facilitate the identification of allelic imbalances such as copy-neutral LOH in the absence of a paired normal DNA reference.

The aberrations in chromosomes 1q, 2, 8, and 20 have been noted as the most commonly occurring aberrations in all previous reports,[21,22] as well as in the present study. In the present study, the most frequently detected aberrations were gains in chromosomes 1q and 2 (or 2q), observed in approximately 50% of the cases.

Trisomy in chromosome 1q is a well-known alteration in HBL.[34] Similar 1q imbalances have also been described in other pediatric neoplastic disorders such as lymphoma,[35] Wilms' tumor,[36] and sarcoma,[37] indicating that these aberrations are related to tumor progression. The candidate genes in 1q included the *NTRK1*, *ABL2*, *CD34*, *DAP3* (death receptor protein-3), and caspase-3 genes.[38] The anomalies in chromosome 2, which almost always result in gains in 2q, are also common in HBL. These imbalances are also commonly found in embryonal rhabdomyosarcoma and other pediatric tumors related to BWS. Translocation involving the *PAX3* gene located in 2q35 has been suggested to play a crucial role in the pathogenesis of alveolar rhabdomyosarcoma.[39] Based on this, a genetic link has been suggested between HBL and alveolar rhabdomyosarcoma. The role of the *PAX3* gene in the pathogenesis of HBL is yet to be determined. Additionally, the 2q24–32 region contains several genes that may also have an oncogenic potential. These include a serine/threonine kinase receptor, *ITRAF*, *FRZB*, a secreted antagonist of WNT signaling, and BRCA1-associated RING domain 1 (*BARD1*) genes. However, no specific gene has been identified in the previous,[21,22] and present studies.

The losses in chromosomes 4q and 11q were comprehensively observed. In hepatocellular carcinoma (HCC) cells, Wong *et al.* demonstrated a growth advantage following the loss in the 4q arm.[40] In HCC, 4q21–q22 and 4q35 have been identified as commonly deleted regions, and allelic losses in 4q35 have been associated with a larger tumor size and an aggressive histological tumor type.[41] Previous studies have not reported a significant correlation between HBL with loss in the distal 4q arm and prognosis, but the underlying oncogenic event might be due to the loss of a gene on the distal 4q arm.

Many minimal regions of amplification and deletion were detected using high-density SNP arrays, although homozygous deletion was not identified in any sample. The SNP loci located in 7q34 and 14q11.2 were found to be highly amplified in sporadic HBL samples. The candidate genes at these loci are *EphB6*, *DAD1*, and BCL-like 2 (*BCL2L2*) genes that encode the proteins associated with the execution of cell apoptosis. Gains as well as high amplifications in this region have not been reported previously; however, such an observation will be of particular interest for the discovery of oncogenes involved in the pathogenesis of HBL.

The UPD regions were identified in five of the 17 samples. This is chiefly important because UPD is being particularly considered as a possible mechanism of tumor initiation. During tumorigenesis, UPD is believed to arise due to a mitotic recombination caused by a rare crossover event during mitotic cell division. The products of mitotic recombination are the regions of the genome exhibiting UPD, and both the genomic regions originate from the same parent. We could identify a common UPD on chromosome 11p that is reminiscent of BWS with paternal UPD; in this case, the loss of function of the 11p15 maternal alleles through various mechanisms may be the critical event associated with tumorigenesis and BWS.[42] BWS is a neonatal overgrowth syndrome that predisposes an individual to cancer,[6] and the importance of the maternally active locus in chromosome 11p15 in tumorigenesis is supported by the finding that the loss of imprinted allele and paternal duplication leads to tissue overgrowth and subsequent tumor development. Methylation analysis was performed for the four HBL samples having UPD within 11p15, and hypermethylation of *H19* DMR was detected in all four HBL samples. Because *H19* DMR was hypermethylated on the paternal allele and hypomethylated on the maternally expressed allele in humans, we consider that the UPD within 11p15 was of paternal origin.

Two candidate genes, namely, *IGF2* and *H19*, are located within the telomeric region of chromosome 11p15.5 and have opposite imprinting patterns.[43] In the majority of human tissues, *IGF2* is expressed only from the paternal allele, whereas *H19* is transcribed only from the maternal allele. *H19* is an untranslated gene but has been suggested to function as a tumor suppressor.[44] In fetal and adult organs, the transcriptionally silent *H19* allele was extensively hypermethylated throughout the entire gene and its promoter. On the maternally expressed *H19* allele, *H19* DMR is unmethylated and can bind to the CTCF protein. On the paternal *H19* allele, *H19* DMR is highly methylated. This not only prevents the expression of the imprinted paternal *H19* alleles but also blocks the binding of the CTCF protein.[43] In general, the outcome of UPD with losses of the 11p15 maternal alleles in HBL is the same as that of the loss of imprinting on the inactivated, imprinted, and maternally expressed genes in BWS. Weksberg *et al.* proposed a dual pathway model for tumor development in BWS, wherein methylation defects at *H19* and/or *IGF2* in 11p15 were found to play a role in Wilms' and HBL tumorigenesis.[45] The combined loss of expressions in various 11p15-imprinted genes may contribute to tumorigenesis.

In the present study, we identified that the expression patterns of *IGF2* and *H19* were opposite between genes with and without the UPD in 11p15. This difference in the expression patterns might influence the clinical features of HBL. Further prospective studies are required to reveal any potential correlations between specific LOH and clinical outcomes.

In summary, the analysis of LOH and CN alterations using the SNP microarray in HBL samples revealed significant areas of allelic imbalance. We hypothesize that UPD, in addition to allelic imbalance, constitutes a novel genetic mechanism involved in tumorigenesis. Therefore, detailed characterizations such as functional studies should be conducted to elucidate the significance of the regions detected in this study, many of which may contain the candidate tumor suppressor genes and oncogenes involved in the pathogenesis of HBL.

