alone did not shift its physiological localization in the cytoplasm (Figure 3B, top). Taken together, these data demonstrated that myeloid immortalization in vitro by *MLL-SEPT6* requires homo-oligomerization in the nucleus, probably dimerization of MLL fusion proteins as reported (11, 12), through both its GTP-binding domain and its coiled-coil region.

*MLL-SEPT6 induces lethal MPD, not acute leukemia, in vivo.* We next examined the leukemogenic potential of *MLL-SEPT6* in vivo using transplantation of the cells immortalized by *MLL-SEPT6* into sublethally irradiated syngeneic mice. Five of 8 mice transplanted with the immortalized cells died with latencies of over 6 months within an observation period of 15 months (Table 1). Morbid mice that received the transplantation exhibited hepatosplenomegaly with leukocytosis, anemia, and thrombocytopenia (Table 1). Notably, histopathologic analysis showed that bone marrow and peripheral blood cells derived from the morbid mice had morphologic and immunophenotypic features of MPD with myeloid hyperplasia consisting predominantly of mature granulocytic elements on FACS analysis, and that these elements infiltrated the spleen and the liver (data not shown). The lethal MPD was also induced by transplantation of murine hematopoietic progenitors transduced with *MLL-SEPT6* into lethally irradiated syngeneic mice directly after retroviral infection (described in detail below), and bone marrow cells harvested from these MPD mice grew and expanded dependently on IL-3 in vitro (data not shown). These results differ from findings in the previous reports in which acute leukemias with latencies of 2–6 months were induced by some MLL fusion proteins (9, 10), which suggested that fusion of MLL with SEPT6 was not sufficient, and required additional genotoxic stress, to induce acute leukemia.

*Secondary genotoxic stress, such as FLT3-ITD, synergistically transforms hematopoietic progenitors transduced with MLL-SEPT6 or MLL-ENL in vitro.* To seek a candidate for additional genotoxic stress required for leukemogenesis by *MLL-SEPT6*, we first analyzed the transforming activity of *MLL-SEPT6*-transduced cells with additional expression of *FLT3*-ITD, using the transformation assay (Figure 4, A and C). The infection efficiencies of *FLT3*-ITD and vector alone were 0.77% ± 0.31% and 9.0% ± 0.67%, respectively. The transduction of *FLT3*-ITD not only enabled the immortalized cells to grow without IL-3 but also turned almost all of the cells (95.0% ± 1.5%) positive for GFP 7 days after the deprivation of IL-3, while transduction of the vector alone did not (Figure 4B). These results suggest that *FLT3*-ITD has the potential to replace the signaling pathways by IL-3 in the cells expressing MLL-SEPT6 in vitro. The transduction of a kinase-active mutant FLT3 with an Asp-835-to-Val mutation ($FLT3^{D835V}$) also transformed the immortalized cells (data not shown) in the same transformation assay, as it has been reported to transform Ba/F3 cells (30).

On the other hand, the myeloid immortalization assay using *FLT3*-ITD in pMYpuro could not detect any myeloid immortalization (Figure 4, A, C, and D), which implies that *FLT3*-ITD alone

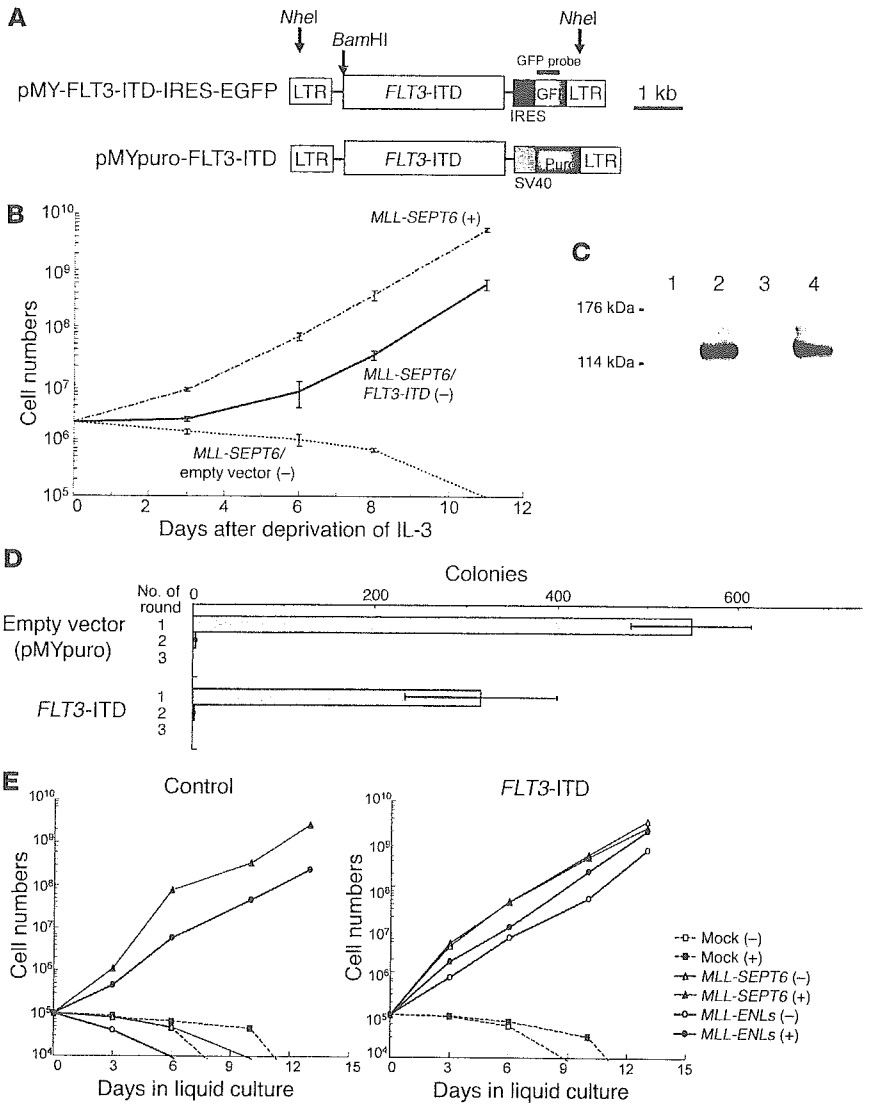


**Figure 4**
Synergistic transformation of murine hematopoietic progenitors by *MLL* fusion genes and *FLT3*-ITD in vitro. (**A**) Schematic representation of the retroviral constructions expressing FLT3-ITD. (**B**) Transformation assay of the cells immortalized by *MLL-SEPT6*, after transduction with *FLT3*-ITD in the pMY-IRES-EGFP construct shown in **A** in the presence (+) or absence (–) of IL-3. (**C**) Western blot analysis of proteins extracted from PlatE cells transfected with the constructs shown in **A** and each vector alone as a control, after immunoprecipitation using the anti-FLT3 Ab (lanes 1–4). Each lysate was blotted with the anti-FLT3 Ab. Lane 1, pMY-IRES-EGFP alone; lane 2, pMY-FLT3-ITD-IRES-EGFP; lane 3, pMYpuro alone; lane 4, pMYpuro-FLT3-ITD. (**D**) Myeloid immortalization assay using the pMYpuro constructs shown in **A**. The bar graph shows numbers of colonies obtained after each round of replating in methylcellulose (average ± SD). (**E**) Myeloid transformation assay using the sequential transduction with *FLT3*-ITD or control (pMYpuro alone) after *MLL-SEPT6*, *MLL-ENL*s, or mock (pMXs-neo alone) in the presence (+) or absence (–) of IL-3.

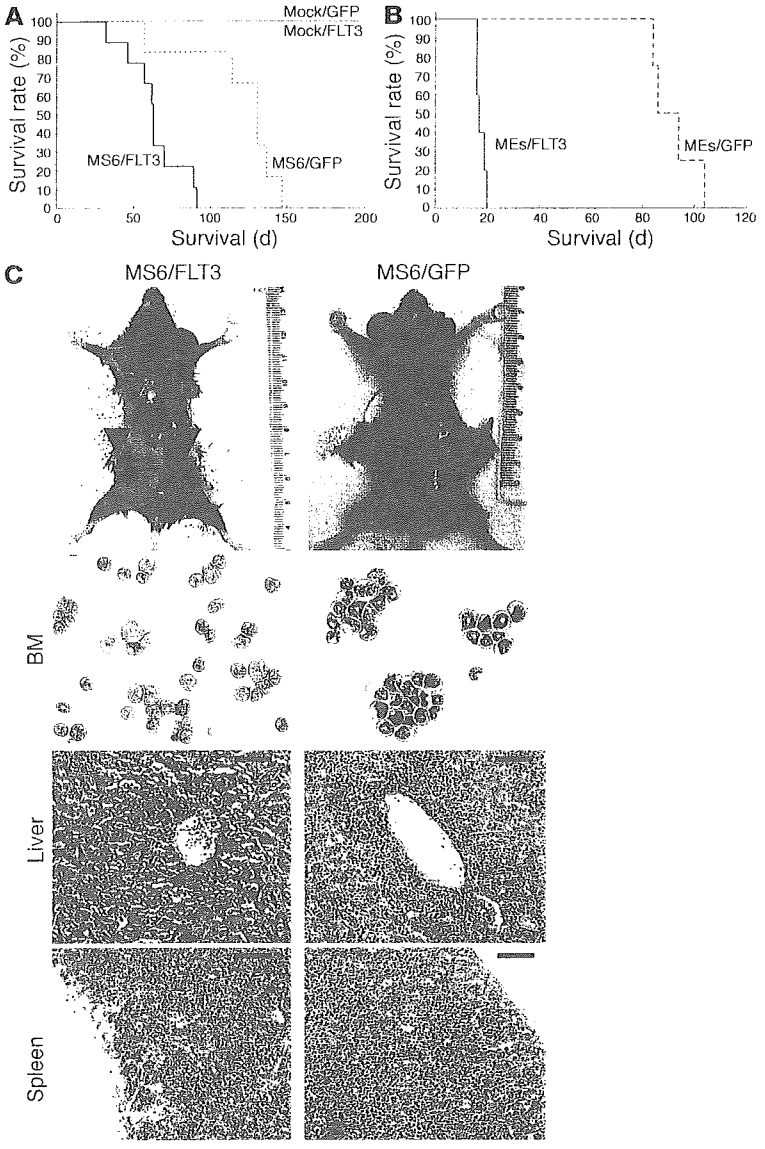


**Figure 5**
Synergistic leukemogenesis induced by *MLL* fusion genes and *FLT3*-ITD in vivo. (**A** and **B**) Survival curves of mice transplanted with mock/GFP ($n$ = 4), *MLL-SEPT6*/GFP (MS6/GFP; $n$ = 6), mock/FLT3 ($n$ = 5), and MS6/FLT3 ($n$ = 9) (**A**), and with *MLL-ENL*s/GFP (MEs/GFP; $n$ = 4) and MEs/FLT3 ($n$ = 5) (**B**). Mice transplanted with *MLL* fusion genes in combination with *FLT3*-ITD showed significantly shorter survival time than those with corresponding *MLL* fusion genes in combination with GFP ($P < 0.05$, log-rank test). (**C**) Representative macroscopic and histopathologic analysis of morbid mice transplanted with MS6/FLT3 and MS6/GFP, respectively. Arrowheads show lymphadenopathy. BM cells were stained with Wright-Giemsa, and paraffin sections of liver and spleen were stained with H&E. Original magnification of BM cells, ×200; scale bars, 200 μm.

