections were repeated every 6 weeks in the first year, every 8 weeks in the second year, and every 12 weeks in the third year. The HHR group patients included in salvage arm 2 received 18 Gy of CRT in addition to 6 and 7 doses of TIT injections until week 22 and 32 of therapy, respectively.

Cumulative doses of the corticosteroids administered in ALL941/2000 and ALL2004 are listed in Table 2.

Identification of ON Patients

Because the significance of this therapy-related toxicity had not been fully appreciated until the early 2000s, case report form in the 3 studies did not request data regarding ON. Thus, cases with ON were collected by the questionnaire specified for ON to the investigators of JCCLSG. Most of the ON patients were identified based on clinical symptoms such as bone pain and further confirmed with diagnostic imaging studies (x-ray/magnetic resonance imaging [MRI]) by the each institutional radiologists, except one who was asymptomatic and diagnosed by imaging studies at the discretion of primary physician.

Statistical Analysis

Differences in the categorical variables of patient characteristics were analyzed using the χ^2 test. The cumulative incidence of ON during the study period was estimated using Kaplan-Meier analysis. The median follow-up periods were 147.4, 100.3, and 43.6 months for patients enrolled in ALL941, ALL2000, and ALL2004, respectively (median follow-up period, 78.7 mo for all patients). Differences in cumulative incidence between patient subsets were tested using the log-rank test.

RESULTS

A total of 1095 patients were enrolled in the present study (ALL941, n = 464; ALL2000, n = 305; and ALL2004, n = 326). Sixteen of 1095 patients developed ON during or after treatment, 4 (0.86%), 2 (0.65%), and 10 (3.1%) were in ALL941, ALL2000, and ALL2004, respectively. In patients with ON, the median age at diagnosis of ALL was 11.5 years (range, 5 to 16 y) and the male to female ratio was 1:3. When patients were evaluated by risk classification, only 1 patient in the SR group and 15 patients in the HR/HHR groups developed ON.

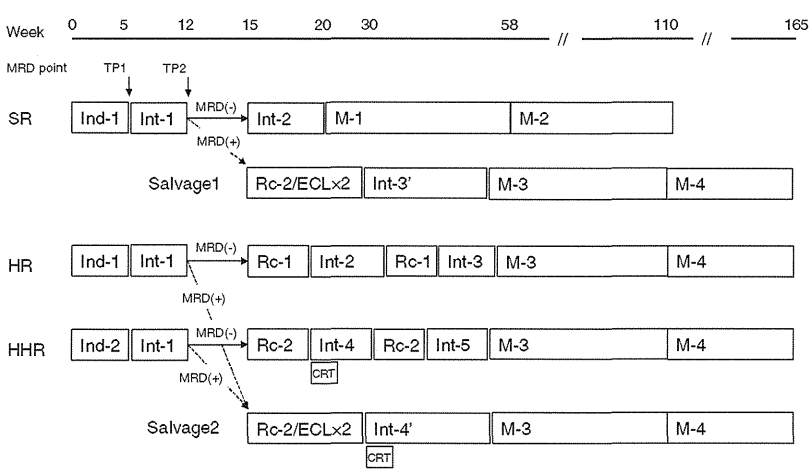

FIGURE 1. Treatment framework and minimal residual disease (MRD) stratification in the ALL2004 study. Patients with MRD levels $\geq 10^{-3}$ at week 12 received salvage therapy (dotted arrows), whereas the remainder continued to receive the initial risk-adapted therapy (solid arrows). Treatment schedules are shown in Table 1. CRT indicates cranial radiotherapy; HR, high-risk group; HHR, high-high-risk group; SR, standard-risk group.

