common to normal stem cells [1-4]. It is believed that only the CSC, but not most of the remaining descendants (constituent cancer cells), are responsible for tumorigenesis, progression, metastasis, and relapse after treatments. Recently, the existence of CSC has been proven for several cancers [4-6]. Stem cells have a long life span with the ability to survive in severe environments, to stay quiescent in G_0 phase most of the time, and to self-renew occasionally. In contrast, progenitors and mature cells have a limited life span and divide rapidly. This implies that multiple oncogenic gene abnormalities are more likely to accumulate in stem cells than in progenitors and mature cells. The CSC can generate their descendants, including progenitors and relatively differentiated cancer cells, producing the heterogeneity of the cancer. Furthermore, only the CSC can survive conventional anticancer therapies that target only the rapidly dividing cancer cells, although their descendants (most remaining constituent cancer cells) can be killed. This could be strongly related to treatment failure as well as cancer relapse. Accordingly, the elimination of the CSC is an important goal for eradicating refractory cancers. Despite recent advances in treatment modalities, some refractory pediatric solid tumors (PSTs) remain a challenge for pediatric oncologists. It is possible that targeting the CSC in refractory PSTs may provide a novel treatment strategy to eradicate them completely. The aim of this study was to identify the possible stem-like population in PSTs.

Side population (SP) cells characterized by the efficient efflux of Hoechst 33342 dye are thought to be enriched for stem cells in many normal tissues [7-15]. Recently, SP cells that showed stem cell characteristics were isolated from several cancers [16-20]. Side population cells express various adenosine triphosphate–binding cassette (ABC) transporter family members that are responsible for drug resistance, including ABCG2 (BRCP1) [21]. In this study, the authors investigated the existence of SP cells in PST cell lines by fluorescence-activated cell sorting (FACS) analysis.

1. Materials and methods

1.1. Cell lines

Seven neuroblastoma (NB) cell lines (SK-N-AS, SK-N-DZ, GOTO, LAN1, LAN5, NB16, and NB19), 4 rhabdomyosarcoma (RMS) cell lines (RMS, RD, SCMC-RM-2, and KYM-1), and 5 Ewing's sarcoma (EWS) cell lines (ES-1-OT, UTP-ES-1, SCMC-ES-1, RD-ES, and SK-ES) were cultured in appropriate media and used for FACS analysis [22-24].

1.2. Isolation of side population cells

Pediatric tumor cells were adjusted to 10^6 cells/mL in Hanks' Balanced Salt Solution (HBSS). Hoechst 33342 dye was added to the cell suspension at a final concentration of 5 μg/mL, and the mixture was incubated for 90 minutes at 37°C. As a negative control, verapamil (Sigma, St Louis, Mo), an inhibitor of ABC transporters, was also added to a final concentration of 50 or 500 μmol/L with Hoechst 33342. After Hoechst 33342 staining, the tumor cells were washed by centrifugation, resuspended at 1 to 2 × 10^7 cells/mL in HBSS, and kept on ice until use. Before FACS analysis, propidium iodide solution was added to a final concentration of 1 μg/mL to identify nonviable cells. Fluorescence activated cell sorting analysis and sorting were performed on a dual laser flow cytometer (Becton Dickinson FACS Vantage SE cell sorter, San Jose, CA). The Hoechst dye was excited by the 355-nm ultraviolet laser, and its fluorescence was measured at 2 wavelengths using a 424/44 (Hoechst blue) band-pass filter and a 585/42 (Hoechst red) band-pass filter. Propidium iodide

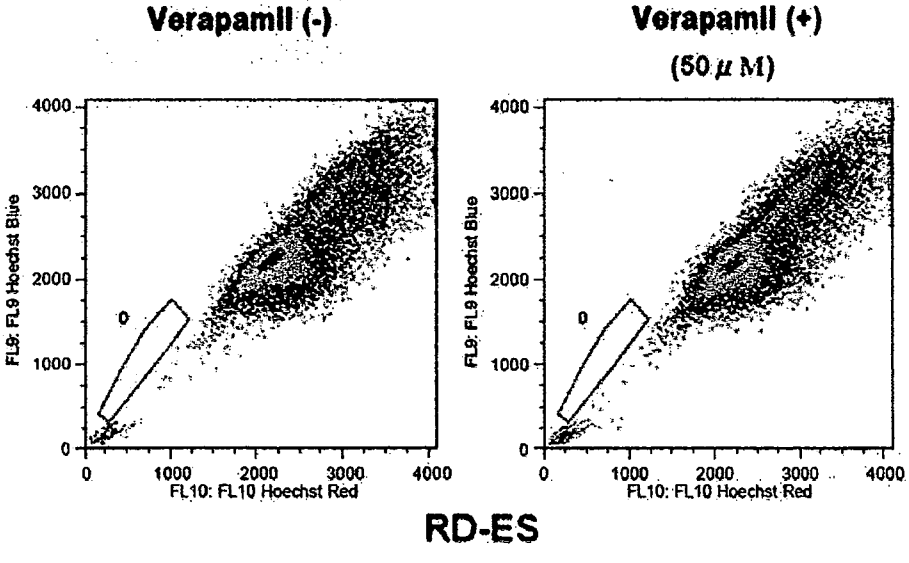

Fig. 1 A EWS cell line, RD-ES, did not show an SP fraction in the absence (left) or presence (right) of verapamil.

fluorescence was excited by 488-nm laser and detected after passing through a 630/22 band-pass filter. Propidium iodide–positive dead cells and debris were excluded.

2. Results

2.1. Side population cells in PST cell lines

One EWS cell line, RD-ES, did not show SP cells in this study (Fig. 1). Side population cells were detected in the remaining 15 cell lines including 7 NB, 4 RMS, and 4 EWS (0.12%-14.6%) (Table 1 and Fig. 2).

2.2. Sensitivity of SP cells to verapamil

The SP fraction cells disappeared upon treatment with 50 μmol/L verapamil in 6 cell lines (GOTO, NB16, SCMC-RM-2, ES-1-OT, SK-ES, and UTP-ES-1) (Table 1, and Fig. 2B and C). In the other 9 cell lines, the SP fraction disappeared in the presence of 500 μmol/L of verapamil (Table 1 and Fig. 2A).

3. Discussion

Macroscopically as well as microscopically, PSTs show a heterogeneous appearance, containing undifferentiated as well as differentiated cells. Furthermore, when the tumor is resected after aggressive chemotherapies and/or radiotherapies, it contains a heterogeneous mixture of tissues, showing not only some viable portions but also other nonviable portions in the same tumor. Certainly, not every cell in the bulk of the tumor behaves in the same manner. How this heterogeneity is generated has been well answered by the *cancer stem cell theory*. Cancer originates from a small fraction of CSC (stem-like cancer cells) with the abilities of self-renewal as well as multipotency as a reservoir. The cancer has a hierarchical organization with only a small number of CSC and a large number of their descendants (constituent cancer cells). Only the CSC are responsible for tumorigenicity, progression, and metastasis in the cancer, whereas their descendants are not. Their descendants will differentiate in various ways, resulting in the heterogeneity of the cancer. Recently, CSC have been isolated from several cancers [4-7]. Normal stem cells and CSC show similar resistance to current therapies, because they both stay in a quiescent state and have a common drug efflux capacity. This means that a small fraction of CSC can survive aggressive therapies, although most remaining constituent cancer cells are responsive to them. This eventually leads to the relapse of the cancer. Accordingly, the real target determining the biological characteristics and the treatment strategy should be the CSC themselves instead of most constituent cancer cells. If this theory is true in the case of PSTs, isolation of the CSC is the first step to better understand their characteristics and to develop novel treatment strategies of eradicating refractory PSTs.