was insufficient for immortalization of murine hematopoietic progenitors in this colony assay or that this assay might be inappropriate to examine the oncogenic proliferative and/or survival advantage, which was considered to be primarily conferred by some oncogenic mutations including *FLT3*-ITD (31). Furthermore, the synergy of *MLL-SEPT6* and *FLT3*-ITD was examined with myeloid transformation assay using either simultaneous or sequential transduction with these genes. To generalize the synergy in the *MLL*-mediated transformation in vitro, *MLL-ENL*s was also applied to the same assay. While the simultaneous transduction failed to cause transformation, probably because of the low efficiency of double transduction and limitation of the sensitivity in this in vitro assay (data not shown), the sequential transduction with *FLT3*-ITD after *MLL-SEPT6* or *MLL-ENL*s, not mock, enabled murine hematopoietic progenitors to grow and expand without IL-3 (Figure 4E). Taken together, these results demonstrated that *MLL* fusion genes *MLL-SEPT6* and *MLL-ENL* can transform murine hematopoietic progenitors in vitro in concert with additional genotoxic stress, such as *FLT3*-ITD, which suggests that *MLL* fusion gene and *FLT3*-ITD may synergistically serve as oncogenes through different pathways.

*MLL fusion genes MLL-SEPT6 and MLL-ENL require secondary genotoxic stress, such as FLT3-ITD, to induce acute leukemias in vivo with short latency.* To address the synergistic leukemogenic potential of *MLL* fusion gene and *FLT3*-ITD in vivo, we directly transplanted Ly-5.1 murine hematopoietic progenitors transduced with *MLL-SEPT6* and *FLT3*-ITD (MS6/FLT3) using a combination of each retrovirus into lethally irradiated syngeneic Ly-5.2 mice. The other combinations of each retrovirus — mock/GFP (pMXs-neo and pMY-internal ribosomal entry site–enhanced GFP [pMY-IRES-EGFP]), MS6/GFP, and mock/FLT3 — were also given as controls. In contrast to the MS6/GFP mice, which died with latencies of 57–147 days (120 ± 32 days), the MS6/FLT3 mice died with significantly shorter latencies of 32–91 days (63 ± 18 days, $P < 0.05$, log-rank test) (Figure 5A and Table 1). The morbid MS6/FLT3 mice exhibited mild hepatomegaly and moderate splenomegaly with various ranges of leukocytosis, anemia, and thrombocytopenia (Table 1). Some of the morbid MS6/FLT3 mice also exhibited remarkable lymphadenopathy (Figure 5C). Histopathologic analysis showed that the majority of bone marrow or peripheral blood cells derived from the morbid MS6/FLT3 mice had morphologic features of immature myelomonocytic blasts, which severely infiltrated the spleen and the liver (Figure 5C). The morphology of the differentiation blockade suggested that the MS6/FLT3 mice developed acute leukemias. On the other hand, the MS6/GFP mice died of MPD that showed features (Figure 5, A and C, and Table 1) similar to those seen in mice transplanted with the cells immortalized by *MLL-SEPT6* in vitro, while the mock/FLT3 mice survived without hematological disorders in peripheral blood for an observation period of 6 months (Figure 5A and Table 1). Histopathologic analysis of 1 mock/FLT3 mouse sacrificed 160 days after the transplantation demonstrated neither significant hepatosplenomegaly nor any disorders in the bone marrow, where 20% of the cells were positive for GFP and hence expressed *FLT3*-ITD (data not shown).

Cytological analysis showed that almost all of the bone marrow cells (94.4% ± 9.0%) derived from the MS6/FLT3 mice originated in donor cells (Ly-5.1), and that the majority of these cells (85.4% ± 14.9%) were positive for GFP and thus expressed *FLT3*-ITD (Figure 6). Southern blot analysis of DNA derived from the

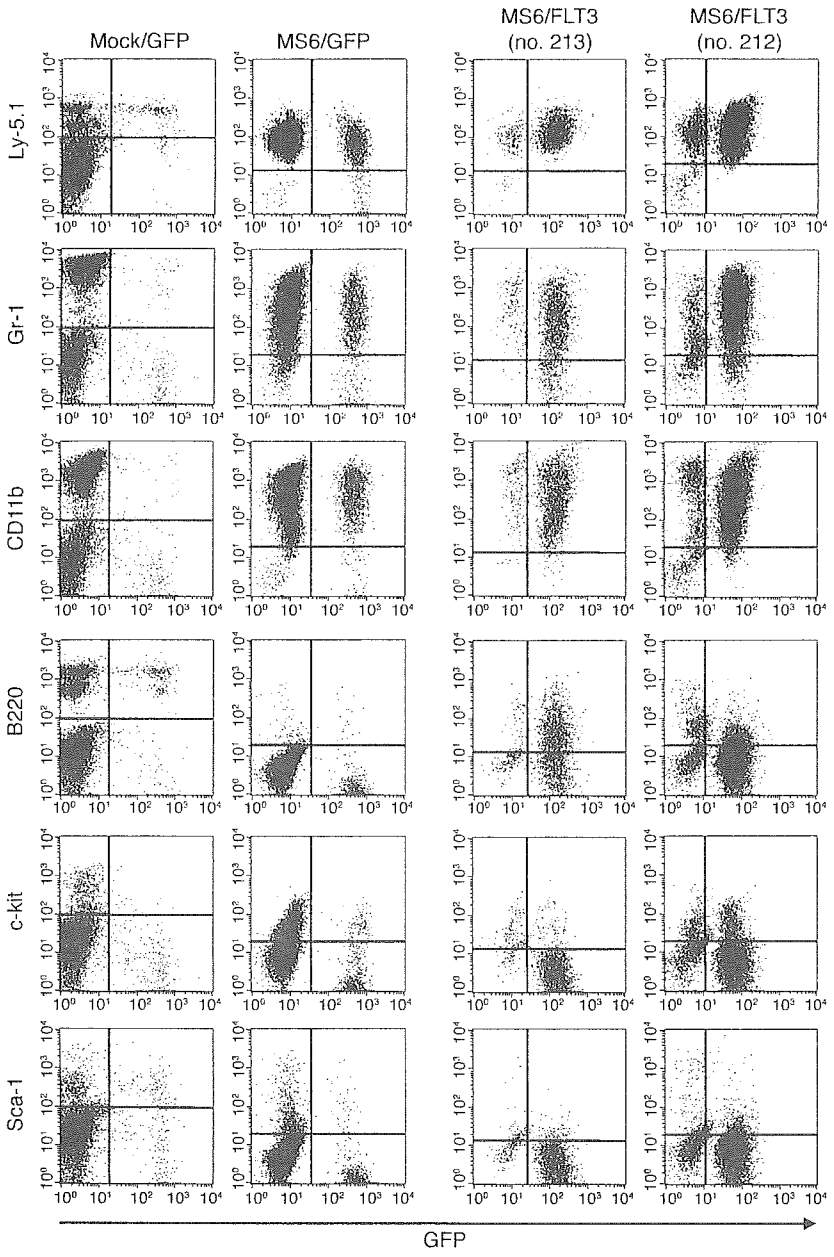


**Figure 6**
Immunophenotype of bone marrow cells obtained from representative mice (nos. 213 and 212) transplanted with mock/GFP, MS6/GFP, and MS6/FLT3. The dot plots show each surface antigen labeled with a corresponding PE-conjugated mAb versus expression of GFP.

spleen of the MS6/FLT3 mice demonstrated that intensities of proviral integration bands of *MLL-SEPT6* and *FLT3*-ITD were almost equal, and that oligoclonal bands of proviral integration sites were also present (Figure 7, A and B). In addition, expression of *MLL-SEPT6* was detected by RT-PCR analysis of total RNA derived from the spleens (data not shown), indicating both cointegration and coexpression of *MLL-SEPT6* and *FLT3*-ITD in the leukemic cells. Immunophenotyping analysis revealed that these leukemic cells were highly positive for Gr-1 and CD11b (Figure 6). Interestingly, the leukemic cells with these myeloid markers sometimes coexpressed B220, but no CD3 (Figure 6 and data not shown), and B220 expression did not always correlate with *IgH* gene rearrangements (Figure 7C). For example, 57.0% and 98.2% of the GFP-positive leukemic cells derived from mouse no. 213 were positive for B220 and CD11b, respectively (Figure 6), which demonstrates that at least half of these leukemic cells were biphenotypic, although more direct evidence from 3- or 4-color flow cytometry analysis could not be obtained because of lack of these leukemic cells. These results suggest that *MLL-SEPT6* and *FLT3*-ITD could induce acute myeloid, or sometimes biphenotypic, leukemia with lineage promiscuity (32). Peroxidase stain of these leukemic cells carrying B220 also corroborated myeloid lineage of these blasts (data not shown).

To generalize the synergetic effect in vivo in *MLL*-mediated leukemogenesis, *MLL-ENL*s was also applied to this leukemogenesis assay. *MLL-ENL*s and *FLT3*-ITD (MEs/FLT3) could induce AML with significantly shorter latency of 17.6 ± 1.8 days ($P < 0.05$, log-rank test), while *MLL-ENL*s (MEs/GFP) induced MPD with latency of 92 ± 9.1 days (Table 1, Figure 5B, and data not shown) as previously described (14). Taken together, these data demonstrate that *MLL* fusion genes *MLL-SEPT6* and *MLL-ENL* can synergize with secondary genotoxic stress such as *FLT3*-ITD to induce acute myeloid or biphenotypic leukemias in vivo with short latency as a clinical feature.

