

Table 2 Distribution of events by *MLL* gene status and treatment phase

	No. of patients	No. of events	No. of events during induction		No. of events before HSCT		No. of events after HSCT	
			No CR	Death	Relapse	Death in CR	Relapse	Death in CR
Overall	102	50	5	1	21	1	14	8
MLL-G	22	1	0	0	1	0	—	—
MLL-R	80 ^a	49	5	1	20	1	14	8

Abbreviations: CR, complete remission; HSCT, hematopoietic stem cell transplantation; MLL-G, patients with germline *MLL*; MLL-R, patients with *MLL* gene rearrangement.

^aNo events were observed among all four patients in the MLL-R group who did not receive HSCT for lack of a suitable donor.

associated with MLL-R. By karyotyping analysis, 11q23 abnormalities were found in 60 of 73 evaluable cases (82.2%) in the MLL-R group, half of whom had the t(4;11)(q21;q23) abnormality. The clinical characteristics of patients enrolled in the MLL96 study were comparable to those in the MLL98 study (data not shown).

Treatment outcome

Remission induction and subsequent events. The numbers and types of events are summarized in Table 2. The overall remission induction rate was 94.1% (96/102). Remission induction rates were high in both the MLL-R and MLL-G groups: 92.5% (74/80 patients) in the MLL-R group and 100% (22/22 patients) in the MLL-G group. There was one induction failure due to a fatal adenoviral infection and five induction failures in the MLL-R group. Two of the latter patients survived for 4.9 and 6.0 years, respectively, without evidence of disease after alternative therapies (either acute myeloid leukemia-directed chemotherapy or second HSCT).

Of the 74 patients in the MLL-R group who achieved CR1, 53 remained in continuous complete remission (CCR) during the postremission phase, 1 patient died of infectious pneumonia and 20 relapsed (19 with isolated marrow relapses and 1 with relapse site not specified) before reaching the timepoint of HSCT. Among these 20 relapsed patients, 12 underwent allogeneic HSCT in second remission (CR2) and 3 underwent HSCT without remission; the 5 of 12 who underwent HSCT in CR2 remain in remission for a median duration of 8.4 years (range, 5.7–10.2 years). Forty-nine of the 53 cases in CCR underwent HSCT in CR1: 2 autologous HSCT, 21 HSCT from a related ($n=12$) or unrelated donor ($n=9$) and 26-unrelated cord blood transplantation. The median time from remission to transplantation was 4 months (range, 0–9 months). Twenty-seven of the 49 patients with HSCT remained in CCR at the time of analysis, 8 died in CCR (four of veno-occlusive disease, one of cytomegalovirus infection, one of bacterial sepsis, one of gastrointestinal hemorrhage due to GVHD and thrombotic microangiopathy and one of an unspecified transplant-related complication) and 14 relapsed (nine with an isolated marrow relapse, one with combined marrow/CNS relapse, two with CNS relapse, one with testicular relapse and one with relapse site not specified). Among the 14 relapsed patients, 3 continue to survive for a median duration of 7.2 years (range, 5.0–10.1 years) after subsequent HSCT, while the remaining 11 patients eventually died, mostly of relapsed disease. The four patients who lacked a suitable donor received chemotherapy only as specified by the protocol and remained in CCR for median duration of 8.7 years (range, 3.6–10.8 years).

Analysis of overall outcome. The estimated 5-year OS and EFS rates for all 102 patients were 60.5% (95% CI, 50.7–70.2%)

and 50.9% (95% CI, 41.0–60.8%), respectively, after a median follow-up of 7.1 years (range, 1.5–10.8 years). Patients in the MLL-R group had a significantly worse outcome than those in the MLL-G group: 5-year OS, 50.8% (95% CI, 39.6–62.0%) vs 95.5% (95% CI, 86.6–100%) (Figure 1, $P<0.001$) and 5-year EFS, 38.6% (95% CI, 27.7–49.5%) vs 95.5% (95% CI, 86.6–100%) (Figure 1, $P<0.001$).

The only difference between the MLL96 and MLL98 protocols was the higher dosages of antileukemic drugs in the latter study, which was not associated with improved outcome as demonstrated by 5-year EFS rates in the MLL-R group: 35.7% (95% CI, 21.0–50.4%) in MLL96 vs 41.8% (95% CI, 25.8–57.8%) in MLL98 ($P=0.67$). Neither conditioning regimen received nor donor source had a significant impact on post-transplantation EFS rates among the 49 patients with a rearranged *MLL* gene who underwent HSCT after CR1 (Table 3).

Treatment outcome in the MLL-R group according to prognostic factors. The prognostic impact of several potential risk factors (Table 3) was determined in the MLL-R group. Infants younger than 6 or 3 months and those with CNS disease at diagnosis had significantly worse 5-year EFS rates than did infants without these features. Gender, leukocyte count and karyotype lacked prognostic significance in this univariate analysis. Further analysis with a Cox regression model indicated that only age less than 6 months exerted independent predictive strength (data not shown).

Long-term side effects. It was possible to evaluate long-term sequelae among 57 of the all 62 survivors of infant ALL treated on the MLL96 and MLL98 studies: 39 in the MLL-R group and 18 in the MLL-G group (complete follow-up data were not available for the remaining five patients). The median age of the 57 patients at analysis was 7.7 years (range, 1.1–10.4 years). Thirty-six of the 57 patients, all in the MLL-R group, underwent HSCT. Twenty-two received the TBI-based conditioning regimen, while 14 received the non-TBI conditioning regimen. In the TBI group, four patients had undergone allogeneic HSCT twice.

Significant late effects were not observed among patients in the MLL-G group. By contrast, various late complications were observed in the MLL-R group as follows: chronic GVHD in 5; hypothyroidism in 5; short stature (defined as a height standard deviation (s.d.) score below -2.0 or a requirement for growth hormone therapy) in 23; skin abnormalities (alopecia, scleroderma, hyper- or hypo-pigmentation) in 12; fasciitis in 1; ophthalmologic complications (dry eye, corneal opacity, retinal vasculitis) in 5; pulmonary complications (interstitial pneumonia, bronchiolitis obliterans) in 6; chronic diarrhea with malnutrition in 1; dental abnormalities in 6; multiple exostosis in 1; epilepsy in 2 and neurocognitive deficits (learning

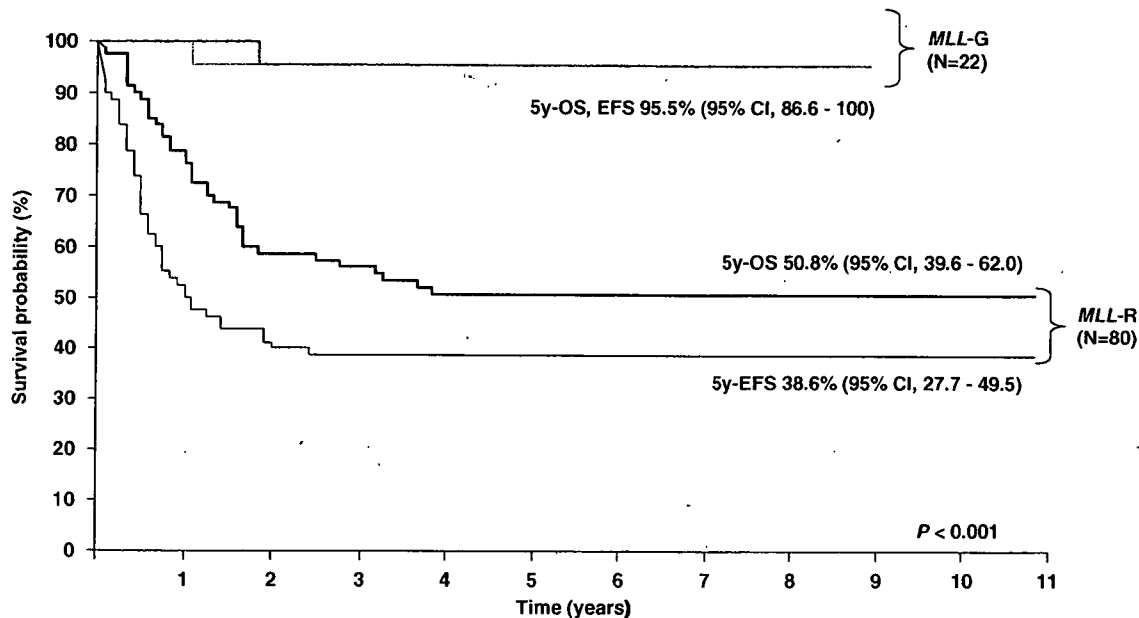


Figure 1 Overall survival (OS) and event-free survival (EFS) rates for infants with ALL treated in the MLL96 or MLL98 study by *MLL* status. Outcome was significantly better in patients with germline *MLL* (MLL-G) than in those with rearranged *MLL* (MLL-R) ($P < 0.001$).

disability, intelligence impairment, autism) in 4. There were no cases of secondary malignancy or symptomatic chronic heart failure. Pubertal development could not be evaluated because all study patients were younger than 12 years old and had not entered puberty.