The CSC in PSTs resembled normal stem cells morphologically as well as immunohistochemically, as found in acute myeloid leukemia, in which CSC were first identified [4]. The CSC are believed to retain properties similar to those of normal stem cells. That means that CSC may be isolated using the same procedures used to isolate tissue stem cells. The major problems in isolating normal stem cells as well as CSC are their rarity and the absence of specific markers for purifying them. Various efforts have been made to isolate CSC from various cancers [4-7]. Cancer stem cells, first identified in acute myeloid leukemia , could reproduce the original cancer with heterogeneous phenotypes and could express the stem cell surface markers $CD34^+$ and $CD38^-$ [4]. In cancers for which specific markers had not been identified, CSC or stem-like cancer cells have been isolated as SP cells that export Hoechst 33342 dye [16-20]. It has been shown that stem cells are enriched in SP cells in various normal tissues, including bone marrow, skeletal muscle, mammary gland, skin, lung, testis, brain, liver, and kidney [7-15]. Recently, SP cells have been isolated as the CSC that possess the stemness characteristics and are responsible for tumorigenesis in several cancers [16-20]. Side population cells are characterized by the rapid efflux of Hoechst 33342 dye via ABC transporters. Side population fractions are known to disappear upon treatment with inhibitors of ABC transporters such as verapamil and rapamycin. A variety of

Table 1 Side population cells in pediatric solid tumor cell lines and their sensitivity to verapamil

Cell lines	SP cells (%)	Verapamil-induced disappearance of SP fraction (concentration of verapamil, μmol/L)
NB		
SK-N-AS	+ (0.43)	+ (500)
SK-N-DZ	+ (3.26)	+ (500)
GOTO	+ (0.55)	+ (50)
LAN1	+ (0.54)	+ (500)
LAN5	+ (9.88)	+ (500)
NB16	+ (1.76)	+ (50)
NB19	+ (8.71)	+ (500)
RMS		
RMS	+ (0.53)	+ (500)
RD	+ (1.51)	+ (500)
SCMC-RM-2	+ (0.83)	+ (50)
KYM-1	+ (14.6)	+ (500)
EWS		
ES-1-OT	+ (0.33)	+ (50)
UTP-ES-1	+ (5.53)	+ (50)
SCMC-ES-1	+ (0.63)	+ (500)
RD-ES	-	-
SK-ES	+ (0.12)	+ (50)

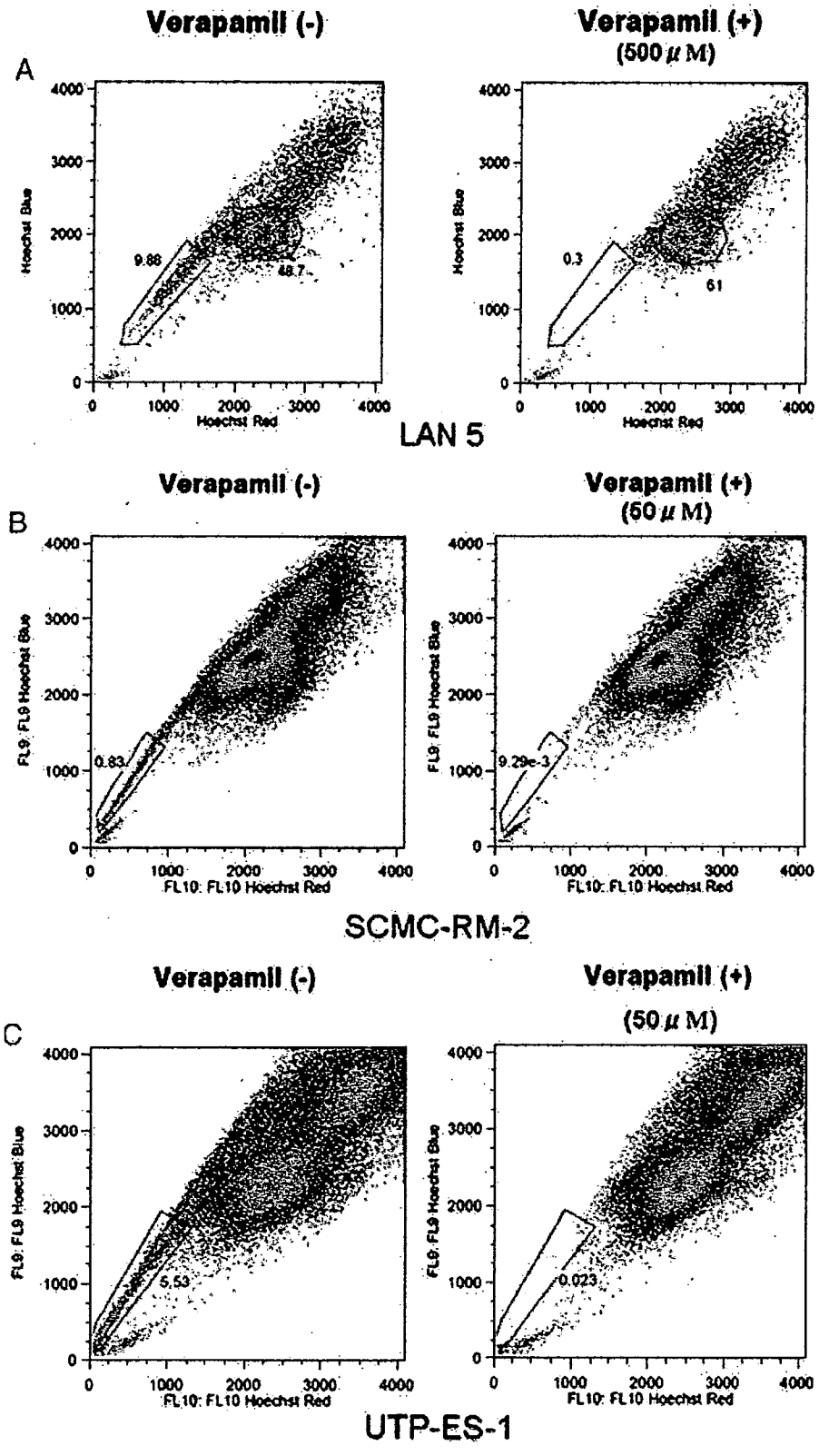

Fig. 2 Side population cells found in PST cell lines. Side population cells identified in a NB cell line, LAN 5 (A, left); a RMS cell line, SCMC-RM-2 (B, left); and a EWS cell line, UTP-ES-1 (C, left). Every SP fraction disappeared with cotreatment of 50 (B and C, right) or 500 μmol/L (A, right) verapamil plus Hoechst 33342.