## Discussion

We demonstrated that homo-oligomerization of MLL-SEPT6 through its intact GTP-binding domain and coiled-coil region in the nucleus is critical for its oncogenic activity. Intrinsic septins, such as Sept6, tend to polymerize into filaments as heteromeric septin complexes in the cytoplasm at least partially through their coiled-coil regions (28, 33), while a recombinant septin, such as Sept2, is able to homopolymerize depending on the binding of GTP, even in the absence of its coiled-coil region (18). Therefore, it is possible that ectopic expression of septin fusion proteins, such as MLL-SEPT6, may result in homo-oligomerization through their GTP-binding domains, and that their coiled-coil regions may contribute to stabilize the homo-oligomerization in the nucleus. Indeed, replacement of the coiled-coil region within MLL-SEPT6 with another inducible dimerization domain, the mutant LBD of hER, did not induce the immortalization in the presence of 4-OHT, which suggests that the coiled-coil region might play some important roles in addition to dimerization (11). It has been revealed that some MLL fusion proteins require oligomerization domains of the partner proteins that normally reside in the cytoplasm and that artificial dimerization of the portion of MLL is sufficient to induce immortalization of hematopoietic progenitors (11, 12), while it had already been disclosed that other MLL fusion proteins require the transactivation

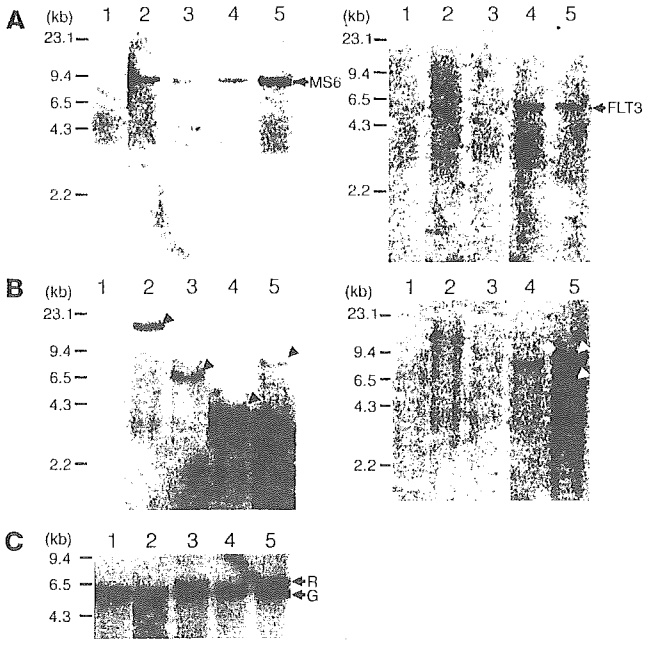


**Figure 7**
Southern blot analysis of spleen DNA obtained from representative mice transplanted with mock/GFP, MS6/GFP, and MS6/FLT3. Genomic DNA extracted from each spleen was digested with *Nhe*I (**A**), *Bam*HI (**B**), or *Eco*RI (**C**) and hybridized with the Neo probe (**A** and **B**, left), the GFP probe (**A** and **B**, right), or the $J_H$ probe (**C**). (**A**) Each single band of intact proviral DNA, including *MLL-SEPT6* (MS6) and *FLT3*-ITD (FLT3), is indicated by an arrow with each abbreviation. (**B**) Oligoclonal bands of proviral integration sites of MS6 or FLT3 are indicated by black or white arrowheads, respectively. (**C**) Germ line (G) or rearrangements (R) of *IgH* gene are indicated by an arrow with each abbreviation. Lane 1, mock/GFP mouse; lanes 2 and 3, MS6/GFP mice; lanes 4 and 5, MS6/FLT3 mice.

domains of the partner proteins that normally reside in the nucleus (9). Our findings that dimerization of MLL-SEPT6 is a prerequisite to develop leukemia also support this notion, but some kinds of simple dimerization of MLL fusion proteins, such as that of MLL-SEPT6$^{\Delta coil\text{-}ER}$, might be insufficient to induce the immortalization in vitro, as has been reported in the MLL-ENL mutant fused with the mutant LBD of hER (34). On the other hand, the mechanism by which oligomerization of MLL fusion proteins results in transcriptional activation of target genes including *HOX*, some of which were upregulated in our results, is less clear. In addition, *Meis1* was expressed in the cells transduced with mock, as in those with *MLL* fusion genes, in our myeloid immortalization assay. Replacement of pMXs-neo with pMXs-puro in this immortalization assay reproduced upregulation of *Meis1* (data not shown), suggesting that retroviral transduction followed by colony assay itself might accumulate the expression of *Meis1*.

*MLL-SEPT6* by itself was able to induce lethal MPD but not to induce acute leukemia in mice, although it did immortalize murine hematopoietic progenitors in vitro. These results differ from previous data (9, 11), suggesting 2 possibilities. One possibility is that *MLL-SEPT6* itself might possess not so strong an oncogenic potential as other *MLL* fusion genes examined previously. However, this hypothesis is inconsistent with the strong (i.e., early onset) phenotype of infant AML characteristic of *MLL-SEPT6*, which implies that secondary essential genotoxic events that might be evoked by interchromosomal gene rearrangement of *MLL* and *SEPT6*, and/or intrinsic unknown genetic instability, are involved in humans (15). It is noteworthy that lethal MPD was recently recapitulated in vivo by Cre-loxP–mediated de novo reciprocal chromosomal translocations bearing *Mll-Enl* (14). Another possibility is that methodological differences, including the use of different retrovirus vectors, account for the inconsistency with previous data. The promoter activity in the retroviral vectors we used may not be so strong as in the murine stem cell virus vector used in previous reports. But high expression of MLL fusion protein is known to be cytotoxic (9).

We herein provided direct evidence for a multistep leukemogenesis by MLL fusion proteins in vitro and in vivo. It had been suggested that in infant acute leukemia, rearrangements of *MLL* arise in utero and result in leukemia after birth (15). The phenotype of *Mll-AF9* knock-in mice that develop overt leukemia with long latency after birth and do not acquire sufficient oncogenic potential prenatally or perinatally (13, 35) supports the multistep leukemogenesis. Other experimental models in vivo (9, 10) did require a longer latency than expected from clinical features of human infant leukemia, which also implies that some secondary genotoxic events are essential for leukemogenesis by MLL fusion proteins. Vigorous investigations using DNA microarray revealed that *FLT3* is associated with *MLL*-rearranged leukemia (36), and there has been an interesting report that *MLL-ENL* induced acute multilineage leukemia in vivo only in combination with an activated receptor tyrosine kinase in a chicken model (37). The mutations of *FLT3* with constitutive tyrosine kinase activity are classified into *FLT3*-ITD or mutations within the activation loop (*FLT3*-mut), such as *FLT3*$^{D835V}$. *FLT3*-ITDs are associated with adult AML with poor prognosis (31), while *FLT3*-muts are found frequently in infant acute lymphoid leukemia with *MLL* rearrangements (38). Therefore, even though infant AML with *FLT3*-ITD has yet to be reported in studies of the limited number of cases of pediatric AML with 11q23 translocations (39), *FLT3*-ITD was assigned to a secondary genotoxic event in our experimental model system by both the clinical feature of infant AML with *MLL-SEPT6* and the presence of the myeloid and biphenotypic leukemic cell lines MOLM-13 and MV4;11, which harbor *FLT3*-ITD as well as rearrangements of *MLL* (38).

Recent reports demonstrated that *FLT3*-ITD induces not only MPD by itself (40), but also acute leukemia in concert with *PML-RARα* which alone causes MPD (41). Indeed, additional transduction of *FLT3*-ITD synergistically led to both in vitro transformation of *MLL-SEPT6*–transduced cells and in vivo development of acute leukemia with shorter latency by *MLL-SEPT6*, although the transduction of *FLT3*-ITD alone was not sufficient to induce lethal MPD with short latency in our experimental model, where we did not dare to intentionally enhance retroviral transduction since the enhancement could lead to *FLT3*-ITD–induced MPD (R. Ono, H. Nakajima, T. Kitamura, and T. Nosaka, unpublished observations) as reported previously (40). Taken together with the findings of stability of *MLL* fusion genes (5) and instability of *FLT3*-ITD in relapse samples (42), our results of the synergistic effects of *MLL-SEPT6* and *FLT3*-ITD on leukemogenesis indicate that *FLT3*-ITD can confer necessary and sufficient secondary genotoxicity on leukemogenesis by *MLL-SEPT6*. The bone marrow cells harvested from the mice developing MPD induced by direct transduction with *MLL-SEPT6* grew and expanded dependently on IL-3 in vitro as reported (43), suggesting that induction of

IL-3 independence by *FLT3*-ITD might be at least partly responsible for the leukemogenesis by *MLL-SEPT6* in concert with *FLT3*-ITD. Furthermore, since *MLL-ENL*, which requires no dimerization but a transactivation domain within ENL for its oncogenic activity (9), also induces transformation of murine hematopoietic progenitors in vitro and AML in vivo, in concert with *FLT3*-ITD, it is likely that MLL fusion proteins serve primarily to impair differentiation and/or to enhance self-renewal of hematopoietic progenitors and that *FLT3*-ITD confers the oncogenic proliferative and/or survival advantage (31). Interestingly, although *FLT3*-ITD is strongly associated with myeloid lineage, acute leukemia induced by *MLL-SEPT6* in concert with *FLT3*-ITD possessed not only myeloid but also biphenotypic lineage markers, as evidenced by the combination of *MLL-ENL* and an activated tyrosine kinase (37). This suggests that the multilineage property of acute leukemia with some rearrangements of *MLL* may require mutations such as constitutively active tyrosine kinases. Finally, these 2-step model systems will provide novel insights into the multistep leukemogenesis of acute myeloid and biphenotypic leukemia with 11q23 translocations.

## Methods

*Retroviral constructs.* The 5′-MLL fragment covering *MLL* exons 1–7 (a kind gift from H. Hirai, The University of Tokyo), and the full length of *SEPT6* type I (16), were inserted into pMXs-neo to generate pMXs-neo-5′-MLL and pMXs-neo-SEPT6, respectively. Several fragments containing portions of *SEPT6*, *ENL* (a kind gift from M. Seto, Aichi Cancer Center Research Institute, Aichi, Japan), or the mutant LBD of hER (29), produced with PCR, were cloned into pMXs-neo-5′-MLL for various pMXs-neo constructs. Each fragment inserted into pMXs-neo was fused with a FLAG or HA epitope tag at the C-terminus. A fragment of *FLT3*-ITD (30) was also inserted upstream of the IRES-EGFP cassette of pMY-IRES-EGFP to generate pMY-FLT3-ITD-IRES-EGFP, and the IRES-EGFP cassette was replaced with the puromycin cassette of pMXs-puro (27) to generate pMYpuro-FLT3-ITD.

*Transfection and retrovirus production.* Transfection was carried out using FuGENE 6 (Roche Diagnostics Corp.) according to the manufacturer's recommendations. Retroviruses were harvested 48 hours after PlatE cells were transfected with retroviral constructs, as previously described (30). Appropriate expression of the transgenes in PlatE cells was confirmed by Western blot analysis.

*Production of a polyclonal Ab against MLL.* The anti-MLL Ab against an oligopeptide within the portion of MLL retained in MLL fusion proteins (44) was manufactured in rabbits, using standard methods.

