

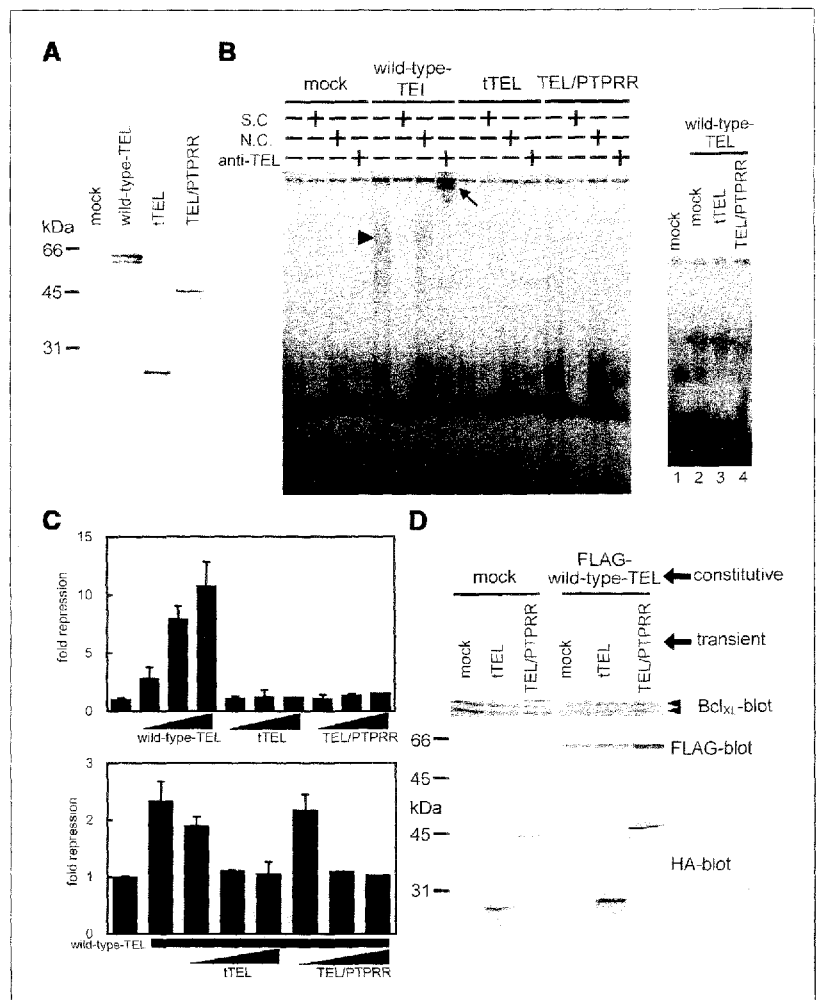
encompassed the acceptor site and mainly resided in the cytoplasm, we examined whether they were modified by SUMO-1. COS-7 cells were transfected with hemagglutinin-tagged wild-type TEL, tTEL, or TEL/PTPRR expression plasmid with or without FLAG-tagged SUMO-1 plasmid. As reported on wild-type TEL, bands corresponding to SUMO-1-modified proteins (wild-type TEL, 75 and 85 kDa; tTEL, 45 kDa; TEL/PTPRR, 65 kDa) were detected in Western blot analysis with anti-hemagglutinin antibody only when SUMO-1 plasmid was cotransfected (Fig. 3C, left). To confirm that these larger-sized bands were indeed derived from SUMO-1-modified proteins, the total cell lysates were immunoprecipitated with anti-hemagglutinin antibody. The precipitated proteins were visualized with anti-FLAG antibody at the expected sizes of each SUMO-1-conjugated protein (Fig. 3C, right). Therefore, we conclude that both tTEL and TEL/PTPRR are subjected to modification with SUMO-1, which may support their cytoplasmic localization. As an additional remark, several proteins of higher molecular weights than sumoylated tTEL were concomitantly immunoprecipitated with anti-hemagglutinin antibody in the presence of exogenous SUMO-1. Because they were not detected with anti-hemagglutinin antibody but with anti-FLAG antibody, a likely scenario is that some other SUMO-1-modified proteins interacting with tTEL were coimmunoprecipitated.

Truncated TEL and TEL/PTPRR heterodimerize with wild-type TEL. Because tTEL and TEL/PTPRR contain the entire helix-

loop-helix domain of TEL, they conceivably heterodimerize with wild-type TEL. To test this hypothesis, we transiently transfected into COS-7 cells hemagglutinin-tagged tTEL or TEL/PTPRR expression plasmid, alone or along with FLAG-tagged wild-type TEL expression plasmid. Cell lysates were subjected to immunoprecipitation with anti-FLAG antibody followed by Western blot analysis with anti-hemagglutinin antibody. As shown in Fig. 3D, both tTEL and TEL/PTPRR were coimmunoprecipitated with anti-FLAG antibody when they were coexpressed with FLAG-tagged wild-type TEL. This coimmunoprecipitation completely disappeared when anti-FLAG antibody was removed from the reaction or when FLAG-tagged wild-type TEL was not coexpressed in COS-7 cells. Moreover, when we applied anti-hemagglutinin antibody for immunoprecipitation, FLAG-tagged wild-type TEL was coimmunoprecipitated with hemagglutinin-tagged tTEL or TEL/PTPRR (data not shown). These data indicate that these aberrant proteins heterodimerize with wild-type TEL *in vivo*.

TEL/PTPRR prevents wild-type TEL from binding to ETS-binding consensus site. Because the breakpoint occurs between exons 4 and 5 of the *TEL* gene, neither tTEL nor TEL/PTPRR encodes the ETS domain of TEL. It is therefore quite likely that these isoforms do not possess EBS-specific DNA-binding property as wild-type TEL does. To clarify this point, wheat germ extracts expressing wild-type TEL, tTEL, and TEL/PTPRR at comparable levels (Fig. 4A) were applied to EMSA in which radioactive EBS

Figure 4. Truncated TEL and TEL/PTPRR confer a dominant-negative effect over wild-type TEL. **A**, expression of hemagglutinin-tagged wild-type TEL, tTEL, or TEL/PTPRR in the lysates prepared by *in vitro* translation system. Western blot analysis was done with anti-hemagglutinin antibody. **B**, EMSA with the lysates and ³²P-labeled EBS probe. **Left**, both tTEL and TEL/PTPRR lack EBS-specific DNA-binding ability. In competition assay, a 300-fold molar excess of specific competitor (S.C.) or nonspecific competitor (N.C.) was added to the reaction mixtures. **Arrowhead**, broad band derived from EBS-TEL complex. In supershift assay, anti-TEL (N-19) antibody was added to the reaction mixtures. **Arrow**, supershifted band. **Right**, TEL/PTPRR inhibits wild-type TEL from binding to EBS. *In vitro* translated lysates were mixed as indicated. **Lanes 2 to 4**, the amount of wild-type TEL lysates was kept constant. **Asterisks**, EBS-TEL complex. **C**, luciferase reporter assays. **Top**, HeLa cells were transfected with 1 μg of (EBS)₃tkLuc reporter plasmid, alone or along with increasing amounts (0.1, 0.5, and 1.0 μg) of the indicated expression plasmids. **Bottom**, HeLa cells were transfected with 1 μg of (EBS)₃tkLuc, alone or along with 0.1 μg of wild-type TEL expression plasmid. Increasing amounts (0.05, 0.5, and 0.9 μg) of tTEL or TEL/PTPRR expression plasmid was also added. To emphasize transcriptional repression, reciprocals of relative luciferase activities presenting average results of duplicate experiments are shown as "fold repression." **D**, aberrant TEL proteins relieve suppressed expression of endogenous Bcl-X_L mediated by wild-type TEL. NIH3T3 cells retrovirally infected with empty or FLAG-tagged wild-type TEL vector were transiently transfected with tTEL or TEL/PTPRR expression plasmid. **Top**, cell lysates were subjected to Western blot analysis with anti-Bcl-X_L antibody. **Middle and bottom**, constitutive expression of FLAG-tagged wild-type TEL and transient expression of hemagglutinin-tagged tTEL and TEL/PTPRR were confirmed by Western blot analysis with anti-FLAG and anti-hemagglutinin antibodies, respectively.



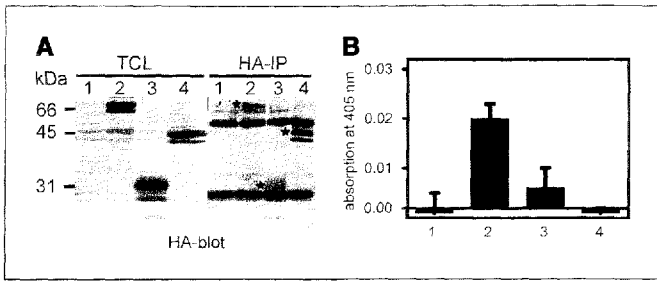


Figure 5. Truncated TEL and TEL/PTPRR lack phosphatase activity. *A*, expression and immunoprecipitation of hemagglutinin-tagged wild-type PTPRR, tTEL, or TEL/PTPRR in COS-7 cell lysates. Western blot analysis was done with anti-hemagglutinin antibody. Lane 1, mock; lane 2, wild-type PTPRR; lane 3, tTEL; lane 4, TEL/PTPRR. *B*, *in vitro* phosphatase assay using *p*-nitrophenyl phosphate as a substrate. Nonenzymatic hydrolysis of *p*-nitrophenyl phosphate was subtracted from the measured values. Columns, averages of duplicate experiments.

oligonucleotide was used as a probe. As previously observed (2, 40), wild-type TEL formed a specific DNA-protein complex that emerged as a somewhat broad band possibly due to weak binding (Fig. 4B, left). This band represented a specific association of wild-type TEL and the EBS probe because it was completely canceled by cold specific competitor but not by nonspecific competitor. Furthermore, this band was supershifted when anti-TEL antibody was preincubated with the lysate. In contrast, neither tTEL nor TEL/PTPRR formed a specific DNA-protein compound. Taken together, we conclude that both isoforms lose EBS-specific DNA-binding capacity.

We next examined whether tTEL and TEL/PTPRR alter the capacity of wild-type TEL to bind with EBS. Whereas mixture of

wild-type TEL and tTEL did not change the intensity of the band derived from possible EBS-TEL complex, mixture of wild-type TEL and TEL/PTPRR obviously diminished it (Fig. 4B, right). We confirmed that the shifted bands contained wild-type TEL protein by the supershift experiment with anti-COOH-terminal TEL antibody (data not shown). This result indicates that TEL/PTPRR prevents wild-type TEL molecule from binding to EBS, possibly by heterodimerizing with it.

Truncated TEL and TEL/PTPRR themselves do not repress transcription through ETS-binding consensus site, but confer a dominant-negative effect over wild-type TEL. TEL is a member of ETS family transcription factors and represses transcription of target genes through EBS. Because both tTEL and TEL/PTPRR lack the ETS domain and thus do not bind to EBS, they could lose activity as a transcription factor. We thus did luciferase reporter assays with (EBS)₃tkLuc and examined the effects of tTEL and TEL/PTPRR on transcription through EBS in HeLa cells. When increasing amounts of wild-type TEL expression plasmid were cotransfected with (EBS)₃tkLuc, the luciferase activities were repressed in a dose-dependent manner (Fig. 4C, top). This transcriptional regulation was EBS specific because no repression was observed when (EBS)₃tkLuc was substituted with a mutant reporter plasmid, (mEBS)₃tkLuc (data not shown). On the other hand, transfection of tTEL or TEL/PTPRR expression plasmid along with (EBS)₃tkLuc yielded no significant changes in luciferase activities even at the highest dose.

Because they were found to associate with wild-type TEL, we then speculated that tTEL and TEL/PTPRR could affect the functions of wild-type TEL by heterodimerizing with it. Thus, increasing amounts of tTEL or TEL/PTPRR expression plasmid were transfected with (EBS)₃tkLuc and wild-type TEL expression plasmid.