The distribution of height s.d. scores is shown in Figure 2. Median scores for the MLL-R and MLL-G subgroups were -2.49 (range, -8.05 to $+2.92$) and $+0.05$ (range, -1.00 to $+0.90$), respectively (Figure 2a, $P < 0.001$). In contrast to the 36 patients who underwent HSCT, height s.d. scores for the three chemotherapy-only MLL-R patients were in the normal range (-1.88 , -1.57 , -1.48 , respectively). The median height s.d. score for patients receiving TBI-based conditioning ($n = 22$) was -3.07 (range, -8.05 to -0.08), which was significantly lower than the median value for those given non-TBI conditioning ($n = 14$, -1.72 (range, -6.13 to $+2.92$)) (Figure 2b, $P = 0.02$). However, 5 of the 14 patients in the latter group had height s.d. scores below -2.0 .

Discussion

The Japanese MLL96 and MLL98 clinical trials are the first published prospective studies to stratify infants with ALL by their *MLL* gene status. The results demonstrated clear differences in clinical features, including treatment outcome, between patients with or without an *MLL* gene arrangement. In most previous reports of EFS rates for infants with ALL, these two subgroups have been combined,²⁻⁸ when results are listed according to *MLL* gene status or CD10 expression, the EFS rates for those with a rearranged *MLL* gene or negative CD10 expression range from only 21 to 40%, and generally do not exceed 30%. Thus, our overall EFS rate of 50.9% justifies the decision to stratify infants with ALL by *MLL* gene status, so that appropriate risk-based treatments can be applied in each group. The method for determining *MLL* gene status is critical to segregating patients

into 'true' *MLL* rearranged and non-rearranged cohorts. Because 16.2% of our *MLL*-rearranged patients lacked abnormalities in band 11q23 by normal karyotypic analysis, we conclude that molecularly based methods such as Southern blotting or split-signal FISH are essential in strategies to determine the accurate *MLL* gene status in infants with newly diagnosed ALL.

Whether allogeneic HSCT has an important role in the treatment of infants with ALL remains controversial because of the limited data on this issue.¹⁹⁻²⁷ Pui *et al.*¹⁹ retrospectively analyzed cooperative group and individual transplant center data for children with ALL and 11q23 abnormalities, concluding that any type of HSCT was associated with a worse outcome than chemotherapy alone for t(4;11)-positive leukemia. In that study, the EFS rate for the 28 infants who underwent HSCT was only $19 \pm 3\%$, which is extremely low compared to our results and those of Sanders *et al.*,²⁷ who reported a 3-year disease-free survival rate of 42.2% among 40 infants with ALL following HSCT. However, this apparent improvement in outcome after HSCT must be interpreted with caution, since both the report of Sanders *et al.* and ours lack adequate retrospective or prospective control groups. Moreover, infants are the age group most vulnerable to intensive cytotoxic therapy, especially HSCT with a myeloablative preparative regimen, as illustrated by the high proportion of post-HSCT events in the current study (36.3%, 8/22) that were due to transplant-related toxicity. It is also notable that all four patients in the MLL-R subgroup, who did not receive HSCT for lack of a suitable donor, are alive without any subsequent events.

Allogeneic HSCT in infants always harbors the risk of late effects. In our analysis, 23 of 39 patients (58.9%) in the MLL-R group, especially those receiving TBI, had short stature after a median follow-up of 7.7 years. Sanders *et al.*²⁷ reported milder growth impairment in their series despite earlier treatment with a TBI-based conditioning regimen. This discrepancy may reflect the hyperfraction method of TBI used by Sanders *et al.*, such that 15.75 Gy was given three times a day over 7 days. Whatever the

Table 3 Five-year EFS by selected prognostic features for infants with a rearranged *MLL* gene

	No. of patients	5-year EFS, % (s.e.)	P-value
Age (months)			
<3	19	26.3 (10.1)	0.04
≥3	61	42.4 (6.3)	
<6	46	27.8 (6.6)	
≥6	34	52.9 (8.5)	
Gender			
Male	32	37.5 (8.5)	0.74
Female	48	39.3 (7.0)	
WBC count (× 10⁹/l)			
<100	25	51.2 (10.1)	0.08
≥100	55	32.7 (6.3)	
<300	57	41.9 (6.5)	0.28
≥300	23	30.4 (9.6)	
CNS disease			
Positive	15	20.0 (10.3)	0.03
Negative	59	47.3 (6.5)	
Karyotype			
t(4;11)(q21;q23)	41	33.8 (7.4)	0.29
Others	39	46.8 (8.8)	
Conditioning regimen^a			
TBI-based	26	47.1 (10.1)	0.24
BU-based	23	65.2 (9.9)	
Donor source^b			
Unrelated cord blood	26	53.8 (9.7)	0.92
Others	23	56.5 (10.3)	

Abbreviations: BU, busulfan; CNS, central nervous system; EFS, event-free survival; HSCT, hematopoietic stem cell transplantation; s.e., standard error; TBI, total-body irradiation; WBC, white blood cell count.

^a5-year EFS by conditioning regimen was compared among patients who had undergone HSCT in CR1.

^b5-year EFS by donor source was compared among patients who had undergone HSCT in CR1.

explanation, we would stress that severe growth impairment was also observed among patients in the non-TBI group, which may indicate that any form of conditioning regimen for infants could increase the risk of growth retardation. We observed several other serious long-term side effects, but additional follow-up is needed before the impact of these complications can be fully assessed. Besides, caution is needed to evaluate some of the late effects reported here, because there is a certain methodological limitation in collecting these data, which is questionnaire-based, that may lead to an underestimation of these events.

Several steps will need to be taken to further improve the prognosis of infant ALL with *MLL* gene rearrangements. First, additional risk stratification may identify important subsets of patients who would benefit from alternative therapy. Our analysis indicated that age at diagnosis can be used to segregate patients into two subgroups with different 5-year EFS rates: 27.8% for infants younger than 6 months and 52.9% for infants 6 months or older. This approach would not only contribute to better control of the leukemic clone, but might also reduce treatment-related toxicity. Second, we would emphasize that nearly half of the events (21/49) in our *MLL*-R group occurred before the use of HSCT. Thus, despite an initial remission rate of more than 90%, effective strategies to prevent early relapse are urgently needed. Pieters *et al.*²⁸ described the *in vitro* drug-resistance data, which demonstrate greater sensitivity to cytarabine (Ara-C) and higher resistance to glucocorticoids and L-asparaginase by leukemic cells from infants. In fact, we did introduce intensive use of Ara-C and dexamethasone in our treatment, which may in part have contributed to improved outcome compared to the historical data. However, one might improve results by further intensifying the early phase of postremission intensification therapy with Ara-C and with intensive use of L-asparaginase, which was not used in the current therapy, to overcome the leukemic cell drug-resistance to this agent. Third, the toxic events and late effects related to HSCT are hardly acceptable for treating infants with ALL. Without clear evidence for benefit of HSCT, it will be necessary to devise an effective chemotherapy regimen without HSCT, at least for the 'lower-risk' *MLL*-R subgroup. Finally, two large

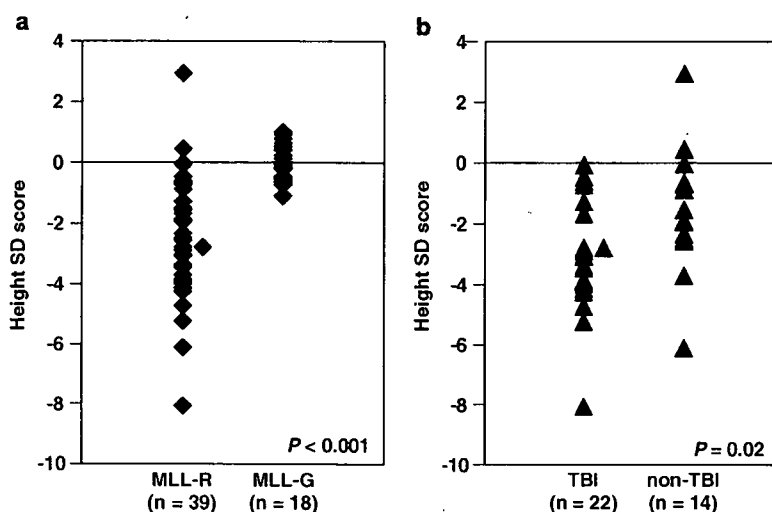


Figure 2 Distribution of height standard deviation (s.d.) scores among survivors of infant ALL in the *MLL*96 and *MLL*98 studies. (a) Comparison of scores for the *MLL*-rearranged (*MLL*-R) vs germline *MLL* (*MLL*-G) patients. (b) Comparison of scores between patients who received total-body irradiation (TBI)- or non-TBI-based conditioning regimens.

