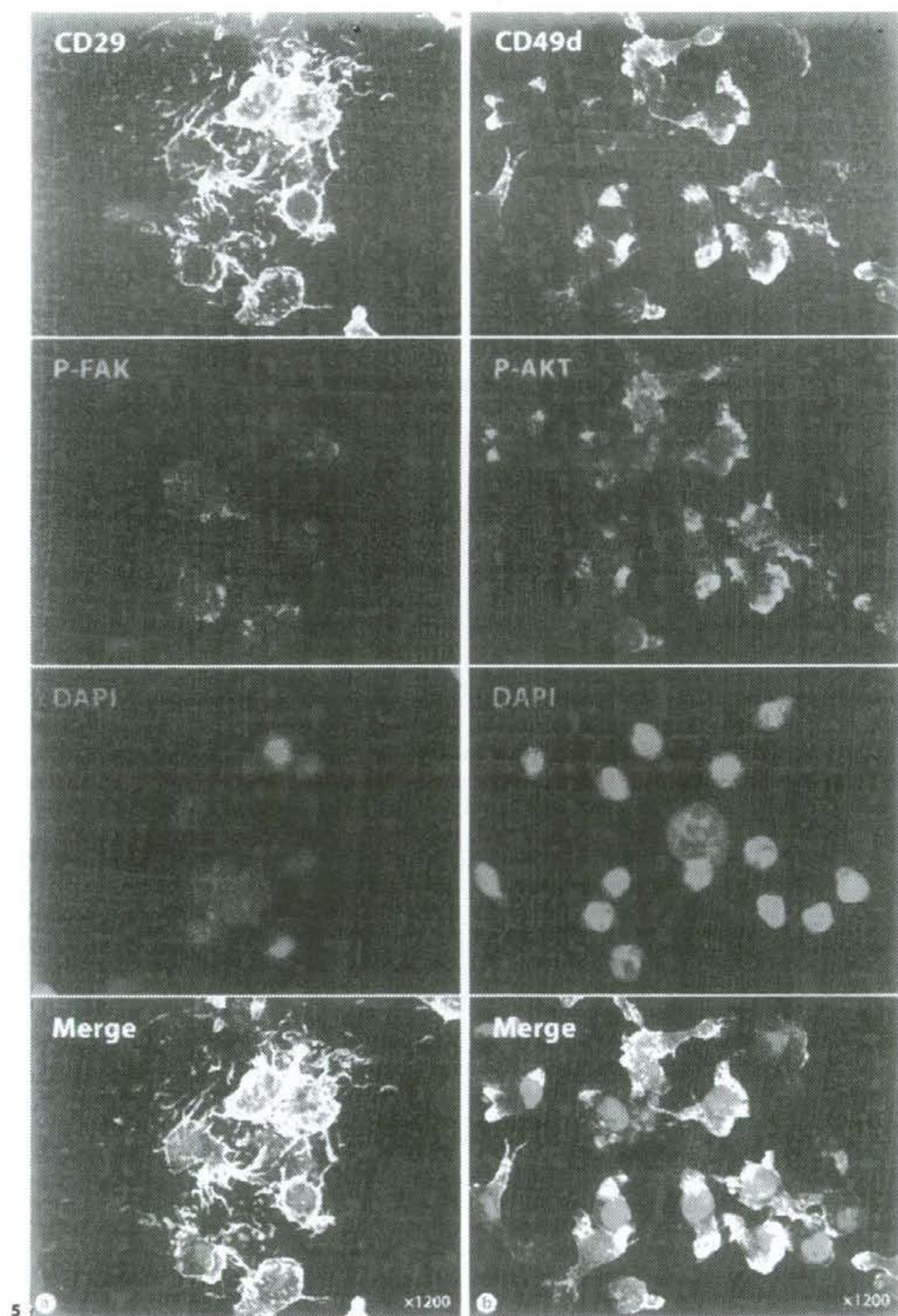

CD29
CD49d
P-FAK
P-AKT
DAPI
DAPI
Merge
Merge
5
×1200
×1200

49d and phosphorylated in hematopoietic cells adhering to osteoblasts, we suspect that cell-to-cell interaction induces activation of integrin-bound kinases, leading to cell survival signals in hematopoietic cells in which AKT is involved. Although CD34+ bone marrow cells were cultured in the presence of 30% of the cultured supernatant of osteoblasts, most cells died over a 4-week culture period (data not shown), suggesting that the soluble factors derived from osteoblasts are not sufficient to support the survival of human CD34+ bone marrow cells, and adhesion to osteoblasts must be important for the survival of hematopoietic cells.

Human osteoblasts have been reported to produce several hematopoietic cytokines, including IL-1β, IL-6, IL-7, G-CSF, M-CSF, GM-CSF, tumor necrosis factor-α, LIF, OPG, receptor activator of NF-κB ligand, SDF-1, VEGF, and osteoclast differentiation factor [1, 2, 10–12], and not to produce IL-1α, IL-3, or SCF [10]. However, in our experiment, human osteoblasts did not produce IL-7, G-CSF, M-CSF, or GM-CSF. Although the precise reason for the discrepancy is not clear, it may be attributable to differences in cell culture conditions or donor age. Alternatively, different subsets or differentiation states related to differential cytokine production may be present among the osteoblasts.

Several cytokines have been shown to contribute to the maintenance, proliferation, and differentiation of HSCs. For example, Flt3-L and SCF play an important role in the early stage of hematopoiesis [13]. An in vivo study has demonstrated that SCF and IL-3 prevent unirradiated hematopoietic progenitors from undergoing apoptosis, and Flt3-L has been demonstrated to induce survival and proliferation of CD34+CD38– cells [14], suggesting the effects of these cytokines on hematopoiesis in vivo to some extent [15], but their effects in vitro, whether alone or in combination, are still a matter of controversy [2]. The results of this study demonstrate that SCF and IL-3, but not Flt3-L, induce proliferation of CD34+ bone marrow cells to some extent in our culture condition. When added to the coculture system of human osteoblasts and human CD34+ bone marrow cells, however, each of them significantly promoted the proliferation of hematopoietic cells. SCF and Flt3-L induced in particular significant growth of hematopoietic cells cultured on osteoblasts. Since our RT-PCR experiments revealed no expression of SCF and IL-3 mRNA in osteoblasts, the major role of osteoblasts in hematopoiesis could be to maintain HSCs as HSCs and therefore the lack of proliferation-inducing cytokines is appropriate for this role. In the context of the microenvironment, other cells should supply these factors to the niche. Alternatively, it is also possible that disaggregated osteoblasts do not produce these factors when they are grown in monocultures but do so in the niche when in the appropriate context.

Fig. 5. Phosphorylation of cell signaling molecules in hematopoietic cells cultured on osteoblasts detected by immunohistochemistry. Human CD34+ bone marrow cells were grown on a primary culture of osteoblasts for 2 weeks and stained with the combination of phospho-specific antibodies and anti-cell adhesion molecule antibodies and examined as in figure 4. **a** CD29 versus phosphorylated FAK; **b** CD49d versus phosphorylated AKT.

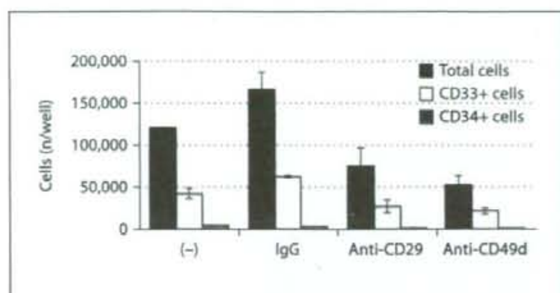
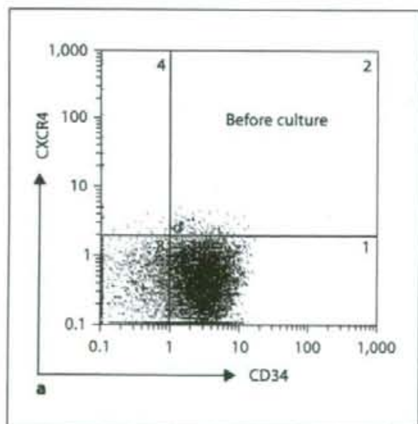


Fig. 6. Effect of anti-integrin antibodies on growth of CD34+ bone marrow cells on human osteoblasts. Human CD34+ bone marrow cells were cultured on osteoblasts for 2 weeks in the presence or absence of antibodies against CD29 or CD49d at a concentration of 5 μg/ml. Following cultivation, hematopoietic cells were collected, counted, and positivity for CD33 and CD34 was determined by flow cytometry (see fig. 3). Purified mouse IgG served as a negative control.