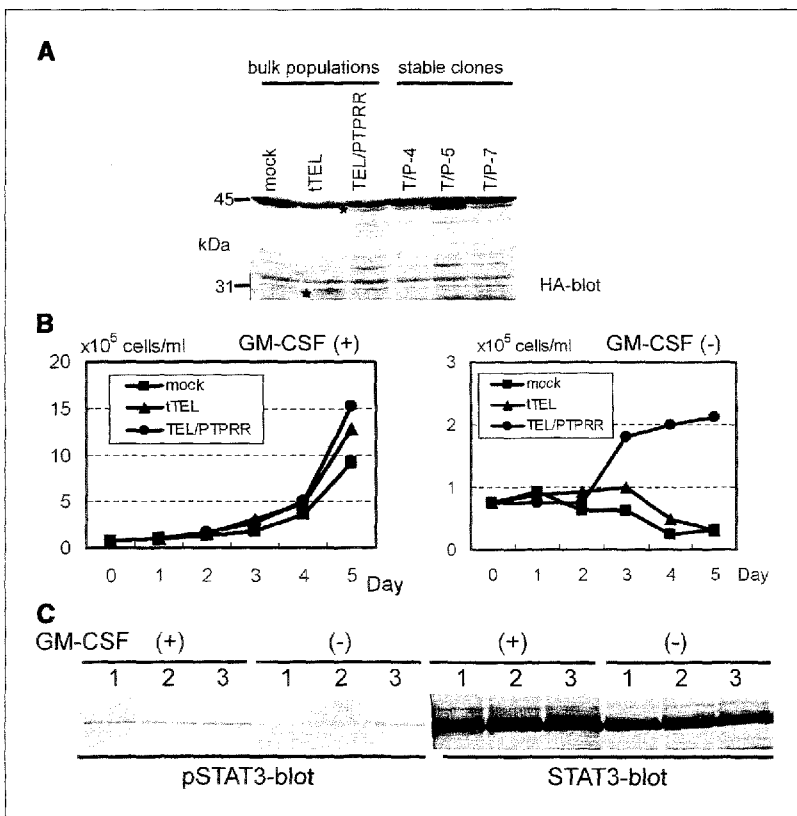


Figure 6. TEL/PTPRR renders UT7/GM cells factor independent. *A*, expression of tTEL and TEL/PTPRR in the bulk and stable populations of UT7/GM cells. These cells were obtained by electroporating the corresponding expression plasmids and selecting cells by G418 resistance. Western blot analysis was done with anti-hemagglutinin antibody. Asterisks, overexpressed proteins. *B*, UT7/GM cells overexpressing TEL/PTPRR show GM-CSF-independent proliferation. UT7/GM cells expressing the indicated proteins were cultured in the presence (left) or absence (right) of GM-CSF. Cells were suspended in culture media at a concentration of 0.8×10^5 cells/mL at day 0, and cell numbers were counted at the indicated time points. *C*, phosphorylated STAT3 is retained after factor withdrawal in tTEL- and TEL/PTPRR-overexpressing UT7/GM cells. Cells were cultured in the presence or absence of GM-CSF for 96 hours before the harvest. Lysates were immunoprecipitated with anti-STAT3 antibody and applied to Western blot analysis with anti-phosphorylated STAT3 (left) or anti-STAT3 (right) antibody. Lane 1, mock; lane 2, tTEL; lane 3, TEL/PTPRR.

Interestingly, this led to a decrease in fold repression induced by wild-type TEL (Fig. 4C, bottom). These data suggest that both tTEL and TEL/PTPRR exert a dominant-negative effect on wild-type TEL-mediated transcriptional repression and that tumor-suppressive functions of wild-type TEL may be abolished in the leukemic cells.

To confirm the dominant-negative effect of tTEL and TEL/PTPRR over wild-type TEL *in vivo*, we evaluated by Western blot analysis the endogenous expression of *Bcl-X_L*, a well-known transcriptional target of TEL. For this purpose, NIH3T3 cells constitutively expressing FLAG-tagged wild-type TEL were transiently transfected with tTEL or TEL/PTPRR expression plasmid, and were subjected to Western blot analysis with anti-Bcl-X_L antibody. As previously reported (11), expression of endogenous Bcl-X_L was found repressed by wild-type TEL (Fig. 4D). Importantly, coexpression of tTEL and TEL/PTPRR recovered suppressed Bcl-X_L expression in the wild-type TEL-expressing cells.

TEL/PTPRR lacks protein tyrosine phosphatase activity. PTPRR, a human homologue of PTPSL/PTPBr7 in mouse and PC12-PTP1/PCPTP1 in rat, is a receptor-type tyrosine phosphatase that contains one cytoplasmic protein tyrosine phosphatase domain at its COOH terminus. Because TEL/PTPRR possesses almost full length of the protein tyrosine phosphatase domain, we analyzed if it retained catalytic activity by *in vitro* phosphatase assay using *p*-nitrophenyl phosphate as a substrate. Lysates from COS-7 cells overexpressing hemagglutinin-tagged wild-type PTPRR, tTEL, or TEL/PTPRR were immunoprecipitated with anti-hemagglutinin antibody. To confirm that each protein was successfully collected, a portion of immunoprecipitates and total cell lysates was subjected to Western blot analysis (Fig. 5A). All these proteins were gathered at comparable levels. Wild-type PTPRR showed catalytic activity as a protein tyrosine phosphatase (Fig. 5B). In contrast, tTEL completely lacking the protein tyrosine phosphatase domain was catalytically inactive, as expected. Surprisingly enough, TEL/PTPRR also lost its activity, probably because it did not express the 13 NH₂-terminal amino acid residues of the protein tyrosine phosphatase domain. We speculate that phosphatase activity is not a requisite for the development of *inv(12)*-carrying leukemia.

TEL/PTPRR endows UT7/GM cells with factor independence. To search for transforming activity of tTEL and TEL/PTPRR, we employed the human megakaryocytic leukemia cell line UT7/GM that requires GM-CSF for proliferation and survival, and analyzed the effects of overexpressed tTEL or TEL/PTPRR on the factor-dependent growth. By using electroporation, we introduced tTEL or TEL/PTPRR expression plasmid into UT7/GM cells. As indicated in Fig. 6A, Western blot analysis with anti-hemagglutinin antibody showed the comparable expression of tTEL and TEL/PTPRR proteins in the bulk populations and we used these uncloned cells in the following experiments. When cultured under the presence of GM-CSF, both tTEL- and TEL/PTPRR-overexpressing cells proliferated slightly faster than mock cells (Fig. 6B). Mock cells expectedly starved to death after depletion of GM-CSF from the media. The tTEL-overexpressing cells did not survive after cytokine removal either. In contrast, the TEL/PTPRR-overexpressing cells continued to proliferate slowly in factor-free media at least beyond day 60 of culture. We also established by limiting dilution three stable lines overexpressing TEL/PTPRR (T/P-4, T/P-5, and T/P-7), and confirmed the factor independence beyond 2 months of culture. The calculated doubling times at day 28 were 45, 34, and 76 hours, respectively. These observations suggest that the GM-

CSF-independent survival is not due to selection and expansion of mutated clones, but results from the expression of TEL/PTPRR itself.

We hypothesized that some growth signalings were constitutively activated in the TEL/PTPRR-overexpressing cells, leading to the factor-independent proliferation. To identify the exaggerated signals in these cells, we next compared the phosphorylation levels of molecules in various signal transduction systems including mitogen-activated protein (MAP) kinase signals, JAK/STAT signals, and phosphatidylinositol 3-kinase signals among mock, tTEL-, and TEL/PTPRR-overexpressing cells. We observed that c-jun-NH₂-kinase (JNK) in the MAP kinase signalings, STAT1 and STAT5 in the JAK/STAT signalings, and Akt1/2/3 in the phosphatidylinositol 3-kinase signalings were almost equally phosphorylated among them and their phosphorylation levels remained unchanged before and after the cytokine removal (data not shown). Extracellular signal-regulated kinase (ERK)-1/2 in the MAP kinase signalings alone became almost thoroughly dephosphorylated within 12 hours after the factor deprivation in all the bulk populations. On the other hand, in spite of the steady expression of STAT3 proteins, phosphorylated STAT3 rapidly declined after GM-CSF withdrawal in the mock cells (Fig. 6C). Notably, however, the levels of phosphorylated STAT3 were unaltered in the tTEL- and TEL/PTPRR-overexpressing cells. These data suggest that overexpressed mutant TEL proteins, tTEL and TEL/PTPRR, might stimulate the JAK/STAT signal via STAT3, but that TEL/PTPRR might activate other pathways to render UT7/GM cells factor independent.

Discussion

We have shown in this study that *inv(12)(p13q13)* results in the formation of the *TEL/PTPRR* chimeric gene, fusing exon 4 in the *TEL* gene with exon 7 in the *PTPRR* gene. Ten isoforms of the *TEL/PTPRR* cDNAs that are generated possibly through alternative splicing have been cloned in RT-PCR assay. Among the isoforms, only one isoform encodes a chimeric product (TEL/PTPRR) connecting the helix-loop-helix domain of TEL and the protein tyrosine phosphatase domain of PTPRR, and the rest express COOH-terminally truncated TEL due to frameshift. As judged from higher levels of amplification in RT-PCR analysis, two isoforms, tTEL (type 1) and TEL/PTPRR, are likely to be dominantly expressed in the leukemic cells. Generation of truncated form of TEL from the *TEL*-related chimeric allele is also reported in the *TEL/JAK2* chimeric gene (22). Importantly, whereas wild-type *TEL* mRNA is detected in the leukemic cells, neither reciprocal *PTPRR/TEL* nor wild-type *PTPRR* mRNA is detected in the sample. This suggests that promoter of the *PTPRR* gene is inactivated in these leukemic cells. These data indicate that tTEL and TEL/PTPRR are implicated in the pathogenesis of *inv(12)*-type leukemia.

To get some insight into the underpinning mechanisms in leukemogenesis by *inv(12)(p13q13)*, we evaluated molecular functions of tTEL and TEL/PTPRR. Because TEL is a tumor suppressor, functional effects of the mutant proteins on wild-type TEL may be one cause of leukemia. We reported that ΔETS isoforms, which lack the ETS DNA-binding domain and are frequently expressed in acute myelogenous leukemia transformed from myelodysplastic syndrome, molecularly and biologically show dominant-negative effects over functions of wild-type TEL (41). As expected from their structural similarity in TEL part to ΔETS isoforms, both tTEL and TEL/PTPRR dominantly interfere with transcriptional repression mediated by wild-type TEL of both

artificial EBS reporter and endogenous *Bcl-X_L* gene. Thus, losing the ETS DNA-binding domain but retaining the helix-loop-helix domain seems critical to bolster the dominant inhibitory ability. Considering that both tTEL and TEL/PTPRR are found to heterodimerize with wild-type TEL, there are three plausible explanations for molecular basis of this effect. First, tTEL and TEL/PTPRR seem to prevent *de novo* translated wild-type TEL from entering the nucleus because wild-type TEL molecule resides mainly in the nucleus on its own, but almost equally in both the nucleus and the cytoplasm when coexpressed with the TEL mutants. Importantly, we observed the cytoplasmic localization of endogenous wild-type TEL in primary leukemic cells carrying *inv(12)*. Second, TEL/PTPRR impairs DNA-binding property of wild-type TEL although tTEL does not. Third, tTEL and TEL/PTPRR may take corepressor mSin3A away from wild-type TEL because they associate with endogenous mSin3A in COS-7 cells (data not shown). The same dominant interfering functions of chimeric protein over authentic TEL have also been reported in TEL/AML1 by t(12;21)(p13;q22) in pediatric acute lymphoblastic leukemia (35) and provide one molecular mechanism (loss of tumor suppressive function of wild-type TEL) in leukemogenesis by 12p13 translocations. Given that *Bcl-X_L* is an antiapoptotic molecule, its derepressed expression by the aberrant TEL proteins could be one of the molecular pathogenesises in *inv(12)*-type leukemia.

PTPRR is the first protein tyrosine phosphatase that was identified as a fusion partner for TEL. It is a receptor-type phosphatase possessing an intracellular catalytic domain at the COOH terminus (46–48). Due to alternative splicing or promoter switch, PTPRR exists as various isoforms in different organs such as brain, placenta, uterus, and colon (49, 50). However, there is no report that PTPRR is expressed in hematopoietic tissues. Notably, wild-type PTPRR is not expressed in the leukemic cells, and TEL/PTPRR does not show any phosphatase activity probably due to incomplete structure of the catalytic domain with loss of its NH₂-terminal part. These observations indicate that alteration in phosphatase activity is not causally related to leukemogenic mechanism in these cells. Despite this indication, one interesting hypothesis is that aberrant expression of a portion of catalytically inactive PTPRR in the chimeric form may protect some phosphorylated tyrosine residues that transmit growth-stimulating signals from dephosphorylation. As a result, this may constitutively activate proliferation-inducing pathways.