*Immunoprecipitation and Western blot analysis.* PlatE or 293T cells transfected with various constructs, or the *MLL-SEPT6*–transduced cells, were harvested with the lysis buffer (500 mM NaCl, 20 mM HEPES [pH 7.4], 0.5 mM EDTA, 2 mM DTT, 0.2% NP-40), supplemented with protease inhibitor cocktail (Sigma-Aldrich), and left on ice for 3 minutes. In mutual-interaction assay of MLL-SEPT6 fusion protein and its mutants, cell lysates were immediately diluted only for NaCl on ice, resulting in 150 mM of NaCl. Cell lysates were subjected to immunoprecipitation with the anti-MLL, monoclonal anti-HA (12CA5) (Roche Diagnostics Corp.), monoclonal anti-FLAG (M2), or polyclonal anti-FLT3 (C-20) (Santa Cruz Biotechnology Inc.) Ab as previously described (45), except that samples were washed with diluted lysis buffer (150 mM NaCl, 20 mM HEPES [pH 7.4], 0.5 mM EDTA, 2 mM DTT, 0.2% NP-40) supplemented with protease inhibitor cocktail in the mutual-interaction assay. Cell lysates transfected with pMXs-neo-SEPT6 were directly mixed with an equal volume of 2× SDS sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.04% bromophenol blue), and boiled for 5 minutes. Western blot analysis of the immunoprecipitate of each sample and the samples obtained from the cells transfected with pMXs-neo-SEPT6 was done using the anti-FLAG, anti-HA, anti-FLT3, or polyclonal anti-SEPT6 (a kind gift from M. Kinoshita, Kyoto University, Kyoto, Japan) (33) Ab to probe membranes as previously described (45).

*Myeloid immortalization assays and transformation assays of hematopoietic progenitors in vitro.* The oncogenic potential of MLL-SEPT6 or MLL-ENLs fusion protein and/or FLT3-ITD in vitro was analyzed in modified myeloid immortalization assays of murine hematopoietic progenitors, as previously described (43). In brief, hematopoietic progenitors were harvested from 6- to 10-week-old C57BL/6 mice 4 days after i.p. administration of 150 mg/kg 5-fluorouracil (5-FU), and cultured overnight in αMEM supplemented with 20% FCS and 50 ng/ml each of mouse SCF, human IL-6, human FLT3 ligand (R&D Systems Inc.), and human thrombopoietin (Kirin Brewery Co.). The prestimulated cells were infected for 60 hours with the retroviruses harboring various *MLL* fusion genes in pMXs-neo, *FLT3*-ITD in pMYpuro, or empty vector as control, in the αMEM supplemented with the same FCS and cytokines, using 6-well dishes coated according to the manufacturer's recommendations with RetroNectin (Takara Bio Inc.). Coinfection in the myeloid transformation assay, using simultaneous transduction, was done with mixtures of each retrovirus produced independently in equal mole ratio of each insert between long-terminal repeats in each retroviral construct. Infected cells ($10^5$ except for $10^6$ in coinfection) were then plated in 1.1 ml of 1% methylcellulose medium (StemCell Technologies) supplemented with 50 ng/ml of SCF and 10 ng/ml each of mouse IL-3, IL-6, and mouse GM-CSF (R&D Systems Inc.) in the presence of the appropriate drug or drugs (i.e., 1 mg/ml of G418, 1 μg/ml of puromycin, or a mixture of both). After culture for 1 week, colonies were counted, and single-cell suspensions ($10^4$ cells) of drug-resistant colonies were subsequently replated under identical conditions without any drug. Every 1 week, replating was repeated in the same way. Experiments with the pMXs-neo construct harboring fusion with the mutant LBD of hER were performed in the presence of either 1 μM of 4-OHT or vehicle control (ethanol). The immortalized cells were generated by seeding of cells from the third round of methylcellulose cultures in RPMI 1640 medium supplemented with 20% FCS and 10 ng/ml of IL-3. In myeloid transformation assays using sequential transduction, the G418-resistant cells harvested at the first round were secondarily infected with retroviruses harboring or not harboring *FLT3*-ITD in pMYpuro using the same protocol as in the first infections, and replated under identical conditions in the presence of puromycin. After 1 week, these transduced cells were cultured in RPMI 1640 medium supplemented with 20% FCS and SCF (20 ng/ml, only for the first 1 week) in the presence or absence of IL-3 (10 ng/ml), and viable cell numbers were counted periodically after standard trypan blue staining.

*Tumorigenicity and leukemogenesis assays in vivo.* For tumorigenicity assays of the cells immortalized by *MLL-SEPT6*, $10^6$ of the immortalized cells, or PBS as negative control, were injected i.v. into 8- to 12-week-old C57BL/6 (Ly-5.2) mice, which had been administered a sublethal dose of 5.25 Gy total-body γ-irradiation ($^{135}$Cs). For assays of leukemogenesis by *MLL-SEPT6* or *MLL-ENLs* in concert with *FLT3*-ITD, hematopoietic progenitors harvested after 5-FU treatment of Ly-5.1 C57BL/6 mice were prestimulated in the same way as in the myeloid immortalization assays. Using 6-well dishes coated with RetroNectin, the prestimulated progenitors were infected with mixtures of retroviruses under identical conditions to those for myeloid immortalization assays. These mixtures consisted of combinations of retroviruses harboring *MLL-SEPT6*, *MLL-ENLs*, or no insert in pMXs-neo, and *FLT3*-ITD or no insert in pMY-IRES-EGFP, as in the coinfection in the myeloid immortalization assay. Infected Ly-5.1 cells ($10^5$) were injected i.v. together with a radioprotective dose of $2 \times 10^5$ Ly-5.2 cells into Ly-5.2 mice, which had been administered a lethal dose of 9.5 Gy total-body γ-irradiation. In both assays, morbid mice were euthanized, and their tissue samples, such as peripheral blood, bone marrow, liver, and spleen, were

analyzed. Circulating blood cells were counted, and tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E, as previously described (46). Probabilities of murine overall survival were estimated using the Kaplan-Meier method and compared using the log-rank test. All animal studies were approved by the Animal Care Committee of the Institute of Medical Science, The University of Tokyo.

*Transformation assays of the cells immortalized by MLL-SEPT6 in vitro.* Cells immortalized by *MLL-SEPT6*, which grew dependently on IL-3, were infected with retroviruses harboring or not harboring *FLT3*-ITD in pMY-IRES-EGFP, as previously described (45). To determine whether the infected cells were transformed, these cells were deprived of IL-3 24 hours after the infection, and viable cell numbers were counted periodically after standard trypan blue staining.

*Morphological and FACS analysis.* Cytospin preparations of the cells immortalized by *MLL-SEPT6* and of bone marrow cells, and blood smears, were stained with Wright-Giemsa or peroxidase stain to assess cell morphology. Immunophenotyping and assessment of GFP-positivity were done with a FACSCalibur (BD Biosciences) as previously described (46).

*Southern blot analysis.* Genomic DNA was extracted from tissues or the immortalized cells and analyzed with a 352-bp GFP probe spanning nucleotides 63–414 (Figure 4A), the Neo probe (46) (Figure 2A), or the $J_H$ probe (a kind gift from C. Coleclough, St. Jude Children's Research Hospital, Memphis, Tennessee, USA) (47) as previously described (46).

*RT-PCR.* Total RNA was extracted from tissues, the immortalized cells, or a murine pro-B cell line, Ba/F3, and reverse-transcribed to cDNA with random hexamers as previously described (16). Conditions, reagents for PCR, and the primers specific for $\beta_2$m were as previously described (46). To detect transcripts of *MLL-SEPT6*, *Hox a7*, *Hox a9*, and *Meis1*, PCR amplification was run for 30 cycles using primers as follows: MLL-S6, 5′-GTGAAGAACGTGGTGGACTCTAG-3′, and S6-MLL, 5′-TGGCTGGCTCCCCTTCGAA-3′; Hoxa7S, 5′-TGCGCCTCCTACGACCAAAACATC-3′, and Hoxa7AS, 5′-CTCTTTCTTCCACTTCATGCGCCGA-3′; Hoxa9S, 5′-TGGCATTAAACCTGAACCGCTCTCG-3′, and Hoxa9AS, 5′-CTTCATTTTCATCCTGCGGT-TCTGGAAC-3′; M1S, 5′-GCATGGGTTCCTCGGTCAATGAC-3′, and M1AS, 5′-CATGGTCTCTATTCC-AAGAGGGCTG-3′.

*Immunostaining.* Immunostaining of 293T cells was done and viewed with a FluoView FV300 confocal microscope (Olympus Corp.), as previously described (48).

## Acknowledgments

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# The *MYO1F*, unconventional myosin type 1F, gene is fused to *MLL* in infant acute monocytic leukemia with a complex translocation involving chromosomes 7, 11, 19 and 22

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**We analysed a complex translocation involving chromosomes 7, 11, 19 and 22 in infant acute monocytic leukemia, and identified that the *MLL* gene on 11q23 was fused to the unconventional myosin type 1F, *MYO1F*, gene on 19p13.2–13.3. *MYO1F* consists of at least 28 exons and was predicted to encode a 1098-amino-acid with an N-terminal head domain containing both ATP-binding and actin-binding sequences, a neck domain with a single IQ motif, and a tail with TH1, TH2 and SH3 domains. Northern blot analysis of RNAs prepared from multiple tissues showed that the expression of approximately 4-kb transcripts appeared constant in most tissues examined. However, *MYO1F* was expressed in only three of 22 leukemic cell lines. The MLL-MYO1F fusion protein contains almost the entire MYO1F, however, C-terminal MYO1F has neither the transactivation domain nor the dimerization domain found in various MLL fusion partners. Further analysis of this novel type of MLL fusion protein would provide new insights into leukemogenesis. MYO1F is the fourth partner gene of *MLL* on 19p13. At the cytogenetic level, it may be difficult to distinguish *MLL-ENL*, *MLL-ELL*, *MLL-EEN* and *MLL-MYO1F* fusions created by t(11;19)(q23;p13), and it is likely that cases of t(11;19) lacking a known fusion gene may result in this gene fusion.**
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**Keywords:** *MLL*; *MYO1F*; acute monocytic leukemia; chromosome translocation

## Introduction

A variety of chromosomal aberrations in hematological malignancies have been identified and characterized. Recent studies have demonstrated that several chromosomal rearrangements and molecular abnormalities are strongly associated with distinct clinical subgroups, and are predictive of clinical features and therapeutic responses (Look, 1997; Rowley, 1998). The 11q23 translocation is a frequent cytogenetic abnormality found in hematological malignancies, occurring in 5–6% of patients with acute myeloid leukemia (AML), 7–10% of patients with acute lymphoblastic leukemia (ALL), 60–70% of infants with acute leukemia, and in most patients with therapy-related leukemia induced by inhibitors of topoisomerase II (Biondi *et al.*, 2000; Bloomfield *et al.*, 2002). The *MLL* gene (also called *ALL-1* or *HRX*) has been cloned in 11q23 translocations, such as t(4;11), t(9;11) and t(11;19) (Ziemin-van der Poel *et al.*, 1991; Gu *et al.*, 1992; Tkachuk *et al.*, 1992), and is translocated to approximately 60 different chromosomal loci (Hayashi, 2000; Daser and Rabbitts, 2004). Cloning of various fusion partners of *MLL* has revealed that the phenotype of leukemia with *MLL* gene rearrangement usually depends on the fusion partner (Hayashi, 2000). Despite the progress of cancer therapy, *MLL* gene rearrangement is strongly associated with a poor outcome in infants with ALL, compared with that of older children with ALL or AML (Biondi *et al.*, 2000; Kawasaki *et al.*, 2001; Kosaka *et al.*, 2004).