CD184, a receptor for CXC subfamily chemokines, was originally identified as an orphan receptor [16]. It was suggested that CD184 and its sole ligand SDF-1 play an important role in hematopoiesis and are required for homing of stem cells and progenitor cells from the liver to the bone marrow [2, 16–18], but their role at the molecular level remains unknown. Tokoyoda et al. [18] stated that contact between the earliest HSCs and SDF-1-expressing cells is necessary for B lymphopoiesis. In our study, the CD184 expression pattern was dramatically altered by cytokines and the presence of osteoblasts. Although the exact mechanism of action remains to be elucidated, the different expression pattern of CD184 may be related to the different function of hematopoietic cells,

Fig. 7. Expression of CD184 in hematopoietic cells grown on human osteoblasts. Human CD34+ bone marrow cells were cultured for 2 weeks (see fig. 3). Hematopoietic cells were collected and examined by flow cytometry. Two-parameter histograms for CD184 versus CD34 (**a**) or CD33 (**b**) are shown.



e.g. homing. Further investigation to identify the role of CD184 expression in hematopoiesis is now underway.

In conclusion, human osteoblasts have the ability to support the survival and differentiation of human CD34+ bone marrow cells. Addition of cytokines to this culture system stimulates human CD34+ bone marrow cells to differentiate into various blood cells. Osteoblasts provide a useful in vitro model of the hematopoietic microenvironment, and further studies are required to elucidate the role of the microenvironment in early hematopoiesis.

Acknowledgments

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CANCER GENETICS AND CYTOGENETICS

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

Machiko Kawamura[a,*], Hidefumi Kaku[a], Takeshi Taketani[b], Tomohiko Taki[c], Akira Shimada[d], Yasuhide Hayashi[d]

[a]Department of Pediatrics, Tokyo Metropolitan Komagome Hospital, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8677, Japan
[b]Department of Pediatrics, Shimane University, Faculty of Medicine, Izumo, Shimane, Japan
[c]Department of Molecular Laboratory Medicine, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan
[d]Department of Hematology/Oncology, Gunma Children's Medical Center, Shibukawa, Gunma, Japan

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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. © 2008 Elsevier Inc. All rights reserved.

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.

* Corresponding author. Tel.: +81-3-3823-2101; fax: +81-3-3824-1552.

E-mail address: m.kawamura@cick.jp (M. Kawamura).

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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

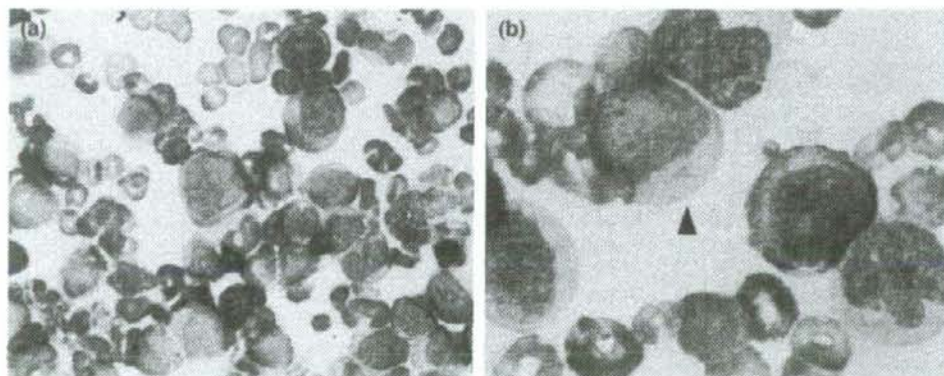

Fig. 1. Initial bone marrow smear at diagnosis. (a) Bone marrow aspirate showing hypercellularity (Giemsa staining). (b) Leukemic cells with Auer bodies (arrowhead).

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, nonconstitutional 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.

Acknowledgments

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Fbxw7 acts as a critical fail-safe against premature loss of hematopoietic stem cells and development of T-ALL

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Fbxw7 acts as a critical fail-safe against premature loss of hematopoietic stem cells and development of T-ALL

Sahoko Matsuoka,[1,2] Yuichi Oike,[1,3,4,13] Ichiro Onoyama,[5] Atsushi Iwama,[6,7] Fumio Arai,[1] Keiyo Takubo,[1] Yoichi Mashimo,[8] Hideyuki Oguro,[7] Eriko Nitta,[1] Keisuke Ito,[1,2] Kana Miyamoto,[1] Hiroki Yoshiwara,[1] Kentaro Hosokawa,[1] Yuka Nakamura,[1] Yumiko Gomei,[1] Hiroko Iwasaki,[1] Yasuhide Hayashi,[9] Yumi Matsuzaki,[10] Keiko Nakayama,[11] Yasuo Ikeda,[2] Akira Hata,[7] Shigeru Chiba,[12] Keiichi I. Nakayama,[5] and Toshio Suda[1,6,14]

[1]Department of Cell Differentiation, The Sakaguchi Laboratory, School of Medicine, Keio University, Tokyo 160-8582, Japan; [2]Department of Internal Medicine, School of Medicine, Keio University, Tokyo 160-8582, Japan; [3]PRESTO, Japan Science Technology Agency (JST), Saitama 322-0012, Japan; [4]Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan; [5]Department of Molecular and Cellular Biology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan; [6]CREST, Japan Science Technology Agency (JST), Saitama 322-0012, Japan; [7]Department of Cellular and Molecular Medicine, Chiba University, Chiba 260-8670, Japan; [8]Department of Public Health, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan; [9]Department of Hematology/Oncology, Gunma Children's Medical Center, Gunma 377-8577, Japan; [10]Department of Physiology, School of Medicine, Keio University, Tokyo 160-8582, Japan; [11]Department of Developmental Biology, Graduate School of Medicine, Tohoku University, Sendai 980-8575, Japan; [12]Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Tokyo 113-8655, Japan

Common molecular machineries between hematopoietic stem cell (HSC) maintenance and leukemia prevention have been highlighted. The tumor suppressor Fbxw7 (F-box and WD-40 domain protein 7), a subunit of an SCF-type ubiquitin ligase complex, induces the degradation of positive regulators of the cell cycle. We demonstrate that inactivation of *Fbxw7* in hematopoietic cells causes premature depletion of HSCs due to active cell cycling and p53-dependent apoptosis. Interestingly, *Fbxw7* deletion also confers a selective advantage to cells with suppressed p53 function, eventually leading to development of T-cell acute lymphoblastic leukemia (T-ALL). Thus, Fbxw7 functions as a fail-safe mechanism against both premature HSC loss and leukemogenesis.