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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

Akira Shimada, MD,[1] Tomohiko Taki, MD,[2] Ken Tabuchi, MD,[3] Takeshi Taketani, MD,[4] Ryoji Hanada, MD,[5] Akio Tawa, MD,[6] Masahiro Tsuchida, MD,[7] Keizo Horibe, MD,[8] Ichiro Tsukimoto, MD,[9] and Yasuhide Hayashi, MD[1]*

Background. *Mixed-lineage leukemia* (*MLL*)-partial tandem duplication (PTD) is associated with poor prognosis in adult acute myeloid leukemia (AML), but its relationship to pediatric AML is unknown. ***Procedure.*** One hundred fifty-eight newly diagnosed AML patients, including 13 FAB-M3 and 10 Down syndrome (DS) patients, who were treated on the Japanese Childhood AML Cooperative Treatment Protocol AML 99 were analyzed for *MLL*-PTD, as well as internal tandem duplication (ITD) and the kinase domain mutation (D835Mt) in the *FLT3* gene. ***Results.*** We found *MLL*-PTD in 21 (13.3%) of 158 AML patients, but not in FAB-M3 or DS patients. The differences between patients with and without *MLL*-PTD were significant for 3-year overall survival (OS) (56.3% vs. 83.2%, $P = 0.018$), disease-free survival (DFS) (41.7% vs. 69.6%, $P = 0.010$), and relapse rate (RR) (54.3% vs. 27.6%, $P = 0.0085$) of 135 AML patients excluding the FAB-M3 and DS patients. Furthermore, ITD and D835Mt in the *FLT3* gene were found in 17 (12.6%) and 8 (5.9%) of these 135 patients, respectively. The differences between patients with *FLT3*-ITD and the wild-type allele were significant for 3-year OS (35.3% and 84.3%, $P < 0.0000001$), DFS (40.0% and 66.9%, $P < 0.003$), and RR (52.4% and 30.3%, $P < 0.005$). Coduplication of both genes was found in only 3 (1.9%) patients. ***Conclusion.*** AML patients with *FLT3*-ITD, but not D835Mt, showed a poor prognosis. AML patients with *MLL*-PTD were also correlated with poor prognosis in this study. Pediatr Blood Cancer 2008;50:264–269. © 2007 Wiley-Liss, Inc.

Key words: AML; childhood; cytogenetics; *FLT3*; *MLL*; *tandem duplication*

INTRODUCTION

Risk classification of acute myeloid leukemia (AML) patients based on cytogenetic abnormalities has been widely accepted in adult and pediatric AML studies [1–4]. AML patients with t(8;21), inv(16), and t(15;17) have been classified into a low risk (LR) group, those with monosomy 5 and monosomy 7 into a high risk (HR) group, and others into an intermediate risk (IR) group [2–4]. Patients with normal karyotype were classified into the IR group and showed various prognoses. Classification by gene alterations other than karyotypic abnormalities would be preferable for improving the treatment outcome of pediatric AML patients.

Chromosome 11q23 abnormalities involving the *mixed-lineage leukemia* (*MLL*) gene are found in about 5% of adult AML patients and in ~50% of infants with AML [5–7]. *MLL*-partial tandem duplication (PTD) is reported in ~10% of adult AML patients, but in 20–50% of adult AML patients with a normal karyotype and trisomy 11 [8–13]. *MLL*-PTD is associated with a poor prognosis in adult AML patients and a high relapse rate (RR) [10–13]. On the other hand, the prevalence and prognosis of *MLL*-PTD in pediatric AML patients remains obscure, although a relatively high prevalence of *MLL*-PTD has been reported in a few articles [14,15].

Fms-related tyrosine kinase 3 (*FLT3*) is one of the class III receptor tyrosine kinases that is normally expressed in hematopoietic stem cells and early progenitor cells [16,17]. Internal tandem duplication (ITD) of the juxtamembrane domain (JM) of the *FLT3* gene occurs in approximately 30% of adult AML patients [18–20] and in ~20% of pediatric AML patients [21–23]. *FLT3*-ITD is strongly associated with poor prognosis, especially in patients with a normal karyotype [18–23]. Furthermore, ~10% of adult AML patients have an activating loop mutation in the kinase domain specifically, a point mutation in aspartic acid residue at codon 835 (D835Mt). These patients show a poor prognosis [19,20,24]. The prevalence and prognostic significance of *FLT3*-D835Mt in pediatric AML patients are controversial [21,23].

We have previously reported the existence of the coduplication of *MLL* and *FLT3* in pediatric AML patients who had poor prognoses [25]. These results were confirmed in adult patients with a normal karyotype and trisomy 11 [12,13,26,27]. We here performed mutation analysis of both *MLL* and *FLT3* genes in 158 unselected pediatric AML patients treated on the Japanese pediatric AML collaborative treatment protocol AML99. These data suggest that *FLT3*-ITD and *MLL*-PTD are both important markers of poor prognosis in pediatric AML patients.

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[1]Department of Hematology/Oncology, Gunma Children's Medical Center, 779 Shimohakoda, Hokkitsu, Shibukawa, Gunma 377-8577, Japan; [2]Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine, Graduate School of Medical Science, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto, Japan; [3]Department of Hematology, Kanagawa Children's Medical Center, Mutsukawa 2-138-4, Minami-ku, Yokohama, Kanagawa, Japan; [4]Department of Pediatrics, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane, Japan; [5]Division of Hematology/Oncology, Saitama Children's Medical Center, 2100, Magome, Saitama, Saitama, Japan; [6]Department of Pediatrics, National Hospital Organization, Osaka National Hospital, 2-1-14, Hoenzaka, Chuoku, Osaka, Japan; [7]Department of Pediatrics, Ibaraki Children's Hospital, 3-3-1, Futabadai, Mito, Ibaraki, Japan; [8]Clinical Research Center, National Hospital Organization, Nagoya Medical Center, 4-1-1, Sannomaru, Nakaku, Nagoya, Aichi, Japan; [9]First Department of Pediatrics, Toho University School of Medicine, 6-11-1, Omori-nishi, Otaku, Tokyo, Japan