Employing factor-dependent UT7/GM cells, we explored the oncogenic potential of tTEL and TEL/PTPRR. Overexpression of TEL/PTPRR renders these cells factor independent, whereas overexpression of tTEL does not prevent apoptotic induction after factor deprivation. Therefore, TEL/PTPRR could exhibit transforming activity in myeloid cells, but tTEL alone could not, although tTEL may support the TEL/PTPRR activity. This suggests

that the dominant-negative effects over wild-type TEL are not sufficient in the development of leukemia. Interestingly, phosphorylated STAT3 does not fade away after factor withdrawal in both tTEL- and TEL/PTPRR-expressing cells, which is in sharp contrast to mock cells. All the other signaling molecules examined, such as ERK1/2 and JNK in MAP kinase pathway, Akt1/2/3 in phosphatidylinositol 3-kinase pathway, and STAT1 and STAT5 in JAK/STAT pathway, show no difference in phosphorylation levels among mock, tTEL-, and TEL/PTPRR-expressing cells both before and after factor removal. Because STAT3 is one of the well-known signal transducers involved in UT7/GM cell growth (51), the maintenance of phosphorylated STAT3 seems to contribute to the leukemic cell growth without GM-CSF. How these mutants preserve phosphorylated STAT3 remains unknown, but it could be possible for TEL/PTPRR to bind STAT3 through the helix-loop-helix domain of TEL and protect its phosphorylated tyrosine residues through the catalytically inactive domain of PTPRR, because it has been reported that wild-type TEL associates with STAT3 through the helix-loop-helix domain (52). Furthermore, wild-type TEL is found to repress STAT3-mediated transcriptional activity in the literature. Although we could not show the association between endogenous STAT3 and overexpressed tTEL or TEL/PTPRR in UT7/GM cells (data not shown), tTEL and TEL/PTPRR may stimulate the STAT3 signals by heterodimerizing with wild-type TEL and thus blocking its inhibitory functions on STAT3.

In summary, tTEL and TEL/PTPRR produced from the rearranged *TEL* allele could be important players in the development of leukemia carrying *inv(12)(p13q13)*. They could block two functions of tumor-suppressive wild-type TEL: transcriptional repression through EBS and inhibition of STAT3-mediated signal. However, common functions of these two molecules may not be sufficient, if any, in the leukemogenesis because overexpression of tTEL does not induce autonomous cell growth of the factor-dependent cells by itself. The PTPRR part in TEL/PTPRR fusion protein might provide additional unknown functions for growth advantage. Further experiments with mouse modeling are needed to prove their leukemogenic roles *in vivo*.

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Dysplastic definitive hematopoiesis in *AML1/EV11* knock-in embryos

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The *AML1/EV11* chimeric gene is created by the t(3;21)(q26;q22) chromosomal translocation seen in patients with leukemic transformation of myelodysplastic syndrome or blastic crisis of chronic myelogenous leukemia. We knocked-in the *AML1/EV11* chimeric gene into mouse *Aml1* genomic locus to explore its effect in developmental hematopoiesis in vivo. *AML1/EV11⁺* embryo showed defective hematopoiesis in the fetal liver and died

around embryonic day 13.5 (E13.5) as a result of hemorrhage in the central nervous system. The peripheral blood had yolk-sac-derived nucleated erythroblasts but lacked erythrocytes of the definitive origin. Although E12.5 fetal liver contained progenitors for macrophage only, E13.5 fetal liver contained multilineage progenitors capable of differentiating into dysplastic myelocyte and megakaryocyte. No erythroid progenitor was de-

tected in E12.5 or E13.5 fetal liver. Hematopoietic progenitors from E13.5 *AML1/EV11⁺* fetal liver were highly capable of self-renewal compared with those from wild-type liver. Maintained expression of *PU.1* gene and decreased expression of *LMO2* and *SCL* genes may explain the aberrant hematopoiesis in *AML1/EV11⁺* fetal liver. (Blood. 2005;106:2147-2155)

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Introduction

The t(3;21)(q26;q22) chromosomal translocation occurs in patients with aggressive transformation of myelodysplastic syndrome (MDS) or chronic myelogenous leukemia (CML).¹⁻⁴ In the joining region of t(3;21)(q26;q22), the *AML1* gene on 21q22 is fused with the *EV11* (ecotropic viral integration site-1) gene on 3q26.⁵ The resultant *AML1/EV11* fusion gene is translated in frame to generate a chimeric transcription factor in which the N-terminal of *AML1*, including its DNA-binding Runt domain, is fused to almost the entire portion of *EV11*. This chimeric transcription factor could be a molecular culprit for the leukemic progression of stem-cell malignancies caused by t(3;21)(q26;q22).

AML1 is involved in transcriptional regulation of a number of hematopoietic cell-specific genes. The Runt domain of *AML1* binds to a specific DNA consensus sequence named polyomavirus enhancer binding protein 2 (PEBP2; ACCRCA), together with a non-DNA-binding β subunit (core-binding factor β [CBF β]/PEBP2 β) to form a heterodimeric active transcription factor complex.⁶⁻¹⁰ *AML1*- or *CBF β* -deficient mice are embryonic lethal at embryonic day 12.5 (E12.5) and show massive hemorrhage in the central nervous system (CNS) with lack of hematopoiesis in the fetal liver.¹¹⁻¹⁵ A recent study demonstrated that inactivation of *AML1* in adult mice results in megakaryocyte maturation arrest, defect in T- and B-lymphocyte development, and increase in hematopoietic precursor cells.¹⁶

The *EV11* gene was initially identified as the frequent retrovirus integration site in myeloid tumors in AKXD mice.¹⁷ *EV11* is less detected in normal hematopoietic cells but highly expressed in some patients of MDS or acute myelogenous leukemia (AML).^{18,19}

EV11 has 2 zinc finger domains, one in the N-terminal and the other in the C-terminal region. *EV11* is reported to interfere with the transforming growth factor β (TGF β) signaling and to antagonize its growth inhibitory effect through targeting an intracellular signal transducer Smad3.²⁰ *EV11* is also known to enhance activator protein 1 (AP-1) activity²¹ or block c-Jun N-terminal kinase (JNK) activity.²² These findings suggest the versatile nature of this molecule in malignant transformation of hematopoietic cells.

The molecular characterization of *AML1/EV11* points to 2 major mechanisms of its leukemogenic effect; one is the dominant suppression of the functions of wild-type *AML1*, and the other is the ectopic expression of *EV11* molecule in the hematopoietic cells.²³ *AML1/EV11* binds to the PEBP2 site through its Runt domain and suppresses the expression of *AML1* target genes by recruiting corepressor C-terminal binding protein by the *EV11* portion.^{24,25} This dominant-negative effect is a common feature among *AML1*-related chimeric molecules, including *AML1/ETO* (eight-twenty-one) in t(8;21)(q22;q22) and translocation ets leukemia (TEL)/*AML1* in t(12;21)(p13;q22).^{9,23,26-29} By suppressing the expression of canonical *AML1* target genes, *AML1/EV11* distorts the normal function of *AML1* and may lead to leukemia development. The second mechanism of leukemogenesis is brought by the *EV11* part; inhibition of TGF β signaling,³⁰ stimulation of AP-1 activity,³¹ or repression of JNK activity.²² Thus, *EV11* portion also contributes to the oncogenicity of *AML1/EV11*.

Of the *AML1*-fusion proteins, *AML1/EV11* and *AML1/ETO* are of particular interest. They show a similar structure; N-terminal half of *AML1*, including the Runt domain, is fused to almost the

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entire of EVI1 or ETO, both of which are the zinc finger protein.^{23,28,29} Moreover, AML1/EVI1 and AML1/ETO mediate dominant-negative effects over wild-type AML1 by recruiting corepressors via EVI1²³ and ETO portions,^{28,29} respectively. However, despite such similarities in molecular structure and function, AML1/EVI1 and AML1/ETO are differentially associated with the disease phenotype. AML1/EVI1 aggravates MDS or CML,¹⁻⁴ while AML1/ETO develops de novo AML subtype M2 of the French-American-British classification.²⁹ A possible explanation for such different disease settings is that AML1/EVI1 and AML1/ETO perturb different signaling pathway. Thus, exploring the molecular functional differences between AML1/EVI1 and AML1/ETO in vivo would advance our understanding toward differential role of the chimeric transcription molecules in causing different forms of leukemia.

For a direct comparison of AML1/EVI1- and AML1/ETO-expressing animals, we created AML1/EVI1 knock-in mice and compared its phenotype with that of AML1/ETO knock-in mice described previously.^{32,33} AML1/EVI1^{+/+} embryos died around E13.5 resulting from hemorrhage in the brain and the spinal cord as a result of impaired definitive hematopoiesis in the fetal liver. However, E13.5 fetal liver contained hematopoietic progenitors with enhanced replating efficiency. These phenotypes are shared between AML1/EVI1^{+/+} and AML1/ETO^{+/+} embryos. On the other hand, unique to AML1/EVI1^{+/+} mice was that hematopoietic progenitors in the fetal liver cannot develop to erythroid cells but can develop into dysplastic myeloid and megakaryocytic cells, whereas hematopoietic progenitors in the AML1/ETO^{+/+} fetal liver produce cells of all 3 lineages with normal morphology. Expression analysis for the crucial genes revealed that AML1/EVI1^{+/+} fetal liver maintained normal level of the *PU.1* gene expression, but lacked expression of the *LMO2* and *SCL* genes. Normal expression of the *PU.1* gene may support maintenance of the progenitor cells capable of differentiating into the myeloid and megakaryocytic lineages, and the low levels of *LMO2* and *SCL* genes expression could lead to blockade of erythroid differentiation in AML1/EVI1^{+/+} fetal liver. Dysplastic hematopoiesis seen in AML1/EVI1^{+/+} embryo partly recapitulates that observed in patients with the t(3;21)(q26;q22) translocation, indicating the pathogenic role for this chimeric protein in t(3;21)(q26;q22)-related leukemia.

Materials and methods

Construction of AML1/EVI1 knock-in targeting vector

Murine *Aml1* genome derived from a genomic library was amplified by polymerase chain reaction (PCR) method to produce a DNA fragment that includes intron 4 and the first 94 base pair (bp) of exon 5 with silent mutations at codons 519 and 522 of exon 5 creating a *SacII* cleavage site. In

the same fashion, another DNA fragment including the last 16 bp of human *AML1* exon 5 and 5' part of subsequent *EVI1* sequence was also amplified using human *AML1/EVI1* cDNA⁵ as a template to create a *SacII* site by introducing silent mutations at identical codons. These 2 PCR products were subcloned into the human *AML1/EVI1* expression plasmid, pME18S-AML1/EVI1,⁵ to give an in-frame fusion of the first 94 bp of murine *Aml1* exon 5 to the following portion of human *AML1/EVI1* cDNA (Figure 1A). The Neo-resistant gene derived from pBK-Neo³² was added downstream of the *AML1/EVI1* sequence. A 4.1-kb (kilobase) *XbaI-AvrII* fragment from intron 4 and a 4.3-kb *EcoRI* fragment from intron 5 of murine *Aml1* genome were used for homologous recombination. To create the final targeting vector, the DNA fragment containing the previously mentioned sequences was inserted into the *NotI* and *Clal* sites of pBluescript-DTA (diphtheria toxin-A gene), in which the *Dta* gene from pMC1DTApolvA (Stratagene, La Jolla, CA) was subcloned into the *Sall* site of pBluescript. The construct was verified by restriction endonuclease mapping and DNA sequencing.

Production of chimeric mice

The linearized targeting vector (50 μ g) was electroporated into TT2 embryonic stem (ES) cells.³⁴ G418-resistant clones were analyzed for homologous recombination by Southern analysis hybridizing *EcoRI*-digested DNA with a probe 5' to the 5' homologous sequence in the targeting vector and a *Neo* gene probe (Figure 1B). Heterozygous AML1/EVI1^{+/+} clones with undifferentiated morphology were injected into morulas of the ICR mouse strain. Chimeric males were bred with C57BL/6 females, and whole-body specimens of resultant embryos were analyzed for the presence of the knock-in allele by Southern analysis.