[*Keywords*: Fbxw7; c-Myc; Notch1; p53; hematopoiesis; T-ALL]
Corresponding authors.
[13]E-MAIL oike@gpo.kumamoto-u.ac.jp; FAX 81-96-373-5145.
[14]E-MAIL sudato@sc.itc.keio.ac.jp; FAX 81-3-5363-3474.
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Stem cells in various organ tissues are governed by general genetic programs that maintain their common features, including self-renewal and multipotency. Recent studies have demonstrated that it is crucial for hematopoietic stem cells (HSCs) to be quiescent for protection against oxidative stress and to sustain self-renewal capacity (Ito et al. 2004; Tothova et al. 2007). Most HSCs remain quiescent when located in the stem cell niche (Calvi et al. 2003; Zhang et al. 2003; Arai et al. 2004). Once HSCs are released from the niche, they enter the cell cycle and start to proliferate. Cell cycle kinetics of HSCs are strictly controlled by various systems to sustain blood cell production throughout life (Cheng et al. 2000). These findings indicate that precise regulation of the cell cycle in stem cells is essential to maintain stem cell phenotype. The ubiquitin–proteasome system plays a critical role in controlling physiologic events—such as cell cycle progression, apoptosis, signal transmission, and repair of DNA damage—through protein degradation (Fuchs 2005; Minella and Clurman 2005; Welchman et al. 2005; Nakayama and Nakayama 2006). Fbxw7 (F-box and WD-40 domain protein 7)—also known as Fbw7, Sel-10, hCdc4, or hAgo—is an SCF ubiquitin ligase component catalyzing ubiquitination of Myc, cyclin E, Notch, and c-Jun, all of which positively regulate the cell cycle. We hypothesized that a protein like Fbxw7 may play a pivotal role in controlling the HSC cell cycle and maintaining normal hematopoiesis. Tetzlaff et al. (2004) and we (Tsunematsu et al. 2004) independently reported that *Fbxw7*-deficient mice die at embryonic day 10.5 and exhibit deficiencies in hematopoietic and vascular development, suggesting that Fbxw7 functions in hematopoiesis. Mutations in *FBXW7* have been detected in certain human malignancies, including T-cell acute lymphoblastic leukemia (T-ALL) (Spruck et al. 2002; Ekholm-Reed et al. 2004; Mao et al. 2004; Maser et al. 2007). Recent reports have highlighted the existence of molecules operating differentially in the self-renewal of both normal tissue stem cells and cancer stem cells (Yilmaz et al. 2006; Zhang et al. 2006). In this study, we addressed this issue by examining the effect of *Fbxw7* deletion on the maintenance of HSCs and leukemogenesis. Here, we demonstrate that inactivation of *Fbxw7* in bone marrow (BM) HSCs leads to premature depletion of normal HSCs due to active cell cycling and promotes T-ALL due to a compromised p53 response. Thus, Fbxw7 acts as a critical fail-safe against premature loss of HSCs and development of T-ALL (Supplemental Fig. S1).

Results and Discussion

We examined *Fbxw7* expression by quantitative RT–PCR in various hematopoietic lineages sorted from adult mouse tissues. *Fbxw7* was expressed abundantly in most of the hematopoietic cells tested, including Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) CD34$^-$ HSCs (Supplemental Fig. S2).

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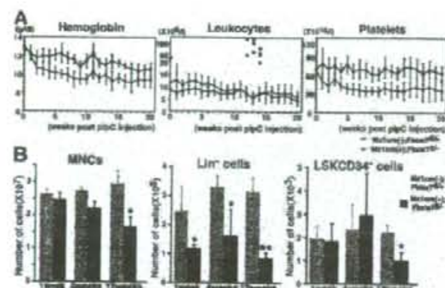

Figure 1. Fbxw7 is essential to maintain the adult hematopoietic pool. (*A*) Peripheral blood cell counts of Fbxw7-deficient (red closed circles, $n = 15$) and control (black closed circles, $n = 15$) mice after pIpC treatment. Results are shown as means ± SD. Red open circles indicate white blood cell counts of mice developing leukemia. (*B*) Absolute numbers of MNCs, Lin⁻ cells, and LSK CD34⁻ HSCs in *Fbxw7*-deficient and control BM at 4, 8, and 12 wk after pIpC treatment. Results are shown as means ± SD from six to eight independent experiments. (*) $P < 0.05$; (**) $P < 0.01$.

To investigate the role of Fbxw7 in adult tissues, we generated $Fbxw7^{fl/fl}$ mice in which *Fbxw7* was deleted conditionally in targeted cells (Onoyama et al. 2007). In this study, we created *Mx-1-Cre(+);*$Fbxw7^{fl/-}$ mice by crossing *Mx-1-Cre(+);*$Fbxw7^{+/-}$ mice and $Fbxw7^{fl/fl}$ mice in order to investigate the role of Fbxw7 in hematopoietic cells. pIpC was injected into the 8-wk-old *Mx-1-Cre(+);*$Fbxw7^{fl/-}$ mice every other day for 1 wk to induce *Cre* expression and thereby delete *Fbxw7* in their hematopoietic cells. *Mx-1-Cre(–);*$Fbxw7^{fl/+}$ littermates treated with pIpC served as controls. *Mx-1-Cre(–);*$Fbxw7^{fl/-}$ and *Mx-1-Cre(+);*$Fbxw7^{fl/+}$ littermates showed no significant difference from control mice in our analysis (data not shown). We confirmed that *Fbxw7* deletion was induced in BM cells as early as 3 d after pIpC treatment (Supplemental Fig. S3A).

We analyzed peripheral blood cell counts of *Fbxw7*-deficient mice. Levels of hemoglobin and platelets were markedly lower compared with controls immediately after pIpC treatment (Fig. 1A). However, these levels stopped decreasing ~12–16 wk after pIpC treatment. Genomic PCR analysis detected a significant amount of unexcised floxed allele in *Fbxw7*-deficient hematopoietic cells 16 wk after pIpC treatment (Supplemental Fig. S3B). These data suggest that hematopoiesis was compromised in the absence of Fbxw7 after pIpC treatment, whereas the few cells that retained the unexcised *Fbxw7* allele gradually competed out the *Fbxw7*-deficient hematopoietic cells.

The number of the Lin⁻ cells was also reduced immediately in *Fbxw7*-deficient BM after pIpC treatment. In contrast, the number of BM mononuclear cells (MNCs) and LSK CD34⁻ HSCs remained within normal range at the beginning of treatment but was decreased significantly by 12 wk of pIpC treatment (Fig. 1B). The size of the thymus was also reduced significantly in *Fbxw7*-deficient mice (data not shown). To determine whether Fbxw7 is essential for HSC differentiation, we examined the proportion of differentiated cells in *Fbxw7*-deficient BM by flow cytometry. Although a portion of lymphoid cells was decreased slightly, populations of cells sufficient to generate both mature myeloid and lymphoid cells were observed in *Fbxw7*-deficient BM 4 wk after pIpC treatment (Supplemental Fig. S4A). Moreover, in vitro assays revealed that colony-forming capacity was comparable between control and *Fbxw7*-deficient LSK cells (Supplemental Fig. S4B). Morphological analysis of colonies demonstrated the full range of differentiation capacity in *Fbxw7*-deficient LSK cells along the myeloid lineage (data not shown). These data indicate that Fbxw7 is dispensable for multilineage terminal differentiation.

To examine the repopulating capability of *Fbxw7*-deficient HSCs, we transplanted 1500 LSK BM cells from *Fbxw7*-deficient mice or littermate controls 4 wk after pIpC treatment into lethally irradiated recipients using 4×10^5 normal BM mononuclear competitor cells. Although there was no significant difference in the proportion of LSK CD34⁻ HSCs within injected LSK cells, *Fbxw7*-deficient LSK cells showed severely impaired repopulating capacity (Fig. 2A, left). To confirm whether Fbxw7 intrinsically regulates HSC repopulating capability, we transplanted 4×10^5 BM MNCs from either *Mx-1-Cre(+);*$Fbxw7^{fl/-}$ mice before pIpC treatment or littermate controls into lethally irradiated recipients with the same numbers of competitor cells. Two months later, we confirmed that donor cells were reconstituted in recipient BM and then injected the recipient mice with pIpC. Within 1 mo following pIpC treatment, *Fbxw7*-deficient HSCs lost long-term repopulating capability and were eventually competed out by wild-type HSCs (Fig. 2A, right).