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

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PATIENTS AND METHODS

Patients

The diagnosis of AML was made according to the French–American–British (FAB) classification. Cytogenetic analysis was performed using the G-banding method. Among 318 newly diagnosed de novo AML patients enrolled from January 2000 to December 2002, 158 samples were available for molecular analysis (Table I). Among the 158 patients, there were 13 patients with FAB-M3 and 10 patients with Down syndrome (DS) who were treated with different treatment protocols [28–30]. There were no significant differences between the 135 analyzed patients without FAB-M3 and DS and the 105 non-analyzed patients in terms of age (median 6 years (range: 0–15 years) vs. 6 years (range: 0–15 years)) and initial WBC count (median 24.8×10^9/L (range: 1.65–621.0×10^9/L) vs. 13.8×10^9/L (range: 1.0–489.0×10^9/L, $P = 0.0764$)). Patients who were younger than 2 years old or had an initial WBC count <100,000/μl were treated with the Induction A regimen (etoposide (VP16), cytarabine (CA) and mitoxantrone (MIT), (ECM)). Patients who were older than 2 years old and had an initial WBC count >100,000/μl were treated with the Induction B regimen (VP16, CA and idarubicin (IDA), (ECI)). If patients achieved complete remission (CR), the patients were classified into three risk groups (62 in low, 57 in intermediate and 10 in high) according to the results of cytogenetic analyses or the achievement of CR after initial 2 courses of chemotherapy [28–30] (Supplemental Fig. 1 which has been reported in Blood [30], http://bloodjournal.hematologylibrary.org/cgi/data/2005-08-3408/DC1/2). AML patients with t(8;21) (except for those with WBC counts >50,000/μl) or inv(16)(p11q22) were classified into the LR group. Patients with monosomy 7, 5q-, t(16;21), or Ph1 were classified into the HR group. Patients were treated with additional chemotherapy or allogeneic stem cell transplantation (allo-SCT) in each risk group (Supplemental Fig. 1).

Informed consent was obtained from the patients or patients' parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center approved this project.

Detection of *MLL*-PTD

Total RNA (4 μg) extracted from the bone marrow or peripheral blood samples at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *MLL*-PTD was examined by simple first round reverse transcriptase-polymerase chain reaction (RT-PCR) with 35 cycles using the primer pair 6.1 (located on exon 9) and E3AS (located on exon 4), according to the conditions previously reported [10,25,31]. We did not use the nested RT-PCR method because a previous report suggested that the *MLL*-PTD transcripts were highly detected in the healthy controls [31]. We used the CTS cell line as a positive control for *MLL*-PTD and water as a negative control for RT-PCR analysis

TABLE I. Clinical Characteristics of Patients With *MLL* or *FLT3* Gene Alterations

	All patients	*MLL*-PTD	*FLT3*-ITD	*FLT3*-D835Mt
Age, median (year)	6 (0–15)	10 (2–15)	9 (2–15)	11 (2–14)
WBC count, median ($\times 10^9$/L)	20.7 (1.0–620.0)	31.4 (3.6–343.4)	33.2 (3.0–620.0)	45.0 (3.3–440.0)
Male/female	89/69	12/9	8/12	7/4
FAB classification				
M0	6	1	1	0
M1	24	7(2[a])	4(2[a])	2
M2	46	5	4	2
M3	13	0	3	3
M4	22	4(1[a])	1(1[a])	1
M5	25	3	5	3
M6	1	0	0	0
M7	19	0	1	0
Unclassified	2	0	1	0
Karyotypic abnormalities				
Normal	33	8(2[a])	9(2[a])	2
t(8;21)	46	4	2	1
11q23 abnormalities	20	5	0	1
t(15;17)[b]	13	0	3	3
inv(16)	7	0	0	2
DS[b]	10	0	0	0
Others[c]	27	4(1[a])	5(1[a])	2
Unknown	2	0	1	0
Total	158	21	20	11
Risk group				
Low	62	4	2	3
Intermediate	57	13(2[a])	8(2[a])	4
High	10	3	2	0
Non-CR	6	1(1[a])	5(1[a])	1
Total	135	21	17	8

[a]Cases who showed *MLL*-PTD and *FLT3*-ITD simultaneously; [b]DS—Down syndrome, patients with FAB-M3 or DS were treated with the different protocol; [c]others contain −7, +8 or complex karyotypes.

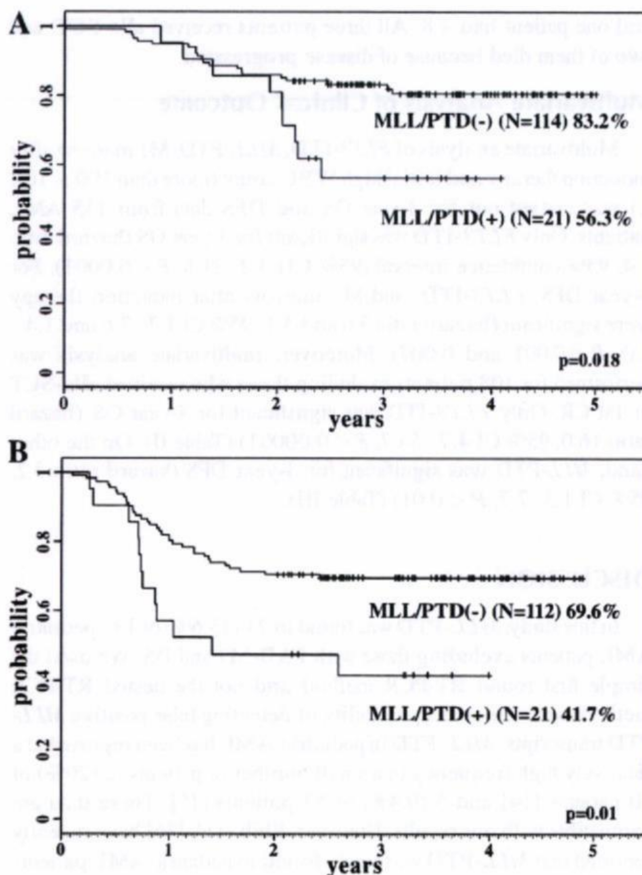

Fig. 1. Probabilities of 3-year OS (A) and 3-year DFS (B) in 135 AML patients excluding those with FAB-M3 and Down syndrome. Kaplan–Meier method estimates for patients with and without *MLL*-PTD are shown. The difference in patient numbers between OS and DFS resulted from the death of two patients during induction therapy.