Recent progress in gene expression monitoring using DNA microarrays has revealed novel leukemia classification with a distinct gene expression in pediatric ALL with *MLL* gene rearrangement (Hayashi, 2003). Clustering algorithms have revealed that, based on their gene expression patterns, acute leukemias with *MLL* rearrangement can clearly be separated from conventional ALL and AML, suggesting that they constitute a distinct disease (Armstrong *et al.*, 2002). We have also found that infant ALL with *MLL* gene rearrangement can be identified from the distinct expression pattern of several genes, including *FLT3*, *CD44*, *HOXA9* and *MEIS1* (Tsutsumi *et al.*, 2003). Intriguingly, point mutations of D835/I836 of the *FLT3* gene have been frequently found in infant ALL with *MLL* gene rearrangement, suggesting that *FLT3* mutation is a

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second genetic event in ALL with *MLL* rearrangement (Taketani *et al.*, 2004). Furthermore, using the gene expression profiles, each of the t(4;11), t(11;19), or t(5;11) found in ALL with *MLL* gene rearrangement could be classified into two distinct groups, with differential prognosis, irrespective of their translocation partner chromosomes (Tsutsumi *et al.*, 2003).

Several cases with complex 11q23 translocations have been identified by molecular analysis (Taki *et al.*, 1996; Chinwalla *et al.*, 2003). Particularly, these translocations were frequently observed in t(10;11)(p12;q23) creating *MLL-AF10* (Beverloo *et al.*, 1995; Shibuya *et al.*, 2001) and t(X;11)(q22-24;q23) creating *MLL-SEPT6* (Ono *et al.*, 2002a; Slater *et al.*, 2002), because the direction of transcription of *AF10* and *SEPT6* is opposite to that of *MLL*. Therefore in t(10;11)(p12;q23) or t(X;11)(q22-24;q23), an inversion of a part of chromosome including *MLL*, *AF10* or *SEPT6* inserts adjacent to the partner gene to form regular head-to-tail fusion transcripts (Beverloo *et al.*, 1995; Fu *et al.*, 2003). In this study, we identified the *MYO1F* gene on chromosome 19p13 as a novel fusion partner of the *MLL* gene in a patient with infant acute monocytic leukemia (AMoL). To date, *ENL*, *ELL/MEN* and *EEN* have been identified as fusion partners of *MLL* in reciprocal t(11;19)(q23;p13) (Hunger *et al.*, 1993; Thirman *et al.*, 1994; Mitani *et al.*, 1995; So *et al.*, 1997). *MYO1F* is the fourth partner gene of *MLL* on chromosome band 19p13.

## Results

### *Rearrangement of the* MLL *gene in an infant AMoL patient with a complex translocation involving chromosomes 7, 11, 19 and 22*

A 2-month-old girl was diagnosed as having AMoL. Cytogenetic analyses of the leukemic cells of the patient using regular G-banding and spectral karyotyping (SKY) analysis revealed 46, XX, der(7)t(7;19)(q11;p13), der(11)t(7;11)(q11;q23), del(19)(p13), der(22)t(11;22)(q23;p13) [17/20] and 46, XX [3/20] (Figure 1). Southern blot analysis of DNA prepared from the leukemic cells of the patient using an *MLL* cDNA probe showed a chromosomal breakpoint within the breakpoint cluster region of the *MLL* gene at 11q23 (Figure 2). These findings likely showed that 5′-*MLL* was fused to a gene on 7q11. Since no partner gene of *MLL* had been identified on chromosome 7q11, we inferred that the *MLL* in this patient was fused to a novel partner gene.

### *Cloning and identification of* MLL *fusion cDNA*

To clone the chimeric transcripts, we performed cDNA panhandle PCR analysis of poly(A)$^+$ RNA from the bone marrow mononuclear cells of the patient at diagnosis. We detected PCR products using primers MLL-3 and ALL-7S for the second PCR (Figure 3a). Sequence analysis of the subcloned PCR product revealed that one clone contained only an 8-bp sequence fused to *MLL* exon 7 (Figure 3c). To clone a longer sequence of the novel partner gene, we next performed second PCR for the same first PCR product using primers MLL-3 and AK76, which contains the junction of *MLL* exon 7 and an unknown 8-bp sequence, and

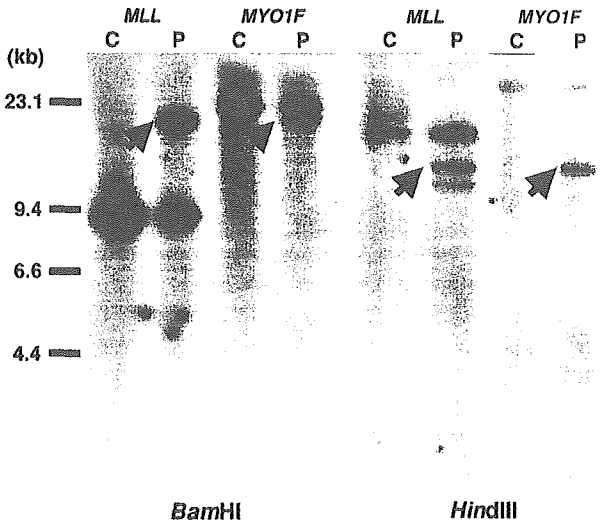


**Figure 2** Southern blotting of the *MLL* and *MYO1F* genes with *Bam*HI and *Hind*III. C, normal peripheral lymphocytes. P, patient's leukemic cells. Arrows indicate the same-sized rearranged bands detected by both *MLL* and *MYO1F* probes

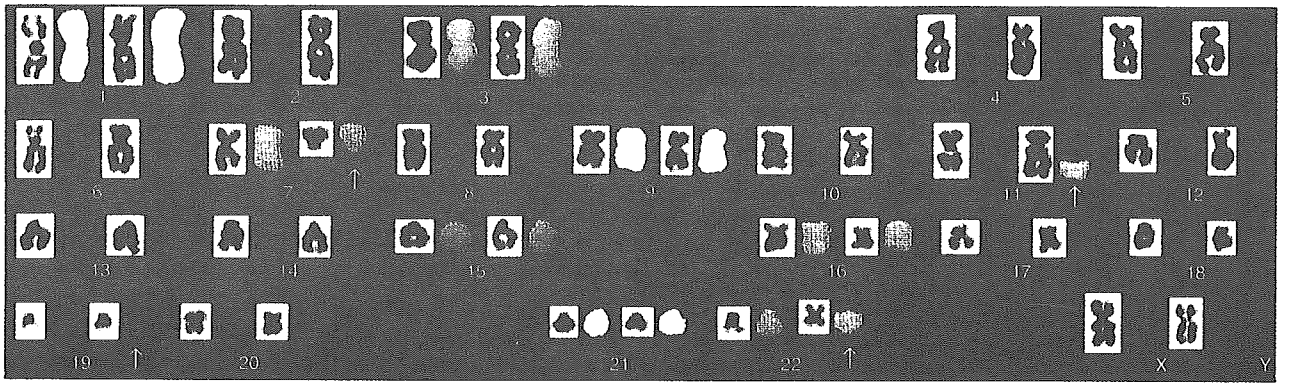

**Figure 1** SKY analysis of the patient's leukemic cells. Rearranged chromosomes are indicated by arrows

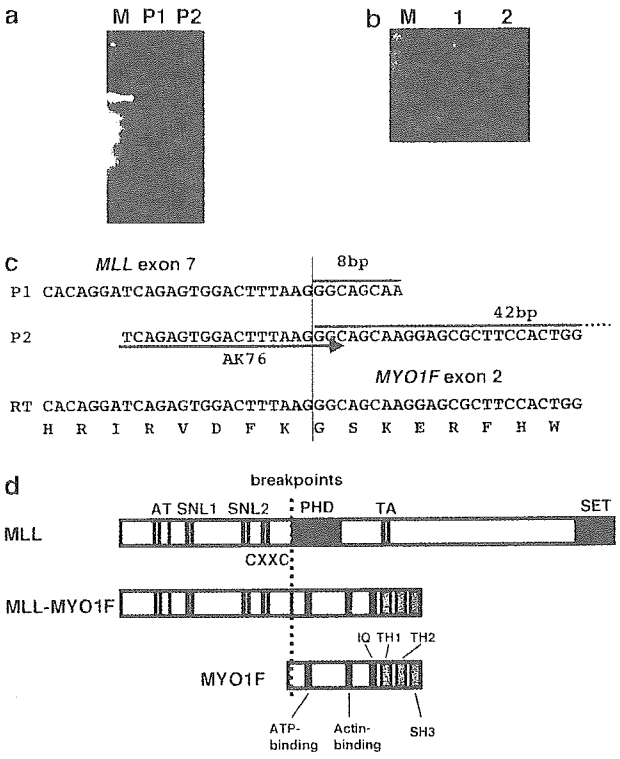


**Figure 3** Identification of the *MLL-MYO1F* chimeric transcripts. (**a**) Analysis of t(7;11;19;22)-AML by cDNA panhandle PCR. M, 1-kb DNA ladder (Life Technologies, Inc.); P1, first-round panhandle PCR product using primers MLL-3 and ALL-7S; P2, second-round panhandle PCR product using primers MLL-3 and AK76. (**b**) Identification of fusion transcripts by RT–PCR for leukemic cells from the patient. M, 1-kb DNA ladder; lane 1, primers used were ALL-7S and SS-2A for detection of *MLL-MYO1F*; lane 2, primers used were SS-1S and ALL-8A for detection of reciprocal *MYO1F-MLL*. P1, first-round panhandle PCR product using primers MLL-3 and ALL-7S; P2, second-round panhandle PCR product using primers MLL-3 and AK76. (**c**) Partial sequence of the panhandle PCR (P1 and P2) and RT–PCR (RT) products. (**d**) Schematic representation of the MLL, MYO1F and MLL-MYO1F fusion proteins. AT, AT hooks; SNL1 and SNL2, speckled nuclear localization signals 1 and 2; CXXC, CXXC domain; PHD, plant homeodomain fingers; TA, transactivation domain; SET, Su (var)3-9, enhancer of zeste, and Trithorax domain; IQ, IQ motif; TH1 and TH2, tail-homology domains 1 and 2; SH3, Src-homology 3 domain

detected PCR products (Figure 3a). Sequence analysis of the subcloned PCR product revealed that one clone contained a fusion product of a sequence of exon 7 in the *MLL* gene at the 5′ region, to an unknown 42-bp sequence. The open reading frame was preserved in this fusion transcript (Figure 3c).