It has been reported that most HSCs remain quiescent and that excessive acceleration of the HSC cell cycle

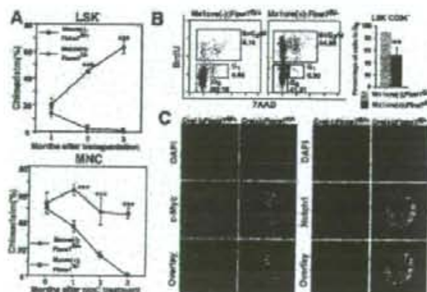

Figure 2. Reconstitution capacity and quiescence are impaired in *Fbxw7*-deficient HSCs. (*A*) Competitive repopulation analysis. (*Left*) Recipient Ly5.1 mice ($n = 6$) were transplanted with 1500 *Fbxw7*-deficient or control LSK cells together with 4×10^5 Ly5.1 × Ly5.2 competitor BM MNCs. Donor-derived chimerism of peripheral white blood cells was analyzed monthly after transplantation. (*Right*) Recipient Ly5.1 mice ($n = 10$) were transplanted with 4×10^5 BM MNCs from *Mx-1-Cre(+);*$Fbxw7^{fl/-}$ mice before pIpC treatment or with controls together with the same number of Ly5.1 × Ly5.2 competitor BM MNCs. pIpC treatment of recipient mice was performed 2 mo after transplantation. Donor-derived chimerism of peripheral white blood cells was then analyzed monthly after pIpC treatment. Results are shown as means ± SD. (*B*) Cell cycle status of LSK CD34⁻ cells of *Fbxw7*-deficient or control mice. BrdU was administered for 3 d to mark cells that entered S phase, and 7-aminoactinomycin D (7-AAD) was administered to detect DNA content. Data shown are representative FACS patterns derived from three independent experiments (*left*) and graphs showing the mean percentage of cells in G_0 (*right*). (*C*) LSK cells isolated from *Fbxw7*-deficient or control mice were stained with DAPI (blue) and anti-c-Myc antibody or anti-Notch1 antibody (green).

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leads to their exhaustion (Hock et al. 2004; Ito et al. 2004). Since Fbxw7 functions as a master regulator of the cell cycle by regulating protein levels of cell cycle-related molecules, we hypothesized that a dysregulated HSC cell cycle leads to a decrease in HSC number in *Fbxw7*-deficient mice. To address this possibility, we examined the cell cycle status of *Fbxw7*-deficient HSCs using a BrdU assay. The population of *Fbxw7*-deficient LSK $CD34^-$ HSCs in G_0 was decreased significantly compared with controls (Fig. 2B). We also monitored proliferation of single LSK $CD34^-$ HSCs in culture for 7 d. The frequency of cell division seen in *Fbxw7*-deficient HSCs was markedly increased in culture (data not shown). These data suggest that Fbxw7 functions to maintain HSC quiescence. It has been reported that c-Myc, an Fbxw7 substrate, promotes re-entry of quiescent HSCs into the cell cycle by inducing release of HSCs from the stem cell niche, leading to loss of self-renewal activity at the expense of differentiation (Wilson et al. 2004). c-Myc protein significantly accumulated in *Fbxw7*-deficient LSK cells (Fig. 2C), suggesting that c-Myc-induced active cell cycling of *Fbxw7*-deficient HSCs largely accounts for premature loss of HSCs. This hypothesis would be strongly supported by the observation that inactivation of c-Myc rescued the phenotype of hyperproliferation of DP T cells and the occurrence of lymphoma in *Fbxw7*-deficient thymocytes (Onoyama et al. 2007). In addition, Notch1 protein, which also accumulated in *Fbxw7*-deficient LSK cells, might accelerate HSC differentiation (Fig. 2C; Pui et al. 1999). The levels of c-Jun, another Fbxw7 target, in *Fbxw7*-deficient LSK cells were equivalent to those in the control cells, suggesting that c-Jun did not contribute to the hematopoietic abnormalities in *Fbxw7*-deficient mice (Supplemental Fig. S5). To clarify the time course of activation of these target genes, we performed immunocytochemical staining for c-Myc and Notch at 0, 72, and 96 h after a single injection of pIpC (700 μg) (Supplemental Fig. S6). Nuclear accumulation of c-Myc began within 72 h, and reached plateau by 96 h after pIpC treatment. On the other hand, marked accumulation of Notch1 in nuclei occurred within 72 h after pIpC treatment. These observations indicated that ubiquitin–proteasome-dependent degradation of Notch1 and c-Myc was inhibited rapidly after *Fbxw7* deletion by an initial single pIpC treatment. Interestingly, the accumulation of Notch1 reached plateau slightly earlier than that of c-Myc. It has been found recently that Notch1 directly regulates c-Myc transcription in normal and leukemic T cells (Weng et al. 2006). These studies suggest that the up-regulation of c-Myc expression in *Fbxw7*-deficient HSCs might be enhanced subsequently by Notch1 at the transcriptional level to some extent, in addition to increased protein stability in the absence of Fbxw7.

About 30% of *Fbxw7*-BM-deficient mice showed extremely severe pancytopenia 12 wk after pIpC treatment (Fig. 3A). These mice showed a marked decrease in the number of BM MNCs, including all lineages of hematopoietic cells. It has been suggested that enhanced c-Myc expression likely activates a p53-dependent checkpoint and induces apoptosis, thereby protecting cells from hyperproliferative oncogenic signals (Zindy et al. 1998). Actually, p53 protein was markedly accumulated in ~80% of *Fbxw7*-deficient LSK cells (Supplemental Fig. S7), while there was no difference in p53 mRNA levels between *Fbxw7*-deficient HSCs and the controls (Supplemental Fig. S8). Instead, Mdm2 expression was markedly down-regulated in *Fbxw7*-deficient HSCs at both the mRNA and protein levels (Supplemental Figs. S7, S8). Since it is believed that Mdm2 suppresses p53 function by promoting protein degradation via its E3 ligase activity, p53 may be up-regulated in *Fbxw7*-deficient HSCs, at least partly, through Mdm2 inactivation, although the mechanism underlying reduced Mdm2 protein levels remains elusive. We therefore hypothesized that the drastic reduction of hematopoietic cell number in these mice was caused not only by the dysregulated cell cycle but also by p53-dependent apoptosis. Indeed, the population of apoptotic cells in BM MNCs and HSCs increased substantially in mice with leukopenia compared with those without leukopenia (Fig. 3B). We next asked whether there is a correlation between apoptosis and p53 expression levels in *Fbxw7*-deficient BM cells. p53 protein levels were up-regulated in BM cells derived from all *Fbxw7*-deficient mice 4 wk after pIpC treatment (Fig. 3C, left). In contrast, p53 protein levels were decreased in BM MNCs of *Fbxw7*-deficient mice that showed no leukopenia at 12 wk after pIpC treatment (Fig. 3C, right). These findings suggest that the variations in white blood cell count data could be attributable to the differential modes of p53-dependent checkpoint responses, which induce apoptosis. This notion was further supported by the absence of abnormal ploidy, which could lead to apoptotic cell death, in *Fbxw7*-deficient BM cells (data not shown). It is well recognized that senescence as well as apoptosis is a tumorigenesis barrier (Collado et al. 2005). However, gene expression of $p16^{Ink4a}$ was not increased in *Fbxw7*-deficient HSCs (Supplemental Fig. S8) and senescence-associated (SA)-β-galactosidase activity was not considerably detected in *Fbxw7*-deficient LSK cells (data not shown), indicating that *Fbxw7* deletion did not promote cellular senescence.