Visual inspection and histologic analysis

Embryos were removed from the uterus, dissected free from the fetal membranes, and inspected under a dissecting microscope for evidence of gross abnormalities. Embryos were fixed in formaldehyde solution and embedded in paraffin. The sections were stained with hematoxylin and eosin solution. Peripheral blood was collected in 10 mM EDTA (ethylenediaminetetraacetic acid), and smears were stained with Wright-Giemsa solution.

Histologic images were obtained on either an Olympus AX80 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP70 digital camera, a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with an Olympus DP12 digital camera, or an Olympus IMT-2 microscope equipped with an Olympus DP12 digital camera. Each microscope was equipped with a 10 \times /22 ocular lens. Images were cropped in Adobe Photoshop CS (Adobe Systems, San Jose, CA) and composed in Canvas 9 (Polaroid, Waltham, MA).

Cell culture, transfection, and induction of megakaryocytic differentiation

K562 cells were maintained in RPMI-1640 supplemented with 10% fetal calf serum. To obtain bulk transfectant of mock or the AML1/EVI1 cDNA, 1 \times 10⁷ K562 cells were electroporated with 50 μ g pCXN2 or pCXN2-AML1/EVI1 at 380 V and 975 μ F using Gene Pulser (Bio-Rad, Hercules, CA) and selected with 0.4 mg/mL G418 (Sigma-Aldrich, St Louis, MO). Resistant cells were screened for expression of AML1/EVI1 chimeric

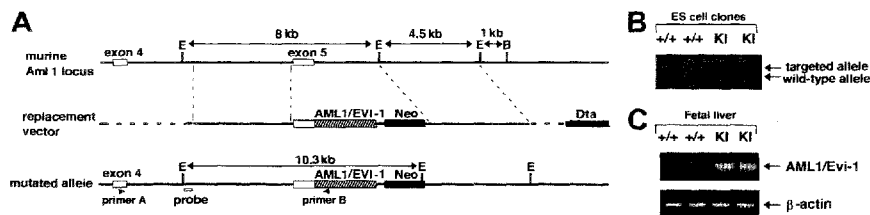


Figure 1. Gene targeting strategy to introduce AML1/EVI1 chimeric gene into the murine AML1 locus. (A) Schematic presentation of partial murine *Aml1* genomic locus (top line), replacement vector containing a partial human *AML1/EVI1* cDNA, a *neomycin resistance* cassette (*Neo*) for positive selection and a *diphtheria toxin-A* cassette (*Dta*) for negative selection (middle line), and the targeted allele (bottom line). (B) Southern analysis of wild-type (+/+) and AML1/EVI1 knock-in (KI) ES cell clones. For detecting homologous recombination, the murine *Aml1*-specific probe indicated in the lower line of panel A was used. (C) RT-PCR analysis on E12.5 fetal liver cells from wild-type (+/+) and KI embryos. AML1/EVI1 mRNA was amplified using primers A and B indicated in the lower line of panel A. Amplification was also performed for β -actin mRNA as a control for the presence of amplifiable RNA.

proteins by Western analysis using anti-AML1 Ab-1 antibody (Oncogene Research Products, San Diego, CA). To induce megakaryocytic differentiation, 100 nM staurosporine (Sigma-Aldrich) was added to the culture.

DNA content analysis

Each mock or *AML1/EVII*-expressing K562 bulk population treated with 100 nM staurosporine was washed, fixed, and stained with 50 mg/mL propidium iodide using Cycle TEST PLUS (BD Biosciences, San Jose, CA). DNA content of nuclei was determined by fluorescence-activated cell sorting analysis.

Ultrastructural study

E13.5 fetal livers of *AML1/EVII*^{+/+} heterozygous embryos and their wild-type littermates were examined with a JEOL 1200CX electron microscope (JEOL, Tokyo, Japan). Each mock or *AML1/EVII*-expressing bulk population treated with 100 nM staurosporine for 48 hours was also examined.

In vitro culture of hematopoietic cells

Fetal livers from E12.5 or E13.5 embryos were disrupted by passing through 21-gauge needles and then 26-gauge needles for 3 times each. Single-cell suspensions with 1×10^4 cells were plated in triplicate in 1.2% methylcellulose in Iscove's modified Dulbecco medium containing 30% fetal calf serum, 0.1 mM β -mercaptoethanol, 2 mM glutamine, and 1% bovine serum albumin, 5 U/mL human erythropoietin, 100 ng/mL murine stem cell factor, 5 ng/mL murine interleukin 3 (IL-3; Kirin Brewery, Tokyo, Japan). Cell aggregates containing more than 50 cells were counted as colonies. Cytocentrifuge preparations of hematopoietic colonies were stained with Wright-Giemsa for morphologic examination, benzidine for the presence of erythroblasts, and choline esterase for the presence of megakaryocytes. In replating experiments, all of the cells from the preceding culture were collected, washed, and then replated at 1×10^4 cells per methylcellulose plate under the same condition as in the preceding culture. Colonies were scored as previously mentioned.

Quantitative reverse transcriptase (RT)-PCR analysis.

E12.5 and E13.5 live embryos were selected on the basis of the presence of heartbeats. Total RNA was isolated from liver cells using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Random hexamer-primed cDNA was prepared from 2 μ g total RNA with mouse mammary leukemia virus reverse transcriptase (Gibco BRL, Grand Island, NY) in a total volume of 25 μ L. An oligonucleotide primer set containing murine *Aml1* exon 5-derived sense (5'-AAGAGCTTCACTCTGACCAT-3') and human *EVII* noncoding region-derived antisense (5'-CCTTTCACCTACTTCGATCT-3') sequences was used for amplification of the *AML1/EVII* fusion sequence. Amplifications of various hematopoietic regulator transcripts were performed with primer sets described previously³⁵⁻³⁹ as well as that for murine *CD11b*, sense (5'-CAGATCAA-CAATGTGACCGTATGG-3') and antisense (5'-CATCATGTCCTTGACTGCCGC-3') primers, using CYBR Green PCR Master Mix and ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster, CA). Parallel reactions were carried out using a β -actin-amplifying primer set to ensure the integrity of the RNA samples.

Quantification of mRNA level was performed by measuring the cycle threshold (CT) that is defined as the fractional cycle number at which the fluorescence encounters a fixed threshold. The CT value of each gene was normalized to that of β -actin (Δ CT; CT value of target gene minus CT value of β -actin). Results were expressed as difference of Δ CT between *AML1/EVII*^{+/+} versus wild-type or *AML1*^{-/-} versus wild-type animals, and fold expression.

Results

Generation of an *AML1/EVII* fusion allele

The t(3;21) breakpoint on chromosome 21 in human leukemic cells occurs in the fifth intron of the *AML1* gene, which is 3' to the exons

encoding the Runt DNA-binding domain.⁵ To mimic in the mouse genome the mutant *AML1/EVII* chimeric gene at the human t(3;21) allele, we made a targeting construct that replaces murine *Aml1* exon 5 with human *AML1* exon 5 fused in frame to the human *EVII* sequence (Figure 1A). This targeting strategy renders generation of the murine/human hybrid *AML1/EVII* gene whose expression is controlled by the endogenous mouse *Aml1* promoter. Using electroporation, we obtained 8 ES cell clones targeted correctly (no. 31, no. 35, no. 37, no. 42, no. 47, no. 50, no. 70, and no. 97), as determined by the presence of both *AML1/EVII* allele and endogenous murine *Aml1* allele in Southern analysis (Figure 1B). These *AML1/EVII*^{+/+} ES cell clones were injected into mouse morulas, and 2 chimeric mice (no. 31 and no. 97) transmitted the mutant allele through the germ line. These chimeric mice were largely healthy up to 7 months but showed sudden death thereafter. The cause of death is unknown despite anatomic examination performed immediately after the death of the 5 chimeric mice; there were no signs of hepatosplenomegaly or lymphadenopathy, excluding at least the leukemic cause of death.

Knock-in expression of *AML1/EVII* fusion gene results in embryonic lethality with CNS hemorrhage and a lack of fetal-liver hematopoiesis

Genotyping analysis of 42 delivered F1 pups of *AML1/EVII* chimeric male and wild-type C57BL/6 female found no *AML1/EVII*^{+/+} genotypes, suggesting that *AML1/EVII*^{+/+} embryos died in the uteri. *AML1*^{+/+} agouti mice appeared normal; thus, designated major mutations did not occur in the ES cells. To examine the timing and cause of embryonic death in *AML1/EVII*^{+/+} heterozygous embryos, we killed pregnant females at various time points between E10.5 and E14.5. The *AML1/EVII* chimeric mRNA was detected in the fetal liver of the heterozygous mice by RT-PCR (Figure 1C). Between E10.5 and E11.5, all the *AML1/EVII*^{+/+} embryos were viable and showed no significant morphologic abnormalities (Table 1). However, 10% of the embryos was found dead at E12.5 and 100% by E14.5, as judged by the absence of heartbeats. The size of the *AML1/EVII*^{+/+} embryos between E12.5 and E13.5 was comparable to that of the control littermates (Figure 2A). However, they had noticeable white livers, and signs of massive hemorrhage in the CNS and intersegmental regions of the presumptive spinal cord. Microscopic analysis revealed that the hemorrhage occurred as early as E12.5 in the cerebral ventricle and the dorsal root ganglia (Figure 2B-E), and such hemorrhagic sites were somewhat conserved in *AML1/EVII*^{+/+} embryos analyzed (n = 58). This hemorrhage and lethality of *AML1/EVII*^{+/+} heterozygous embryos were observed in 2 independent ES cell lines (no. 31 and no. 97). These data indicate that *AML1/EVII*^{+/+} embryos die around E13.5 from the massive hemorrhage in the CNS. We focused our further analysis on embryos derived from line no. 97

Table 1. Genotype and phenotype of embryos derived from *AML1/EVII* chimeric males mated with normal females

Stages	No. of Pups	Genotype		Phenotype of KI* mice	
		+/+	+KI*	Hemorrhage	Death
E10.5	9	1	8	0	0
E11.5	14	11	3	0	0
E12.5	98	67	31	27	3
E13.5	78	62	16	16	10
E14.5	32	21	11	11	11

Genotype analysis was performed by Southern analysis of DNA extracted from embryos. Surviving embryos were defined as those with beating hearts at the time of dissection.

*KI indicates *AML1/EVII* knock-in.