[32,33]. Furthermore, we analyzed *MLL*-PTD in 10 normal bone marrow samples. Five microliter of the PCR products were electrophoresed in a 3% agarose gel. The amplified products were purified and directly sequenced.

Detection of *FLT3*-ITD and D835Mt

Using 1 μl of the cDNA, PCR amplification was performed for the JM and tyrosine kinase domain of the *FLT3* gene. The PCR procedure has been reported previously using primer pairs R5, R6, and 17F, TKR [30,34,35]. If a longer size product was found, the product was cut from the gel, purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA), and directly sequenced on a DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). D835Mt was confirmed using *Eco*RV digestion and followed by direct sequencing as previously reported [24,30,34,35].

Statistical Analysis

Estimation of the survival distributions was performed using the Kaplan–Meier method and the differences were compared using the log-rank test. Disease-free survival (DFS) was defined as the time from diagnosis until the date of relapse. Overall survival (OS) was defined as the time from diagnosis until death owing to any cause or the last follow-up. Statistical difference analysis was performed using the χ^2 test. The prognostic significance of the clinical variables was assessed by using Cox proportional hazards model. These statistical analyses were performed with statistical software R. For all analyses, the *P*-values were two-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

MLL-PTD

MLL-PTD was found in 21 (13.3%) of 158 pediatric AML patients (Table I). One type of fusion transcript (exon 9 and exon 3) was found in 10 patients, and the other type (exon 10 and exon 3) was found in 11 patients. Only one patient showed both fusion transcripts corresponding to alternatively spliced exons 10 and 11 to exon 3 (Supplemental Fig. 2). Furthermore, 10 normal bone marrow samples did not show *MLL*-PTD transcripts. *MLL*-PTD was frequently found in FAB-M1, M4 and patients with normal karyotype or 11q23 abnormalities (Table I). *MLL*-PTD was not found in FAB-M3 and DS patients. Patients with trisomy 11 were not found in this study. Remarkably, more than half of the patients with *MLL*-PTD were classified into the IR group (13 of 21 (61.9%)). The median age of patients with *MLL*-PTD was 10 years old (2–15) and no patients with *MLL*-PTD under 2 years old were found. Excluding the FAB-M3 and DS patients, the statistical differences in the clinical outcome between patients with and without *MLL*-PTD were significant for 3-year OS (56.3% vs. 83.2%, $P = 0.018$), DFS (41.7% vs. 69.6%, $P = 0.01$), and RR (54.3% vs. 27.6%, $P = 0.0085$) (Fig. 1). Allo-SCT was performed in 18 (85.7%) of 21 *MLL*-PTD patients, and 9 (50.0%) of them have been alive for a median of 42.0 months. The three patients without allo-SCT are all alive. Notably, six of the eight patients who received allo-SCT in the 1st CR and three of four patients who received allo-SCT in the 2nd CR are still alive.

FLT3-ITD and D835Mt

FLT3-ITD was found in 20 (12.7%) of 158 patients (Table I). All patients except for one showed both *FLT3*-ITD and *FLT3*-WT transcripts by RT-PCR. Half of the *FLT3*-ITD consisted of an in-frame tandem repeat of exon 11 (12–147 bp). The other half of *FLT3*-ITD showed insertions of 1–15 bp between the duplicated regions. *FLT3*-D835Mt was found in 11 (7.0%) of 158 patients. D835Mt consisted of D835Y (seven patients), D835V (two patients) and D835H (two patients). Differences in the median age of patients with *FLT3*-ITD, D835Mt, and the wild-type gene (WT) were not statistically significant (9, 11, and 5 years old, respectively). All patients with *FLT3*-ITD or D835Mt were older than 2 years old. The difference in the median initial WBC count between patients with *FLT3*-ITD and WT was significant ($P = 0.014$). Excluding FAB-M3 and DS patients, the differences between AML patients with *FLT3*-ITD, D835Mt, and WT were significant for the 3-year OS (35.3%, 100% and 84.3%, $P < 0.0000001$), DFS (40.0%, 87.5%, and 66.9%, $P < 0.003$), and RR (52.4%, 11.8% and 30.3%, $P < 0.005$) (Fig. 2). *FLT3*-ITD was found in five (83.3%) of six patients who did not

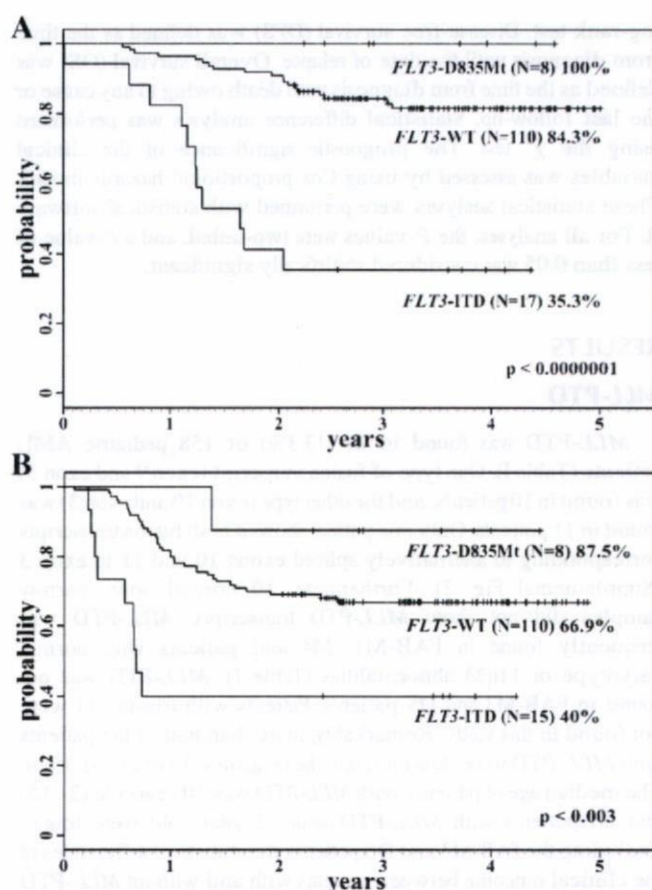

Fig. 2. Probabilities of 3-year OS (A) and 3-year DFS (B) in 135 AML patients, excluding those with FAB-M3 and Down syndrome. The Kaplan–Meier method for patients with *FLT3*-ITD, D835Mt, and WT is shown. The difference in patient numbers between OS and DFS resulted from the death of two patients during induction therapy.

attain CR. Allo-SCT was performed in 12 (70.6%) of 17 *FLT3*-ITD patients; of whom 4 (33.3%) were still alive for a median 43.5 months. The remaining eight patients died. Furthermore, four of seven patients who received allo-SCT in the 1st CR are still alive. Three of five patients without allo-SCT are also alive.