ABC transporters, including multiple drug resistance protein (MDR1, ABCB1), MDR related protein (MRP1, ABCC1), and breast cancer–resistant protein (BRCP1, ABCG2), have been shown to contribute to the drug resistance in cancers [25-29]. Interestingly, some of these ABC transporters have also been shown to be expressed in various kinds of normal stem cells [21,25-30]. In particular, BRCP1 (ABCG2) is known to contribute to the exclusion of Hoechst 33342 dye in SP cells, which are enriched in stem cells [21]. ABCG2 expressed in normal stem cells and SP cells is believed to play a physiological role in the protection of both of them. This suggests that the SP cells in cancers possess drug resistance because of expression of the same transporters. Transcriptional profiling studies of SP and non-SP cells in several tissues showed that the genes up-regulated in SP cells are implicated in the quiescent status, the maintenance of pluripotency, and the capacity to undergo asymmetric division [31]. Side population cells have also been identified in several tumor cell lines as well as fresh tumor samples, including NB [16-20]. Hirschmann-Jax et al [16] demonstrated that SP cells constituted 0.8% to 51% of the cells in 15 of 23 NB tumor samples as well as 4% to 37% of the cells in 5 NB cell lines. Side population cells in NB were $Gd2^+$, $c\text{-}kit^+$, $CD133^-$, $CD71^-$, and $CD56^{\pm}$ and expressed ABC transporter proteins, ABCG2 and ABCA3, at high levels, supporting the possibility that they were CSC. We have investigated SP cells in PST cell lines, including NB, RMS, and EWS. Unlike fresh tumor samples, cell lines do not contain any contaminating non-CSC such as bone marrow–derived stem cells. Accordingly, the SP cells identified in cell lines are definitely derived from cancer cells. The stem-like population in PSTs may be enriched in SP cells. Our data showed that 15 of 16 PST cell lines contained a small fraction of SP cells (0.12%-14.6%). The percentage of SP fractions is lower than that in the previous study on NB [16]. This may be partly owing to the differences in the gated regions for the SP fraction or in the types of the cell lines used. It is likely that the cells with stemness constitute a smaller fraction. One EWS cell line did not show an SP fraction. Side population formation was blocked by 50 or 500 μmol/L verapamil (an inhibitor of ABC transporters), although the sensitivity of the cell lines varied. The absence of SP cells in the presence of verapamil confirmed the identity of the SP cells as an enriched stem-like population. This also suggested that verapamil-sensitive ABC transporters are involved in the Hoechst dye efflux of these SP cells. Of 15 cell lines, 9 were more resistant to verapamil because verapamil is generally used at a concentration of 50 μmol/L (Table 1). Different mechanisms may be involved in the Hoechst dye efflux activity of these cell lines. Further characterization of the SP cells as candidates of the CSC will be essential to understand the mechanisms of tumorigenesis, progression, metastasis, treatment failure, and tumor relapse in PSTs. Side population analysis should also be done in fresh tumor samples because the cell lines may be a collection of immortalized and rather modified cancer cells after many passages.

Our study demonstrated that most PSTs contained SP cells (possible stem-like population). Conventional treatments used for PSTs have targeted rapidly growing cancer cells, which are synthesizing DNA. The survival of the CSC in PSTs despite such treatments may be responsible for treatment failure and tumor relapse. The target of future treatments of PSTs should be the CSC themselves. New treatment strategies for targeting the CSC will be required to eradicate the refractory PSTs completely. Although how to selectively attack only the CSC without any influence on the normal tissue stem cells will be a difficult problem, complete removal of the pool of CSC as a reservoir will result in the ability to eradicate refractory PSTs in the near future.

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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

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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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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([a])	4([a])	2
M2	46	5	4	2
M3	13	0	3	3
M4	22	4([a])	1([a])	1
M5	25	3	5	3
M6	1	0	0	0
M7	19	1	1	0
Unclassified	2	0	1	0
Karyotypic abnormalities				
Normal	33	8([a])	9([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([a])	5([a])	2
Unknown	2	0	1	0
Total	158	21	20	11
Risk group				
Low	62	4	2	3
Intermediate	57	13([a])	8([a])	4
High	10	3	2	0
Non-CR	6	1([a])	5([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.

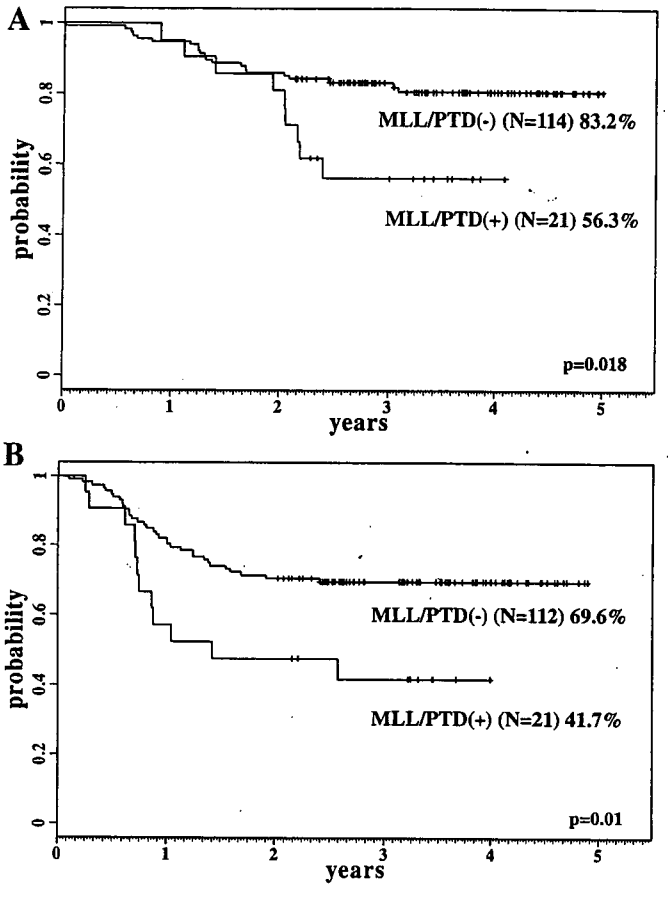

Fig. 1. Probabilities of 3-year OS (**A**) and 3-year DFS (**B**) in 135 AML patients excluding those with FAB-M3 and Down syndrome. Kaplan–Meier method estimates for patients with and without *MLL*-PTD are shown. The difference in patient numbers between OS and DFS resulted from the death of two patients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

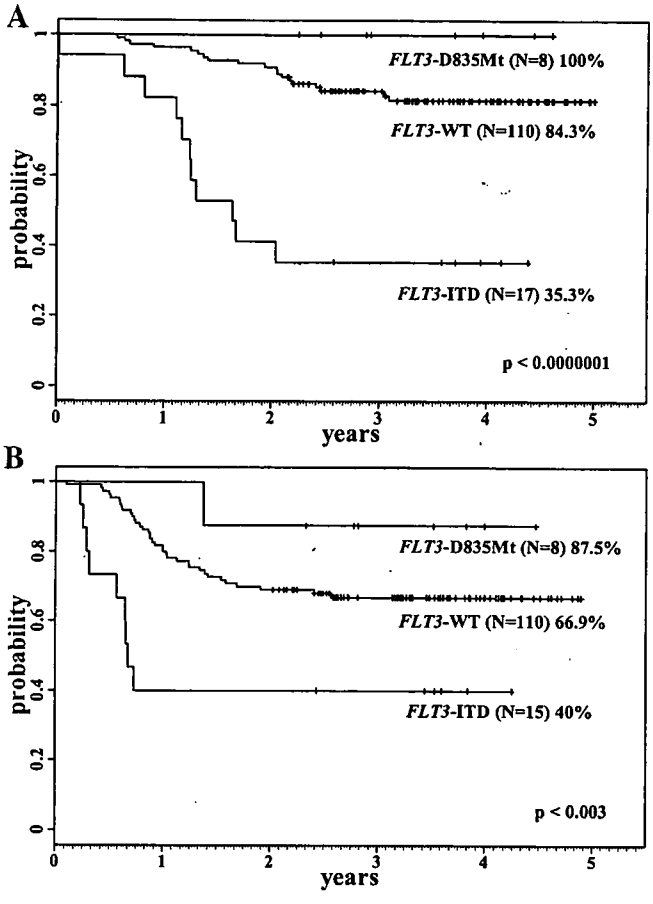

Fig. 2. Probabilities of 3-year OS (A) and 3-year DFS (B) in 135 AML patients, excluding those with FAB-M3 and Down syndrome. The Kaplan–Meier method for patients with *FLT3*-ITD, D835Mt, and WT is shown. The difference in patient numbers between OS and DFS resulted from the death of two patients during 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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Pediatr Blood Cancer 2008;50:213–217