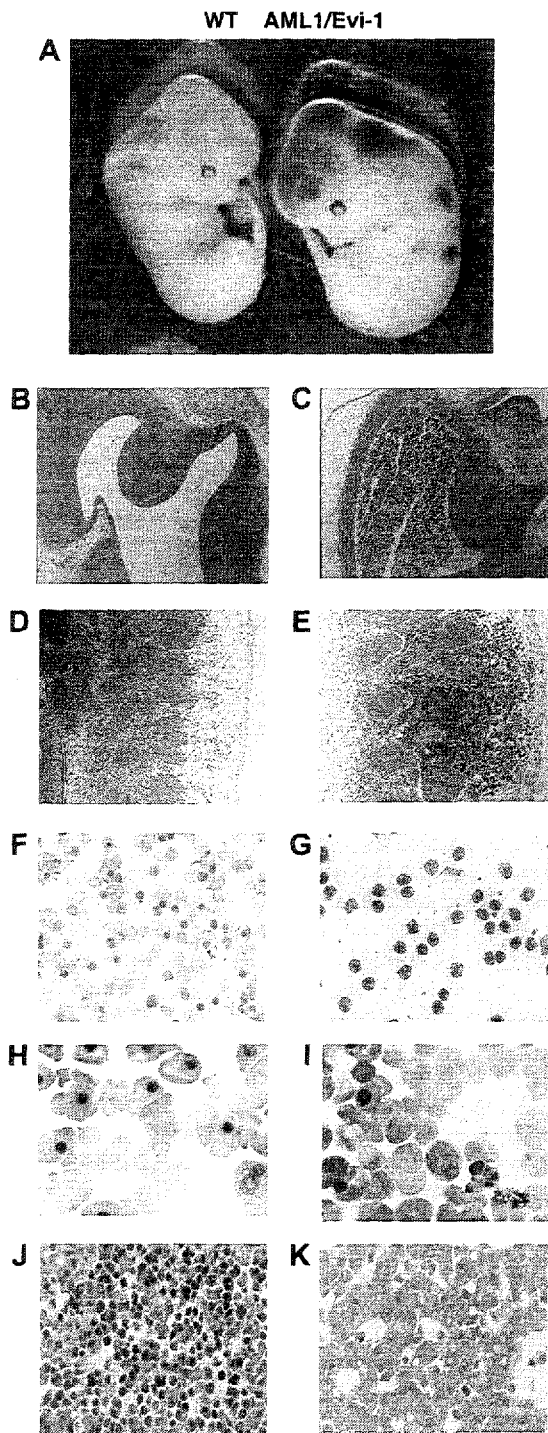


Figure 2. Morphologic comparison of E12.5 wild-type (WT) and *AML1/EVI1*^{+/+} heterozygous embryos. (A) External appearance of WT and *AML1/EVI1*^{+/+} littermates. *AML1/EVI1*^{+/+} embryo on the right is similar in size to WT littermate on the left but is clearly identifiable by the presence of fetal liver pallor and massive hemorrhage within the CNS and soft tissues in the back (objective lens [OL], 2 × 0.05; original magnification [OL], × 20). (B-C) Sections of the cerebral ventricle from WT (B) and *AML1/EVI1*^{+/+} (C) embryos. *AML1/EVI1*^{+/+} embryo shows massive hemorrhage into the ventricle (OL, 10 × 0.40; OM, × 100). (D-E) Sections of the dorsal root ganglia from WT (D) and *AML1/EVI1*^{+/+} (E) embryos. *AML1/EVI1*^{+/+} embryo shows hemorrhage in the ganglia (OL, 10 × 0.40; OM, × 100). (F-G) Smears of the peripheral blood from WT (F) and *AML1/EVI1*^{+/+} (G) embryos. *AML1/EVI1*^{+/+} embryo shows the absence of definitive erythrocytes, while WT littermate shows numerous enucleated definitive erythrocytes (OL, 40 × 0.65; OM, × 400). (H-I) Smears of the peripheral blood from WT embryo. Only WT littermate shows monocyte (H) and neutrophil (I) (OL, 100 × 1.40; OM, × 1000). (J-K) Sections of the fetal liver from WT (J) and *AML1/EVI1*^{+/+} (K) embryos. *AML1/EVI1*^{+/+} embryo shows a near complete absence of hematopoietic precursors, while WT littermate shows numerous hematopoietic precursor cells (OL, 20 × 0.70; OM, × 200).

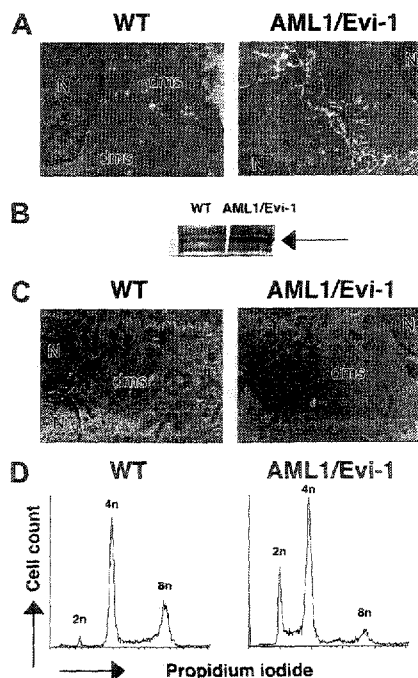


Figure 3. Morphology and DNA content of megakaryocytic cells overexpressing *AML1/EVI1*. (A) Electron micrographs of megakaryocytes in E13.5 fetal liver. N indicates nucleus; dms, demarcation membrane (OM, × 3000). (B) Expression of *AML1/EVI1* chimeric protein in transfected K562 cells. An arrow indicates overexpressed *AML1/EVI1* proteins. (C) Electron micrographs of mock or *AML1/EVI1*-overexpressing K562 cells after staurosporine treatment for 48 hours. N indicates nucleus; dms, demarcation membrane (OM, × 3000). (D) DNA content of mock or *AML1/EVI1*-overexpressing K562 cells after staurosporine treatment for 48 hours.

because of the efficient germ line transmission and propagation relative to those from line no. 31.

Yolk sac hematopoiesis appeared largely intact in *AML1/EVI1*^{+/+} embryos, with normal number and morphology of the yolk sac-derived nucleated erythroblasts circulating in the peripheral vessel (Figure 2G). Erythroblasts in E12.5 *AML1/EVI1*^{+/+} embryos looked similar to those in E11.5 wild-type littermates, suggesting that they normally developed in the yolk sac. In contrast, fetal liver hematopoiesis was severely impaired, as indicated by markedly whitish liver resulting from a lack of red cells. The microscopic examination showed near complete absence of the erythroid, myeloid, or megakaryocytic progenitors of the definitive origin (Figure 2K). Postnucleated erythrocytes were absent in the peripheral blood from E12.5 *AML1/EVI1*^{+/+} embryos, whereas it was abundantly observed in the peripheral blood of E12.5 wild-type littermates (Figure 2F-G). Also, monocytes and neutrophils were absent in the smears from *AML1/EVI1*^{+/+} embryos, whereas they were easily observed in the smears from wild-type littermates (Figure 2H-I). These findings indicate that *AML1/EVI1*^{+/+} heterozygous mice fail to establish definitive hematopoiesis in the fetal liver but maintain primary hematopoiesis in the yolk sac, recapitulating hematopoietic defects observed in *AML1*^{-/-} 11,12 or *AML1/ETO*^{+/+} mice.^{32,33} We further performed electron microscopic examination to search for hematopoietic progenitors that would have been missed by histologic microscopic examination in the E13.5 fetal liver. In addition to decreased erythroid and myeloid progenitors, we observed megakaryocytes that were defective in their demarcation membrane (Figure 3A), which resemble those in *AML1* conditional knock-out mice.¹⁶ Thus, although there is massive defect in fetal liver hematopoiesis, some hematopoietic progenies do exist, although dysplastic, in the *AML1/EVI1*^{+/+} fetal liver.

Table 2. Hematopoietic progenitors in E12.5 and E13.5 fetal liver

Stages/genotype	No. of Pups	BFU-E	CFU-GM	CFU-GEMM	CFU-M
E12.5					
+/+	13	10 ± 3	32 ± 8	20 ± 9	8 ± 2
<i>AML1/EV11</i> ^{+/+}	6	0	0	0	25 ± 11
E13.5					
+/+	9	8 ± 3	58 ± 14	25 ± 11	10 ± 3
<i>AML1/EV11</i> ^{+/+}	5	0	0	154 ± 54	4 ± 3

Numbers represent colonies per 1×10^4 fetal liver cells, mean \pm SD.

BFU-E indicates erythroid burst-forming unit; CFU-GM, granulocyte/macrophage colony-forming unit; CFU-GEMM, granulocyte/erythrocyte/macrophage/megakaryocyte colony-forming unit; CFU-M, macrophage colony-forming unit.

To test for the direct effect of *AML1/EV11* molecule in megakaryocyte morphology, we expressed *AML1/EV11* cDNA in human leukemic cell line K562 (Figure 3B) and differentiated one bulk population into the megakaryocytic lineage by exposing to staurosporine.⁴⁰ The *AML1/EV11*-expressing K562 cells treated with staurosporine showed poorly developed demarcation membrane and lower level of polyploidy as compared with control mock cells (Figure 3C), indicating that the expression of *AML1/EV11* molecule is causative for the aberrant maturation of megakaryocytes in *AML1/EV11*^{+/+} mice.

***AML1/EV11* generates dysplastic hematopoietic progenitors in the fetal liver**

To analyze for the defective hematopoiesis in the *AML1/EV11*^{+/+} embryos, we performed methylcellulose colony-forming assay using fetal liver cells from E12.5 or E13.5 embryos. Because the total number of cells recovered from E12.5 or E13.5 *AML1/EV11*^{+/+} fetal livers was 20-fold less than that recovered from wild-type fetal livers, we adjusted the number of plated fetal liver cells (1×10^4 cells) to assess for the precursor frequencies. In day 10 of culture, wild-type fetal liver gave rise to multilineage colonies, whereas E12.5 *AML1/EV11*^{+/+} fetal liver cells generated macrophage colonies only, with no detectable erythroid, myeloid, and mixed colonies (Table 2). The E13.5 *AML1/EV11*^{+/+} cell culture looked similar to that of E12.5, except for some mixed-like colonies that were 5-fold more numerous than those from wild-type cell culture in microscopic examination. These data indicate that expression of *AML1/EV11* leads to severe defects in definitive hematopoiesis, but the fetal liver contains some progenitors capable of differentiating into multilineage hematopoietic progenies.

To gain more insights into the mixed-lineage-like colonies derived from E13.5 *AML1/EV11*^{+/+} fetal liver, we performed time-course microscopic analysis of the methylcellulose culture. On day 7 of culture, colonies from E13.5 *AML1/EV11*^{+/+} fetal liver

consisted mainly of myelocytic blastoid cells, while those from wild-type fetal liver contained numerous erythroblasts, hypergranular myeloid cells, and megakaryocytes (Figure 4). Benzidine staining confirmed the absence of erythroblasts in *AML1/EV11*^{+/+} cell-derived colonies. However, choline esterase-positive megakaryocytes were observed in both types of the colonies. On day 10, colonies from *AML1/EV11*^{+/+} fetal liver contained a large number of dysplastic myelocytes with bilobulated or multilobulated nuclei along with scanty cytoplasmic granulation. These findings suggest that hematopoietic progenitors in the *AML1/EV11*^{+/+} fetal liver are completely blocked for differentiation into the erythroid lineage but are capable of differentiating into the myeloid and megakaryocyte lineages, although immature and dysplastic.

Hematopoietic progenitor in the *AML1/EV11*^{+/+} fetal liver is highly capable of self-renewal

To examine self-renewal capacity of hematopoietic progenitors in the E13.5 *AML1/EV11*^{+/+} fetal liver, we performed a serial methylcellulose replating assay using bulk population of fetal liver-derived colonies under the conditions optimal for the development of multipotential hematopoietic progenitors. Cells from *AML1/EV11*^{+/+} fetal livers were able to replat beyond 7 passages, whereas cells from wild-type fetal livers lost their ability to form colonies after 5 passages (Table 3). During each replating, *AML1/EV11*-expressing colonies showed increase in size and number (Figure 5, left). The cytospin samples from the fourth passage colony identified a polymorphic population of dysplastic cells, including agranular myelocytes and megakaryocytes (Figure 5, right). We conclude that expression of *AML1/EV11* in the fetal liver produces multilineage potential progenitors that are capable of more self-renewal than the wild-type progenitors and have a dysplastic maturation tendency. However, even after amplification of the cells through passage, we failed to demonstrate expression of *AML1/EV11* chimeric proteins in the colony-forming cells by Western analysis with anti-*AML1* antibody, possibly because of low expression and high molecular weight (190 kDa) of the proteins.

Expression of *PU.1* is maintained in *AML1/EV11*^{+/+} fetal liver cells but significantly decreased in *AML1*^{-/-} cells

To get the molecular underpinning for the dysregulated hematopoiesis caused by *AML1/EV11*, we assessed the expression of various genes implicated in definitive hematopoiesis. The total RNA was extracted from E12.5 fetal liver cells from wild-type, *AML1*^{-/-}, and *AML1/EV11*^{+/+} embryos, and mRNA was quantified using semiquantitative RT-PCR analysis. The level of expression in

Figure 4. Morphology of dysplastic mixed-like hematopoietic colonies in the *AML1/EV11*^{+/+} fetal liver culture. On days 7 and 10 of culture, cytocentrifuge preparations of cells from mixed-like colonies were stained with Wright-Giemsa for morphologic examination (OL, $100 \times / 1.40$; OM, $\times 1000$), benzidine for the presence of erythroblasts (OL, $10 \times / 0.30$; OM, $\times 100$), or choline esterase for the presence of megakaryocytes (OL, $60 \times / 1.40$; OM, $\times 600$). *AML1/EV11*^{+/+} mixed-like colonies in the bottom panels show retarded maturation and hypogranulation of myeloid cells as well as the absence of erythroid cells. In contrast, WT mixed colonies in the top panels show normal tri-lineage differentiation.