FLT3-ITD and D835Mt were found in 3 (23.1%) of 13 patients with FAB-M3. Both alterations of the *FLT3* gene did not influence the clinical outcome of FAB-M3 patients. Furthermore, these *FLT3* alterations were not found in DS patients.

Coduplication of the *MLL* and *FLT3* Genes

Coduplication of the *MLL* and *FLT3* genes were found in only 3 (1.9%) of 158 patients (Table I). Two patients had normal karyotype and one patient had +8. All three patients received allo-SCT, and two of them died because of disease progression.

Multivariate Analysis of Clinical Outcome

Multivariate analysis of *FLT3*-ITD, *MLL*-PTD, M1 marrow after induction therapy and initial high WBC count (more than 100×10^9/L) was carried out for 3-year OS and DFS data from 135 AML patients. Only *FLT3*-ITD was significant for 3-year OS (hazard ratio 8.4, 95% confidence interval (95% CI) 3.2–21.8, $P < 0.0001$). For 3-year DFS, *FLT3*-ITD, and M1 marrow after induction therapy were significant (hazard ratio 3.6 and 3.1, 95% CI 1.7–7.6 and 1.4–7.0, $P < 0.001$ and 0.007). Moreover, multivariate analysis was performed for 108 patients excluding those who received allo-SCT in 1st CR. Only *FLT3*-ITD was significant for 3-year OS (hazard ratio 16.0, 95% CI 4.7–54.7, $P < 0.00001$) (Table II). On the other hand, *MLL*-PTD was significant for 3-year DFS (hazard ratio 3.2, 95% CI 1.3–7.7, $P < 0.01$) (Table III).

DISCUSSION

In this study, *MLL*-PTD was found in 21 (15.6%) of 135 pediatric AML patients excluding those with FAB-M3 and DS. We used the simple first round RT-PCR method and not the nested RT-PCR method to minimize the possibility of detecting false positive *MLL*-PTD transcripts. *MLL*-PTD in pediatric AML has been reported at a relatively high frequency in a small number of patients: 2 (20%) of 10 patients [14] and 5 (9.4%) of 53 patients [15]. These data are compatible with our results. However, Shih et al. [36] have recently reported that *MLL*-PTD was rarely found in pediatric AML patients (one of 123, 0.8%). The difference of these frequencies in pediatric AML remains unknown but it may be partially due to the patient's age; although the median age of 16 patients with *MLL* rearrangements, including one *MLL*-PTD, is 1.3 years (1 day to 5.5 years) in the paper by Shih et al. [36], that of 21 patients with *MLL*-PTD is 10 years (2–15 years), and 17 of 21 patients with *MLL*-PTD is more than 6 years old in our study.

Patients with *MLL*-PTD showed a poor prognosis, a short duration of remission, and a high RR, as previously reported for adult AML patients [10–14,26]. Multivariate analysis suggested that *MLL*-PTD was a marker of poor prognosis for 3-year DFS, but not for 3-year OS, in AML patients excluding those who received allo-SCT in 1st CR in this study. This result may be explained by the effectiveness of allo-SCT in 2nd CR for patients with *MLL*-PTD. Indeed, four patients received allo-SCT in 2nd CR, and three of these patients are still alive.

Regarding karyotypic abnormalities, our results also confirmed that *MLL*-PTD was frequently found in AML patients with a normal karyotype as reported for adult patients [10–14]. Interestingly, *MLL*-PTD was found in AML patients with 11q23 translocations in this study. Moreover, *MLL*-PTD was also found in AML patients with

TABLE II. Prognostic Factors for 3-year Overall Survival in 108 AML Patients Treated on AML99 Protocol, Excluding Those Who Received Allo-SCT in 1st CR

Variable	*P*-values	Hazard ratio	95% CI
FLT3-ITD	<0.00001	16.0	4.7–54.7
MLL-PTD	0.25	2.1	0.6–7.4
M1 marrow after induction therapy	0.092	5.3	0.8–37.3
WBC > 100×10^9/L	0.14	0.19	0.02–1.7

TABLE III. Prognostic Factors for 3 Year Disease-Free Survival in 108 AML Patients Treated on AML99 Protocol, Excluding Those Who Received Allo-SCT in 1st CR

Variable	*P*-values	Hazard ratio	95% CI
FLT3-ITD	<0.0001	7.7	2.9–20.6
MLL-PTD	0.0099	3.2	1.3–7.7
M1 marrow after induction therapy	0.028	9.3	2.1–40.1
WBC > 100 × 10^9/L	0.013	3.1	1.3–7.5

t(8;21), which has not previously been reported in adult AML [10–14,26]. Unfortunately, we could not analyze DNA because genomic samples were not available. Two of the 4 t(8;21)-AML patients with *MLL*-PTD were also found to have *KIT* mutations in our previous study [30], suggesting that some patients possibly had genetic instability. We must await further studies to clarify these issues.

As for *FLT3* gene, multivariate analysis also strongly suggested that *FLT3*-ITD was an independent marker of poor prognosis in pediatric AML as previously reported [18,20,22]. D835Mt did not represent a poor prognosis in this study, confirming a previous report of pediatric AML [21], although D835Mt has been reported to be associated with poor prognosis in adult AML [18–20,24]. The difference between adult and pediatric AML remains unknown.