multicenter studies investigating effective chemotherapy regimens for infant ALL (Interfant 99 and POG/COG9407) have been completed in Europe and in the United States, and these results will be important in designing future protocols for this age group, as will studies to develop innovative targeted therapies for infants with *MLL*-rearranged ALL, now underway.^{29,30}

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Supplementary Information accompanies the paper on the Leukemia website (<http://www.nature.com/leu>)

Identification of a Novel Fusion Gene *MLL-MAML2* in Secondary Acute Myelogenous Leukemia and Myelodysplastic Syndrome with *inv(11)(q21q23)*

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We have identified a novel fusion partner of *MLL*, namely the mastermind like 2 (*MAML2* gene), in secondary acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) with *inv(11)(q21q23)*. RT-PCR and sequencing revealed that exon 7 of *MLL* was fused to exon 2 of *MAML2* in the AML and MDS cells. The *inv(11)(q21q23)* results in the creation of a chimeric RNA encoding a putative fusion protein containing 1,408 amino acids from the NH₂-terminal part of *MLL* and 952 amino acids from the COOH-terminal part of *MAML2*. The NH₂-terminal part of *MAML2*, a basic domain including a binding site of the intracellular domain of NOTCH, was deleted in *MLL-MAML2*. *MLL-MAML2* in secondary AML/MDS and *MECT1-MAML2* in mucocystic carcinoma, benign Wartin's tumor, and clear cell hidradenoma consist of the same COOH-terminal part of *MAML2*. A luciferase assay revealed that *MLL-MAML2* suppressed *HES1* promoter activation by the NOTCH1 intracellular domain. *MAML2* involving a chimeric gene might contribute to carcinogenesis in multiple neoplasms by the disruption of NOTCH signaling. © 2007 Wiley-Liss, Inc.

INTRODUCTION

11q23 translocations are frequent in hematologic malignancies, occurring in 5–6% of acute myeloid leukemia (AML), 7–10% of acute lymphoblastic leukemia, 60–70% of acute leukemias in infants, and in most patients with therapy-related leukemias induced by inhibitors of topoisomerase II (Rowley, 1998). The *MLL* gene is rearranged as a consequence of 11q23 translocations, and at least 50 partner genes for *MLL* have so far been identified (Meyer et al., 2006).

Although the leukemogenic effect of *MLL* fusion proteins has been well established in a number of instances lack of functional information about the *MLL* partners has made it difficult to address their contribution to the oncogenic potential of *MLL* fusion proteins. A few observations indicated that fusion partners play essential roles in determining the oncogenic capacity of the *MLL* fusion proteins (So and Cleary, 2003; Liu et al., 2004). In the present study, we have identified the mastermind like 2 (*MAML2*) gene as a novel fusion partner of *MLL* in therapy-related AML and myelodysplastic syndrome (MDS) with *inv(11)(q21q23)*, and provide evidence that the *MLL-MAML2*

fusion suppresses a promoter activation of the NOTCH target gene, *HES1*.

MATERIALS AND METHODS

Case Reports

Case 1. Details of the patient, a 48-year-old female, have been previously published (Takei et al., 2006). She initially had AML (M2 in the FAB classification) with *t(8;21)(q22;q22)*. She achieved complete remission by chemotherapy. Consolidation and maintenance chemotherapies containing etoposide (VP-16; total dose 1,150 mg) were administered. Seven months after chemotherapy, a chromosomal abnormality, *inv(11)(q21q23)*, appeared and was constantly detected in bone

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TABLE I. Karyotype Analysis of Bone Marrow Cells (Patient 1)

Date	Number of 46,XX,inv(11) in 20 metaphases	Other abnormality	Status of the bone marrow
Jan 21, 1993	0	+	AML
Jun 14, 1994	20	—	CR
Apr 3, 1995	19	—	CR
Dec 11, 1995	14	—	CR
Oct 6, 1997	4	—	NHL
Nov 14, 1997	1	—	CR
Feb 23, 1998	19	—	CR
Apr 21, 1998	17	—	CR
Apr 21, 1999	6	+	CR
Mar 13, 2000	19	+	t-AML
Apr 10, 2000	19	+	t-AML
Apr 24, 2000	12	+	t-AML
Jun 27, 2000	19	—	t-AML

*indicates t(8;21), 9q-. AML, acute myeloid leukemia; CR, complete remission; t-AML, therapy-related AML; NHL, non-Hodgkin's lymphoma.

marrow cells during hematologic remission (Table 1). After 6 years, she developed secondary AML with inv(11)(q21q23), and died in 2001.

Case 2. A 69-year-old male was admitted to a regional hospital due to phlegmon of the left thigh in March 2006. He had been diagnosed with rheumatoid arthritis and treated with bucillamine, prednisolone, salazosulfapyridine, and methotrexate (total dose 50 mg) for 8 years. He had no history of malignancy. After recovery of the phlegmon, mild anemia, leukocytosis (peak $136.4 \times 10^9/l$) and thrombocytopenia ($30-40 \times 10^9/l$) persisted. He was referred to Hitachi General Hospital for further examination. His bone marrow was hypercellular with micromegakaryocytes and hypersegmentation of granulocytes. The karyotype was 46,XY,inv(11)(q21q23)[20]. Fluorescence in situ hybridization (FISH) analysis for *BCR-ABL1* was negative, whereas FISH analysis for *MLL* on peripheral blood revealed that 71% of the white blood cells had deletion of the 3' part of the *MLL* gene. MDS was diagnosed, and he died of pneumonia in January 2007.

cDNA Panhandle Polymerase Chain Reaction (PCR)

Total RNA was extracted from bone marrow or peripheral blood cells using the acid guanidine thiocyanate-phenol chloroform method (Chomczynski and Sacchi, 1987) and analyzed using a modified cDNA panhandle PCR method (Meganigal et al., 2000; Suzukawa et al., 2005). In brief, first-strand cDNAs were synthesized with *MLL*-

random hexamer oligonucleotides, *MLL*-N. After primer 1 extension with *MLL*-1, and extension in stem-loop templates, the sample was amplified by first PCR with *MLL*-1 and *MLL*-2. Then, 1/25 of the products were used for nested PCR with *MLL*-3 and *MLL*-4. The *MLL*-random hexamer oligonucleotides and primers used were as follows: *MLL*-N, 5'-TCG AGG AAA AGA GTG AAG AAG GGA ATG TCT CNN NNN N-3'; *MLL*-1, 5'-TGA AGA ACG TGG TGG ACT CT-3'; *MLL*-2, 5'-GTC CAG AGC AGA GCA A AC AGA-3'; *MLL*-3, 5'-GTC AGA AAC CTA CCC CAT CA-3'; and *MLL*-4, 5'-TGT GAA GCA GAA AAT GTG TGG-3'.

Reverse Transcriptase PCR

Five μ g of total RNA was reverse transcribed to cDNA in a total volume of 33 μ l with random hexamers using the Ready-To-Go You-Prime First-Strand beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), after which 1/30 of the cDNA was amplified using PCR in a total volume of 50 μ l with 50 mM KCl, 1.5 mM MgCl₂, 10 mM TAPS Buffer (pH 9.3 at room temperature), 0.4 μ M of each primer, 0.2 mM of each dNTP, and 1 unit of Ex *Taq* polymerase (Takara-Bio, Siga, Japan). After 35 rounds of PCR (30 sec at 94°C, 30 sec at 55°C and 1 min at 72°C), 5 μ l of the PCR product was electrophoresed in a 3% agarose gel. The primers used were as follows: *MLL*7s: 5'-TCC TCA GCA CTC TCT CCA AT-3'; *MAML2*R: 5'-GTC ATT TGG CCA TCC ATG TG-3'.

Plasmid Construction

The KIAA1816 cDNA clone in pBluescript II SK(+) vector was obtained from the KAZUSA DNA Research Institute in Japan. The GenBank accession number for KIAA1816 is AB058719. Full-length *MAML2* and the *MAML2* part of the *MLL*-*MAML2* fusion gene (C-*MAML2*) cDNA were constructed by replacing the 5' and 3' untranslated region with PCR-amplified fragments. *MLL* exon 1-7 cDNA (N-*MLL*) was provided by Dr. H. Hirai (University of Tokyo, Tokyo, Japan). Both ends of N-*MLL* were replaced with PCR fragments for subsequent cloning into the mammalian expression vector, pcDNA3.1. FLAG(M2)-tagged MKK6 expression vector in pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was provided by Dr. T. Sudo (RIKEN, Saitama, Japan). MKK6 cDNA was replaced with *MAML2*, C-*MAML2*, and N-*MLL* for the construction of FLAG tagged genes. The 3' end of FLAG-tagged N-*MLL* was replaced with an RT-PCR product containing an

MLL-MAML2 fusion sequence from the leukemia cells and C-MAML2 to construct full-length MLL-MAML2 fusion. All PCR-amplified fragments were confirmed by nucleotide sequence analysis. Further information regarding the primer sequences and cloning is available upon request. *Notch1* intracellular domain tagged V5 (N1ICD) expression vector was provided by Dr. F. Ito (Tsukuba University, Japan) (Itoh et al., 2004). The luciferase reporter containing the promoter of the HES1 gene (pHES1-luc) was provided by Dr. R. Kageyama (Kyoto University, Japan) (Takebayashi et al., 1994).