### *Isolation of the* MYO1F *gene*

A BLAST database search for the novel 42-bp sequence identified two highly homologous expressed sequence tag (EST) clones (GenBank Accession numbers BI022672 and BI911874), but no known gene sequences at that time. After several rounds of database search and reverse transcription–polymerase chain reaction (RT–PCR) followed by sequencing, we identified a more than 2-kb sequence with a continuous open reading frame. The 3′ part of the sequence was found to match the partial sequence of the *MYO1F* gene (GenBank Accession numbers X98411 and U57053). More recently, two complete *MYO1F* sequences have been deposited to GenBank (BC028071, AJ310570). The *MYO1F* gene was predicted to encode a 1098-aminoacid with an N-terminal head domain containing both ATP-binding and actin-binding sequences, a neck domain with a single IQ motif, and a tail with TH1, TH2 and SH3 domains. Surprisingly, the *MYO1F* gene has been mapped to chromosome band 19p13.2–p13.3, the border of 19p13.2 and 19p13.3 (Hasson *et al*., 1996), but not 7q11, as we expected. We also confirmed that the BAC clone including the partial *MYO1F* sequence (RP11-79F15) was assigned on chromosome band 19p13.2–p13.3 by fluorescence *in situ* hybridization analysis for normal metaphase chromosomes (data not shown).

### *Detection of the* MLL-MYO1F *fusion transcript and genomic junctions*

To verify that this fusion product was indeed expressed in the leukemic cells, we performed RT–PCR analysis using primers ALL-7S and SS-2A, and obtained a fusion product of an 83-bp sequence of exon 7 in the *MLL* gene at the 5′ region to a 96-bp sequence at the 3′ region (Figure 3b).

We next performed a database search for the *MYO1F* sequence and identified three overlapped genomic clones (LLNLR-269C7, LLNLR-282E1, and LLNLR-309F9). Restriction mapping by Southern blot analysis using the *MYO1F* probe, as well as the *MLL* cDNA probe (Figure 2), allowed us to locate both breakpoints of *MLL* and *MYO1F* (Figure 4a). We cloned the genomic junction of the breakpoint by genomic PCR using a set of primers (ALL-7S and SS-18AG) followed by sequencing (Figure 4b). Cloning of the genomic junction of *MLL* and *MYO1F* suggested that the chromosome translocation that occurred in the leukemic cells of this patient was not a simple 4-way translocation, but a complex translocation that could not be detected by spectral karyotyping analysis. Although the direction of transcription of *MYO1F* is the same as that of *MLL*, one possible explanation of this complex translocation is that a small portion of chromosome 19 could have been inserted into chromosome 7, which when it translocated to chromosome 11, carried 19p with it. However, we could not analyse the cytogenetic change of this complex translocation further, because samples could no longer be obtained.

### *Expression of the* MYO1F *gene in normal human tissues and leukemic cell lines*

To examine expression of the *MYO1F* gene, we performed Northern blot analysis of poly(A)$^+$ RNA from various human tissues using the *MYO1F* cDNA probe spanning exons 1–2, and detected a transcript of

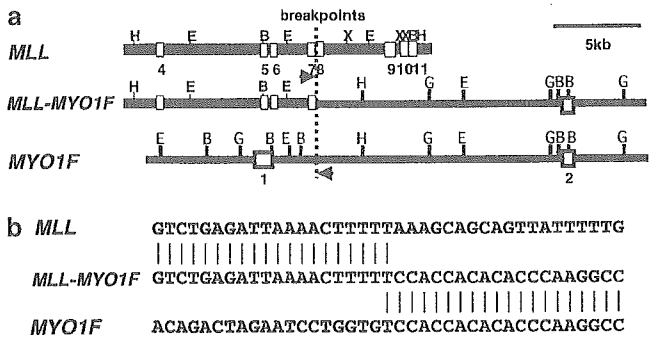


**Figure 4** Cloning of the breakpoint junction. (**a**) Physical maps of the breakpoint regions. Restriction sites are indicated by capital letters: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; X, *Xba*I. (**b**) Sequences of the breakpoints in the patient cells

approximately 4kb (Figure 5). The expression of transcripts was found in all tissues examined, particularly in peripheral blood lymphocytes at a high level (Figure 5a). On the other hand, expression of the transcript was found in only three leukemic cell lines (Figure 5b). Two of the three cell lines have *MLL* gene rearrangement (KOCL-45 and THP-1).

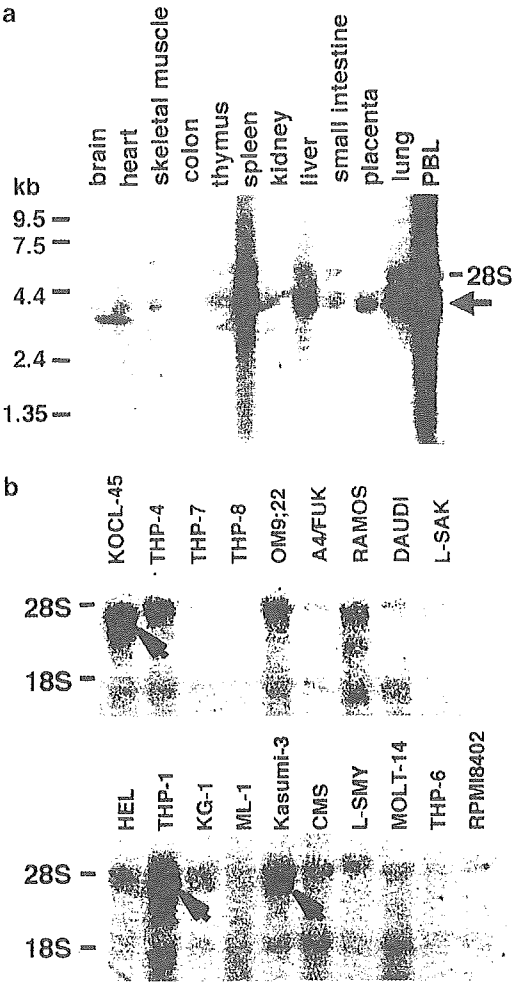


**Figure 5** Northern blot analysis of RNAs from normal human tissues (**a**), and leukemic cell lines (**b**)

## Discussion

In the present study, we isolated a novel fusion partner of the *MLL* gene, *MYO1F*, on chromosome band 19p13.2–p13.3, in *de novo* infant AMoL with chromosomal abnormalities involving chromosomes 7, 11, 19 and 22. *MYO1F* is the fourth partner gene of *MLL* on chromosome band 19p13. t(11;19)(q23;p13) is a recurring chromosomal translocation frequently observed in both ALL and AML. To date, three partner genes of *MLL*, *ENL*, *ELL/MEN* and *EEN*, have been identified and characterized (Hunger *et al.*, 1993; Thirman *et al.*, 1994; Mitani *et al.*, 1995; So *et al.*, 1997). ENL has transcriptional transactivation properties (Slany *et al.*, 1998), and ELL/MEN is an RNA polymerase II elongation factor (Shilatifard *et al.*, 1996). EEN contains a central α-helical coiled-coil region and a C-terminal SH3 domain, suggesting an association with the dimerization of MLL-EEN chimeric protein (Liu *et al.*, 2004). These three gene products and MYO1F share no homology with each other except that EEN and MYO1F have an SH3 domain, and are predicted to be functionally different. At the cytogenetic level, it may be difficult to distinguish *MLL-ENL*, *MLL-ELL/MEN*, *MLL-EEN*, and *MLL-MYO1F* fusion, and it is likely that other cases of t(11;19) lacking known fusion genes may result in this gene fusion. That no amplified product was detected by RT–PCR in t(11;19) may be due not only to the heterogeneity of the breakpoints of the same gene, but also to the different partner genes.

*MYO1F* encodes the unconventional myosin type 1F (Crozet *et al.*, 1997), and is considered a candidate gene for nonsyndromic deafness, DFNB15 (Chen *et al.*, 2001). The function of MYO1F, however, is less well characterized. Myosins are molecular motors that, upon interaction with actin filaments, utilize energy from ATP hydrolysis to generate mechanical force (Oliver *et al.*, 1999; Soldati, 2003). The myosin superfamily of mechanoenzymes comprises 18 classes. The human genome encodes about 40 myosin genes, among which about 25 are unconventional and come from at least 11 classes. Each myosin has a conserved N-terminal motor domain (25–40% identical at the amino-acid level) that contains both ATP-binding and actin-binding sequences. Following the motor domain is a light-chain-binding 'neck' region containing 1–6 copies of a repeat element, the IQ motif, which serves as a binding site for calmodulin or other members of the EF-hand superfamily of calcium-binding proteins. At the C-terminus, each myosin class has a distinct tail domain that serves in dimerization, membrane binding, protein binding, and/or enzymatic activities, and targets each myosin to its particular subcellular location.

The MLL-MYO1F fusion protein contains almost the entire MYO1F protein. Recent functional analyses of various MLL fusion proteins demonstrated two different mechanisms for leukemogenesis by MLL fusion proteins (Hsu and Look, 2003; Daser and Rabbitts, 2004). One mechanism leads to the aberrant activation of target genes including *HOX* by the fusion of MLL with transcriptional activation domains within translocation partners that are transcriptional factors and located in the nucleus, such as ENL, ELL, AF10, and AFX (Lavau *et al*., 1997; Luo *et al*., 2001; DiMartino *et al*., 2002; So and Cleary, 2002). The other mechanism leads to the similar aberrant activation of target genes by the dimerization of MLL fusion proteins, through oligomerization domains within various translocation partners that are located in the cytoplasm, such as GAS7, AF1p and Gephyrin (Martin *et al*., 2003; So *et al*., 2003; Eguchi *et al*., 2004). MYO1F has neither a transcriptional transactivation domain nor a dimerization domain. It remains unclear whether MLL-MYO1F leads to the development of leukemia through either mechanism, or another mechanism. In MLL-MYO1F fusion protein, C-terminal region of MLL including PHD fingers, taspase cleavage sites, transactivation domain, CBP (CREB-binding protein)-binding region, and SET (Su(var)3-9, Enhancer-of-zeste, Trithorax) domain, which has consequences for target gene activity as the MLL-mediated H3 and H4 methylation of the *HOXA9* and *Hoxc8* promoter, is lost, to be replaced by MYO1F (Daser and Rabbitts, 2004). Loss of function of these domains of MLL may also be associated with leukemogenesis.

Expression of the *MYO1F* gene was found in all tissues examined, as previously described (Crozet *et al*., 1997). The expression was particularly found in peripheral blood lymphocytes at a high level (Figure 5a), but only in three leukemic cell lines (Figure 5b). These findings suggest that gain-of-function by C-terminal MYO1F in fusion protein, but not loss-of-function of normal MYO1F, plays some role in leukemogenesis. Further analysis is needed to clarify the role of MYO1F in normal development and leukemogenesis.