Expression of KIT and PDGFR Is Associated With a Good Prognosis in Neuroblastoma

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Background. The clinical outcome of neuroblastoma (NB) depends on age, stage, and *MYCN* amplification. Receptor tyrosine kinases (RTKs) promote cell growth, migration, and metastasis in cancer cells, including NB. However, the correlation of the expression profile of RTKs with prognosis in NB remains controversial. ***Procedure.*** Expression and mutation analysis of *KIT, PDGFR, FLT3, RET,* and *TRKA* mRNAs were performed in 24 NB cell lines and 40 tumor samples using RT-PCR followed by direct sequencing. Immunohistochemical analysis of KIT and PDGFR protein expression was also examined in 38 paraffin sections of NB tumor samples. ***Results.*** The expression of *KIT, PDGFRβ,* and *FLT3* mRNA was associated with NB in patients under 1 year ($P < 0.02$) and *TRKA* expression ($P < 0.001$). The loss of expression of these kinases was associated with *MYCN* amplification ($P < 0.02$) and advanced stages of disease in patients over 1 year of age ($P < 0.005$). *PDGFRα* mRNA expression was detected in all cell lines and tumor samples, and *RET* mRNA expression was not associated with any clinical parameters. Immunohistochemistry results showed the similar findings. We did not find any activating mutations in *KIT, PDGFR, FLT3,* or *RET.* Notably, the GNNK$^-$ isoform of *KIT* was predominant in all cell lines and clinical samples. ***Conclusion.*** Expression of *KIT, PDGFRβ,* and *FLT3* was associated with a good prognosis in NB. The loss of expression of these RTKs might correlate to the disease progression of NB. Pediatr Blood Cancer 2008;50:213–217.

Key words: *FLT3; KIT;* neuroblastoma; *PDGFR;* receptor tyrosine kinase

INTRODUCTION

The receptor tyrosine kinases (RTKs) play an important role in the growth, migration, metastasis and angiogenesis in varieties of malignancies [1–3]. KIT is one of the type III RTKs and is well known to have roles not only in hematopoiesis, but also in germ cell and melanocyte development and differentiation as well as in neuroectodermal tumor cells [1–9]. Recently, KIT expression in NB has been reported to be associated with a poor prognosis with MYCN amplification [4,9]. On the other hand, another report suggested that KIT expression was associated with a good prognosis [7]. Moreover, a tyrosine kinase inhibitor, imatinib, has been shown to have an inhibitory effect for NB cell growth in vitro and in vivo [4–6]; however, imatinib was suggested not to inhibit the stem cell factor (SCF)/KIT pathway in NB cells [6]. Therefore, the therapeutic mechanism of imatinib in NB remains undetermined. *KIT* mutations have been frequently found in gastrointestinal stromal tumor (GIST) [10] and a subtype of acute myeloid leukemia (AML) [11], but not in NB [8]. The platelet derived growth factor receptor (PDGFR)-α has important roles in the development of neural crest-derived cells [12]. *PDGFRα* mutation has been frequently found in GIST [13]. PDGFRβ is overexpressed in metastatic medulloblastoma, and has been considered to have a more oncogenic potential than *PDGFRα* [14]. The roles of *PDGFRα* and *PDGFRβ* remain to be elucidated in NB.

FLT3 and RET have been reported to have roles in proliferation and differentiation in NB [15,16]. Although *FLT3*-internal tandem duplication (ITD) is a poor prognostic factor in AML [17], *FLT3*-ITD or kinase domain mutations have not yet been reported in NB. The RET receptor signal pathway is functional in most NB [16,18]. *RET* gene mutations have been identified in multiple endocrine tumors [19]. The expression of TRKA has been associated with good clinical outcome in NB. On the other hand, Tacconelli et al. [20] reported that the alternative spliced isoform III of TRKA has oncogenic potential. Therefore, we performed expression and mutation analysis of these 5 RTK (*KIT, PDGFRs, FLT3, RET,* and *TRKA*) genes in 24 NB cell lines and 40 clinical specimens.

Here we described that the expression of *KIT, PDGFRβ,* and *FLT3* is associated with NB in patients under 1 year of age and with a good prognosis. The loss of expression of these RTKs may be associated with NB disease progression.

MATERIALS AND METHODS

Cell Lines and Clinical Samples

Twenty-four NB cell lines were examined in this study (Supplemental Table I). RNAs were extracted from 40 frozen tumor samples using a QIAGEN RNA extraction kit (Qiagen, Chatsworth, CA), which were obtained before chemotherapy from January 2001 to December 2005. Twenty of these samples were taken from patients under 1 of age, and they received surgical resection and chemotherapy. All patients except for one are alive. Five patients were stages I or II and over 1 year of age and received surgical resection and chemotherapy and were alive. Fifteen patients had advanced stage and were over 1 year of age; they received surgical resection, radiation therapy, and intensive chemotherapy including autologous-SCT [21] (Table I); however, five patients (33.3%) died due to the disease progression after autologous-SCT. Informed consent was obtained from parents. The institutional review board of Gunma Children's Medical Center approved this project.

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TABLE I. Expression of *KIT, PDGFRβ, FLT3, RET* in 40 Clinical NB Samples by RT-PCR

	No. of patients	*KIT* (%)	*PDGFRβ* (%)	*FLT3* (%)	*RET* (%)
Age					
<1 year	20	20 (100)	19 (95)	19 (95)	8 (40)
>1 year	20	12 (60)	10 (50)	13 (65)	9 (45)
		($P=0.0016$)	($P=0.0014$)	($P=0.0177$)	ns
MYCN status					
>5 copies	6	2 (33.3)	2 (33.3)	2 (33.3)	1 (16.7)
1 copy	34	30 (88.2)	27 (79.4)	30 (88.2)	16 (47.1)
		($P=0.0006$)	($P=0.0198$)	($P=0.0019$)	ns
Clinical stage					
III, IV, and over 1 year old	15	7 (46.7)	7 (46.7)	8 (53.3)	4 (26.7)
I, II, IVs at any age	25	25 (100)	22 (88)	24 (96)	13 (52)
		($P<0.0001$)	($P=0.0046$)	($P=0.0011$)	ns
TRKA					
Positive	28	27 (96.4)	25 (89.3)	27 (96.4)	12 (42.9)
Negative	12	5 (41.7)	4 (33.3)	5 (41.7)	5 (41.7)
		($P<0.0001$)	($P=0.0003$)	($P<0.0001$)	ns
Total	40	32 (80)	29 (72.5)	32 (80)	17 (42.5)

P-value is analyzed for the correlation between RTK expression and age, *MYCN* amplification, clinical stage, and TRKA expression, respectively. ns represents not significant.

Expression and Mutation Analysis of *KIT* and *PDGFR*

The procedure was reported previously. Briefly, a total of 4 μg of RNA was reverse transcribed to cDNA. Using 1 μl of the cDNA, polymerase chain reaction (PCR) was performed using primer pairs for extracellular (EC), juxtamembrane (JM), transmembrane (TM), and the second tyrosine kinase (TK2) domains of *KIT* and *PDGFR* using an ABI 2700 thermal cycler (Applied Biosystems, Tokyo, Japan; Supplemental Table II) [11,22]. If the PCR-product was found as the estimated size and confirmed by sequencing directly, we evaluated it as positive expression of mRNA.