Cell Lines and Antibodies

HEK293, a human embryonic kidney cell line, and KG-1, a leukemic cell line established from an AML patient, were purchased from RIKEN Bio-Resource Center (Tsukuba, Ibaraki, Japan). The HEK293 cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (SIGMA-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin sulfate. The KG-1 cells were grown at 37°C in RPMI 1640 Medium (SIGMA-Aldrich) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/ml penicillin, and 100 µg/ml streptomycin sulfate.

The anti-FLAG polyclonal antibody (F7425) and the anti-MLL1 (BL1289) polyclonal antibody were purchased from Sigma and Bethyl Laboratory Inc. (Montgomery, TX), respectively.

Transfection and Immunoblotting

1×10^6 cells/well of HEK293 cells were seeded in six well plates. The next day, the cells were transfected with FLAG-tagged MLL-MAML2, MAML2, C-MAML2, or N-MLL expression vector using Lipofectamine 2000 (Invitrogen). The next day, the cells were washed with PBS twice and then lysed with RIPA buffer (1% TritonX-100, 0.1% SDS, 1% Sodium deoxycholate, 158 mM NaCl, 1 mM Na_3VO_4 , 5 mM EGTA, 10 mM Tris-HCl, and pH 7.4). The cell lysates were centrifuged at 15,000 rpm for 30 min, and the supernatants were boiled and denatured in Sample buffer containing SDS and DTT (Invitrogen) followed by electrophoresis using SDS-PAGE gradient gel (2–15%) in Tris-Glycin buffer. The proteins were electro-transferred to nitrocellulose membrane (GE Healthcare Bioscience, Tokyo, Japan) using a semi-dry transfer cell (Trans-Blot SD, Bio-Rad Japan, Tokyo, Japan). The resulting protein-bound membrane was blotted with Anti-FLAG ($\times 1,000$) or anti-MLL ($\times 1,000$) antibody and visualized

using ECL reagents (GE Healthcare Bioscience). The cells for all samples were cultured and the protein extracts were also prepared at the same time.

Transfection and Luciferase Assay

The HEK293 cells were seeded (1×10^5 cells per well) in 24-well plates and transfected 48 hr later using Lipofectamine 2000 (Invitrogen) with reporter construct (HES1-luciferase, 100 ng), expression vector (300 ng), ICD expression vector (10 ng) and pRL-TK internal control vector (5 ng) (Promega, Madison, WI, USA). When increasing amounts of the expression vector were transfected, the amount of total DNA was kept constant by adding empty pcDNA3.1 vector to the transfection mixture. Forty-eight hours after transfection, the cells were washed with PBS twice, 100 µl of PLB lysis buffer of the Dual Luciferase Reporter Assay kit (Promega) was added. The samples were gently rocked for 15 min at room temperature. Luciferase activity was determined with 10 µl aliquots of the sample with Dual-Luciferase Reporter assay system (Promega) and Lumat LB9501 luminometer (Berthold Japan, Tokyo, Japan) by following the manufacturer's protocol.

2×10^6 of the KG-1 cells were suspended in 100 µl of Nucleofector solution R (Amaxa biosystems, Cologne, Germany). Reporter construct (HES1-luciferase, 500 ng), expression vector (1.5 µg), ICD expression vector (50 ng), and the pRL-TK internal control vector (50 ng) were added to the suspension in the cuvette followed by electrotransfection using Nucleofector II Device (Amaxa biosystems). Transfected cells were incubated in culture medium for 48 hr, after which the cells were washed with PBS and centrifuged at 1,200 rpm for 5 min. PLB lysis buffer (100 µl) was added to the cells; then the samples were rocked for 15 min at room temperature. About 20 µl aliquots of cell lysate were used for quantification of luciferase activity. All experiments were performed in triplicate. Firefly luciferase activity was normalized by reference to the Renilla luciferase activity expressed by the pRL-TK vector.

Exon Nomenclature

The exon nomenclature for *MLL* was taken from a report (Rasio et al., 1996).

RESULTS

Isolation of MLL-MAML2 Fusion Transcript from AML cells with *inv(11)(q21q23)*

FISH and Southern blot analyses revealed rearrangement of the *MLL* gene in case 1 (Takei et al.,

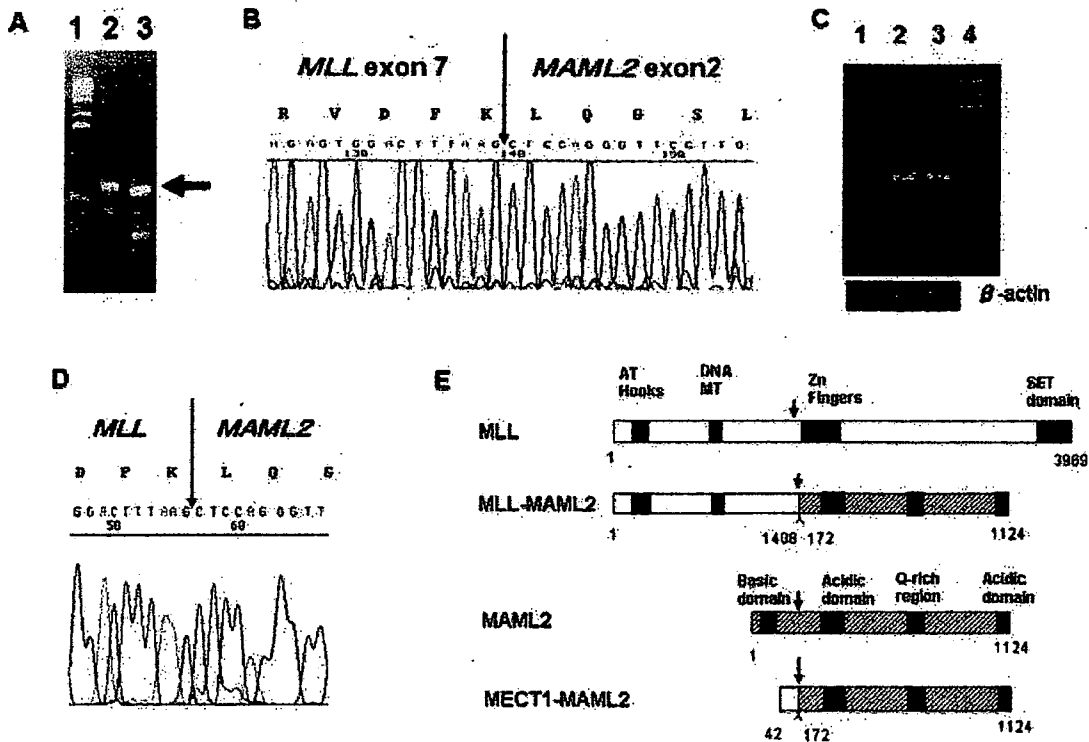


Figure 1. Identification of *MLL-MAML2* fusion transcript. (A) Panhandle PCR for cDNA product. Lane 1: λ *HindIII* digest (marker); lane 2: first PCR product; lane 3: second (nested) PCR product. An arrow indicates a DNA fragment whose sequence is shown in panel B. (B) Sequence analysis of the amplified panhandle PCR product from case 1 revealed an in-frame fusion between *MLL* exon 7 and *MAML2* exon 2 (arrow; GenBank accession no. 828759). (C) RT-PCR of two cases with *inv(11)(q21q23)*. Lane 1: cDNA from normal peripheral blood; lane 2:

cDNA from leukemic cells in case 1; lane 3: cDNA from peripheral blood in case 2; lane 4: 1 kb ladder marker. (D) Sequence analysis directly performed on the amplified RT-PCR product from case 2 revealed the same fusion between *MLL* and *MAML2*. (E) Schematic structures of wild-type *MLL*, *MLL-MAML2* fusion gene, wild-type *MAML2* and *MECT1-MAML2* fusion gene. *MT*, DNA methyltransferase homology domain; *Q-rich region*, glutamine rich region; Arrow, fusion point. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

2006). To identify the *MLL* partner gene at 11q21, we performed cDNA panhandle PCR for total RNA from the patient's bone marrow cells. First and second PCR gave a specifically amplified DNA fragment (Fig. 1A). Sequence analysis of the fragment revealed an in-frame fusion between *MLL* exon 7 and *MAML2* exon 2 (Fig. 1B).