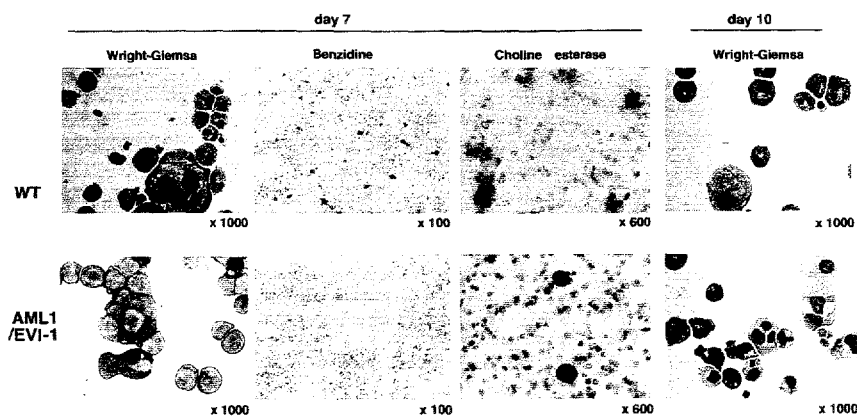


Table 3. Serial in vitro replating assays of E13.5 fetal liver hematopoietic progenitors

Genotype	In vitro passage						
	1	2	3	4	5	6	7
+/+	99 ± 23	384 ± 86	164 ± 41	59 ± 76	11 ± 9	0	0
AML1/EV11/+	162 ± 59	388 ± 131	365 ± 150	403 ± 89	436 ± 104	407 ± 114	626 ± 3

Cells from methylcellulose culture were disaggregated, washed, and replated at 1×10^4 cells per methylcellulose plate under the same condition every 7 days. Numbers represent colonies per 1×10^4 cells, mean \pm SD.

AML1^{-/-} and AML1/EV11^{+/+} fetal liver cells was compared with that in wild-type cells. Table 4 revealed the calculated fold expression of the indicated genes in AML1^{-/-} and AML1/EV11^{+/+} embryos relative to that of wild-type littermates along with the cycle threshold differences. The expression of the *PU.1*⁴¹ gene in AML1/EV11^{+/+} fetal liver cells was maintained to the normal, whereas its expression level was markedly decreased in AML1^{-/-} fetal liver cells, as has been reported previously.⁴² Consistent with this, the expression of the *CD11b* gene, one of the target genes of PU.1 (an ets transcription factor family member), was maintained in AML1/EV11^{+/+} knock-in liver, whereas markedly decreased in AML1^{-/-} liver. The expression of the critical transcription factor such as PU.1 in AML1/EV11^{+/+} fetal liver may support multilineage hematopoietic progenitors up to E13.5 and also aids the monocyte/macrophage lineage differentiation. The myeloid lineage-specific transcription factor *C/EBPA*⁴³ was expressed several fold higher in AML1/EV11^{+/+} fetal liver cells than in wild-type cells, and this may bolster the myeloid-lineage differentiation along with PU.1. The expression levels of cytokine receptors transcripts such as G-CSF receptor and GM-CSF receptor were largely unchanged across wild-type, AML1^{-/-} and AML1/EV11^{+/+} fetal liver cells. On the other hand, the expression of erythroid lineage-specific genes such as *LMO2*⁴⁴ and *SCL*⁴⁵ was approximately 10-fold lower in AML1/EV11^{+/+} fetal liver cells than in wild-type cells. The decreased expression of *LMO2* and *SCL* could impinge on successful differentiation along the erythroid lineage in AML1/EV11^{+/+} fetal liver cells, and the similar decrease was observed in AML1^{-/-} fetal liver cells that lack erythroid cells as well. Other erythroid lineage-specific transcripts such as δ -aminolevulinic acid synthase-erythroid (*ALASE*) and β -globin were more decreased in AML1/EV11^{+/+} fetal liver cells than in AML1^{-/-} cells, despite equivalent expression of their master regulator GATA binding protein 1 (GATA-1) in both cell types. The net GATA-1 activity was presumably repressed in AML1/EV11^{+/+} fetal liver cells because of

maintained expression of PU.1 that inhibits DNA binding of GATA-1.⁴⁶ The suppressed expression of *ALASE* and β -globin could also contribute to the complete defect of erythroid differentiation in AML1/EV11^{+/+} progenitor cells. These expression analyses provide a logical explanation for the distinct hematopoietic capacity intrinsic to AML1^{-/-} and AML1/EV11^{+/+} fetal liver cells. Our data point that the sufficient expression of the *PU.1* gene may be the first critical prerequisite for the definitive hematopoiesis to be set off in the fetal liver.

Discussion

AML1-CBF β transcription factor complex is one of the critical regulators of the hematopoietic system. A substantial amount of evidence highlights the central role for this transcription factor system in normal hematopoiesis as well as in abnormal hematopoietic disorders. Four gene-ablated animals including ours, AML1-null,^{11,12} CBF β -null,¹³⁻¹⁵ AML1/*ETO* knock-in,^{32,33} and AML1/*EV11* knock-in mice, show normal hematopoiesis in the yolk sac but share common defects in definitive hematopoiesis in the fetal liver. These 4 embryos also show massive hemorrhage in the CNS and spinal cord and die during midgestation. A series of biochemical analyses in AML1-CBF β transcription factor, or AML1/*ETO* or AML1/*EV11* fusion molecules^{23,28,29} indicate that such in vivo phenotypes arise from shutting off or suppressing AML1 function in the early hematopoietic tissues. We speculate that such phenotype in AML1/*EV11* knock-in mice also comes from dominant-negative effect against wild-type-AML1 in vivo.

The microscopic analysis of the fetal liver of the 4 mutant embryos shows common lack of visible hematopoietic cells.

Table 4. Expressional changes of various hematopoietic regulators in E12.5 AML1/EV11^{+/+} and AML1^{-/-} fetal liver cells

	Δ CT _{KI} - Δ CT _{WT}	Fold (KI/WT)	Δ CT _{KO} - Δ CT _{WT}	Fold (KO/WT)
<i>C/EBPA</i>	1.4 ± 0.1	2.7 ± 0.1	2.9 ± 0.0	7.2 ± 0.1
<i>LMO2</i>	3.5 ± 0.3	0.1 ± 0.6	3.3 ± 0.1	0.1 ± 0.3
<i>SCL</i>	3.9 ± 0.8	0.1 ± 1.7	2.4 ± 0.2	0.2 ± 0.3
<i>c-MYB</i>	-0.5 ± 0.2	1.4 ± 1.5	-2.4 ± 0.3	5.4 ± 0.6
<i>GATA1</i>	-0.4 ± 0.7	1.4 ± 1.3	-1.6 ± 0.5	3.0 ± 1.0
<i>PU.1</i>	-0.4 ± 0.1	1.3 ± 0.1	8.2 ± 0.1	0.0 ± 0.3
<i>NFE2</i>	2.9 ± 0.5	0.1 ± 1.0	0.3 ± 0.2	0.6 ± 0.2
<i>CD11b</i>	1.2 ± 0.4	0.4 ± 0.8	5.0 ± 1.8	0.0 ± 3.6
<i>MCSFR</i>	0.1 ± 0.2	0.9 ± 0.4	-1.5 ± 0.9	2.8 ± 1.8
<i>GCSFR</i>	-0.6 ± 0.1	1.5 ± 0.2	0.0 ± 0.0	1.0 ± 0.0
<i>GMCSFR</i>	0.1 ± 0.2	1.1 ± 0.4	-0.6 ± 0.1	1.5 ± 0.2
<i>MPO</i>	1.0 ± 1.1	0.0 ± 2.2	7.0 ± 0.7	0.0 ± 1.3
<i>β-globin</i>	3.9 ± 0.9	0.1 ± 1.7	1.0 ± 0.4	0.5 ± 0.8
<i>FOG1</i>	2.3 ± 0.2	0.2 ± 0.3	2.0 ± 0.2	0.3 ± 0.3
<i>ALASE</i>	5.6 ± 0.4	0.0 ± 0.7	1.9 ± 0.4	0.3 ± 0.8

Δ CT indicates mean CT of indicated gene - mean CT of β -actin; KI, AML1/EV11 knock-in; KO, AML1 knock-out; fold, fold difference relative to that of WT litters calculated by $2^{-(\Delta$ CT_{KI} or _{KO} - Δ CT_{WT})}. Data are means \pm standard deviation.

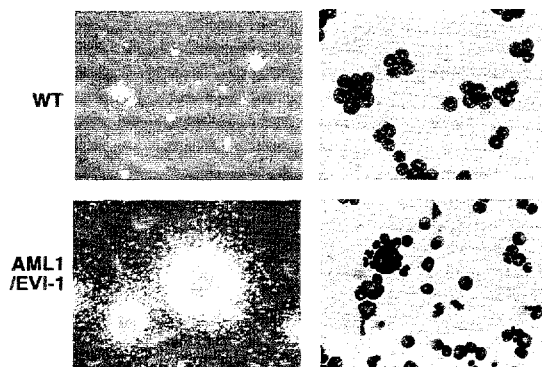


Figure 5. Morphology of serially replated AML1/EV11^{+/+} hematopoietic colonies. (Left) WT and typical dysplastic multilineage AML1/EV11^{+/+} colonies after the fourth passage. The size of AML1/EV11^{+/+} hematopoietic colonies is significantly larger than that of WT colonies. (Right) Wright-Giemsa-stained cytocentrifuge preparations of the WT and AML1/EV11^{+/+} colony. AML1/EV11^{+/+} hematopoietic colonies maintain the dysplastic nature. Scales bars equal 300 μ m (OL, 100 \times /1.40; OM, \times 1000).

Despite such gross similarity, close examination of the hematopoiesis reveals some intriguing differences. First, although *AML1*^{-/-11,12} or *CBFB*^{-/-13-15} fetal liver has no hematopoietic progenitors that would have produced colonies, *AML1/EV11*^{+/+} and *AML1/ETO*^{+/+}^{32,33} fetal livers have progenitors that can give rise to macrophage colonies. *AML1/EV11*^{+/+} fetal liver (E12.5) contains 3-fold more progenitors than wild-type fetal liver capable of differentiating into the monocyte/macrophage lineage, and a similar finding is reported for *AML1/ETO*^{+/+} yolk sac where Yergeau et al³² observed a marked increase in the number of macrophage colonies. Therefore, the capacity to differentiate along the monocyte/macrophage lineage seems a characteristic feature of hematopoietic progenitors expressing *AML1/EV11* or *AML1/ETO* in the fetal hematopoietic system. Second, in contrast to *AML1*^{-/-} or *CBFB*^{-/-} fetal liver, *AML1/EV11*^{+/+} and *AML1/ETO*^{+/+}³³ fetal livers (E13.5) can also give rise to colonies committed to erythrocyte/megakaryocyte and myelocyte lineages. The appearance of megakaryocytic cells in the colony assay would suggest some production of platelets in these embryos, and this might contribute to 1 day-longer survival of *AML1/EV11* and *AML1/ETO* knock-in embryos (lethal at E13.5) than *AML1* or *CBFB* knock-out embryos (lethal at E12.5). Third, the serial colony-forming assay demonstrates that *AML1/EV11*⁻ or *AML1/ETO*³³-expressing progenitors show more self-renewal than those of the wild-type animals, although these progenitors cannot establish functional hematopoiesis in the fetal liver. These differences between *AML1/EV11*⁻ or *AML1/ETO*-expressing cells versus *AML1* or *CBFB* knock-out cells may arise from either incomplete suppression of wild-type-*AML1* or additional functions brought by the individual fused molecules.