Mutation analyses of *KIT* and *PDGFR* in 24 NB cell lines were performed by direct sequencing using an ABI prism 310 sequence analyzer (Applied Biosystems). The mRNA expression of each ligand (*SCF, PDGFA*, and *PDGFB*) was also analyzed by RT-PCR.

Expression and Mutation Analysis of *FLT3*

Using 1 μl of the cDNA, PCR amplification was performed for the JM or TK2 domain of the *FLT3* gene. The PCR procedure has been reported previously using primer pairs R5, R6 and 17F, TKR [23]. If more than two bands were found, the amplified products were cut from the gel, purified with a QIAquick gel extraction kit (Qiagen) and directly sequenced.

Expression and Mutation Analysis of *RET*

Using 1 μl of the cDNA, PCR amplification was performed for the TM and TK domain of the *RET* gene. PCR was performed using previously reported condition and primer pairs RET-TM(+) and RET-TK2(−) [24]. *RET* isoforms, RET9 and RET51, were analyzed as previously reported [25].

Expression and Mutation Analysis of *TRKA*

TRKA mRNA expression was analyzed using newly designed primer pairs, TRKA-F and TRKA-R (Supplemental Table II). This primer pair could distinguish the alternative spliced form I (deleted exon 9), II (no-deletion), and III (exons 6, 7, and 9) [24] by the forward primer in exon 5 and reverse primer in exon 10.

Protein Expression Analysis

Paraffin sections were obtained from 38 NB samples (Table II). Eight samples were classified as advanced stage and older than 1 year old. Ten RNAs and ten paraffin sections were obtained from the same patients. The expression of KIT, PDGFRα and PDGFRβ proteins was analyzed using the avidin–biotin–peroxidase complex method on paraffin sections [26]. Antibodies of KIT (DAKO, A4502, diluted 1:80), PDGFRα (SantaCruz, CA, USA, sc-338,

TABLE II. Expression of KIT and PDGFRβ in NB Tumor Specimens by Immunohistochemistry

	Number of patients	KIT (%)	PDGFRβ (%)
Age			
<1 year	27	20 (74)	20 (74)
>1 year	11	3 (27.3)	4 (36.4)
		($P=0.0074$)	($P=0.019$)
MYCN status			
>5 copies	6	1 (16.2)	0
1 copy	32	22 (68.8)	24 (75)
		($P=0.017$)	($P=0.0052$)
Clinical stage			
III, IV, and over 1 year old	8	2 (25)	1 (12.5)
I, II, IVs in any age	30	21 (70)	22 (73.3)
		($P=0.014$)	($P=0.0011$)
Shimada's Histology			
Favorable	27	19 (70.4)	21 (77.8)
Unfavorable	11	4 (36.4)	3 (27.3)
		($P=0.052$)	($P=0.0021$)
Total	38	23 (60.5)	24 (63.2)

P-value is analyzed for the correlation between each RTK expression and age, *MYCN* gene amplification, clinical stages, and histology.

diluted 1:200) and PDGFRβ (SantaCruz, sc-6252, diluted 1:200) were used. We also analyzed the expression of KIT and PDGFRβ in 6 ganglioneuroma samples (Table III). GIST specimens were used for the positive controls. The evaluation of immunohistochemistry was performed by two independent observers (AS and JH). We evaluated the complete cytoplasm and membrane staining in more than 30% of cells as positive, and cytoplasm or membrane staining in less than 30% of cells as negative. We considered that the positive specimens showed the expression of the protein.

Statistical Analysis

Statistical analysis was performed using Statview software (SAS). The χ^2-test was used to correlate the categorical variables. The prognostic significance of the clinical variables was assessed by using Cox proportional hazards model. For all analyses, the *P* values were 2-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Expression and Mutation Analysis of *KIT*

KIT mRNA expression was found in 22 (91.7%) of 24 cell lines with RT-PCR (Supplemental Table I). All cell lines predominantly showed a 12 bp (GGTAACAACAAA) deleted product ($GNNK^-$ isoform) at the end of the extra cellular domain (exon 9) compared to the wild-type of *KIT* (Supplemental Fig. 1) [27]. We could not find any activating mutations as previously reported in GIST and AML [10,11]. Two single nucleotide polymorphisms (SNPs) were found [541aa, A > C of 1642 bp in exon 10 (Reference SNP (refSNP) Cluster Report: rs 3822214 by NCBI) in SCMC-N4 and SKNSH, 862aa, G > C of 2,607 bp in exon 18 (rs 3733542) in SJNB-5 and SKNSH]. A silent mutation was also found (I798I, ATC > ATT of 2,414 bp in exon 17 in SJNB-8). All cell lines, except for one, expressed *SCF*. Both soluble and membranous bound forms of *KIT* mRNA were found.

KIT expression was detected in 32 (80.0%) of 40 tumor samples by RT-PCR (Table I) and 23 (60.5%) of 38 paraffin sections of tumor samples by immunohistochemistry (Table II). The expression of mRNA and protein was measured in ten patients using both RT-PCR and immunohistochemistry. The expression of *KIT* mRNA and protein was associated with NB patients under 1 year ($P = 0.0016$, 0.0074, respectively) and inversely associated with *MYCN* amplification ($P = 0.0006$, 0.017, respectively) and Stages 3 or 4 NB patients over 1 year old ($P < 0.0001$, 0.014, respectively). *KIT* mRNA expression was significantly associated with *TRKA* mRNA expression ($P < 0.001$). Multivariate analysis showed the coefficient of correlation between *KIT* mRNA and *TRKA* mRNA was 0.627 (0.398–0.785, $P < 0.001$) and between KIT mRNA and survival was 0.665 (0.446–0.809, $P < 0.001$). The KIT protein expression was found in two of four differentiating NB samples and five of six samples of ganglioneuroma (Table III). The difference of expression rate of KIT protein between neuroblastoma (NB) and ganglioneuroma or between differentiating and poorly differentiated NB were not statistically significant.

Expression and Mutation Analysis of *PDGFRs*

PDGFRα mRNA was detected in all cell lines and tumor samples by RT-PCR (Supplemental Table I). As for the mutation of *PDGFRα*, no activating mutations were found. Three SNPs were found (567aa A > G of 1,849 bp in exon12 (rs 1873778) in SJNB4, 603aa G > A of 1957 bp in exon13 (rs 10028020) in SJNB4, NB16, NB69, LAN2, and SKNSH, 824aa C > T of 2,620 bp in exon 18 (rs 2228230) in SJNB-5, SJNB-8, NB-19, LAN-1, LAN-5, and SKNSH). Silent mutation was found in GOTO (V533V, GTG > GTA of 1,747 bp in exon 11). PDGFRα protein was strongly expressed in almost all tumor samples by immunohistochemistry.