Detection of *MLL-MAML2* in Two Hematologic Malignancies with *inv(11)(q21q23)*

To confirm the presence of the *MLL-MAML2* fusion mRNA in the leukemic cells of the case 1 and peripheral blood of case 2, we performed RT-PCR analysis with *MLL7s* and *MAML2R*, which successfully yielded specific DNA fragment (Fig. 1C). We confirmed that the RT-PCR product from case 2 was an *MLL-MAML2* fusion transcript by sequencing (Fig. 1D). The putative *MLL-MAML2* fusion protein of 2,389 amino acids (aa) contained 1,408 aa, from the NH₂-terminal part of

MLL and 981 aa from the COOH-terminal part of *MAML2* (Fig. 1E).

MLL-MAML2 Suppresses *HES1* Promoter Activation by *NIICD*

To investigate the function of the *MLL-MAML2* fusion gene, we constructed FLAG-tagged *MLL-MAML2* (M-M2), *MLL* part of *MLL-MAML2* (N-*MLL*), *MAML2* part of the *MLL-MAML2* (C-M2), and full-length *MAML2* gene (M2) (Fig. 2A). The expression of recombinant proteins was confirmed by Western blot analysis (Fig. 2B). Immunohistochemical experiment using anti-FLAG antibody revealed that both M2 and M-M2 localized in the nucleus (data not shown). We evaluated the ability of *MLL-MAML2* to participate in NOTCH signaling by examining the activation of a NOTCH target gene, *HES1*, the best characterized member of the *HES* gene family (Leong and Karsan, 2006). M-

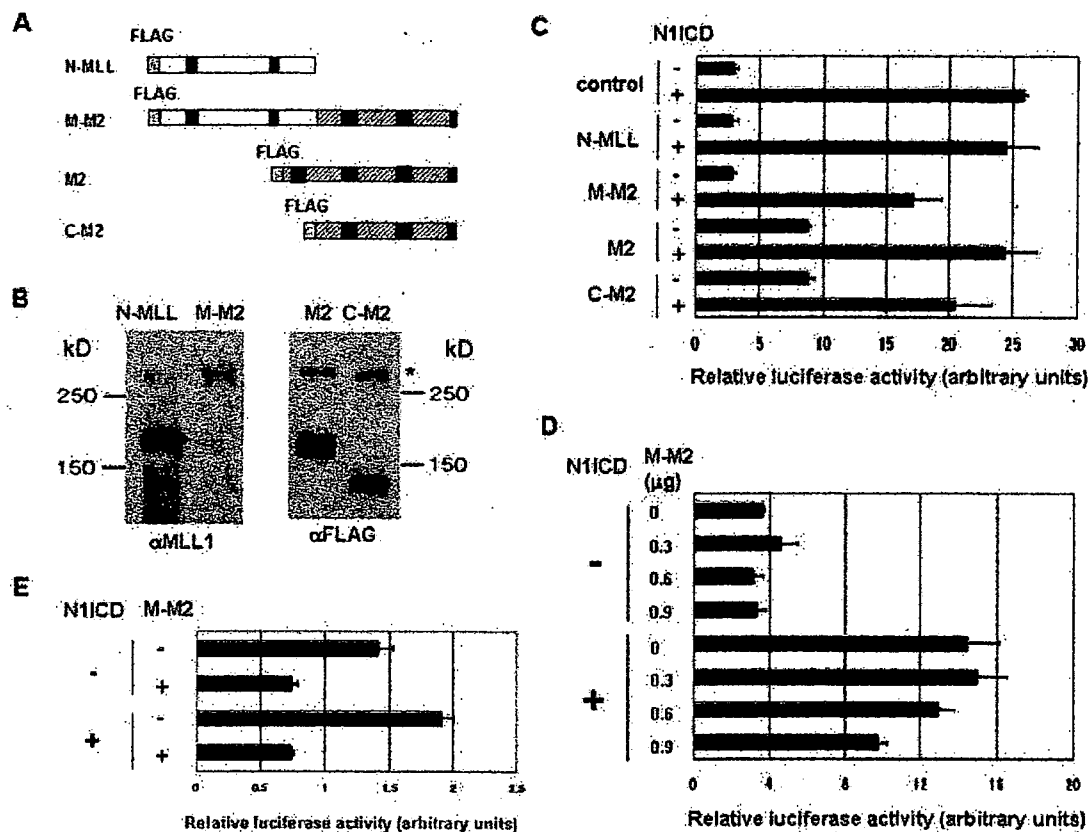


Figure 2. Functional analysis of the fusion gene. (A) a schematic presentation of recombinant genes. FLAG tag was fused to the NH2 terminal end of each recombinant construct. N-MLL: NH2 terminal part of MLL-MAML2; M-M2: MLL-MAML2 fusion gene; M2: full length MAML2; C-M2: COOH terminal part of MLL-MAML2. (B) Detection of recombinant proteins by Western blot analysis. Each protein was detected either anti MLL (left panel) or anti FLAG antibody (right panel). Asterisk

in the right panel indicates a nonspecific band. (C) Comparison of basal (N1ICD-) and N1ICD-induced activation (N1ICD+) of HES1 promoter activity. Each recombinant gene was transfected as indicated. Empty pcDNA3.1 vector was used as control. (D) Dose escalation of MLL-MAML2 fusion gene. (E) Luciferase assay using KG-1 leukemia cell line. The error bars indicate the standard deviations (n = 3).

M2 did not increase the basal HES1 promoter activity, but suppressed N1ICD-induced HES1 promoter activation in a dose dependent manner (Figs. 2C and D). In contrast, MECT1-MAML2 was reported to activate the HES1 promoter, and this was independent of NOTCH stimulation (Tonon et al., 2003). Thus, the MAML2 containing fusion gene might disrupt NOTCH signaling by both activation and inhibition. This is not surprising because NOTCH activation can be both oncogenic and tumor suppressive in different tumors (Leong and Karsan, 2006), although there is a possibility that the difference is due to the different cell lines used for the experiments. N-MLL changed neither the basal nor N1ICD-induced HES1 promoter activity, suggesting that this part is not involved in the NOTCH signaling pathway. M2 and C-M2 enhanced the basal HES1 promoter

activity. C-M2 suppressed the N1ICD-induced activation of the HES1 promoter, whereas M2 did not. Next, we used a leukemia cell line, KG-1, for luciferase assay. The N1ICD-induced activation of the HES1 promoter in KG-1 was less compared to the one in HEK293. M-M2 suppressed both basal and N1ICD-induced HES1 promoter activity to the same level (Fig. 2E).

DISCUSSION

The mammalian *MAML1*, *MAML2*, and *MAML3* genes are widely expressed in adult tissues and localize to nuclear bodies. They share a conserved basic domain in their N termini that binds to the ankyrin repeat domain of NOTCH, and contain a transcriptional activation domain in their C termini. They function as transcriptional co-activators for NOTCH, forming a complex in the nucleus

with the intracellular domain of an activated NOTCH receptor (ICN) and the CSE family of transcription factors, resulting in the activation of NOTCH downstream targets, such as *HES1* and *HES5* (Lin et al., 2002; Wu et al., 2002).

The acquisition of novel properties by the combination of MLL with the fusion partners, rather than the loss of wild-type MLL function leads to the generation of an active oncoprotein. Recent studies suggest that different mechanisms might be involved in the leukemogenesis by MLL fusion proteins (Li et al., 2005). The MLL fusion partners can be divided into nuclear and cytoplasmic factors, according to their compartment of protein expression/function. There is increasing evidence that the nuclear factors have transcription activity themselves and belong to important chromatin remodeling and transcription modulating complexes. A subgroup of fusion partners have oligomerization/dimerization domains, such as leucine zippers and α -helical coiled-coil domains. Recent reports indicate that the dimerization of the N-terminal portion of MLL fusion protein is oncogenic (Martin et al., 2003; So et al., 2003). In the case of MLL-MAML2, the N-terminal portion of MLL may acquire oncogenic activity by transcriptional activation, since the transcriptional activation domain of MAML2 is retained (Wu et al., 2005) and no self-association domain has been identified.