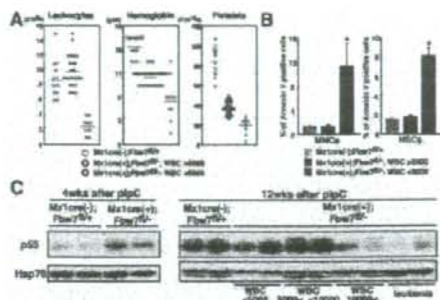

Figure 3. The fate of *Fbxw7*-deficient hematopoietic cells is determined by p53 expression. (*A*) Peripheral blood cell counts of *Fbxw7*-deficient (WBC < 5000, red circle, $n = 24$; WBC > 5000, blue circle, $n = 10$) and control (gray circle, $n = 13$) mice 12 wk after pIpC treatment. Horizontal lines indicate mean values. Closed circles indicate the data for mice that developed leukemia ultimately. (*B*) Percentage of annexin V-positive apoptotic cells in Fbxw7-deficient (WBC < 5000, red bar; WBC > 5000, blue bar) and control (gray bar) BM MNCs (*left*) or LSK $CD34^-$ HSCs (*right*) 12 wk after pIpC treatment. Results are shown as means ± SD from four independent experiments. (*) $P < 0.05$. (*C*) Western blot analysis of p53 in BM MNCs from control and Fbxw7-deficient mice 4 wk (*left*) and 12 wk (*right*) after pIpC treatment. Hsp70 was used as a loading control.

Interestingly, more than half of the *Fbxw7*-deficient mice developed T-ALL (20 of 34; 59%) within 16 wk of

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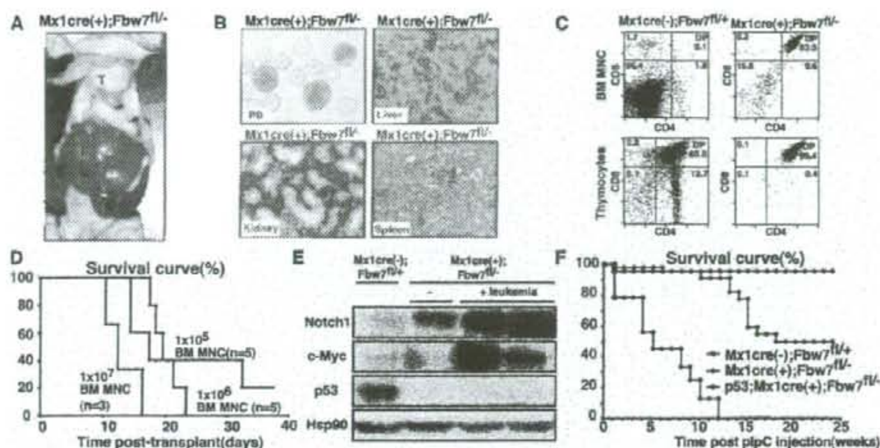

Figure 4. Fbxw7 deficiency in adult hematopoietic cells leads to T-ALL. (*A*) Representative autopsy of leukemic *Fbxw7*-deficient mice. Thymus (T), liver (L), spleen (S), and lymph nodes (arrowheads) were massively swollen. (*B*) Representative histology of peripheral blood (PB), liver, kidney, and spleen from leukemic *Fbxw7*-deficient mice. (*C*) FACS analysis of BM MNCs and thymocytes from leukemic *Fbxw7*-deficient and control mice. Data shown are representative FACS patterns derived from five independent experiments. (*D*) Survival curves for mice injected with graded doses (1×10^5, blue; 1×10^6, red; or 1×10^7 B, green) of leukemic *Fbxw7*-deficient BM MNCs. The average portion of $CD4^+CD8^+$ leukemic blasts in leukemic *Fbxw7*-deficient BM MNCs was 92.4 ± 3.1%. (*E*) Western blot analysis of Notch, c-Myc, and p53 in thymocytes from control, *Fbxw7*-deficient, and leukemic *Fbxw7*-deficient mice. (*F*) Survival curves for *Fbxw7*-deficient (red, $n = 23$), $p53^{-/-}$;*Fbxw7*-deficient (blue, $n = 9$), and control (black, $n = 25$) mice after pIpC treatment.

pIpC treatment (Fig. 4A). In the leukemic mice, lymphoid blasts aggressively invaded BM, liver, spleen, thymus, and kidney (Fig. 4B). Flow cytometry analysis showed that blasts expressed both CD4 and CD8 (Fig. 4C). Furthermore, irradiated mice transplanted with these leukemia cells died of T-ALL within 1 mo (Fig. 4D), indicating *Fbxw7* deletion promoted generation of T-ALL-initiating cells. Most of the *Fbxw7*-deficient mice that did not exhibit leukopenia at 12 wk after pIpC developed T-ALL (20 of 24; 83%) within 16 wk of pIpC treatment, while no *Fbxw7*-deficient mice that exhibited leukopenia led to leukemia (Fig. 3A).

Importantly, leukemic cells of *Fbxw7*-deficient mice displayed significant accumulation of Notch1 and c-Myc proteins (Fig. 4E). Interestingly, the Notch1/c-Myc signaling axis is causally linked to T-ALL development (Weng et al. 2006). These findings suggest that accumulated Notch1 and c-Myc proteins in *Fbxw7*-deficient BM cells caused extrathymic development of T-lineage cells and induced T-ALL. Of note was that p53 protein expression was repressed during leukemogenesis (Fig. 4E). *Fbxw7* deficiency in hematopoietic cells caused only T-cell malignancy, presumably due to Notch1 accumulation, which induces T-lineage commitment in immature cells (Look 2004; Grabher et al. 2006). Constitutive Notch1 activation in hematopoietic stem or progenitor cells blocks B-cell differentiation and expands extrathymic DP T cells in BM, eventually leading to development of T-ALL in BM (Pui et al. 1999). In human T-ALL, Notch1 is the most commonly mutated gene (Weng et al. 2004). While *Fbxw7*-deficient mice did not show a block in B-cell differentiation (data not shown), they did exhibit extrathymic development of DP T cells and T-ALL. In this regard, T-ALL development can be attributed largely to an activated Notch pathway in *Fbxw7*-deficient mice. In contrast, deletion of *Fbxw7* in the thymus of *Lck-cre(+);Fbxw7*$^{fl/fl}$ mice (*Fbxw7*-thymus-deficient mice) promotes development of $CD4^+CD8^+$ lymphoblastic thymic lymphoma without BM invasion (Onoyama et al. 2007), suggesting that the developmental stage at which *Fbxw7* deletion occurs defines T-lineage tumor types; i.e., leukemia versus lymphoma.

To determine whether p53 inactivation promotes leukemogenesis in *Fbxw7*-deficient mice, we generated $p53^{-/-}$;*Mx-1-Cre(+);Fbxw7*$^{fl/-}$ mice ($p53^{-/-}$;*Fbxw7*-deficient mice). These mice developed milder anemia and thrombocytopenia than did *Fbxw7*-deficient mice and exhibited considerably greater numbers of peripheral leukocytes after pIpC treatment (Supplemental Fig. S9), indicating that p53-dependent apoptosis contributes to the reduction of *Fbxw7*-deficient BM. All $p53^{-/-}$;*Fbxw7*-deficient mice developed T-cell malignancies with a much shorter latency and died within 12 wk, suggesting that a p53-dependent checkpoint suppresses leukemogenesis in *Fbxw7*-deficient mice (Fig. 4F). During our observation of 25 wk, two of 13 $p53^{-/-}$ mice died of T-cell thymic lymphoma, but none of them developed T-ALL (data not shown). These findings suggest that deletion of *Fbxw7* provides strong selection of hematopoietic cells that harbor suppressed p53 function. Several studies demonstrate that p53 expression or function is suppressed during tumorigenesis by oncogenes including *Notch* and *c-Myc* (Eischen et al. 1999; Beverly et al. 2005). Eischen et al. (1999) have reported that c-Myc activates the $p19^{Arf}$–Mdm2–p53 tumor suppressor pathway, enhancing p53-dependent apoptosis, and strongly selects for subsequently spontaneous p53 inactivation, resulting in canceling its protective checkpoint function and the occurrence of tumorigenesis, such as lymphoma. These findings support the idea that p53 inactivation is

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an essential event in leukemogenesis in *Fbxw7*-deficient hematopoietic cells. Although p19^{Arf}, an upstream p53 regulator, was significantly elevated and Mdm2 was reduced, p53 was significantly inactivated in *Fbxw7*-deficient leukemic cells (Supplemental Fig. S10). p53 exerts negative feedback control on p19^{Arf} through its interactor, CARF (Kamrul et al. 2007). Thus, elevated p19^{Arf} protein levels seen in T-ALL cells further support the fact that p53 function is strongly inhibited during leukemogenesis. Although the precise mechanism underlying reduced p53 protein levels remains elusive, p53 degradation via a proteasome-dependent pathway is likely compromised in the absence of Fbxw7. Loss of Fbxw7 also induces genomic instability and genomic alterations (Rajagopalan et al. 2004), which may also initiate leukemogenesis in *Fbxw7*-deficient mice.