To date, a small number of myosin genes have been reported to be associated with chromosomal translocation in hematological malignancies. Smooth muscle myosin heavy chain 11, MYH11, is fused to core-binding factor beta, CBFb, in AML-M4Eo with inv(16)(p13q22) (Liu *et al*., 1993), and nonmuscle myosin heavy chain (MYH9) is fused to ALK in anaplastic large cell lymphoma with t(2;22)(p23;q11.2) (Lamant *et al*., 2003). Furthermore, MYO18B was identified as a candidate tumor suppressor gene at chromosome 22q12.1, and was found to be deleted, mutated and methylated in human lung cancer (Nishioka *et al*., 2002). Myosin family genes might play some roles in oncogenesis. However, it remains unclear whether the gain of function of a fusion partner, the truncation of *MLL* or some other mechanism leads to the development of leukemia.

## Materials and methods

### *Patient*

A 2-month-old girl with anemia (Hb 7.4 g/dl), a low platelet count (21 000/μl) and a high leukocyte count (155 000/μl) containing 95% blasts in peripheral blood was diagnosed as having AMoL (FAB M5b). A bone marrow smear was hypercellular with 90% monocytic blasts, and positive for myeloperoxidase and combined esterase stain (NaF inhibition-negative). The leukemic cells expressed CD4 (26.8%), CD13 (45.3%), CD14 (45.9%), CD33 (90.1%), CD56 (92.9%) and HLA-DR (87.6%) in the bone marrow cells. She was treated with a low dose of etoposide (VP-16) (25 mg/m$^2$), but showed no effect in the reduction of leukemic cells. She was also treated with blood exchange to reduce the blast cells. Unfortunately, she developed respiratory distress, which was controlled by mechanical ventilation. Moreover, brain echography showed intracranial hemorrhage. She died of cardiovascular failure 5 days after admission.

### *Leukemic cell lines*

B-precursor ALL (KOCL-45, THP-4, THP-7, OM9;22 and L-SAK), B-ALL (THP-8, A4/FUK, RAMOS and DAUDI), T-ALL (L-SMY MOLT-14, THP-6 and RPMI8402), AMOL (THP-1), AML (HEL, KG-1, ML-1 and Kasumi-3) and AMKL (CMS) cell lines were analysed by Northern blot analysis (Ono *et al*., 2002a, b).

### *Southern blot analysis*

High-molecular-weight DNA was extracted from the bone marrow at diagnosis, and 10 μg of DNA was analysed, as reported previously (Ono *et al*., 2002b). A 0.9-kb *MLL* cDNA probe (Ono *et al*., 2002b) and a 243-bp *MYO1F* genomic probe within intron 1 (nucleotides 11 300–11 542, GenBank Accession number AC130469) were used.

### *RNA extraction and cDNA panhandle PCR*

Poly(A)$^+$ RNA was extracted from the bone marrow cells at diagnosis using QuickPrep Micro mRNA Purification Kit (Amersham Biosciences, Tokyo, Japan), and analysed by a modified cDNA panhandle PCR method as previously described (Megonigal *et al*., 2000; Ono *et al*., 2002a, b). In brief, first-strand cDNAs were synthesized with *MLL*-random hexamer oligonucleotide, MLL-N. After primer 1 extension with MLL-1, and extension in stem-loop templates, the sample was amplified by first PCR with ALL-6A and MLL-1. Then, one-twenty fifth of the product was used for nested PCR with MLL-3 and ALL-7S or AK76. Five microliters of the product was electrophoresed in a 3% agarose gel. The *MLL*-random hexamer oligonucleotides and primers used were as follows: MLL-N, 5′-TCGAGGAAAAGAGTGAAGAAGGGAATG TCTCNNNNNN-3′; MLL-1, 5′-TGAAGAACGTGGTGGA CTCT-3′; ALL-6A, 5′-GTCCAGAGCAGAGCAAACAGA -3′; MLL-3, 5′-GTCAGAAACCTACCCCATCA-3′; ALL -7S, 5′-TCCTCAGCACTCTCTCCAAT-3′; and AK76, 5 ′-TCAGAGTGGACTTTAAGGGC-3′.

### *Nucleotide sequencing*

Nucleotide sequences of the PCR products, or if necessary the subcloned PCR products, were analysed as previously described (Ono *et al*., 2002a, b; Hiwatari *et al*., 2003).

*RT–PCR and genomic PCR*

Of the total RNA, 4 μg was reverse-transcribed to cDNA in a total volume of 33 μl with random hexamers using Ready-To-Go You-Prime First-Strand Beads. The conditions and reagents for PCR have been already described (Ono *et al.*, 2002a, b; Hiwatari *et al.*, 2003). The primers used were as follows: ALL-7S and SS-2A, 5′-AATGGCGTCTTCGGT GATCTG-3′.

In all, 40 ng of genomic DNA derived from the patient's leukemic cells was amplified under conditions identical to those for RT–PCR. The primers used were as follows: ALL-7S and SS-18AG, 5′-TTGTGGTGAAGGTGATGGTG-3′.

*Northern blot analysis*

Aliquots (10 μg) of total RNA derived from each cell line and multiple human tissue Northern blots (CLONTECH Laboratories, Inc., CA, USA) were analysed with a $^{32}$P-labeled *MYO1F* cDNA probe (Ono *et al.*, 2002a, b; Hiwatari *et al.*, 2003). A 174-bp *MYO1F* cDNA fragment (nucleotides 39–213 from sequences of *MYO1F* cDNA, GenBank Accession number BC028071) was used.

**Abbreviations**

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; AMoL, acute monocytic leukemia; RT–PCR, reverse transcription–polymerase chain reaction.

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# Features and Outcome of Neonatal Leukemia in Japan: Experience of the Japan Infant Leukemia Study Group

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***Background.*** Neonatal leukemia characterized by early stem cell origin and extramedullary infiltration in the first 4 weeks of life is rare. We analyzed the features and outcome of neonatal leukemia in Japan to establish an appropriate treatment strategy for this rare disorder. ***Procedure.*** Patients with infant leukemia registered and treated in the Japan Infant Leukemia Study between 1996 and 2001 were analyzed. ***Results.*** Among 162 infant leukemia patients, 11 exhibited neonatal leukemia; frequencies for all infant leukemias were 6.9% (8/116) for acute lymphoblastic leukemia (ALL) and 7.3% (3/41) for acute myeloid leukemia (AML). Positive *MLL* gene rearrangement was observed in all eight patients with ALL; a single patient with AML displayed germline configuration. Acute monoblastic leukemia was apparent in all three patients with AML (M5a in the FAB classification). Most of the patients demonstrated hepatoplenomegaly and hyperleukocytosis at diagnosis. Cutaneous and central nervous system involvement were detected in half of the patients. Four patients (one with AML, and three with ALL) have survived following stem cell transplantation (SCT); however, growth impairment related to SCT was observed in these patients. ***Conclusions.*** These results suggest an improvement attributable to treatment of neonatal leukemia. International-based collaborative studies are necessary to investigate the biology of this condition and to establish appropriate therapeutic strategies. Pediatr Blood Cancer © 2005 Wiley-Liss, Inc.

**Key words:** acute leukemia; extramedullary leukemia; *MLL* gene rearrangement; neonate

## INTRODUCTION

Leukemia occurring in infants less than 12 months of age is often accompanied by hyperleukocytosis, hepatosplenomegaly, and extramedullary leukemic infiltration at initial diagnosis [1]. Biologically, leukemic cells are characterized by positive 11q23 translocations/*MLL* gene rearrangements, which are associated with poor outcome in instances of acute lymphoblastic leukemia (ALL) in most treatment programs [2]. However, we previously demonstrated the effect of intensive chemotherapy followed by stem cell transplantation (SCT) in infant ALL

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patients displaying *MLL* gene rearrangements [3,4]. Infant acute myeloid leukemia (AML) also exhibits clinical and biological features distinct from those of childhood AML, which include monoblastic or myelomonoblastic phenotypes with 11q23 translocations/*MLL* gene rearrangements [2]. Although the outcome and prognostic factors in infants with AML is obscure, we confirmed the effect of intensive chemotherapy without SCT in cases of infant AML, despite the presence of *MLL* gene rearrangements or other possible prognostic factors [5].

Neonatal leukemia, which is also classified as congenital leukemia, is rare and accounts for <1% of all childhood leukemias [6]. The following diagnostic criteria have been proposed for this rare disorder: presentation in the first 4 weeks of life, proliferation of immature myeloid, lymphoid or erythroid cells, and infiltration into extramedullary tissues [7,8]. Greaves et al. clarified the etiology of infant leukemia; analysis of *MLL* gene rearrangements of identical leukemic twins suggested that the *MLL* gene undergoes prenatal rearrangement and that leukemic cells are present in the blood of newborns [9]. *In utero* exposure to some drugs and foods that possess functional similarity to topoisomerase-II inhibitors and to certain environmental factors may affect *MLL* gene rearrangements, which contributes to leukemogenesis [10]. Based on these findings, features and outcome of neonatal leukemia were analyzed in order to clarify pathogenesis and to establish more appropriate treatment modalities.

## PATIENTS AND METHODS

All infants less than 12 months of age at diagnosis presenting with acute leukemia were registered in the Japan Infant Leukemia Study Group [3–5]. The period of registration was December 1995 to June 2001 for ALL, and December 1995 to December 1998 for AML. Informed consent was obtained from the parents of all patients at the time of registration. Each patient was evaluated in terms of characteristics of leukemic cells in bone marrow or alternatively, in peripheral blood in case bone marrow was not obtained due to dry tap, which included immunophenotypic analysis, cytogenetics and assessment of *MLL* gene rearrangements via Southern blotting. Acute leukemia was diagnosed when leukemic cells exceeded 30% of total nuclear cells in bone marrow. ALL or AML was defined according to morphological, cytochemical, and immunological analyses. Acute mixed lineage leukemia (AMLL) was also diagnosed according to the criteria proposed by the European Group for the immunological Classification of Leukemia (EGIL) [11]. Central nervous system (CNS) invasion was defined as follows: >5/μl mononuclear cells with obvious blasts present in the cerebrospinal fluid at onset. Cutaneous involvement was defined as clinically apparent skin infiltration, which disappeared with treatment.

Neonatal leukemia was diagnosed with respect to specific symptoms evident during the first 4 weeks (28 days) of life in accordance with criteria documented in several reports [7,8]. Some of these patients, who were considered congenital leukemia due to the observation of symptoms at birth or within the first few days of life, were classified as having neonatal leukemia. Patients with ALL received either the MLL96 (December 1995 to December 1998) or the MLL98 (January 1999 to June 2001) protocol. The protocol schedule was described in previous publications [3,4]. Those exhibiting AML were treated with the ANLL91 protocol (December 1995–1998), which has also been previously described [5]. Triple intrathecal chemotherapy with methotrexate, cytarabine, and hydrocortisone as prophylaxis for CNS leukemia was also administered during each course of the induction or consolidation therapy; five times in the MLL96 or MLL98, and nine times in the ANLL91 protocols.