Although *AML1/EV11*^{+/+} and *AML1/ETO*^{+/+} fetal liver showed similar results in colony counts, there are some striking differences in their contents of colonies that are unlikely to result from minor differences in culture conditions. We used higher concentrations of erythropoietin and stem cell factor than those reported in *AML1/ETO*^{+/+} fetal liver culture.^{32,33} Nonetheless, *AML1/EV11*^{+/+} hematopoietic progenitors fail to generate erythroid cells, whereas *AML1/ETO*^{+/+} hematopoietic progenitors give rise to mixed colonies containing numerous erythroblasts.³³ The observed resistance to the erythroid-lineage differentiation is highly characteristic of *AML1/EV11*^{+/+} hematopoietic progenitors. This is consistent with the reported retroviral expression of EV11 in hematopoietic progenitor cells that blocks erythroid differentiation *in vitro*⁴⁷ or the transgenic expression of EV11 impairing erythropoiesis *in vivo*.⁴⁸ Therefore, the expression of EV11, either in canonical form or as the fusion chimeric protein, is detrimental to erythroid-lineage differentiation. Another distinct finding between the 2 cell types is the more severe dysplasticity in *AML1/EV11*^{+/+} cells. In *AML1/EV11*^{+/+} progenitors, the arising myeloid cells arrest at the myelocyte stage with no granulation, and the arising megakaryocytes show ill-developed demarcation membrane in the cytoplasm. In contrast, *AML1/ETO*^{+/+} progenitors exhibited far more differentiated morphologies, although reported dysplastic in the text.³³ Overexpressed EV11 protein in G-CSF-treated 32Dcl3 cells completely blocks myeloid differentiation.⁴⁹ Thus, the adverse effect of EV11 in myeloid differentiation may contribute to the more severe defect in the myeloid maturation of the *AML1/EV11*-expressing progenitors than that of the *AML1/ETO*-expressing cells. Finally, the abnormal expression of the *AML1/EV11* gene causes disturbance of megakaryocytic maturation, which is not observed in *AML1/ETO*-expressing animals. This effect seems direct as evi-

dent by *AML1/EV11*-expressing K562 cells showing poor demarcation membrane and lower DNA ploidy when treated with staurosporine. In this case, however, the result seems to be caused by the dominant-negative effect over wild-type-*AML1*, as *AML1* conditional knock-out mice show similar maturation abnormality in megakaryocytes.

AML1/EV11 causes broad perturbation of gene expression that is key to normal hematopoietic cell development. The first key gene is *PU.1* that is known to play a pivotal role in B cell/monocyte development⁵⁰⁻⁵⁴ and the maintenance of hematopoietic stem cell in the fetal liver.⁵⁵ *PU.1* transcripts are markedly decreased in *AML1*^{-/-} fetal liver cells, as reported previously,⁴² whereas its expression is normal in *AML1/EV11*^{+/+} fetal liver cells, and this expression pattern is paralleled by one of its direct target gene *CD11b*. The maintained expression of *PU.1* may afford *AML1/EV11*^{+/+} hematopoietic progenitors a capacity to produce macrophage and mixed colonies. Importantly, our data suggest that the reported complete lack of hematopoietic progenitors in *AML1*^{-/-} fetal liver may result from the absence of *PU.1*, and the recent finding underscores the critical role for *PU.1* in supporting hematopoietic stem cell maintenance in the fetal liver.⁵⁵ Thus, *PU.1* seems to be the key transcription factor that explains the phenotypic difference between *AML1/EV11*^{+/+} and *AML1*^{-/-} animals. The second key gene *CEBPA* is involved in early granulocytic differentiation.⁵⁶ The expression of *CEBPA* is increased in both *AML1/EV11*^{+/+} and *AML1*^{-/-} fetal livers. In *AML1/EV11*^{+/+} liver, the increased expression of *C/EBPα* may enhance myeloid/monocytic differentiation of the existing hematopoietic progenitor supported by *PU.1*. In *AML1*^{-/-} fetal liver, however, the lack of *PU.1* expression dissipates the hematopoietic progenitor; thus, there is no myeloid/monocytic progeny arising from even the increased expression of *C/EBPα*. Third, the expression of *LMO2* and *SCL* is severely repressed in *AML1/EV11*^{+/+} fetal liver. The *LMO2* and *SCL* genes are essential for erythroid lineage differentiation,^{44,45} and low expression of these genes could account for the absence of erythroid cells in *AML1/EV11*^{+/+} fetal liver, even though the hematopoietic progenitor cells are supported by the presence of *PU.1*. The lack of *LMO2* and *SCL* expression is also observed in *AML1*^{-/-} fetal liver. This suggests that the defective expression of *LMO2* and *SCL* in *AML1/EV11*^{+/+} cells is due to dominant-negative effect of *AML1/EV11* over wild-type *AML1*, and that *LMO2* and *SCL* might be candidate target genes for wild-type *AML1*. This possibility might be worth exploring, although no PEBP2 binding sites are found at least in the proximal regulatory region of the *LMO2* or *SCL* gene.^{57,58} Finally, it is worth emphasizing that *GATA1* expression is kept comparable across wild-type, *AML1*^{-/-}, and *AML1/EV11*^{+/+} fetal livers, and yet its target genes *ALASE* and β -globin are much more repressed in *AML1/EV11*^{+/+} fetal liver cells than in *AML1*^{-/-} cells. These differences in expression of *ALASE* and β -globin genes could be due to the interplay between *PU.1* and *GATA-1*,⁴⁶ providing a case that some of the altered gene expressions are the consequences of the combinatory effect of involved transcription factors.

In summary, we have demonstrated that *AML1/EV11* knock-in mice almost completely lack effective hematopoiesis in the fetal liver, but they retain progenitors with increased self-renewal capacity and potential to differentiate into the myeloid/monocytic and megakaryocytic lineages with some dysplasticity. We provide correlative evidence that *PU.1* plays a critical role in the maintenance of hematopoietic progenitors in the *AML1/EV11*^{+/+} fetal liver. Collectively, our study suggests that *AML1/EV11* is not only a

dominant-negative suppressor of wild-type-AML1 but also has more a proliferative and dysplastic effect added on by the fusing EVI1 part. Clinical evidence shows that the emergence of AML1/EVI1 is associated with the progression of hematopoietic stem cell disorder, and our results explain the aggressive and dysplastic transformation of the disease course observed in patients with AML1/EVI1-expressing leukemia. Inducible expression of AML1/ETO in mouse hematopoietic tissue shows that the expression of AML1/ETO per se is not leukemogenic^{59,60} and requires a second hit introduced by alkylating agents to cause leukemia.^{60,61} It certainly needs to be tested whether the expression of AML1/EVI1 is sufficient to cause leukemia when expressed in an inducible fashion in adult mice. Our prediction would be that AML1/EVI1 expression is leukemogenic on its own, or at least more prone to leukemia when a second hit comes in. A conditional project of AML1/EVI1 knock-in expression is in progress in our laboratory.

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Clinical characteristics and prognostic implications of *NPM1* mutations in acute myeloid leukemia

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Recently, somatic mutations of the nucleophosmin gene (*NPM1*), which alter the subcellular localization of the product, have been reported in acute myeloid leukemia (AML). We analyzed the clinical significance of *NPM1* mutations in comparison with cytogenetics, *FLT3*, *NRAS*, and *TP53* mutations, and a partial tandem duplication of the *MLL* gene (*MLL*-TD) in 257 patients with AML. We found *NPM1* mutations, including 4 novel sequence variants, in 64 of 257 (24.9%) patients.

NPM1 mutations were associated with normal karyotype and with internal tandem duplication (ITD) and D835 mutations in *FLT3*, but not with other mutations. In 190 patients without the M3 French-American-British (FAB) subtype who were treated with the protocol of the Japan Adult Leukemia Study Group, multivariate analyses showed that the *NPM1* mutation was a favorable factor for achieving complete remission but was associated with a high relapse rate. Sequential

analysis using 39 paired samples obtained at diagnosis and relapse showed that *NPM1* mutations were lost at relapse in 2 of the 17 patients who had *NPM1* mutations at diagnosis. These results suggest that the *NPM1* mutation is not necessarily an early event during leukemogenesis or that leukemia clones with *NPM1* mutations are sensitive to chemotherapy. (Blood. 2005;106:2854-2861)

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Introduction

Acute myeloid leukemia (AML) is characterized by autonomous proliferation and impaired differentiation of hematopoietic progenitors but is a genetically and phenotypically heterogeneous disease. A number of genetic mutations, such as point mutations, gene rearrangements, and chromosomal translocations, which are involved in the pathogenesis of leukemia, have been documented. Recently, it was suggested that AML is the consequence of 2 broad complementation classes of mutations: those that confer a proliferative or a survival advantage to hematopoietic progenitors (class 1)—including activating mutations in tyrosine kinases such as *BCR-ABL1*, *ETV6-PDGFRB*, *KIT*, and *FLT3* or their downstream effectors such as *NRAS*—and those that impair hematopoietic differentiation and confer properties of self-renewal (class 2)—including rearrangements or point mutations of core binding factor (*CBF*) genes and *PML-RARA*.¹ Mutations in *FLT3*, *NRAS*, and *KIT* have been found in approximately 30% to 35%, 15% to 20%, and

5% to 10% of adult patients with AML, respectively, indicating that mutations in these 3 genes are the most frequent genetic alterations in AML.²⁻¹² *FLT3* and *KIT* mutations are often found in AML patients with *PML-RARA* and *CBF* gene translocations, respectively,^{13,14} whereas *FLT3* mutations have been preferentially found in AML patients with normal karyotype. Because mutated *FLT3* reportedly induces myeloproliferative disease (MPD), but not AML, in primary hematopoietic progenitors in the murine bone marrow transplantation model and MPD does not have serial transplantability, *FLT3* mutations alone are not sufficient for the development of AML.¹⁵ Therefore, it has been suggested that additional mutations are involved in the pathogenesis of AML with *FLT3* mutations.

Recently, Falini et al¹⁶ reported that the nucleophosmin gene (*NPM1*) is mutated in a high proportion of adults with AML, resulting in an aberrant cytoplasmic localization of the product

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(NPMc+). Of note is that NPMc+ is associated with a wide spectrum of morphologic subtypes of AML, a normal karyotype, and *FLT3* mutations. Furthermore, NPMc+ AML is clinically associated with better responsiveness to induction chemotherapy, although its prognostic implications for long-term outcome remain unclear. Although the prevalence and significance of several genetic abnormalities in patients with AML have been reported to date, the most powerful prognostic factor in AML has been the karyotype of the leukemia cells.¹⁷ Three cytogenetic risk groups (favorable, intermediate, and poor) are widely accepted, but there is a practical limitation to the definition of cytogenetic risk, especially in patients in the intermediate group. Additional prognostic factors are, therefore, required. We and several groups^{9,10,12,18} have demonstrated that *FLT3* mutations are a strong prognostic factor in AML, especially in patients with normal karyotype, but they do not affect responsiveness to induction chemotherapy. Therefore, *NPM1* mutations seem to characterize a distinct disease entity not only of AML with normal karyotype but also of AML with *FLT3* mutations.

NPM is a ubiquitously expressed phosphoprotein, continuously shuttles between the nucleus and the cytoplasm,^{19,20} and is involved in the oncogenesis of some types of leukemia and lymphoma because the *NPM* gene is a partner in several tumor-associated chromosomal translocations.²¹⁻²³ However, it is also thought to have a tumor-suppressor function and to regulate the p53 pathway through its chaperoning activity.²⁴⁻²⁶ It is suggested that a loss of nuclear NPM function caused by mutation might impair the p53 pathway and that a lack of p53 might induce genetic instability²⁷; hence, *NPM1* mutations seem to cause AML cells to acquire additional genetic alterations.

In this study, we analyzed the prevalence and clinical characteristics of *NPM1* mutations in comparison with cytogenetics, *FLT3*, *NRAS*, and *TP53* mutations and a partial tandem duplication of the *MLL* gene (MLL-TD) in 257 patients with newly diagnosed de novo AML. The prognostic implications of *NPM1* mutations were evaluated in 190 patients with AML, excluding those with the M3 FAB subtype, who were treated according to the protocol of the Japan Adult Leukemia Study Group (JALSG). Furthermore, to clarify the stability of *NPM1* mutations and the potential effect on genetic instability in AML cells during disease progression, we compared the mutational status of these genes in 39 paired samples obtained at initial diagnosis and first relapse.

Patients, materials, and methods

Patients and samples

The diagnosis of AML was based on the French-American-British (FAB) classification. The study population included 257 patients with newly diagnosed de novo AML, as follows: 9 with the M0, 54 with the M1, 89 with the M2, 15 with the M3, 54 with the M4, 19 with the M5, 8 with the M6, and 9 with the M7 FAB subtype. In 39 of the 257 AML patients, paired samples obtained at diagnosis and first relapse were available. Furthermore, in 6 patients, samples obtained at diagnosis and complete remission (CR) were available. Bone marrow (BM) samples from patients with AML were subjected to Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation. All samples taken at diagnosis or relapse were confirmed to contain more than 90% leukemia cells after enrichment by centrifugation. Informed consent was obtained from all patients to use their samples for banking and molecular analysis, and approval for these studies was obtained from the Nagoya University institutional review board.