These mouse phenotypes led us to analyze *FBXW7* mutations in human T-ALL patients. Mutation of *FBXW7* in the WD40 domain, a crucial site for protein targeting, was observed in eight of 44 cases, and most mutations (seven of eight; 88%) were heterozygous (Supplemental Table S1), suggesting that FBXW7 loss of function is tightly associated with T-ALL development in humans and that *FBXW7* acts in a haplo-insufficient manner as a tumor suppressor gene in humans as in mice. On the other hand, gain-of-function mutations in *NOTCH1* (N-terminal [HD-N] and C-terminal [HD-C] heterodimerization domains and PEST domains) (Weng et al. 2004; Grabher et al. 2006) were observed in 19 of 44 cases. Interestingly, most T-ALL cases with the *FBXW7* mutation (six of eight; 75%) harbored the *NOTCH1* mutations as well, but which mutation is more primary in these T-ALL cases could not be determined sufficiently in the current study. *c-Myc* is a direct Notch1 target in Notch-dependent T-ALL (Weng et al. 2006). Since NOTCH and c-MYC are FBXW7 targets, NOTCH1-independent c-MYC up-regulation could further contribute to leukemogenesis in T-ALL with both *NOTCH1* and *FBXW7* mutations. Moreover, given the two samples with *FBXW7* mutations had wild-type *NOTCH1* (two of eight; 25%), *FBXW7* mutations do not necessarily require the *NOTCH1* mutations as we presented in a mouse model in this study.

This is the first report of *FBXW7* mutations in Japanese T-ALL patients, representing a quite even population compared with the other races that were reported previously. It has been well established that Notch1 gain-of-function mutation is considered as a primary cause to develop T-ALL, because somatic activating mutations of Notch1 have been identified in >50% of all T-ALL cases and are found in all previously defined T-ALL subtypes (Grabher et al. 2006). On the other hand, our analysis in this study revealed that *FBXW7* single mutation was seen in 4.5% (two of 44) of Japanese T-ALL patients. This frequency is slightly lower than that of T-ALL patients in North America: 6.5% (six of 92) (Thompson et al. 2007) and 6.3% (two of 32) (Maser et al. 2007). These findings indicate that there is a genomic type of *FBXW7* single mutation in T-ALL patients infrequently but universally, suggesting the existence of T-ALL that does not bear a *NOTCH1* mutation. We believe that it is noteworthy that we clearly demonstrated, through our analysis of a murine T-ALL model, the mechanism in which *FBXW7* loss of function could be a primary cause for developing T-ALL similar to a *NOTCH1* gain-of-function mutation.

Recently, an array-based comparative genome hybridization (array-CGH) study indicated that *FBXW7* and *PTEN* are commonly mutated in human T-ALL (Maser et al. 2007). It was reported previously that *Pten* deletion induces abnormal active cell cycling of HSCs, leading to their premature loss. *Pten* deletion also results in generation of leukemia-initiating cells. Inhibition of mTOR by rapamycin not only depletes leukemia-initiating cells but also restores normal HSC function (Yilmaz et al. 2006). Similar to *Pten*, *Fbxw7* has distinct effects on normal stem cells and cancer stem cells within the same tissue (Supplemental Fig. S1). Loss-of-function mutations in these genes in normal HSCs are likely prerequisites for generation of cancer stem cells.

Materials and methods

Mx-1-Cre(+);Fbxw7$^{+/-}$ mice were obtained by mating *Fbxw7*$^{+/-}$ mice (Tsunematsu et al. 2004) with interferon-inducible *Mx-1-Cre* transgenic mice. To generate *Mx-1-Cre(+);Fbxw7*$^{fl/-}$ (Fbxw7-deficient) and *Mx-1-Cre(-);Fbxw7*$^{+/fl}$ (control) mice, *Mx-1-Cre(+);Fbxw7*$^{+/-}$ mice were crossed with *Fbxw7*$^{fl/fl}$ mice (Onoyama et al. 2007). To induce *Cre*, mice received 500 µg of pIpC intraperitoneally on three alternate days. C57BL/6-Ly5.1 congenic mice were purchased from Sankyo-Lab Service and C57BL/6-Ly5.1/Ly5.2 F_1 mice were used for competitive reconstitution assays. Animal care was in accordance with the guidance of Keio University for animal and recombinant DNA experiments. See the Supplemental Material for additional procedures.

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Clinical features and outcome of *MLL* gene rearranged acute lymphoblastic leukemia in infants with additional chromosomal abnormalities other than 11q23 translocation

Hisamichi Tauchi[a], Daisuke Tomizawa[b], Mariko Eguchi[c], Minenori Eguchi-Ishimae[c], Katsuyoshi Koh[d], Masahiro Hirayama[e], Noriko Miyamura[f], Naoko Kinukawa[g], Yasuhide Hayashi[h], Keizo Horibe[i], Eiichi Ishii[a,*]

[a] *Department of Pediatrics, Ehime University, Toon, Ehime, Japan*
[b] *Department of Pediatrics and Developmental Biology, Tokyo Medical and Dental University, Tokyo, Japan*
[c] *Department of Hematology, Dokkyo Medical University, Tochigi, Japan*
[d] *Department of Pediatrics, The University of Tokyo, Tokyo, Japan*
[e] *Department of Pediatrics, Mie University, Tsu, Mie, Japan*
[f] *Department of Pediatrics, Okayama University, Okayama, Japan*
[g] *Department of Medical Informatics, Kyushu University, Fukuoka, Japan*
[h] *Gumma Children's Hospital, Gumma, Japan*
[i] *Clinical Research Center, National Nagoya Hospital, Nagoya, Japan*

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Abstract

The treatment outcome for infant acute lymphoblastic leukemia (ALL) with positive *MLL* gene rearrangements remains poor. We analyzed whether additional chromosomal abnormalities (ACA) other than 11q23 translocation could affect the disease behavior and its prognosis.

Eighteen of seventy-four patients with infant acute lymphoblastic leukemia showed ACA, including three-way translocations in four, other novel translocations in four, and complex structural chromosomal changes in four. Only age less than 6 months and positive central nervous system leukemia were significant prognostic factors by multivariate analysis. However, overall survival rates were worse in patients with ACA compared to those with non-ACA. Genetic alterations induced by additional chromosomal changes may be associated with disease progression and poorer overall survival rates in infants with *MLL*-rearranged ALL.
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Keywords: Acute lymphoblastic leukemia; Infants; *MLL* gene rearrangements; Additional chromosomal abnormalities; Prognostic factor

Abbreviations: ALL, acute lymphoblastic leukemia; *MLL*, *mixed lineage leukemia*; MLL-R, *MLL* gene rearranged; FISH, fluorescence in situ hybridization; ACA, additional chromosomal abnormalities other than 11q23 translocation; EFS, event-free survival; OS, overall survival; SEs, standard errors; CIs, confidence intervals.

* Corresponding author. Department of Pediatrics, Ehime University Graduate School of Medicine, Shitsukawa, Too, Ehime 791-0295, Japan. Tel.: +81 89 960 5320; fax: +81 89 960 5941.
E-mail address: ishiei@m.ehime-u.ac.jp (E. Ishii).