## RESULTS

One hundred sixty infants were enrolled in this study: 116 displaying ALL, 41 presenting with AML, and 3 demonstrating AMLL. The number of AML patients was relatively low as AML registration was limited to the period from 1996 to 1998. Eleven patients were diagnosed with neonatal leukemia, which included 8 with ALL and 3 with AML, while none with AMLL was observed (Table I). Frequencies of neonatal leukemia for all infant leukemias were 6.9% (8/116) for ALL and 7.3% (3/41) for AML.

The association between age at onset and the presence of *MLL* gene rearrangements was analyzed in infant leukemia. In ALL, all 24 patients less than 2 months of age exhibited positive *MLL* gene rearrangements, whereas only two-thirds in late infancy (9–11 months of age) (16/23) displayed this genetic rearrangement. No relationship was detected between age at onset and incidence of positive *MLL* gene rearrangements with respect to AML in this investigation. Outcome of infant leukemia was also analyzed. ALL infants less than 5 months of age demonstrated poor outcome; in particular, most of those less than 1 month of age died due to progressive disease or to complications associated with treatment. In AML, an apparent correlation was not detected between age at onset and outcome.

Clinical and laboratory findings of 11 patients with neonatal leukemia are summarized in Table I. Although the onset of disease varied among the patients, three of nine patients diagnosed after the first week of life exhibited skin involvement (petechiae or cutaneous eruption) at birth, suggesting these patients also had leukemia prenatally. All eight patients with ALL exhibited 11q23 translocation associated with *MLL* gene rearrangements. They revealed negative expression of CD10 antigen,

TABLE I. Features and Outcome of 11 Patients With Neonatal Leukemia

| Age/ gender | Type | *MLL* | Karyotype | Skin inv. | Liver (cm) | Spleen (cm) | WBC (/μl) | PL (/μl) | CNS inv. | Marker | Chemotherapy[a] | CR | HSCT[b] | Outcome |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19d/F | M5a | + | t(4;11) | − | 0 | 0 | 66,500 | 50,000 | − | CD33, CD14, CD13, CD15 | P, C | − | − | Dead (1mo) |
| 3d/M | M5a | − | 46,XY | − | 4 | 0 | 91,810 | 70,000 | + | CD7, CD33, CD14, CD13, DR, CD56 | ANLL91 | + | AlloPBSCT (CR1, BU) | Alive in CR (7y+) |
| 15d/F | M5a | + | 46,XX | − | 6 | 2 | 209,400 | 116,000 | − | CD4, CD13, CD15, CD33, DR, MPO | ANLL91 | + | − | Dead (1mo) |
| 12d/F | ALL | + | t(4;11) | + | 5 | 5 | 198,200 | 47,000 | − | CD19, CD79, CD33, CD41, CD34, TdT, DR | MLL96 | + | AlloBMT (noCR, TBI) | Alive in CR (6y+) |
| 8d/F | ALL | + | t(11;19) | + | 3 | 0 | 72,000 | 24,000 | + | CD19, CD34, CD38, DR/CD33, CD38, CD45 | ANLL91 | + | AlloPBSCT (CR2, BU) | Alive in CR (3y+) |
| 0d/F | ALL | + | t(11;19) | + | 3.5 | 3 | 898,000 | 21,000 | + | CD19, DR | MLL96 | − | − | Dead (4mo) |
| 24d/F | ALL | + | t(4;11;15) | + | 3 | 2.5 | 121,600 | 82,000 | + | CD19, CD22, DR, CD15 | MLL96 | + | AlloBMT (CR1, BU) | Dead (9mo) |
| 28d/M | ALL | + | t(4;11) | − | 6 | 3 | 20,200 | 45,000 | − | CD19, CD22, DR, CD79a, CD15 | P, V, D | − | − | Dead (1mo) |
| 25d/F | ALL | + | t(4;11) | − | 7 | 6 | 421,500 | 20,000 | − | CD19, DR | MLL96 | + | − | Dead (2mo) |
| 22d/F | ALL | + | t(9;11) | + | 2 | 0 | 97,400 | 22,000 | − | CD19, CD79, CD7, DR | MLL96 | + | UCBT (CR1, TBI) | Alive in CR (6y+) |
| 13d/M | ALL | + | t(4;11) | + | 4 | 2 | 23,900 | 63,000 | + | CD19, CD34, DR | MLL98 | + | UCBT (CR2, BU) | Dead (15mo) |

[a]MLL96, MLL98, and ANLL91 protocols were described elsewhere. [3–5]P, prednisolone; C, cytarabine; V, vincristine; D, daunorubicine.
[b]Timing and preparative regimens of HSCT were shown in parenthesis. CR1, first CR; CR2, second CR; TBI, total body irradiation-containing regimen; BU, Busulfan-containing regimen.

whereas four displayed simultaneous expression of myeloid (CD33) or monocytoid (CD15) antigen. Acute monoblastic leukemia was apparent in all three AML patients (M5a in FAB classification); however, one demonstrated the *MLL* gene germline configuration. Hepatosplenomegaly and hyperleukocytosis were observed in most of the infants with neonatal leukemia at presentation. Cutaneous and CNS involvement was detected in six (55%) and five (45%) patients, respectively. Of the eight patients achieving complete remission following chemotherapy, six underwent SCT including allogeneic bone marrow transplantation (alloBMT) (2), allogeneic peripheral blood stem cell transplantation (alloPBSCT) (2), and unrelated cord blood transplantation (UCBT) (2). The preparative regimen for SCT consisted of total body irradiation (TBI) in two and busulfan (BU) in four patients, respectively. Two of five patients who relapsed following complete remission (CR) died without SCT. Consequently, four patients (1 with AML and 3 with ALL) have survived after SCT. Timing of transplantation in these patients was first CR in two, second CR in one, and noCR in one.

Long-term side effects were analyzed in four patients who have survived for an extended period. All patients with neonatal leukemia demonstrated apparent growth impairment with respect to body height and body weight; −1.5 SD and −1.2 SD at 1 year, −1.6 SD and −1.7 SD at two years, and −2.4 SD (body weight was not tested) at 3 years after the completion of therapy, respectively. Other late sequelae were observed in two patients, one with hypothyroidism and the other with chronic GVHD. Lung involvement, cardiac dysfunction, psychosomatic deterioration, or secondary malignancy were not detected in any patient in this study.

## DISCUSSION

Neonatal leukemia displays numerous characteristic features that facilitate differentiation from other infant leukemias. Although it has been reported that the frequency of AML is greater than that of ALL in the neonatal period [7,12,13], frequencies of ALL and AML were nearly identical in the current study. Leukemic cells of all patients presenting with AML exhibited monoblastic features consistent with M5a morphology of the FAB classification, which was described in the literature [14,15]. Although no patients with AMLL were observed, there was a relatively high frequency of ALL with myeloid antigen execution noted in this investigation. Co-expression of lymphoid and myeloid antigens or phenotypic switch indicates that leukemic cells in neonatal leukemia develop from an early stem cell that can differentiate in both myeloid and lymphoid directions [13,16,17].

The characteristic clinical feature of neonatal leukemia is a high frequency of cutaneous or CNS involvement

at presentation; the former is known as "leukemia cutis" or "blueberry muffin baby." In several investigations, 20–60% of patients with neonatal leukemia exhibited cutaneous infiltration, which appeared more often in AML than in ALL [8,13,18]. In the current study, cutaneous infiltration at presentation, which disappeared with chemotherapy, was observed in half of the patients. Spontaneous waxing and waning of leukemia cutis has been documented; however, the form involving leukemia cutis usually displays an aggressive clinical course [19]. CNS involvement is also common in neonatal leukemia: the incidence was 50% [12]. Half of the patients in this study were characterized by CNS involvement at onset, which was correlated with poor prognosis [3].

The high frequency of 11q23 translocations/*MLL* gene rearrangements in neonatal leukemia was also evident in this investigation. In a larger study, however, only 30.6% of patients displayed 11q23 translocation; in contrast, *MLL* gene rearrangement was confirmed in most of the patients [13].The frequency of *MLL* gene rearrangements is correlated strongly with age in infant ALL [9]. High frequency of neonatal patients exhibiting *MLL* gene rearrangements strongly suggests prenatal rearrangement of the *MLL* gene. In fact, some of the patients who were diagnosed after the first week of life also displayed symptoms of disease at birth. *MLL* gene rearrangements can be detected in blood samples at birth in most infant leukemia patients, which suggests a prenatal origin of leukemic cells in infant leukemia [20,21]. It has been speculated that exposure to specific topoisomerase-II inhibitors could form cleavage complexes leading to facilitation of illegitimate recombination [22].

The prognosis of neonatal leukemia has been generally poor. In the aforementioned larger study, overall survival for 69 AML and 22 ALL patients was 24.4% and 13.6%, respectively; in particular, disease-free survival of ALL patients was dismal (0%) [13]. Poor prognosis in neonatal ALL has been attributed to unfavorable features at presentation, which include positive *MLL* gene rearrangements, hyperleukocytosis and extramedullary (cutaneous and CNS) involvement [19]. Co-expression of myeloid antigen may also be correlated with poor outcome in these patients [23]. In the current study, three of eight ALL patients survived with intensive chemotherapy followed by SCT, suggesting that improvement in the treatment of these patients is essential in order to achieve high remission rates in infant ALL.

One of three patients presenting with AML has also survived following myeloid-oriented chemotherapy (ANLL91) and SCT in this study. Despite the unfavorable features, infants with AML demonstrate outcomes similar to those of older children with AML upon implementation of intensive multi-agent chemotherapy regimens [5,19]. Pui et al. noted that only two factors predicted a favorable prognosis: M4 or M5 leukemia and the t(9;11) [24]. We recently reported that infant ALL involving t(9;11) possesses a feature with respect to poor prognosis distinct from infant AML with the identical karyotype; furthermore, prognosis of these patients can be improved only via combination of intensive chemotherapy and SCT [25]. Therefore, we hypothesize that a strategy for infant ALL different from that of infant AML should be established.

A major concern related to the utility of SCT in infants is the late toxicity including growth impairment. In particular, TBI was also reported to be a risk factor for short stature, hypopituitarism, and cataract formation; furthermore, these toxicities were inversely correlated with age [26,27]. In this investigation, patients with neonatal leukemia exhibited apparent growth impairment in terms of body height and body weight. In our previous report, outcome of infant ALL did not differ between patients undergoing the TBI- or BU-based regimen [4]. These data indicate that SCT in conjunction with less toxic preparative regimens involving non-myeloablative drugs should be included in the treatment of future neonatal leukemia patients.

To date, the best treatment strategy in terms of improving survival and reducing late toxicity in neonatal leukemia remains elusive. Since limited numbers of neonatal leukemia appear in the literature, international-based collaborative studies are necessary for acquisition of information regarding biology, treatment and natural history of this rare disease.

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