TABLE 1. Drug Dosage and Schedule for ALL2004

	Regimen	Daily Dose	Administration	Days
Induction phase				
Ind-1	VCR	2 mg/m^2	IV	8, 15, 22, 29
	LASP	2000 U/m^2	IM	9-34 (3/wk)
	PSL*	60 mg/m^2	Oral	1-28
	DOX	25 mg/m^2	IV	8, 15, 22
Ind-2	Same as in Ind-1 except for CY (1200 mg/m^2)			8
Intensification phase				
Int-1				
AA	THP	20 mg/m^2	IV	1, 43
	VCR	2 mg/m^2	IV	1, 43
	PSL	120 mg/m^2	Oral	1-5, 43-47
	6MP	250 mg/m^2	Oral	1-5, 43-47
C	CY	400 mg/m^2	IV	15
	CA	50 mg/m^2 × 2	IV	15-18
	6MP	125 mg/m^2	Oral	15-19
BH	MTX	3000 mg/m^2	IV	29
Weekly LASP	LASP	6000 U/m^2	IM	1-50 (1/wk)
Int-2				
C + BH × 3 + A	VCR	2 mg/m^2	IV	43
	PSL	120 mg/m^2	Oral	43-47
	6MP	250 mg/m^2	Oral	43-47
Int-3				
CH + BH × 3	CY	500 mg/m^2	IV	1, 15
	CA	75 mg/m^2 × 2	IV	1-4, 15-18
	6MP	60 mg/m^2	Oral	1-28
AA + C + BH†				63-91
Weekly LASP†				63-98 (1/wk)
Int-4				
CHs	CY	500 mg/m^2	IV	1, 15
	CA	75 mg/m^2 × 2	IV	1-4, 15-18
	6MP	30 mg/m^2	Oral	1-28
AA + C + BI‡	MTX	500 mg/m^2	IV	63
Weekly LASP‡				35-70 (1/wk)
Int-5				
ECL + AA + C + BI	E	100 mg/m^2	IV	1-4
	CA	2000 mg/m^2 × 2	IV	1-4
	LASP	6000 U/m^2	IM	5
Weekly LASP				21-98 (1/wk)
Reinduction phase				
Rc-1	VCR	2 mg/m^2	IV	1, 8, 15, 22
	LASP	2000 U/m^2	IM	2-20 (3/wk)
	DEX*	10 mg/m^2	Oral	1-14
	DNR	25 mg/m^2	IV	1, 8, 15
Rc-2	VCR	2 mg/m^2	IV	1, 8, 15, 22
	LASP	6000 U/m^2	IM	9-20 (3/wk)
	DEX*	10 mg/m^2	Oral	1-14
	DNR	40 mg/m^2	IV	1, 8, 15
	CY	1200 mg/m^2	IV	1
Maintenance phase				
M-1 (C + B + A)	MTX	225 mg/m^2	IV	15
	LASP	2000 U/m^2	IM	15
M-2 (B + As)	VCR	2 mg/m^2	IV	15
	PSL	80 mg/m^2	Oral	15-19
M-3 (AA-C-B)				1-29
M-4 (Bs + As)	MTX	225 mg/m^2	IV	1
CNS prophylaxis				
TIT	MTX	12 mg/m^2	IT	
	CA	30 mg/m^2		
	HDC	50 mg/m^2		
DIT	Same as TIT except for methotrexate			

*PSL and DEX were tapered off (PSL; 30 mg/m^2 for 3 d and 15 mg/m^2 for 4 d, DEX; 5 mg/m^2 for 3 d and 2.5 mg/m^2 for 4 d).
†Repeat 2 cycles in Int-3′ for salvage 1.
‡Repeat 3 cycles in Int-4′ for salvage 2.
6MP indicates 6-mercaptpopurine; CA, cytarabine; CY, cyclophosphamide; DEX, dexamethasone; DNR, daunorubicin; DOX, doxorubicin; E, etoposide; HDC, hydrocortisone; LASP, L-asparaginase; MTX, methotrexate; PSL, prednisolon; THP, pirarubicin; VCR, vincristine.

TABLE 2. Cumulative Dose of Corticosteroid in Trials ALL941/2000 and ALL2004

	ALL941/2000		ALL2004	
	PSL (mg/m²)	DEX (mg/m²)	PSL (mg/m²)	DEX (mg/m²)
Induction	1830	—	1830	—
Reinduction				
SR	—	—	—	—
HR/HHR	1830	—	—	330
Intensification				
SR	600	—	1200	—
HR/HHR	1200	—	2400	—
Maintenance				
SR	18,000	—	9200	—
HR/HHR	15,000	—	12,200	—
Total*				
SR	20,430		12,230	
HR/HHR	19,860		18,575	

*Calculated in PSL equivalents (1 mg of DEX = 6.5 mg of PSL).
DEX indicates dexamethasone; HHR, high-high risk; HR, high risk; PSL, prednisolone; SR, standard risk.