The coduplication of both genes was found in 3 (1.9%) of 158 patients in this study, which is compatible with previous reports (4 (1.6%) of 250 and 16 (1.7%) of 956 adult AML patients) [12,26]. The mechanism of formation of *MLL*-PTD and *FLT3*-ITD remains unknown. *MLL* and *FLT3* loci demonstrate similar susceptibilities to agents that modify chromatin configuration, including topoisomerase II inhibitors [27]. We conclude that the coduplication of *MLL* and *FLT3* genes is rare in pediatric AML as well as adult AML.

There was no definitive result as to the effectiveness of allo-SCT for the pediatric patients with *MLL*-PTD or *FLT3*-ITD. In this study, the majority of patients received allo-SCT due to the protocol agreement or relapse (18 (85.7%) of 21 *MLL*-PTD and 12 (70.6%) of 17 *FLT3*-ITD). Eight *MLL*-PTD patients and seven *FLT3*-ITD patients received allo-SCT in the 1st CR. Although similar results for 3-year DFS were found in patients with *MLL*-PTD (41.7%) and *FLT3*-ITD (40.0%), there was a difference in the 3-year OS between *MLL*-PTD (56.3%) and *FLT3*-ITD (35.3%) ($P = 0.024$). This difference was possibly due to the effectiveness of allo-SCT for the patients with *MLL*-PTD rather than those with *FLT3*-ITD as a lack of effectiveness of allo-SCT has been recently reported for patients with *FLT3*-ITD [37].

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ORIGINAL ARTICLE

Clinical significance of minimal residual disease in patients with t(8;21) acute myeloid leukemia in Japan

Hiroto Narimatsu · Masaki Iino · Takuji Ichihashi · Toshiya Yokozawa · Masaya Hayakawa · Hitoshi Kiyoi · Takaaki Takeo · Akiyo Sawamoto · Hiroatsu Iida · Motohiro Tsuzuki · Masamitsu Yanada · Tomoki Naoe · Ritsuro Suzuki · Isamu Sugiura

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Abstract To examine the prognostic significance of minimal residual disease (MRD) in t(8;21) acute myeloid leukemia (AML), 96 bone marrow samples from 26 Japanese patients in complete remission (CR) were analyzed regarding the *RUNX1/MTG8* transcript using real-time reverse transcriptase polymerase chain reaction assay. All patients were treated with intensive chemotherapy. The median copy number of the *RUNX1/MTG8* transcript, measured after each treatment course decreased over time. However, an increase in the MRD level was documented in three patients after the second consolidation, and all of them subsequently relapsed. The relapse-free survival (RFS) did not differ between the patients whose MRD levels were below or above 1,000 copies/μg after the first consolidation, with respective 2-year rates of 62 and 86% ($P = 0.21$). With respect to the MRD level after induction therapy, our data also failed to show any favorable effect of a lower MRD on RFS. Although these findings need to be confirmed with a larger number of patients, our data indicate that the MRD level at a given time during the early course in CR does not predict the outcome in Japanese patients.

Keywords Acute myeloid leukemia · t(8;21) · *RUNX/MTG8* · Minimal residual disease · Prognosis

1 Introduction

t(8;21)(q22;q22) is one of the most common karyotype abnormalities in acute myeloid leukemia (AML), occurring

H. Narimatsu · A. Sawamoto · I. Sugiura
Department of Hematology and Oncology,
Toyohashi Municipal Hospital, Toyohashi, Japan

H. Narimatsu (✉) · M. Yanada · T. Naoe
Department of Hematology and Oncology,
Nagoya University Graduate School of Medicine,
65 Tsurumai-cho, Showa-ku, Nagoya,
Aichi 466-8550, Japan
e-mail: narimt54@med.nagoya-u.ac.jp

M. Iino
Department of Hematology,
Yamanashi Prefectural Central Hospital, Kofu, Japan

T. Ichihashi
Department of Hematology, Okazaki City Hospital,
Okazaki, Japan

T. Yokozawa
Department of Hematology/Oncology,
Clinical Research Center, National Hospital Organization
Nagoya Medical Center, Nagoya, Japan

M. Hayakawa
Department of Hematology,
Komaki City Hospital, Komaki, Japan

H. Kiyoi
Department of Infectious Diseases,
Nagoya University School of Medicine, Nagoya, Japan

T. Takeo
Department of Hematology,
Yokkaichi Municipal Hospital, Yokkaichi, Japan

H. Iida
Department of Hematology, Meitetsu Hospital,
Nagoya, Japan

M. Tsuzuki
Department of Internal Medicine, Fujita Health University
School of Medicine, Toyoake, Japan

R. Suzuki
Department of HSCT Data Management,
Nagoya University School of Medicine, Nagoya, Japan

in 7–8% of adult patients [1–3]. This translocation leads to the formation of the chimeric *RUNX1(AML1)/MTG8(ETO)* transcript, which enables detection by polymerase chain reaction (PCR) assay. Since the introduction of real-time reverse transcriptase (RT)-PCR [4], prognostic significance of minimal residual disease (MRD) quantified using this method has been intensively investigated. Several studies from Western countries showed that MRD levels during or after treatment are associated with a risk of relapse on the basis of results from 21–51 patients [5–9].

We previously reported that Japanese patients with t(8;21) AML could have a more favorable outcome than the Western patients [10]. Marcucci et al. [11] also showed the difference in the outcome between the white and non-white patients enrolled in successive Cancer and Leukemia Group B trials. Given that clinical characteristics of t(8;21) AML can differ according to ethnicities, prognostic significance of MRD may also differ between Japanese and Western patients.

Here we examine the relationship between MRD status during intensive chemotherapy and the outcome in Japanese patients with t(8;21) AML.

2 Patients and methods

2.1 Study patients

We retrospectively reviewed the medical records of a total of 46 adults, who were newly diagnosed to have t(8;21) AML, at nine collaborating hospitals between January 2000 and December 2005. Induction therapy was given to 45 patients, and 41 (91%) achieved complete remission (CR). Data on MRD after the first or second consolidation were available for 27 of the 41 CR patients. We excluded one patient who received low-dose cytarabine-containing therapy, leaving 26 patients eligible for this study. We did not exclude any patient who relapsed after the first consolidation therapy. All patients provided their informed consent before the initiation of any medical procedure.