PDGFRβ mRNA was expressed in 14 (58%) of 24 cell lines and 29 (73%) of 40 NB samples using RT-PCR (Tables I and II). *PDGFRβ* was expressed in 24 (63%) of 38 tumor samples by immunohistochemistry (Table III). The expression of *PDGFRβ* mRNA and protein was associated with NB patients under 1 year ($P = 0.0014$ and 0.019, respectively) and inversely associated with *MYCN* amplification ($P = 0.0198$ and 0.0052, respectively), advanced stage patients one year old and over ($P = 0.0046$ and 0.0011, respectively). The correlation between *PDGFRβ* and *TRKA* mRNA expression was significant ($P = 0.0003$). Multivariate analysis showed the coefficient of correlation between *PDGFRβ* mRNA and *TRKA* mRNA was 0.574 (0.320–0.751, $P < 0.001$) and between *PDGFRβ* mRNA and survival was 0.525 (0.256–0.719, $P = 0.004$). The correlation between PDGFRβ protein expression and a favorable histology was also significant ($P = 0.0021$). The

TABLE III. Correlation of KIT and PDGFRβ Expression to Histopathology of NB According to INPC System

INPC system	Number of patients	KIT (%)	PDGFRβ (%)
Neuroblastoma (Schwannian stroma-poor)			
Undifferentiated	1	0	0
Differentiating	4	2 (50)	4 (100)
Poorly differentiated	27	17 (63)	16 (59.3)
Ganglioneuroblastoma			
Intermixed (Schwannian stroma-rich)	4	2 (50)	4 (100)
Nodular	2	2 (100)	1 (50)
Total	38	23 (60.5)	25 (65.8)
Ganglioneuroma (Schwannian stroma-dominant)	6	5 (83.3)	6 (100)

The difference of expression rate of KIT or PDGFRβ protein between neuroblastoma and ganglioneuroma was not statistically significant. The difference of expression rate of KIT or PDGFRβ protein between differentiating and poorly differentiated neuroblast.

expression of PDGFRβ was found in all four differentiating NB samples and all five ganglineuroblastoma samples (Table III). The difference of expression rate of PDGFRβ protein between NB and ganglioneuroma or between differentiating and poorly differentiated NB were not statistically significant.

Expression and Mutation Analysis of *FLT3*

FLT3 mRNA expression was detected in 19 (79.2%) of 24 cell lines and in 32 (80%) of 40 tumor samples by RT-PCR (Table I and Supplemental Table I). No ITDs or kinase domain mutations were observed in any cell lines. *FLT3* expression was associated with NB patients under 1 year ($P=0.0177$) and *TRKA* expression ($P<0.0001$; Table I). Inverse correlations were observed for *MYCN* amplification ($P=0.0019$) and advanced stage patients over one year old ($P=0.0011$). FLT3 protein expression was not examined.

Expression and Mutation Analysis of *RET*

RET expression was detected in 22 (91.6%) of 24 cell lines and in 17 (42.5%) of 40 tumor samples by RT-PCR (Table I and Supplemental Table I). However, no mutations were found in this study. We identified SNPs (691aa or 769aa) of the *RET* gene. *RET* expression was not associated with any clinical findings (Table I). Furthermore, we examined the expression of both isoforms RET51 and RET9. There were no correlations between the RET isoforms and the clinical findings.

Expression and Mutation Analysis of *TRKA*

TRKA expression was detected in 7 (29.2%) of 24 cell lines and in 28 (70.0%) of 40 tumor samples by RT-PCR (Table I and Supplemental Table I). *TRKA* expression was associated with NB in patients under age 1 year ($P=0.0006$) and with good prognosis (Table I). We examined the expression of the TRKA isoform, but did not detect isoform III in any cell lines or tumor samples [20]. On the other hand, we found another novel isoform (deletion of exons 7–9) in 6 (25%) in 24 cell lines (SJNB-2, SJNB-6, NB1, TGW, SKNSH, SCMC-N4) with the coexpression of isoforms I or II, which we referred to as isoform IV in this article (Supplemental Fig. 2). However, we could not find this isoform IV in any of 40 tumor samples.

DISCUSSION

The aberrant expression of KIT and SCF has been reported in several solid tumors, such as small cell lung cancer [28], gynecological tumors [29], and breast cancer [30]. However, *KIT* mutations are rarely reported in other cancers [31–33] except for GIST [10] and the core-binding factor AML [11]. An autocrine or paracrine loop of KIT and SCF has been hypothesized in NB cell proliferation [34]. Moreover, the $GNNK^-$ isoform of *KIT* has been shown to be predominantly expressed in varieties of tumors, such as AML and germ cell tumor [35,36], and the $GNNK^-$ isoform has a growth advantage compared with the $GNNK^+$ isoform and phosphorylates downstream signals, such as MAP and STAT kinases [27]. In this study, KIT expression was associated with NB patients under 1 year of age and good prognosis as previously reported [7]. The $GNNK^-$ isoform was predominantly expressed in NB patients. An inverse correlation between KIT expression and *MYCN* amplification was observed and it supported the observation of Krams et al. [7]. On the other hand, KIT expression has been reported to be associated with a poor prognosis and with *MYCN* amplification in NB [4,9]. These different results may due to the differences of experimental method, race or the number of patients analyzed. Moreover, the loss of KIT expression has also been reported in advanced cancer, including breast cancer [32], melanoma [37], thyroid cancer [38], and ovarian cancer [39]. The loss of KIT expression may be associated with NB tumor progression.

PDGFRs and their ligands, PDGFA and PDGFB, have an important role not only in embryogenesis, but also in the progression of some tumors, suggesting the presence of an autocrine or paracrine mechanism [40,41]. PDGFRs can become potent oncoproteins when they are overexpressed or mutated [40–42]. The intensive expression of PDGFRα protein was detected in this study, suggesting that expressed PDGFRα may be the therapeutic target for the kinase inhibitor, imatinib. On the other hand, the expression pattern of PDGFRβ was associated with good clinical outcome in NB similar to KIT. PDGFRβ has been considered to have oncogenic potential compared to PDGFRα [14].

FLT3 expression was associated with a good clinical outcome of NB in our study. Our results may provide the evidence that neuroectodermal and hematopoietic cells share common regulatory pathways, as previously reported [15]. It was reported that the RET and TRKA pathways collaborate to regulate NB differentiation [16], but RET expression was not associated with TRKA expression or any clinical parameters in present study. We could not find the alternative spliced variant form of TRKA, TRKAIII, which was reported to have the oncogenic potential [20]. We found another new isoform (deletion of exons 7–9) in 6 (25%) of 24 cell lines. Further study is needed to clarify the function of this new isoform.

In conclusion, our data suggest that the loss of expression of several RTKs may be related to disease progression and poor clinical outcome in NB.

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Short communication

Mutations of *GATA1*, *FLT3*, *MLL*-partial tandem duplication, *NRAS*, and *RUNX1* genes are not found in a 7-year-old Down syndrome patient with acute myeloid leukemia (FAB-M2) having a good prognosis

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Abstract The prognosis of leukemia developed in Down syndrome (DS) patients has improved markedly. Most DS leukemia occurs before 3 years of age and is classified as acute megakaryocytic leukemia (AMKL). Mutations in the *GATA1* gene have been found in almost all DS patients with AMKL. In contrast, it has been shown that occurrence of DS acute myeloid leukemia (DS-AML) after 3 years of age may indicate a higher risk for a poor prognosis, but its frequency is very low. Age is one of the significant prognostic indicators in DS-AML. The prognostic factor of gene alterations has not been reported in older DS-AML patients. We here describe the case of a 7-year-old DS boy with AML-M2, who had no history of transient abnormal myelopoiesis or any clinical poor prognostic factors, such as high white blood cell counts or extramedullary infiltration. We molecularly analyzed the *GATA1*, *FLT3*, *MLL*-partial tandem duplication, *NRAS*, and *RUNX1* (previously *AML1*) genes and did not detect any alterations. The patient has lived for more than 5 years after treatment on the AML99-Down protocol in Japan. This suggests that a patient lacking these genes alterations might belong to a subgroup of older DS-AML patients with good prognosis. Accumulation of more data on older pediatric DS-AML patients is needed.