Comparisons of the characteristics of patients with and without ON are presented in Table 3, which shows a predominance of females aged 10 years and above, treatment with ALL2004, and high risk ($P < 0.01$) in patients with ON. Notably, 9 of the 12 female and 3 of the 4 male patients with ON were aged 10 years and above, the latter was marginally significant ($P = 0.044$). ON was diagnosed at median treatment weeks 56.5 (range, 32 to 264) and 66 (range, 37 to 120) in ALL941/2000 and ALL2004, respectively. The median cumulative corticosteroid doses at the time of ON onset were as follows: PSL, 5700 mg/m² (range, 3480 to 13,880 mg/m²) in ALL941/2000 and PSL, 6030 mg/m² (range, 3480 to 13,800 mg/m²) and DEX, 330 mg/m² (range, 240 to 330 mg/m²) in ALL2004. As described in Table 2, SR patients in ALL2004 originally did not receive DEX, and despite the cumulative dose of PSL far exceeded the median doses for patients with ON at onset, none of them eventually developed ON. To obtain total PSL equivalents, DEX was multiplied by a conversion factor of 6.5[14]; therefore, a relatively higher steroid dose

TABLE 3. Comparison of Patient Characteristics Between With and Without ON

	Patients With ON (%)	Patients Without ON (%)	P (χ^2)
All	16	1079	
Sex			< 0.01
Male	4 (25)	606 (56)	
Female	12 (75)	473 (44)	
Age (y)			< 0.01
Male			0.044
1-5	1 (6)	345 (32)	
6-9	0	124 (11)	
>10	3 (19)	137 (13)	
Female			< 0.01
1-5	0	271 (25)	
6-9	3 (19)	102 (10)	
>10	9 (56)	100 (9)	
WBC			
< 10,000	6 (38)	571 (53)	
10,000-100,000	9 (56)	398 (37)	
> 100,000	1 (6)	110 (10)	
Immunophenotype			
BCP	9 (56)	739 (68)	
T	3 (19)	99 (9)	
Others	3 (19)	158 (15)	
NK	1 (6)	90 (8)	
Treatment			0.015
ALL941	4 (25)	460 (43)	
ALL2000	2 (12)	303 (28)	
ALL2004	10 (63)	316 (29)	
Risk			< 0.01
SR	1 (6)	629 (58)	
HR/HHR	15 (94)	450 (42)	

BCP indicates B-cell precursor; HHR, high-high risk; HR, high risk; NK, not known; ON, osteonecrosis; SR, standard risk; WBC, white blood cell count.

was used in ALL2004 compared with that used in ALL941/2000.

The cumulative 5-year incidence of ON was 0.76% (SE, 0.43%), 0.35% (SE, 0.35%), and 3.6% (SE, 1.1%) in ALL941, ALL2000, and ALL2004, respectively (Fig. 2), with a significant difference between ALL2004 and ALL941/2000 ($P < 0.01$). To assess the contribution of sex and age to ON incidence in patients receiving DEX-containing protocols, the cumulative incidence of ON was estimated in ALL2004 (Figs. 3A, B). Both sex and age were significantly associated with the 5-year ON incidence rate ($P < 0.01$), whereas female sex and age 10 years and above were HR factors for ON. The cumulative 5-year incidence of ON for girls over 10 years of age was 25.6% (SE, 8.4%), which was extremely higher than the rest of patients in ALL2004 ($P < 0.0001$) (Fig. 3C).

The characteristics of the 16 patients who eventually developed ON are listed in Table 4. All patients showed typical imaging findings on MRI except 1 (case 941-3) who underwent only x-ray that showed bilateral flattened femoral head. The most commonly affected joints and bones were the hip joint (44%), the knee joint (25%), and the femur (13%). Three patients (19%) exhibited multiple lesions. Nine (56%) continued to receive the planned steroid therapy despite the diagnosis of ON, whereas the doses were decreased or withdrawn in 7 (44%). ON management varied for each patient depending on the physician discretion. Most patients (75%) received supportive care only and were advised to avoid lifting heavy weights (grade 2 according to Common Terminology Criteria for Adverse Event version 4.0). Three patients (19%) underwent surgical intervention (grade 3) and 1 was treated with oral bisphosphonates (grade 2). With the median follow-up times of 33 months (range, 4 to 194), the clinical outcomes of ON were as follows: 12 with amelioration of ON and 3 with stable disease, except 1 who suffered a relapse of leukemia.