MLL is the second fusion partner of *MAML2* in human neoplasm. It has been reported that the same part of *MAML2* as in MLL-MAML2 is fused to the NH₂-terminal part of *MECT1* in mucoepidermoid carcinoma, Warthin's tumor, and clear cell hidradenoma (Tonon et al., 2003; Enlund et al., 2004; Behboudi et al., 2005). A recent report demonstrated that not only *MECT1*, but also the *MAML2* component is required for the transformation of RK3E cells (Wu et al., 2005). A common structural alteration of *MAML2* in solid tumors and leukemia suggests that common functional aberration(s) of *MAML2* contribute to carcinogenesis in multiple tissues. There are a few genes that are found as part of fusion genes in both leukemia and solid tumors. For example, the *ETS* family gene *ERG* is found as part of a fusion gene with different partner genes in myeloid leukemia, Ewing sarcoma, and prostate cancer (Ichikawa et al., 1994; Tomlins et al., 2005). The common alteration of *ERG* in these tumors is the overexpression of the COOH-terminal part, including the *ETS* domain. Especially, the same part of *ERG* is fused to either *TLS/FUS* or *EWSR1* in myeloid leukemia or Ewing sarcoma, respectively. Thus,

overexpression of the COOH-terminal portion of *MAML2* may be required for common carcinogenesis. Functional alteration of partner gene might be required in tissue-specific carcinogenesis. In accordance with this, MLL-MAML2 did not transform RK3E cells (unpublished data), while transformation by *MECT1*-*MAML2* was been reported (Wu et al., 2005). Intriguingly, both M-M2 and C-M2 lack the ICN binding site in the basic domain (Wu and Griffin, 2004).

Detection of chromosomal translocations involving *MAML2* in not only benign tumors, Warthin's tumor (Enlund et al., 2004) and clear cell hidradenoma (Behboudi et al., 2005), but also in bone marrow cells before overt leukemia (Takei et al., 2006) and MDS suggests that the disruption of NOTCH signaling by the emergence of a *MAML2* containing fusion gene is an early event of carcinogenesis. The *inv(11)(q21q23)* is rare in hematologic malignancies. There is another case report of secondary AML with *inv(11)(q21q23)* and rearrangement of the *MLL* (Obama et al., 1998). It is likely that the leukemia cells of the patient have MLL-MAML2 fusion gene. Although the clinical effect of this infrequent fusion hence may be limited, their characterization will certainly provide additional mechanistic insights into both MLL- and MAML2-mediated carcinogenesis.

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To investigate further the oncogenic function of BRAF^{L597Q}, we examined its transforming ability by comparing colony formation of various BRAF transfectants of NIH3T3 cells on soft agar. We found significant colony formation with the two activating mutants, BRAF^{V600E} and BRAF^{L597Q}, in a similar manner (Figures 1b and c). No colony formation occurred with vector transfections (control). We also did not see colony formation with the BRAF^{WT} transfectant. This was an expected result as the BRAF^{WT} itself is not oncogenic and BRAF^{WT} was unable to overactivate the MAP kinase pathway (Figure 1a). These data demonstrated the transforming ability of the T1790A BRAF mutation and, together with its constitutive kinase activation of BRAF (Figure 1a), demonstrated its oncogenic function.

In this study, we have for the first time explored the functionality of the T1790A BRAF mutation and demonstrated that the BRAF gene with this mutation is an oncogene. In addition to childhood ALL,⁷ this mutation was reported also in cutaneous melanoma with a lower prevalence (3%).¹² The relative common occurrence (10%) of the T1790A mutation in childhood ALL suggests that this mutation plays an important role in a subpopulation of patients with this blood malignancy and may represent a novel therapeutic target for these patients.

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N822 mutation of *KIT* gene was frequent in pediatric acute myeloid leukemia patients with t(8;21) in Japan: a study of the Japanese childhood AML cooperative study group

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Recently, *KIT* mutations were found in 12.7–48.1% of adult acute myeloid leukemia (AML) patients.^{1–6} Our previous analysis revealed that *KIT* mutations were found in 8 (17.4%) of 46 pediatric t(8;21)-AML patients and these patients had the poorer prognosis than those without *KIT* mutations.⁷ Recent Berlin-Frankfurt-Münster study and Dutch Childhood Oncology Group study revealed that 5 (31.3%) of 16 t(8;21)-AML patients had *KIT* mutations.⁸ On the other hand, Children's Cancer Group study revealed that *KIT* mutations were not found in eight t(8;21)-AML patients.⁹ There remains the possibility of the different frequency about *KIT* mutations in ages and races. Thus, we further investigated *KIT* mutations in a larger number of pediatric t(8;21)-AML patients.

We examined for *KIT* mutations in 42 newly diagnosed t(8;21)-AML patients who were treated on the same AML99 protocol from January 2003 to December 2006 after AML99 study closed.⁷ We found *KIT* mutations in 6 (14.3%) of 42 t(8;21)-AML patients (D816mutation (Mt) ($n=4$), N822Mt ($n=1$), deletion of D419 ($N=1$)).

Furthermore, we searched for other *KIT* mutations and found novel mutations in transmembrane domain (exon 10) in 3 (3.4%) of total 88 t(8;21)-AML patients, including previous 46 patients⁷ (Table 1). Two patients having the same I538V (ATT to GTT) in *KIT* have been in complete remission for 30 and 48 months, respectively. However, the remaining patient having V540L (GTC to CTC) in *KIT* relapsed 4 months after diagnosis. V540L was also found in relapsed bone marrow sample. These mutation sites were in the vicinity of the codon 541, which was reported as single nucleotide polymorphism (SNP) site (ATG to CTG, M541L). Therefore, we examined these mutations (I538V

Table 1 KIT gene mutation in 88 pediatric t(8;21)-AML

	No. of patients	%
Kinase domain (exon 17)	13	14.8
D816Mt	6	
D816H	1	
D816V	3	
D816Y	2	
N822Mt	5	
N822K	4	
N822T	1	
A814S	1	
V825A	1	
Extracellular domain (exon 8)	1	1.1
del. D419	1	
Transmembrane domain (exon 10)	3	3.4
I538V	2	
V540L	1	
Total	17	19.3

Abbreviations: AML, acute myeloid leukemia; Mt, mutation.

and V540L) and M541L (SNP) in 42 normal peripheral blood samples, and detected only M541L (SNP) in 4 (9.5%) samples. Internal tandem duplication of *KIT*¹⁰ was not found in this study.

In total, *KIT* mutations were found in 17 (19.3%) of 88 t(8;21)-pediatric AML patients (Table 1). This frequency was lower than that of several adult reports including Japan.^{1-3,5} Interestingly, in Europe, D816Mt and N822Mt were found in 5 and 0 of 47 adult patients in Netherlands,¹ 3 and 0 of 56 adult patients in France,⁵ 12 and 0 of 42 adult patients in Italy, respectively.⁶ On the other hand, in east Asia, D816Mt and N822Mt were found in 7 and 3 of 37 adult patients in Japan,³ 9 and 10 of 54 adult patients in China,² respectively. Our study revealed that D816Mt and N822Mt were found in 6 and 5 of 88 t(8;21)-pediatric AML patients, respectively. The frequency of N822Mt in *KIT* is different between Europe (Caucasian) and east Asia (oriental people). Boissel *et al.*⁵ suggested that ethnic and geographic variations may also be responsible for such disparities. N822Mt in *KIT* was frequent in Japanese and Chinese t(8;21)-AML patients. Notably, Wang *et al.*² suggested that Imatinib, a tyrosine kinase inhibitor, has the efficacy for the leukemic cells with N822Mt, but not with D816Mt. Further larger study is needed to clarify N822Mt and the efficacy of Imatinib.

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No nucleophosmin mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group

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Acute myeloid leukemia (AML) with normal karyotype had a heterogeneous prognosis. In this subgroup, *FLT3*-internal tandem duplication (ITD) was strongly associated with a poor prognosis.^{1–3} Recently, it was reported that mutations of nucleophosmin (*NPM*) gene occur in 50–60% of adult AML with normal karyotype and were frequently associated with *FLT3*-ITD. In the AML patients with normal karyotype and *FLT3*-ITD, patients with *NPM* gene mutations showed a better prognosis than those without *NPM* gene mutations.^{4–6} However, the frequency and clinical impact of *NPM* gene mutations in pediatric AML patients with normal karyotype remained uncertain because there were a few number of reports.^{7,8}

We searched for *NPM* gene mutations in 33 (20.9%) of 158 patients with normal karyotype who were treated on Japanese Childhood AML Cooperative protocol, AML 99 (0–15 years old, median 8 years old).⁹ We amplified exon 12 of *NPM* gene using the primers; *NPM* cDNA For, 5'-AAAGGTGGTCTCTTCCC AAA-3' and *NPM* cDNA Rev, 5'-GCATTATAAAAAGGACAGCC ACA-3' and directly sequenced on a DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems).⁹ We could not find any *NPM* gene mutations in this study.