1. Introduction

Children with Down syndrome (DS) have a ~20-fold higher incidence of leukemia than do unaffected children. Most DS leukemia is diagnosed as acute megakaryocytic leukemia (AMKL), which occurs before 3 years of age, and the prognosis has markedly improved [1–3]. Infants with DS and transient abnormal myelopoiesis are at high risk for later development of AMKL, usually by 3 years of age. Recently, it has been reported that mutations of *GATA1* are present in virtually all cases of DS acute myeloid leukemia (DS-AML) [4,5]. The same mutations are seen in transient abnormal myelopoiesis cases as well [5]. Furthermore, in paired samples from transient abnormal myelopoiesis and AMKL in the same children, identical *GATA1* mutations were found [4–6], suggesting that DS with transient abnormal myelopoiesis and AMKL are within a biologically homogeneous group. *GATA1* mutation is a very early event in the development of DS-AMKL and in the process of multistep leukemogenesis [4,7].

On the other hand, DS-AML occurring after the age of 3 years may be completely different from that occurring before the age of 3 years, and may instead be biologically similar to de novo AML in non-DS patients. Multivariate analysis of data showed that children with DS aged ≥2 years at diagnosis had an increased risk of relapse after treatment [2]. There has been no good classification of DS-AML patients between the age of 2 and 4 years. Classification of the biological differences would probably be more useful than a better age cut.

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Here we describe the case of a 7-year-old boy with DS-AML who lacked mutations of *GATA1*, *FLT3*, *MLL*-partial tandem duplication (PTD), *NRAS*, and *RUNX1* (previously *AML1*) genes. The prognostic factors for DS-AML, particularly in older children, are still unknown. The present case supports the hypothesis that DS-AML patients who do not have alteration of these genes have a good prognosis.

2. Case report

A 7-year-old boy with DS presenting with a persistent fever was admitted to our hospital because of anemia and thrombocytopenia. On admission, he had a pale face and systemic petechiae and purpuras. No cervical lymphadenopathy or hepatomegaly was noted. Blood testing revealed a white blood cell count of 7,500/μL with 9% myeloblasts, 8% segmented neutrophils, 15% monocytes, 49% lymphocytes, and 6% blasts, a hemoglobin concentration of 6.1 g/dL, and a platelet count of 41.2×10^4/μL. Bone marrow examination revealed 66% blasts (Fig. 1a) with 39.2% monocytoid blasts and 18.8% myeloblastic cells with Auer bodies (Fig. 1b) and azurophilic granules. The diagnosis of AML-M2 was made according to the morphological and immunophenotypic criteria of the French–American–British (FAB) classification in combination with other laboratory data.

Even though the differential count showed a predominance of monocytic cells, myeloblasts (15.2%) and myeloblastic cells (18.8%) were 34% of total. These cells were positive for peroxidase staining (73.5%), and both nonspecific (5.8%) and specific (55%) esterase staining. Nonspecific esterase-positive cells were <20% among blasts, which matches the criteria of FAB-M2. Immunophenotypic analysis of CD45+ cells showed the presence of CD13 (56.8%), CD33 (86%), CD38 (95.2%), and HLA-DR (26.7%) antigens and the absence of CD34 (2.7%), CD11b (11.7%), and CD14 (0.6%). CD11b and CD14 presented on monocytes were negative in this patient. Cytogenetic analysis demonstrated the 47,XY,+21c karyotype in 20 bone marrow cells.

The serum and urine lysozyme level has been used as an aid in distinguishing AML with maturation (FAB-M2) from acute myelomonocytic leukemia (M4). In this patient, the count of monocytes in peripheral blood was 1,125/μL, which is less than the 5,000/μL of the FAB-M2 criteria. The serum lysozyme level was 25 μg/mL (normal range, 5–10 μg/mL) and the urine lysozyme level was 0 μg/mL. The level of lysozyme of this patient in peripheral blood was less than threefold of the normal range. Collectively, these data led us to diagnose this patient with AML-M2.

The patient was treated on the Japanese Childhood AML Cooperative Study Group Protocol for DS patients (AML99-Down protocol), which consists of pirarubicin (THP-ADR) (25 mg/m^2 on days 1 and 2), etoposide (150 mg/m^2 on days 3–5), and cytosine arabinoside (Ara-C) (100 mg/m^2 on days 1–7) at five cycles every month [8,9]. No prophylaxis for the central nervous system was performed.

On the first cycle of chemotherapy, he had severe mucositis and high fever for 5 weeks. On the second cycle, he had high fever during therapy. We considered this fever a side effect of Ara-C, and therefore methylprednisolone was given for 30 minutes prior to drip infusion of Ara-C. The patient obtained complete remission after the first cycle of chemotherapy and has continued in complete remission for 5 years without any reoccurrence.

3. Analysis of *GATA1*, *FLT3*, *MLL*, *NRAS*, and *RUNX1* genes

Written informed consent was obtained from the parents of the patient. RNA extracted from his bone marrow cells at

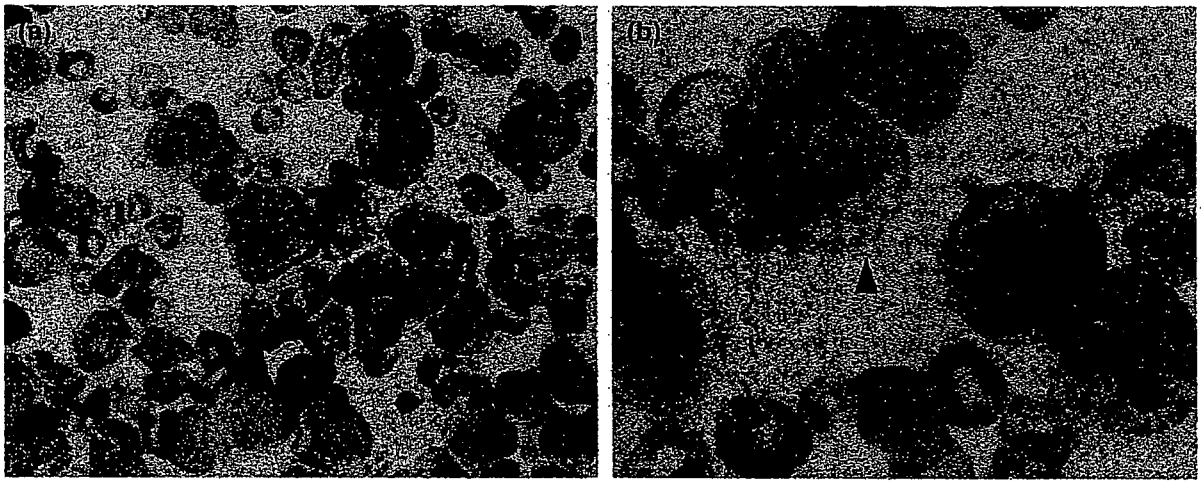

Fig. 1. Initial bone marrow smear at diagnosis. (a) Bone marrow aspirate showing hypercellularity (Giemsa staining). (b) Leukemic cells with Auer bodies (arrowhead).

diagnosis was reverse transcribed to cDNA and alterations of *GATA1*, *FLT3*, *MLL*-PTD, *NRAS*, and *RUNX1* genes were examined as previously described [10–13]. Briefly, mutational analysis of *GATA1* within exon 2, where there are hot spots, was performed with reverse transcription-polymerase chain reaction (RT-PCR) followed by direct sequencing [11]. Point mutations of *FLT3*-D835/I836 were examined with restriction fragment length polymorphism (RFLP)-PCR [12] and *FLT3*-internal tandem duplication (ITD) was analyzed with RT-PCR [11,13]. *MLL*-PTD was examined with simple first-round RT-PCR using the primer pair located between exon 9 and exon 4 [14]. Mutation of *NRAS* and *RUNX1* genes was examined with PCR-single strand conformation polymorphism analysis (SSCP) and direct sequencing [15].