DISCUSSION

In the 3 most recent JCCLSG ALL studies, we found that a significant number of patients developed ON during or after treatment. ALL2004 study was conducted to evaluate the efficacy of DEX usage as a corticosteroid in the context of intensification of reinduction phase, comparing with the preceding 2 studies wherein PSL was the only corticosteroid adopted. This strategy also enabled us to compare the DEX toxicity with that of PSL. The results clearly demonstrated the higher incidence of ON in

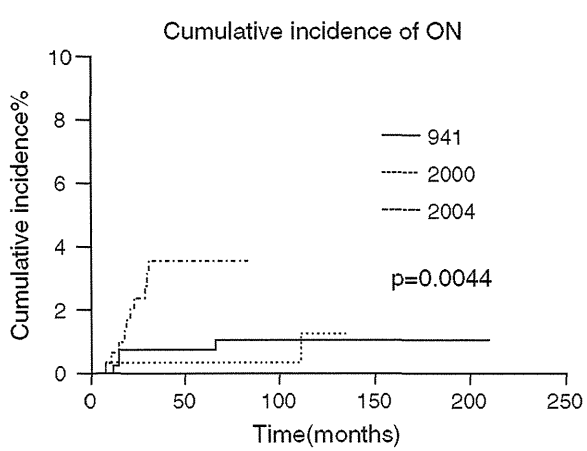

FIGURE 2. The cumulative incidence of osteonecrosis (ON) in the 3 Japanese Childhood Cancer and Leukemia Study Group studies on acute lymphoblastic leukemia (ALL). ALL941: 0.76%, SE, 0.43%; ALL2000: 0.35%, SE, 0.35%; ALL2004: 3.6%, SE, 1.1%.

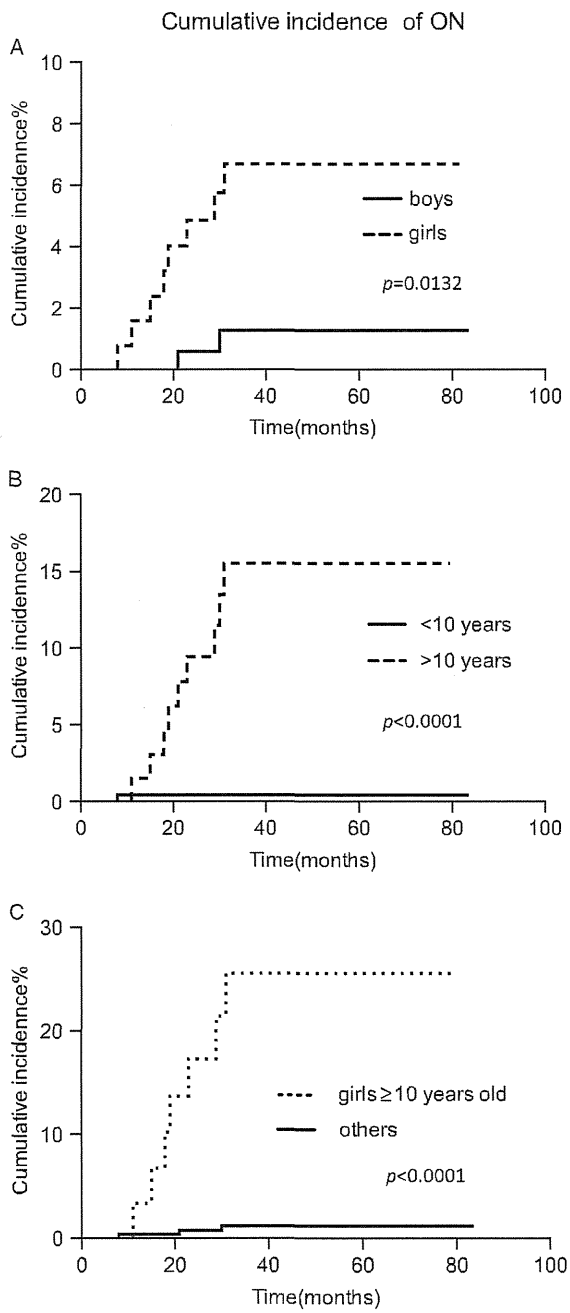

FIGURE 3. The cumulative incidence of osteonecrosis (ON) in ALL2004 according to sex (a), age (b), and combined (c). A, Boys (n=2/190): 1.3%, SE, 0.9%; girls (n=8/136): 6.7%, SE, 2.3%. B, Age below 10 years (n=1/249): 0.42%, SE, 0.42%; age 10 years and above (n=9/77): 15.6%, SE, 4.8%. C, Girls 10 years and above (n=7/33): 25.6%, SE, 8.42%; others (n=3/293): 1.19%, SE, 0.68%.