It was reported that the frequency of *NPM* gene mutations in children (<18 years old) was very low (children 1 out of 47 (2.1%) versus adults 32 out of 126 (25.4%), $P < 0.001$).⁸ Furthermore, they suggested that *NPM* gene mutations were also rarely detected in patients younger than 40 years old (3 (3.5%) out of 85). On the other hand, it was reported that *NPM* mutations were found in seven (27.1%) of 26 pediatric AML patients with normal karyotype from Italy.⁷ These seven patients ranged from 5.0–17.9 years old, 10 years old ($n = 2$), 11 years old ($n = 2$) and 5, 8, 17 years old ($n = 1$). Notably, two AML patients with *NPM* gene mutations have been reported in adult Japanese AML study (15 and 16 years old).⁵

We also analyzed *FLT3*-ITD and *RAS* gene alterations in these patients and found *FLT3*-ITD in nine (27.3%), *NRAS* mutation in two (6.1%) and *KRAS* mutation in three (9.1%). The frequencies of these gene alterations were compatible with those of previous reports.^{2,3}

We considered that *NPM* gene mutations may be infrequent in Asian pediatric AML patients with normal karyotype, especially less than 15 years old. Frequency of *NPM* gene mutations depends on age and may depend on races. Further larger studies of *NPM* gene analysis are needed to clarify this item.

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Pro-inflammatory cytokinemia is frequently found in Down syndrome patients with hematological disorders

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Abstract

Down syndrome (DS) patients are frequently complicated with infections, autoimmune phenomena and hematological disorders, including transient abnormal myelopoiesis (TAM) in infancy and acute megakaryoblastic leukaemia (AMKL) in later life. In this study, serum levels of cytokines from 23 TAM and 15 AMKL patients were examined using the highly sensitive microsphere fluorescence system. Statistical differences between DS neonates with or without TAM were found in IL-1 β [median 7.0 pg/ml (0.34–271.6) versus 0.05 pg/ml (0.0–2.4), $p=0.034$], TNF- α [8.11 pg/ml (0.1–253.0) versus 0.41 pg/ml (0.1–1.5), $p=0.041$], and IFN- γ [20.0 pg/ml (0.14–406.3) versus 1.5 pg/ml (0.14–5.79), $p=0.036$]. Moreover, abnormal inflammatory cytokinemia was also found in myelodysplastic syndrome (MDS) and AMKL with DS. These abnormal cytokinemia may have a role in the pathophysiology of TAM, MDS and AMKL in DS, especially in liver fibrosis or myelofibrosis.

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1. Introduction

Down syndrome (DS) patients are frequently complicated with infections [1], thyroid dysfunction [2], and autoimmune phenomena [3]. These immunological abnormalities were partially explained by the dysfunction and altered subsets of T-lymphocytes [4–7]. Mental retardation in DS resembles that of Alzheimer's disease, and the neuronal degeneration in DS was partially explained by the existence of abnormal pro-inflammatory cytokines [8–11]. Acceler-

ated aging was observed in DS patients [12]. Immunological abnormalities, including sustained inflammatory cytokinemia, are involved in the various pathophysiology in DS patients.

On the other hand, DS patients may also be highly complicated by various hematological disorders, including transient abnormal myelopoiesis (TAM) or transient myeloproliferative disorder (TMD) in infancy, myelodysplastic syndrome (MDS), and acute myeloid leukaemia (AML), especially acute megakaryoblastic leukaemia (AMKL) within 3 years [13–15]. TAM occurs in about 10% of DS infants, and most cases resolve spontaneously with unknown reasons within a few months after birth [13]. However, recent studies have

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revealed that about 20% of TAM patients are life-threatening or fatal cases with respiratory distress or liver failure [16–18]. Our previous report demonstrated that sustained cytokinemia is likely to be involved in the pathophysiology of TAM, and very high serum concentrations might predict a poor outcome [19]. In this study, we assayed several cytokine levels in a larger number of TAM, MDS, and AMKL patients with DS. Our data revealed that abnormal inflammatory cytokinemia existed not only in TAM patients, but also in MDS and AMKL patients, suggesting that abnormal pro-inflammatory cytokinemia contributes to the disease symptoms in TAM and AMKL, including liver fibrosis and myelofibrosis.

2. Patients, materials and methods

Twenty three TAM and 15 AMKL patients with DS were enrolled in this study. All patients had the clinical features of DS and had trisomy 21 in the peripheral blood cultured for 72 h with phytohaemagglutinin. Three TAM patients (13.0%) died within 4 months of birth because of the severe complications, including liver fibrosis, and the remaining 20 TAM patients were alive with or without complications. Sera from 9 DS neonates and 6 DS patients under 2 years old without hematological disorders were used as DS-controls. Sera from 10 neonates without DS were also used as controls. Six patients with less than 20% of blasts in the bone marrow nucleated cells were diagnosed as having myelodysplastic syndrome (MDS) according to the French-American-British (FAB) classification [20]. All patients were enrolled in this study after their parents gave informed consent. The sera were stored at -80°C until use. No IgM antibodies specific for microorganisms, including rubella, cytomegalovirus, herpes simplex virus, or toxoplasma could be detected in the sera at birth from any of the patients. Maternal infection by human T cell lymphotropic virus type 1, human immunodeficiency virus, or hepatitis B or C virus was not detected during pregnancy. AMKL was also diagnosed according to the FAB classification, and TAM was diagnosed according to a previous report [13,21].

3. Cytokine assay

Various pro-inflammatory cytokines, including interleukin (IL)-1 β , IL-2, IL-6, tumor necrosis factor (TNF)- α , interferon (INF)- γ , granulocyte and macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), anti-inflammatory cytokines (IL-4, IL-10), and chemokine (IL-8) in the serum were assayed using a highly sensitive microsphere fluorescence system. Fluorescence, a measure of the number of microspheres per unit volume, was measured using the Multiplex Suspension Array System (BioLad Laboratories) [22,23]. The cases that were under the detection limit of this assay system were considered to be 0 pg/ml.

4. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test, Bonnferroni test, and Pearson's correlation coefficient test. A two-tailed *p*-value less than 0.05 was accepted as indicating significance.

5. Results

5.1. Cytokine/chemokine levels in DS with or without TAM and non-DS healthy neonates

Cytokine and chemokine levels were elevated to some extent in DS neonates with or without TAM compared with normal healthy neonates (Fig. 1a–c). A statistical difference between DS neonates with or without TAM was found in IL-1 β [median 7.0 pg/ml (0.34–271.6) versus 0.05 pg/ml (0.0–2.4), $p=0.034$], TNF- α [8.11 pg/ml (0.1–253.0) versus 0.41 pg/ml (0.1–1.5), $p=0.041$], and IFN- γ [20.0 pg/ml (0.14–406.3) versus 1.5 pg/ml (0.14–5.79), $p=0.036$] according to Bonnferroni analysis. The median serum GM-CSF level in healthy neonates and TAM were 8.00 pg/ml and 132.27 pg/ml ($p=0.017$), respectively.

5.2. Cytokine/chemokine levels in DS-AMKL and DS controls

A significant difference between DS children with or without AMKL was observed in IL-1 β [median 6.88 pg/ml (0.02–108.5) versus 0.02 pg/ml (0.02–6.76), $p=0.032$], IL-4 [8.98 pg/ml (4.27–219.97) versus 0.0 pg/ml (4.27–46.42), $p=0.033$], IL-6 [141.0 pg/ml (7.62–1695.7) versus 18.2 pg/ml (3.21–317.6), $p=0.0081$], IL-8 [25.79 pg/ml (1.5–247.5) versus 2.02 pg/ml (0.75–18.75), $p=0.0051$], IFN- γ [34.47 pg/ml (0.14–566.7) versus 1.43 pg/ml (0.14–146.8), $p=0.042$], and TNF- α [3.13 pg/ml (0.1–573.7) versus 0.10 pg/ml (0.1–27.3), $p=0.049$] according to the Mann–Whitney *U*-test (Fig. 1d and e). The differences were not significant in other cytokines.

5.3. Cytokine/chemokine levels in DS-MDS and DS controls

The cytokine/chemokine levels are elevated in MDS patients compared with those of DS controls. The difference was statistically significant between DS-MDS and DS controls in IL-1 β [median 54.56 pg/ml (48.36–79.26) versus 0.02 pg/ml (0.02–6.76), $p=0.0037$], in IL-4 [27.80 pg/ml (8.53–60.54) versus 4.27 pg/ml (4.27–46.42), $p=0.022$], in IL-8 [10.67 pg/ml (9.09–27.12) versus 2.015 pg/ml (0.75–18.75), $p=0.025$], in IFN- γ [45.98 pg/ml (24.31–879.43) versus 1.43 pg/ml (0.14–146.79), $p=0.024$], and in TNF- α [112.53 pg/ml (75.31–980.37) versus 0.1 pg/ml (0.1–27.3), $p=0.0037$] according to the Mann–Whitney *U*-test. The significant difference between DS-MDS and