4. Discussion

Lange et al. [16] studied 1,206 children with AML, including 118 (9.8%) DS patients. Among these, >95% of AML patients with DS were <5 years old. FAB-M7 (AMKL) was found in 62%, and FAB-M1or M2 in 10%. Children under 2 years (n = 94) treated on Children's Cancer Group (CCG) studies 2861 and 2891 had a 6-year EFS of 86%; those aged 2–4 years (n = 58), 70%; and those older than 4 years (n = 9), 28%. Outcome of children with DS-AML is excellent with standard induction therapy, but declines with increasing age; this report, however, gives no information about patients >4 years old [16].

Although white blood cell count at diagnosis is a significant predictor of outcome in non-DS AML, this is not the case for either DS or antecedent myelodysplastic syndrome patients. Extramedullary infiltration, which includes tumor nodules, skin infiltration, meningeal infiltration, gingival infiltration, or hepatosplenomegaly, has been discussed as a prognostic factor and is generally thought to indicate poor outcome in non-DS AML [8].

Monosomy 7 (−7) or deletion of the long arm of chromosome 7 [del (7q)] is found in only 4–5% of pediatric patients with AML. Although, cytogenetically, −7 is generally associated with a dismal prognosis in AML, even this may not be as unfavorable in those with DS [17]. Our patient did not have an acquired chromosomal abnormality in addition to trisomy 21 at diagnosis. Having no additional chromosomal abnormalities, including absence of −7, might be one of the good prognostic factors.

Our patient had no prior history of transient abnormal myelopoiesis or of the *GATA1* mutation in leukemic cells. In this respect, the leukemogenesis of this patient may differ from that typical of DS-AMKL patients <3 years old. DS-AMKL patients >3 years old at diagnosis often show the absence of a prior history of transient abnormal myelopoiesis. An age of >3 years at diagnosis may indicate only a different biological origin from those with a prior history of transient abnormal myelopoiesis and the *GATA1* mutation. In other words, there may be age-related biologic differences in the nature of AML in DS patients. We suggest that a better way to predict their prognosis would be by analyzing for the presence or absence of *GATA1* mutations and screening for the groups with good prognosis, rather than by the age at diagnosis, because the *GATA1* mutations are tightly associated with AMKL in DS patients, who are mostly younger children and have a good prognosis [1].

There is little clinical and genetic information on older pediatric patients with DS-AML with a poor prognosis. AML-M7 with *GATA1* mutations has a good prognosis among DS patients. This patient was 7 years old and his prognosis was good, suggesting that leukemogenesis in this case was not due to *GATA1* mutation.

DS-AML in older pediatric patients is considered to be similar to de novo non-DS AML. We therefore analyzed the same genetic prognostic factors in this patient as have been reported in de novo pediatric AML. There are no large studies of the genetic prognostic factors associated with older pediatric DS-AML, however, which made it difficult to compare the incidence of those mutations between non-DS AML and DS-AML among children. Particularly for older children with DS-AML, more accumulation of data is needed.

We examined ITD and D835/I836 mutations of *FLT3*. The prevalence and prognostic significance of these features are unknown in DS-AML. *FLT3*-ITD occurs in ~30% of adult AML patients and ~20% of pediatric AML patients [18–21]. *FLT3*-ITD is considered to predict poor prognosis in adult and pediatric AML patients [19,22–24]. On the other hand, ~10% of adult and pediatric AML patients have *FLT3*-D835/I836 mutations. AML patients with *FLT3*-D835/I836 mutations tend to have a poor prognosis as adults, but not as children [25,26]. Alterations of *FLT3* were not detected in the present patient. Given that this case was considered to be the same as de novo AML in a non-DS patient, the absence of *FLT3* alterations suggests a good prognosis.

We analyzed other possible prognostic factors, such as *MLL*-PTD, *NRAS*, and *RUNX1* mutations. *MLL*-PTD was detected in ~10% of AML patients with normal karyotype and in 90% of AML patients exhibiting trisomy 11 as the sole chromosome abnormality. The *MLL*-PTD was reported to be a subgroup of patients with an unfavorable prognosis in adult AML [14]. In a study of the Japanese Childhood AML Cooperative study group, AML patients with *MLL*-PTD comprised 13.3% and correlated with poor prognosis [21]. The prognostic impact of *NRAS* mutations, reported in 11–30% of AML patients, is still under discussion [27,28]. As for *RUNX1* mutation, we have reported that the mutations in pediatric hematologic malignancies are infrequent, but may be related to AML-M0, acquired trisomy 21, and leukemic transformation [10]. Furthermore, non-constitutional chromosome 21 in the leukemic clone may also lead to an unfavorable prognosis. No mutations of these genes were found in our patient, suggesting a good prognosis.

Table 1
Frequency of Down syndrome acute myeloid leukemia and myelodysplastic syndrome patients in published studies, including pediatric patients older than 4 years

Study group	Accrual period, mo/yr	DS-AML/AML patients, no./no. (%)	DS-AML patients >4 yr old, no.	References
POG8498	July 1984–July 1989	12/285 (4.2)	0	Ravindranath et al., 1992 [29]
Nagoya	Sept. 1986–Aug. 1992	9/NI	0	Kojima et al., 2000 [1]
NOPHO84/NOPHO88	July 1984–Dec. 1992	23/223 (10.3)	2	Lie et al., 1996 [30]
BFM 87/BFM 93	July 1987–Dec. 1994	40/633 (6.3)	3	Creutzig et al., 1996 [31]
CCG 2861/2891	Mar. 1988–Oct. 1995	118/1206 (9.8)	3	Lange et al., 1998 [16]
Japan AT group/Down	Sept. 1987–Aug. 1997	33/NI	0	Kojima et al., 2000 [1]
CCG 2891	Oct. 1989–Oct. 1999	161/1108 (14.5)	9[a]	Gamis et al., 2003 [2]
AML99	Jan. 2000–Dec. /2003	66/418 (15.8)	2	Kobayashi et al., 2006 [8]

Abbreviations: DS-AML/MDS, Down syndrome acute myeloid leukemia and myelodysplastic syndrome; NI, no information.
[a] Nine patients are older than 5 years; data are shown separately for patients aged 2–5 years and older than 5 years.

Table 1 presents the frequency of DS-AML/MDS in children >4 years old from previous reports [1,2,8,16,29–31]. In BFM 87/BFM93, there were three such patients among 40 patients with DS-AML [31]. These three patients were 12, 15, and 16 years old at diagnosis, their FAB classification was M0, M2, and M4, and their white blood cell count at diagnosis was 2,600/μL, 22,600/μL, and 1,400/μL, respectively. The 12-year-old girl died from sepsis after four weeks of consolidation therapy; the other two patients were not treated [31]. In the CCG-2861 and CCG-2891 studies, three patients were reported to be >5 years old [16], two of whom died of disease and one from toxicity. On the AML99-Down protocol, there were two patients >4 years old (one being the present patient) [8]. A 4-year-old boy with AML FAB-M5a who failed to obtain complete remission after two courses of induction therapy and received cord blood stem cell transplantation was, at writing, still alive [32].

To date, there are only a few individual case reports of children >4 years old [32,33]. For DS patients, immunologic disorders, congenital heart disease, and other factors possibly caused disease-related and treatment-related mortality. Considering the high incidence of therapy-related mortality, overtreatment should be avoided.

No alterations in *GATA1*, *FLT3*, *MLL*-PTD, *NRAS*, or *RUNX1* were found in our patient, suggesting that he belongs to a subgroup, among older DS-AML patients, with good prognosis. Because the prognostic factors for DS-AML are still unknown, particularly in older children, further data accumulation is needed.

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