2.2 Diagnosis of t(8;21) AML and MRD evaluation

The diagnosis of t(8;21) AML was established based on chromosomal analysis (G-banding) and/or detection of the *RUNX1/MTG8* fusion gene by real-time RT-PCR. The molecular quantification of the *RUNX1/MTG8* fusion gene was performed as described previously [12]. The results were reported as the number of transcript copies, which were normalized by means of *GAPDH* and then converted into copies/μg RNA. The molecular quantification of the *RUNX1/MTG8* fusion gene was conducted each time after the induction and consolidation therapies. Bone marrow samples were used for all the MRD analyses.

2.3 Statistical analysis

The relapse-free survival (RFS) was calculated as the time from diagnosis to relapse or death, using the Kaplan–Meier product limit method. A log rank test was applied to assess the difference between the groups. The estimated survival was calculated as of 7 May 2008. Differences in distribution of categorical variables were compared with the Fisher's exact test. All analyses were conducted using the STATA version 9.2 software program (StataCorp, College Station, TX).

3 Results

3.1 Patient characteristics

The characteristics of the 26 patients are shown in Table 1. The median age was 50 years (range, 25–64 years), with 19 males and 7 females. Details of treatments are also summarized in Table 1. For induction therapy, 24 received idarubicin and cytarabine, and 2 received daunorubicin and cytarabine. Consolidation therapy included high-dose cytarabine in 12, and standard-dose cytarabine in 14 patients. The median follow-up of the surviving patients was 39.2 months (range, 14.0–92.4 months).

3.2 Clinical outcome

Of the 26 patients, 17 had continued first CR until the time of last observation. Relapse occurred in the remaining nine patients at a median of 9.9 months (range, 7.5–81.6 months). Five patients died due to the primary disease ($n = 3$), sudden cardiac disorder ($n = 1$) and cardiac arrhythmia ($n = 1$). The probability of RFS was 73% at 2 years for the entire population. The rate was 67% for patients who received high-dose cytarabine for consolidation therapy, whereas it was 79% for patients who received standard-dose cytarabine ($P = 0.87$).

3.3 Kinetics of MRD of each patient

The MRD levels were measured in a total of 96 samples from the 26 patients. Samples were available from 18 patients after induction therapy, 20 patients after the first consolidation, 18 patients after the second consolidation, and 13 patients after the third consolidation. The kinetics of MRD of each patient is shown in Fig. 1. The median copy number of the *RUNX1/MTG8* transcript decreased over time, for example 4,750 copies/μg after induction, 480 copies/μg after the first consolidation, 240 copies/μg after the second consolidation, and <100 copies/μg after the third consolidation. All the 15 patients whose MRD data

Table 1 Characteristics of the patients with t(8;21) AML at diagnosis

Variables		Number
Age (years)	Median, range	50 (25–64)
Sex	Male/female	19/7
Karyotypic abnormality[a]		
(A) t(8;21)(q22; q22) without additional karyotypic abnormality		7
(B) t(8;21)(q22; q22) with loss of sex (Y) chromosome		6
(C) t(8;21)(q22; q22) with abnormal chromosome 9		2
(D) t(8;21)(q22; q22) with ≥3 additional abnormalities		7
(E) t(8;21)(q22; q22) with loss of X chromosome		1
(F) Other karyotypic abnormality[b]		1
White blood cell count (/μL)	Median, range	7750 (900–54970)
Lactate dehydrogenase level (IU/L)	Median, range	441 (186–3354)
Extramedullary involvement	Present/absent	5/21
Induction therapy		
Idarubicin 12 mg/m² d1–3 + cytarabine 100 mg/m² d1–7		24
Daunorubicin 50 mg/m² d1–5 + cytarabine 100 mg/m² d1–7		2
Consolidation therapy	High-dose cytarabine-based chemotherapy	12
	No. of courses (2/3/4)	1/8/3
	Standard cytarabine-based chemotherapy	14
Hematopoietic stem cell transplantation	In first complete remission (autologous/allogeneic)	2/1
	In other stage (autologous/allogeneic)	0/3[c]

[a] Two patients were diagnosed by the detection of *RUNX1/MTG8* fusion gene using reverse transcriptase-polymerase chain reaction

[b] 46,XX,t(2;19)(q37;p13),t(8;21)(q22;q22)

[c] Patients who underwent allogeneic stem cell transplantations in second complete remission

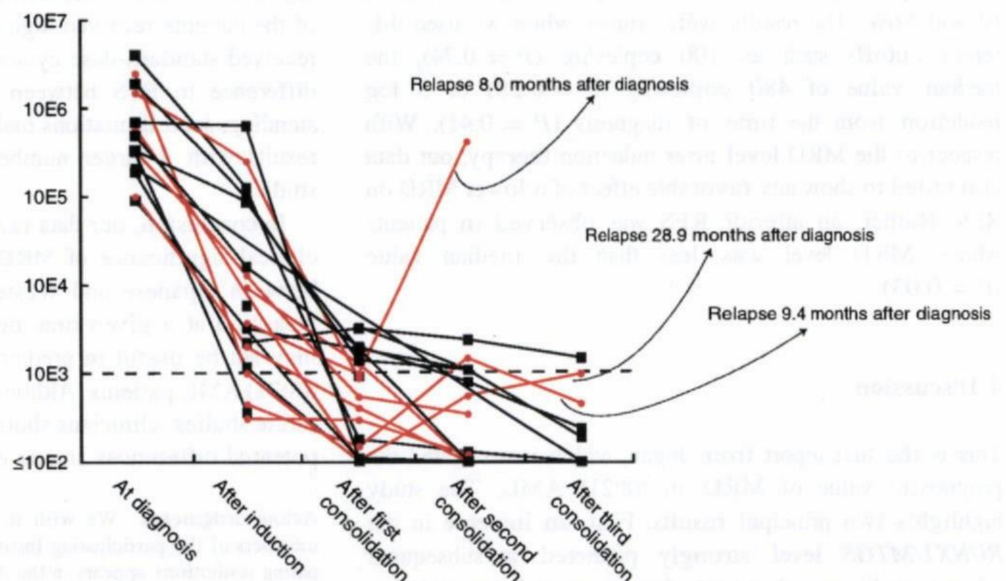

Fig. 1 Kinetics of the *RUNX1/MTG8* level in bone marrow. Kinetics of the *RUNX1/MTG8* level (copies/μg RNA) is shown for the 17 patients who remained in remission (*squares*) and for the 9 patients who had experienced a relapse (*circles*). The increases of the *RUNX1/MTG8* level were documented in three patients and all of them subsequently relapsed

were available both after induction and first consolidation showed reduction in varying degrees. On the other hand, the increments were documented in 3 of the 16 patients who had MRD levels measured both after the first and second consolidation, and all of them subsequently relapsed (Fig. 1).