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1. Introduction

Efforts in clinical trials to improve the outcome for infants with acute lymphoblastic leukemia (ALL), one of the subtypes of childhood ALL with poor outcome, enabled overall survival rates of 40% or higher [1–3]. However, outcomes for infants with positive *mixed lineage leukemia* (*MLL*) gene rearrangements, found in 70–80% of infant ALL cases studied with molecular techniques, remain poor, despite the use of intensive multiagent chemotherapy in combination with hematopoietic stem cell transplantation [1,4,5]. Multivariate analyses on recently conducted large-scale clinical studies have revealed several risk factors among infants with ALL, including a rearranged *MLL* gene, younger age (<3 or 6 months), very high white blood cell count (≥300,000/μL), and poor response to initial prednisone therapy [2,3]. Among these factors, presence of *MLL* gene rearrangement is the most important, significantly correlated with both the adverse clinical features and the poor prognosis that is characteristic of this distinct subtype of childhood ALL [4].

The *MLL* gene is disrupted by 11q23 translocation and fuses to more than 55 different partner genes; mainly, *AF4/FEL* in 4q21, *AF9* in 9p22, *ENL* in 19p13, *AF6* in 6q27 and *ELL* in 19p13.1 [6,7]. The partner genes encode nuclear proteins with transcriptional activities or proteins with dimerization/oligomerization motifs, suggesting that the impaired transcriptional activity by the fusion with *MLL* gene could be associated with leukemogenesis in infant leukemia [8]. In addition to these translocations, partial duplication or deletion of the 11q23 locus disrupts the function of the *MLL* gene [9]. In fact, several previous studies demonstrated that different types of *MLL* gene rearrangements, especially the presence of t(4;11)(q21;q23), the most common *MLL* gene translocation in infant ALL, confer a poor outcome in infants [10–13]. However, we have demonstrated that different 11q23 translocations are not associated with inferior prognosis in *MLL* positive infant ALL [4,5].

Although the rearranged *MLL* gene plays an essential role in leukemogenesis of infant ALL, it is still obscure whether rearrangement of the *MLL* gene is sufficient for leukemic transformation. The murine knock-in model of t(9;11)(p22;q23) (*MLL-AF9*) required a long period to the onset of leukemia [14]. It has been known that some cases harbor additional chromosomal abnormalities other than 11q23 or complex chromosomal changes in *MLL* positive ALL infants [15,16]. Thus, it is possible that several unknown genes located in these chromosomal changes are disrupted, and are associated with leukemogenesis or progression of the disease. Recently, Moorman et al. has reported that no prognostic effect of additional chromosomal abnormalities was observed in a cohort of infants and children with ALL and 11q23 abnormalities in a large collaborative retrospective study [17]. On the other hand, to further improve the outcome of this subset of ALL, it is necessary to identify appropriate prognostic factors for additional risk stratification along with an improvement in anti-leukemic therapy. We therefore conducted a study investigating the prognostic relevance of complex chromosomal abnormalities in infants with ALL and a *MLL* gene rearrangement treated with Japanese MLL96 and MLL98 protocols.

2. Materials and methods

2.1. Patients

Between December 1995 and December 2001, 102 consecutive infants with ALL, younger than 12 months, were registered and treated with two protocols, designated MLL96 (55 patients) and MLL98 (47 patients). Five other patients were also treated with MLL98 protocol without registration in the study. Prior to treatment, each patient was evaluated with respect to the characteristics of their leukemic cells, including immunophenotype, cytogenetics, and *MLL* gene rearrangement. Among the enrolled patients, 86 were identified as *MLL* gene-rearranged (MLL-R). The details of the therapeutic regimens used in the MLL96 and MLL98 studies are described elsewhere [4,5]; briefly, all the 86 patients in the MLL-R group were assigned to receive induction therapy and three courses of postremission intensification therapy followed by allogeneic hematopoietic stem cell transplantation in first remission if a suitable donor was available [1,4,5]. Written informed consent, provided according to the Declaration of Helsinki, was obtained from the guardians of the patients, with institutional review board approval of the study enrollment.

2.2. Cytogenetics

The *MLL* gene status in each patient was determined by Southern blot analysis and/or fluorescence *in situ* hybridization (FISH) as previously published [4]. Two genomic probes were used to detect *MLL* gene rearrangement by FISH analysis: the S1363 probe located in the 5′ region of the *MLL* gene, including *MLL* exon 1, and the LB140 probe in the 3′ region of the *MLL* gene (kindly provided by Dr. Misao Ohki, National Cancer Institute, Japan). BAC clone 216H7 (Research Genetics, Huntsville, AL), which is located on 4q21 and covers introns 3 and 4 of the *AF4* gene, was used for the detection of a *MLL-AF4* fusion gene in combination with the S1363 and LB140 cosmid probes. The karyotypes of leukemic cells were determined by cytogenetic analysis performed by a G-banding technique, also as previously described [4]. Briefly, mononuclear cells were separated from the bone marrow or peripheral blood. After 24 h of incubation without external stimulation, the samples were fixed in Carnoy's fixative solution (3:1 methanol and acetic acid). Slides for cytogenetic analysis were prepared using the trypsin-G banding technique. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN2005) [18].

2.3. Classification

Among the 86 MLL-R infants, only the patients with complete karyotypic data were included in the current analysis ($n = 83$).

Nine patients with normal karyotype were excluded from the study, because these patients had *MLL* gene rearrangements that were not detected by conventional cytogenetics. The remaining 74 were therefore classified into two subgroups: "ACA group", comprising those with additional chromosomal abnormalities other than 11q23 translocation, and "non-ACA group", comprising patients with sole 11q23 translocation with *MLL* gene rearrangements. Three-way 11q23 translocations and simple or complex structural chromosomal changes other than 11q23 abnormalities were also included in the additional chromosomal abnormalities (ACA) group, because several genetic changes in addition to *MLL* could be involved in these cases, as described in previous reports [16,17].

2.4. Statistical analysis

The analysis of treatment outcome was updated on 30 September 2007. Event-free survival (EFS) and Overall survival (OS) rates were estimated by the method of Kaplan–Meier and standard errors (SEs) with the Greenwood formula, and then were compared with the log-rank test. Confidence intervals (CIs) were computed with a 95% confidence level. The clinical and biologic features of patients in the two different subgroups were compared with χ^2 tests for homogeneity. A Cox regression model was used for the multivariate analysis. *P*-values, when cited, are two sided, with a value of 0.05 or less taken to indicate statistical significance.

Table 1
Eighteen *MLL* rearranged ALL infants with additional chromosomal abnormalities