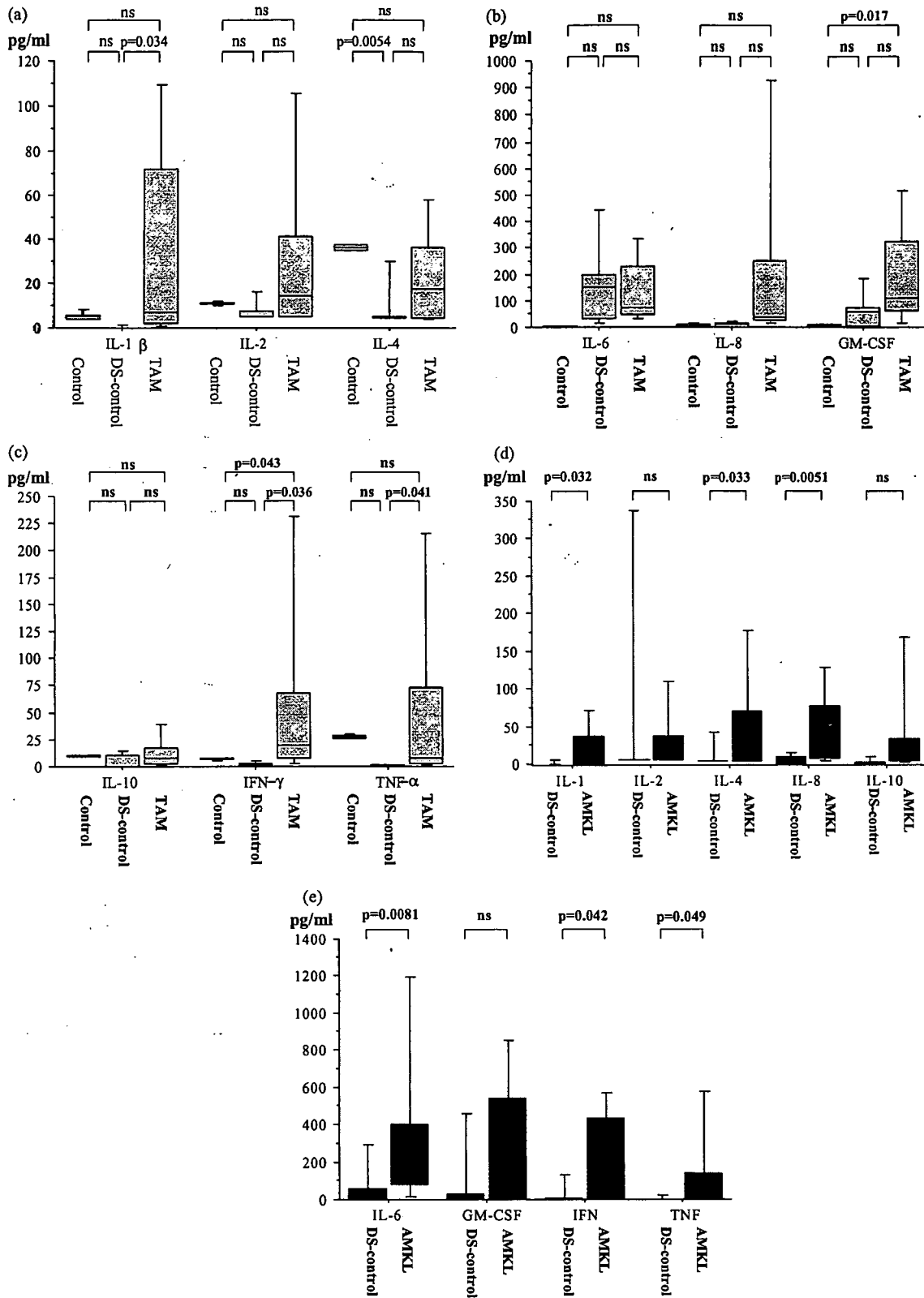


Fig. 1. (a–c) The cytokine levels in the sera of DS neonates with or without TAM and non-DS. A statistical difference between DS neonates with or without TAM was found in IL-1 β , TNF- α and IFN- γ . (d and e) The cytokine levels in the sera of DS children with or without AMKL. A significant difference between DS children with or without AMKL was observed in IL-1 β , IL-4, IL-6, IL-8, IFN- γ and TNF- α .

DS-AMKL was observed in only IL-1 β (median 54.56 pg/ml verses 6.88 pg/ml, $p = 0.035$).

6. Discussion

The production of several abnormal cytokines has been reported to be associated with the pathophysiology of DS [8–11]. For example, IL-1 was overexpressed throughout life in DS and contributed to neuronal degeneration [9,11]. Serum IL-6 levels correlated with the severity of dementia in DS [8]. Overexpression of TNF- α and IFN- γ was observed in the thymus of DS patients [24]. DS patients showed the consistent overexpression of superoxide [SOD]1 on chromosome 21 [25,26]. A high level of inflammatory cytokines may play an important role in several pathophysiologies, including mental retardation [8–11], a high susceptibility to infections [1], and autoimmune phenomena in DS [3]. These abnormal cytokinemia could cause neuronal cell death, via apoptosis [27]. Furthermore, these abnormal cytokinemia were partially explained by the dysfunction of T-lymphocytes in DS patients, but the details remain unknown [4–7].

In contrast, the relationship between inflammatory cytokines and leukemogenesis has been rarely reported so far [28]. High expressions of platelet-derived growth factor and TGF- β 1 were observed in blast cells from TAM patients with liver fibrosis [29,30]. Our previous report demonstrated that sustained cytokinemia was found in 4 TAM patients, and very high serum concentrations might predict a poor outcome [19]. Liver fibrosis in fatal TAM was considered to be due to the sustained pro-inflammatory cytokinemia and the imperfect remodeling [28,31,32]. Pro-inflammatory cytokinemia may cause the cellular apoptosis that resulted in the spontaneous regression of TAM blasts. The inflammatory process, which lost its auto regulatory capacity, was frequently found in neurodegeneration [33]. However, DS neonates without TAM also showed the slightly increased levels of inflammatory cytokines in this study, suggesting that these elevated cytokines might be partially due to the existence of heart failure or other complications in these DS neonates without TAM. Furthermore, abnormal cytokine production in amniotic fluid has been reported to be found in DS pregnant women [34,35], suggesting that abnormal cytokine production has begun in utero. DS-TAM was also suggested to develop in utero [36] and hydrops fetalis with DS-TAM was reported [17,37]. What is the cause of this inflammatory cytokinemia in DS-TAM? It was reported that blasts themselves produce several cytokines [30,38]. Moreover, IFN- γ producing T cells are abundant in DS [7]. Further studies are needed to resolve these issues.

Furthermore, we found that inflammatory cytokinemia was also found in DS-MDS and AMKL in this study. The function of inflammatory cytokines, having pro-apoptotic or anti-apoptotic effects, differs according to the situation. IL-1, IL-6, GM-CSF and TNF- α are pro-inflammatory cytokines, but stimulate the growth of megakaryocytes

[38–42]. Myeloid leukaemia cells are known to produce cytokines that stimulate their proliferation [43]. GM-CSF induces pro-apoptotic and anti-apoptotic signals in AML [44]. Abnormal inflammatory cytokines might have a role in the stimulation of leukemogenesis in DS patients.

In conclusion, pro-inflammatory cytokinemia is frequently found in DS patients with hematological disorders. Further studies are needed to clarify the role of these immunological disturbances in leukemogenesis with DS.

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Identification of side population cells (stem-like cell population) in pediatric solid tumor cell lines[☆]

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Abstract

Purpose: Recent evidence has supported the *cancer stem cell theory* that cancer contains a small number of cancer stem cells (CSC) as a reservoir of cancer cells. Only the CSC, but not most of the remaining constituent cancer cells, are thought to be responsible for tumorigenesis, progression, and metastasis as well as cancer relapse, suggesting that the CSC should be targeted to eradicate the cancer. Side population (SP) cells isolated by fluorescence-activated cell sorting (FACS) using Hoechst dye are known to be enriched in stem cells in various normal tissues as well as cancers. The authors investigated whether such stem-like SP cells may exist in pediatric solid tumors (PSTs).

Materials and Methods: Sixteen pediatric tumor cell lines including 7 neuroblastomas, 4 rhabdomyosarcomas, and 5 Ewing's sarcomas were used for FACS analysis. Analysis of SP cells based on the exclusion of the DNA binding dye, Hoechst 33342, with and without verapamil using FACS was performed.

Results: One Ewing's sarcoma cell line did not show an SP fraction, and only a small fraction of SP cells (0.12%–14.6%) was detected in the other 15 cell lines. These SP cells were all sensitive to verapamil.

Conclusions: This study suggested that most PSTs would contain a small fraction of SP cells (possible stem-like population). Targeting the CSC will provide a novel treatment strategy to eradicate refractory PSTs.

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Stem cells are characterized by the properties of self-renewal and multipotency that are called *stemness*. Normal tissue comprises a hierarchical organization composed of a small fraction of stem cells as a reservoir and their descendants. Recent evidence has shown that cancer may also be maintained by a small fraction of cancer stem cells (CSC) (stem-like cancer cells) that retain the properties