3.4 Effect of MRD level on relapse-free survival

We next evaluated the prognostic relevance of the MRD level at a specific time point. Given that an increase in the MRD level was observed in none of the patients after the first consolidation, but in three patients after the second

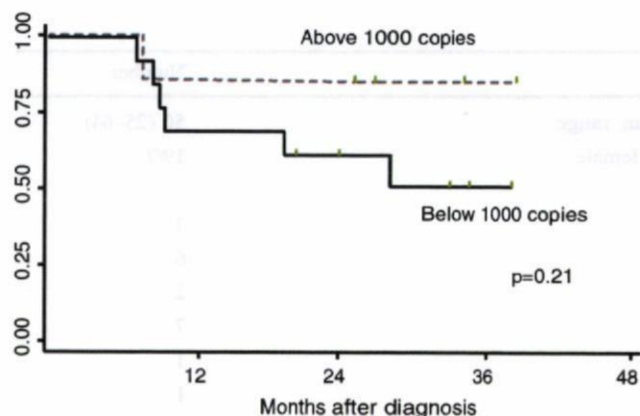

Fig. 2 Relapse-free survival according to the level of minimal residual disease after the first consolidation course. No difference was found between the patients with the *RUNX1/MTG8* level above ($n = 7$) and below 1,000 copies/μg RNA ($n = 13$)

consolidation, we examined the effect of MRD level after the first consolidation on RFS. The copy number of the *RUNX1/MTG8* transcript at this time point was less than 1,000 copies/μg in 13 patients (65%). Six patients (30%) exhibited less than 100 copies/μg. Figure 2 compares RFS according to the MRD level after the first consolidation. Here, a cutoff of 1,000 copies/μg was chosen in accordance with the findings of Tobal et al. [6]. RFS did not differ between the patients with an MRD level below or above 1,000 copies/μg ($P = 0.21$), with respective 2-year rates of 62 and 86%. The results were similar when we used different cutoffs such as 100 copies/μg ($P = 0.74$), the median value of 480 copies/μg ($P = 0.28$) or 3 log reduction from the time of diagnosis ($P = 0.41$). With respect to the MRD level after induction therapy, our data also failed to show any favorable effect of a lower MRD on RFS. Rather, an inferior RFS was observed in patients whose MRD level was less than the median value ($P = 0.03$).

4 Discussion

This is the first report from Japan, which investigated the prognostic value of MRD in t(8;21) AML. The study highlights two principal results. First, an increase in the *RUNX1/MTG8* level strongly predicted a subsequent relapse, and it was observed after the second consolidation or later. Second, unlike previous studies from Western countries [5–9], the MRD data obtained during the early course in CR did not correlate with outcome. A lack of difference in RFS by MRD level in this study might be attributable to the relatively favorable outcome of the patients with a higher *RUNX1/MTG8* level. Although the cutoffs of the MRD level vary from study to study, the RFS rate of 69% at 2 years for the patients with lower MRD level was closely comparable with other studies [5–9]. In contrast, the 2-year RFS rate was 85% for our patients with higher *RUNX1/MTG8* level, which was much better than 10–40% in those reports [5–9]. Although it is not clear why the prognosis of such "poorer responders" was different between the Western reports and ours, this difference might contribute to the more favorable overall outcome observed in Japanese patients with t(8;21) AML [10]. Recent studies have shown that the kinase domain mutations of the *KIT* gene are detected in a substantial proportion of patients with t(8;21) AML and are associated with poor prognosis [13–15]. Further investigations on molecular pathogenesis may therefore provide further insights into this issue. On the other hand, it has been well documented that non-leukemia stem cells in t(8;21) AML patients during CR possess the AML1-MTG8 fusion gene.[16] Therefore, in patients with a high MRD, AML1-MTG8 transcripts might derive from non-leukemia cells. Further basic research on the leukemia genesis of t(8;21) AML are thus warranted.

It should be noted that the patients were not treated with uniform regimens due to the retrospective nature of the study. We therefore restricted the analysis to patients who were given intensive chemotherapy. Accordingly, all but two patients received the same induction therapy consisting of idarubicin and cytarabine, and the other two received daunorubicin and cytarabine, another standard induction regimen for AML. Regarding consolidation therapy, 46% of the patients received high-dose cytarabine, while others received standard-dose cytarabine. However, there was no difference in RFS between these two groups. Notwithstanding, such limitations make it necessary to confirm our results with a larger number of patients in prospective studies.

In conclusion, our data raise an important issue that the clinical significance of MRD in t(8;21) AML may differ between Japanese and Western patients. The MRD level measured at a given time during the early course in CR may not be useful in predicting the outcome of Japanese t(8;21) AML patients. Although this needs to be verified by future studies, clinicians should note the possibility of such potential differences among ethnicities.

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Appendix

This study was conducted at the following institutions: Toyohashi Municipal Hospital, Toyohashi; Yamanashi Prefectural Central Hospital, Kofu; National Hospital Organization Nagoya Medical Center, Nagoya; Komaki

City Hospital, Komaki; Nagoya University Hospital, Nagoya; Yokkaichi Municipal Hospital, Yokkaichi; Okazaki City Hospital, Okazaki; Meitetsu Hospital, Nagoya; Fujita Health University Hospital, Toyoake, Japan.

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