Patient #	Karyotype	Sex	Age (month)	WBC, $\times 10^6$/L	CNS[a]	HSCT in CR1	Outcome
1	46,XX,add(11)(q25) [6]/46,XX [11]	F	4	193.8	–	No	BM relapse. DOD (2nd relapse) after UBMT
2	46,XY,t(4;11)(q21;q23),t(2;4)(q31;q32) [20] 46,XY,t(4;11)(q21;q23),t(2;4)(q31;q32) (2qter → 2q31::4q32 → 4q21::11q23 → cen → 11pter)	M	3	169.9	–	No	BM relapse. TRD after BMT
3[b]	46,XX[18].ish ins(4;11)(q21;q23.3q23.3)(RP11-216H7+, MLL5′+; MLL5′–,MLL3′+)[10]	F	2	953.0	+	No	BM relapse. DOD (2nd relapse) after UCBT
4	46,XX,t(4;11;15)(q21;q23;q22) [9]/46,XX [1]	F	0	121.6	+	RBMT	Death in CCR (TRD)
5	46,XX,add(1)(q32),der(2)t(2;4)(p1?;q21),add(4)(q21), del(11)(q?),add(16)(p11) [20]	F	8	7.7	–	No	CCR
6[b]	46,XX[20]. ish ins(4;11)(q21;q23.3q23.3)(RP11-216H7+, MLL5′+,MLL3′+; MLL5′-,MLL3′-)	F	2	500.0	–	RBMT	CCR
7	48,XX,+X,t(4;11)(q21;q23),+der(4)t(4;11)(q21;q23) [20]	F	0	421.5	–	No	BM relapse. DOD
8	46,XY,der(9)t(9;11)(p22;q13),add(11)(q13) [20]	M	1	473.5	+	No	Induction failure. TRD after RBMT
9	46,XY,t(4;11;5)(q21;q23;p11) [20]	M	3	1000.0	–	UCBT	Death in CCR (TRD after 2nd UCBT because of rejection)
10	46,XX,t(2;9)(p10;q10),add(7)(p22),add(9)(p13), add(11)(p11) [20]	F	7	1.7	–	UCBT	CCR
11	46,XX,t(4;11;9)(q21;q23;q22) [20]	F	9	250.7	+	UCBT	Death in CCR (TRD)
12	46,XX,add(4)(q11) [4]/46,XX [6]	F	5	12.1	+	ABMT	Relapse. TRD after UCBT
13	46,XY, t(6;11)(p10;q10),add(11)(q23) [20]	M	5	NA	NA	No	CNS relapse. TRD after RBMT
14	48,XY,+X,add(2)(p21),del(2)(p?),+6,der(7)add(7)(p11) add(7)(q32),del(11)(q?),add(12)(q13),-17,-17,add(19)(p13), +der(?)t(?;17)(?;q21),+mar1 [20]	M	2	25.6	+	No	DOD before initial therapy
15[b]	46,XY[20].ish ins(10;11)(p12;q23.3q23.3) (MLL5′+,MLL3′+;MLL5′, MLL3′–)	M	2	537.0	–	No	BM relapse. CCR after UBMT in CR2
16	47XX,t(4;11)(q21;q23),+?i(8)(q10) [20]	F	5	59.0	NA	No	BM relapse. DOD
17	47XX,+5,t(9;11)(p22;q23) [5]/46,XX [2]	F	3	22.8	–	UCBT	CCR
18[b]	46,XX,t(4;11)(q21;q23)[20].ish t(4;11;21)(q21;q23;q22) (216H7+; 216H7+,MLL5′+,MLL3′-; MLL3′+)	F	0	198.2	–	No	Induction failure. CCR after UBMT

F, female; M, male; WBC, white blood cell; BM, bone marrow; CNS, central nervous system; CR1, first complete remission; CCR, continuous complete remission; ABMT, autologous bone marrow transplantation; RBMT, related donor bone marrow transplantation; UCBT, unrelated cord blood transplantation; UBMT, unrelated bone marrow transplantation; DOD, death of disease; TRD, treatment-related death; NA, data not available.

[a] CNS disease was diagnosed if more than five leukemic cells/μl were found in cerebrospinal fluid.

[b] FISH analysis has proven complex chromosomal abnormality in these patients. Cloning of the breakpoint regions revealed that patient #6 had 46,XX, ins(4;11)(4pter → 4q21::11q24.1 → 11q23.3(MLL3′)::11q23.3 → 11q23.3(MLL5′)::4q21 → 4qter;11pter → 11q23.3:: 11q24.1 → 11qter), and patient #15 had 46,XY, ins(10;11)(10pter → 10p12::11q23.3 → 11q23.3(MLL3′)::11q23.3 → 11q23.3(MLL5′)::10p12 → 10qter;11pter → 11q23.3:: 11q23.3 → 11qter).

3. Results

Among the 74 eligible infants, 18 (24.3%) were classified as the ACA group, as shown in Table 1. Four patients (patients #4, #9, #11, and #18) had three-way 11q23 translocation. Other novel translocations were also observed in four patients: t(2;4)(q31;q32) in patient #2, t(9;11)(p22;q13) in patient #8, t(2;9)(p10;q10) in patient #10, and t(6;11)(p10;q10) in patient #13. FISH analysis confirmed complex structural chromosomal changes in four patients including insertion of 4q21 fragment to 11q23 locus and *vice versa* resulting in *MLL-AF4* fusion gene (patients #3, #6, and #18) or insertion of 10p12 to 11q23 locus resulting in *MLL-AF10* fusion gene (patient #15). Other frequent chromosomal changes were +X in two patients, involvement in chromosome 4 in two, chromosome 5 in two, chromosome 7 in two, and chromosome 11 except 11q23 in four.

The clinical and biologic findings were compared between the ACA and non-ACA groups, including age at disease onset, sex, initial white blood cell (WBC) count, central nervous system (CNS) involvement, and type of 11q23 translocation. As shown in Table 2, the frequency of sole t(4;11)(q21;q23) was significantly higher in the non-ACA group than in the ACA group. The frequency of positive central nervous system leukemia or young age at onset also tended to be higher in the ACA group than the non-ACA group, although the difference was not statistically significant.

Among the 18 patients in the ACA group, a total of 14 events were observed: one leukemic death before initiating therapy (patient #14); two induction failure (patients #8, and #18); eight relapses (patients #1, #2, #3, #7, #12, #13, #15, and #16); three treatment-related deaths (patients #4, #9, and #11). Only four patients in this group survived without any evidence of disease (patients #5, #6, #10, and #17) (Table 1).

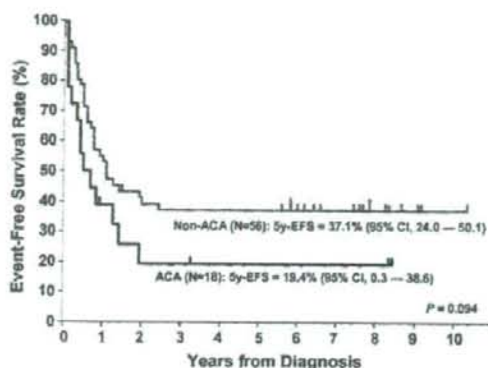

Fig. 1. Event-free survival estimates for 74 infants with ALL and *MLL* gene rearrangements in the MLL96 and MLL98 studies; a comparison between patients with additional chromosomal abnormalities and patients with sole 11q23 abnormality excluding normal karyotype with *MLL* gene rearrangements. Median follow-up period: 78 months (range, 8–124 months).

The EFS and OS rates were also compared between two groups. The 5-year EFS rate in the ACA group tended to be worse than that in the non-ACA group, without a statistically significant difference between two groups (Fig. 1). The 5-year OS in the ACA group was significantly worse than that in the non-ACA group; 26.7% (95% CI, 4.7–48.8%) *vs.* 52.1%

Table 2
Comparison in clinical and laboratory findings between the ACA and non-ACA groups

	Total number of Pt. (%)	ACA group number of Pt. (%)	Non-ACA group number of Pt. (%)	*P*-value[a]
Total number of patients	74	18	56	
Age, month				0.136
<3	21 (28.4)	8 (44.4)	13 (23.2)	
≥ 3, <6	29 (39.2)	7 (38.9)	22 (39.3)	
≥ 6	24 (32.4)	3 (16.7)	21 (37.5)	
Sex				0.650
Male	28 (37.8)	6 (33.3)	22 (39.3)	
Female	46 (62.2)	12 (66.7)	34 (60.7)	
WBC count, $\times 10^9$/L				0.599
<100	23 (31.1)	6 (33.3)	17 (30.3)	
≥ 100, <300	29 (39.2)	5 (27.8)	24 (42.9)	
>300	21 (28.4)	6 (33.3)	15 (26.8)	
NA	1 (1.3)	1 (5.6)	0 (0.0)	
CNS disease[b]				0.131
Positive	16 (21.6)	6 (33.3)	10 (17.9)	
Negative	52 (70.3)	10 (55.6)	42 (75.0)	
Unknown	6 (8.1)	2 (11.1)	4 (7.1)	
Karyotype				0.012
t(4;11)(q21;q23)	47 (63.5)	7 (38.9)	40 (71.4)	
Other 11q23	27 (36.5)	11 (61.1)	16 (28.6)	

ACA, additional chromosomal abnormalities other than 11q23 translocation; Pt., patients; WBC, white blood cell; CNS, central nervous system; NA, data not available.

[a] Comparison between two different groups.

[b] CNS disease was diagnosed if more than five leukemic cells/μL were found in cerebrospinal fluid.