Cytogenetic G-banding analysis was performed according to standard methods. In this study, cytogenetic risk groups were stratified according to the criteria adopted by the Medical Research Council.²⁸

Screening for mutations of the *FLT3*, *NRAS*, and *TP53* genes and of *MLL*-TD

High molecular weight DNA and total RNA were extracted from the samples using standard methods. *FLT3* gene mutations of the ITD (*FLT3*/ITD) and activation loop (*FLT3*/D835Mt), *NRAS* gene mutations of codons 12, 13, and 61, and *TP53* gene mutations of exons 5 to 8 were examined as reported and were confirmed by the sequencing procedure.^{4,29,30} MLL-TD was examined by reverse transcription-polymerase chain reaction (RT-PCR), as described previously.³¹

Screening for mutations of the *NPM1* gene

For the screening of *NPM1* mutations, we amplified genomic DNA corresponding to exon 12 of *NPM1* by PCR using the primers NPM1-F, 5'-TTAACTCTCTGGTGGTAGAATGAA-3' and NPM1-R, 5'-CAAGAC-TATTGCCAATCCTAAC-3', as previously reported.¹⁶ Amplified products were separated through agarose gel, purified using a QIAquick gel extraction kit (Qiagen Inc, Chatsworth, CA), and directly sequenced on a DNA sequencer (310; Applied Biosystems, Foster City, CA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). If mutations were found by direct sequencing, the fragments were cloned into a pGEM-T Easy vector (Promega, Madison, WI), then transfected into the *Escherichia coli* strain DH5 α . At least 4 recombinant colonies were selected, and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen Inc) and sequenced.

Analysis of clinical characteristics

It was necessary to analyze the clinical characteristics in a well-documented group. Among the 257 patients analyzed, 15 had acute promyelocytic leukemia (APL). APL has been considered a separate disease entity among AML, and the introduction of all-*trans* retinoic acid (ATRA) has dramatically improved its clinical outcome.³² In addition, 52 patients with AML, excluding those with APL, were treated with independent regimens. We, therefore, analyzed the clinical characteristics of 190 patients with AML, excluding those with APL, who were treated with the AML87, AML89, and AML92 protocols of JALSG.³³⁻³⁵ (Each protocol is presented in Document S1; see the Supplemental Document link at the top of the online article, at the *Blood* website.)

Statistical analysis

Differences in continuous variables were analyzed using the Mann-Whitney *U* test for distribution between 2 groups. Analysis of frequencies was performed using the Fisher exact test for 2 \times 2 tables or the Pearson χ^2 test for larger tables. Multivariate analysis to identify risk factors for achieving CR was performed using the logistic regression model. Survival probabilities were estimated by the Kaplan-Meier method, and differences in survival distributions were evaluated using the log-rank test. Overall survival was defined as the time from the first day of therapy to death or last visit. Relapse-free survival was defined as the time from the first day of CR to relapse, death, or last visit. Patients undergoing hematopoietic stem cell transplantation were censored at the time of transplantation. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistical analyses were performed with StatView-J 5.0 (Abacus Concepts Inc, Berkeley, CA). For all analyses, the *P* values were 2-tailed, and *P* < .05 was considered statistically significant.

Results

NPM1 mutations were frequently found in AML

We first screened for mutations within exon 12 of the *NPM1* gene through direct sequencing in 257 patients with AML, then confirmed

each type of mutation by cloning. We found the *NPM1* mutation in 64 of 257 (24.9%) patients (Table 1). Importantly, direct sequencing revealed that all AML cells with *NPM1* mutations retained the wild-type allele. Given that all samples contained more than 90% AML cells, all mutations seemed to occur in only one allele. Previously, 6 kinds of mutants, designated mutations A to F, were identified. In this study, we found 49 mutations of type A, 7 mutations of type B, 4 mutations of type D, and 4 novel mutants that were designated mutations G to J (Figure 1). All novel mutations included distinct 4-bp insertions (mutation G, TTTG; mutation H, CTTG; mutation I, TAAG; mutation J, TATG) at position 960, resulting in the same frameshift as mutations A to D. Predicted mutant proteins of mutations G and H and of mutation J were the same as those of mutations A and B, respectively. The protein of mutation I contained a lysine residue at position 289, although the other residues were conserved.

Table 1. Relationship among *NPM1* mutation, FAB type, and genetic alterations in AML

	Total no. patients	<i>NPM1</i>		P
		Mutation, no. (%) [*]	Wild type, no. (%) [†]	
FAB subtype				.005
M0	9	2 (22.2)	7 (77.8)	
M1	54	17 (31.5)	37 (68.5)	
M2	89	12 (13.5)	77 (86.5)	
M3	15	0 (0)	15 (100)	
M4	54	21 (38.9)	33 (61.1)	
M5	19	9 (47.3)	10 (52.7)	
M6	8	2 (25.0)	6 (75.0)	
M7	9	1 (11.1)	8 (88.9)	
Cytogenetics				< .001
t(8;21)	31	0 (0)	31 (100)	
inv(16)	8	1 (12.5)	7 (87.5)	
t(15;17)	15	0 (0)	15 (100)	
t(9;22)	3	1 (33.3)	2 (66.7)	
del(5)	7	0 (0)	7 (100)	
del(7)	5	0 (0)	5 (100)	
Others	43	5 (11.6)	38 (88.4)	
Normal	97	46 (47.4)	51 (52.6)	
Unknown	48	11 (22.9)	37 (77.1)	
FLT3				
Mutations, total	67	39 (58.2)	28 (41.8)	< .001
ITD	58	35 (60.3)	23 (39.7)	< .001‡
D835	9	4 (44.4)	5 (55.6)	.028‡
Wild type	190	25 (13.2)	165 (86.8)	
TP53				NS
Mutation	16	2 (12.5)	14 (87.5)	
Wild type	227	58 (25.6)	169 (74.4)	
Not done	14	5 (35.7)	10 (64.3)	
NRAS				NS
Mutation	34	9 (26.5)	25 (73.5)	
Wild type	202	48 (23.8)	154 (76.2)	
Not done	21	7 (33.3)	14 (66.7)	
MLL-TD				NS
Mutation	17	2 (11.8)	15 (88.2)	
Wild type	130	33 (25.4)	97 (74.6)	
Not done	110	29 (26.4)	81 (73.6)	

Numbers and percentages of 257 patients with AML are shown by FAB type; cytogenetics; and *FLT3*, *TP53*, *NRAS*, and MLL-TD mutations, according to *NPM1* mutation.

NS indicates not significant.

*n = 64; 24.9%.

†n = 193; 75.1%.

‡These variables were compared with those of the wild type.

In 2 patients whose leukemia cells had *NPM1* mutations at diagnosis, the mutations were lost at CR, indicating that these were somatic mutations.

Morphologic and genotypic characteristics of AML with *NPM1* mutations

NPM1 mutations were found in patients with AML of all FAB subtypes except M3 (Table 1). Cytogenetic data were available for 209 patients. Consistent with findings of a previous report,¹⁶ the *NPM1* mutation was preferentially found in patients with normal karyotype (46 of 97; 47.4%), but it was not found in patients with t(8;21), t(15;17), del(5), or del(7). In total, there was a significant difference in the frequency of the *NPM1* mutation among patients with (7 of 112; 6.3%) and without (46 of 97; 47.4%) cytogenetic abnormalities ($P < .001$). Moreover, an *NPM1* mutation was found in each of 8 and 3 patients with inv(16) and t(9;22), respectively.

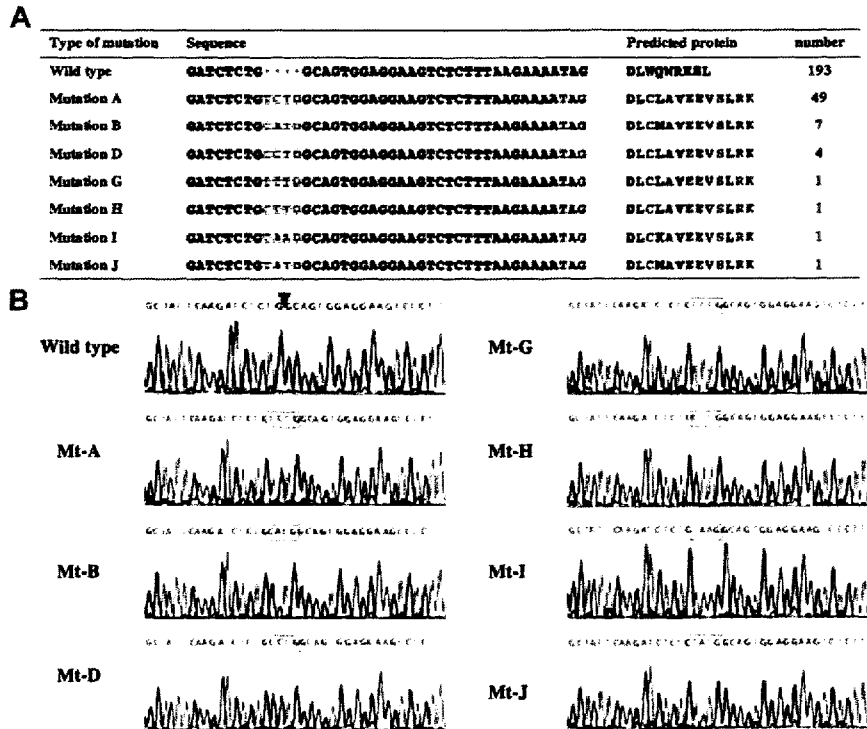
FLT3/ITD and *FLT3/D835Mt* were found in 58 (22.6%) and 9 (3.5%) of 257 patients, respectively (Table 1). Both *FLT3* mutations were significantly associated with *NPM1* mutations: 35 of 58 (60.3%) *FLT3/ITD* ($P < .001$) and 4 of 9 (44.4%) *FLT3/D835Mt* ($P = .028$) were found in the patients with *NPM1* mutations. *TP53*, *NRAS*, and MLL-TD mutations were found in 16 of 243 (6.6%), 34 of 236 (14.4%), and 17 of 147 (11.6%) patients, respectively, although there was no significant correlation between these mutations and the *NPM1* mutation (Table 1).

Clinical characteristics and prognoses of AML patients with or without *NPM1* mutations

Among the 257 patients with AML, 190 patients (excluding those with M3 who were treated with the AML87, AML89, and AML92 protocols of the JALSG) were evaluated for clinical characteristics and initial response to therapy (Table 2). Of these patients, 49 (25.8%) had *NPM1* mutations. The presence of *NPM1* mutations was related neither to sex nor to the occurrence of hepatosplenomegaly or extramedullary involvement. Patients with *NPM1* mutations (median, 58 years; range, 15-77 years) were significantly older than those without mutations (median, 47 years; range, 15-85 years) ($P = .003$). White blood cell (WBC) counts and peripheral blood blast cells were significantly higher in the *NPM1* mutation group than in the wild-type group ($P = .002$ and $P = .029$, respectively). According to the FAB classification, the *NPM1* mutation was infrequent in the M2 subtype ($P = .004$). According to the cytogenetic risk groups, the *NPM1* mutation was preferentially found in the intermediate risk group ($P < .001$). *NPM1* mutations were associated with *FLT3/ITD* ($P < .001$) and *FLT3/D835Mt* ($P = .018$), but not with *TP53* and *NRAS* mutations. Because we could analyze MLL-TD in only 118 of the 190 patients, we excluded MLL-TD from the variables for statistical analysis.

Of the 190 patients, 139 (73.2%) achieved CR after induction chemotherapy. The CR rate was significantly higher in the patients with *NPM1* mutations (42 of 49; 85.7%) than without them (97 of 141; 68.8%) ($P = .025$). In addition, Fisher exact test showed that FAB subtypes other than M2, cytogenetic findings other than good risk, and the presence of *NRAS* and *TP53* mutations were unfavorable factors for achieving CR ($P < .001$, $P = .002$, $P = .030$, and $P = .034$, respectively). Age (older than 60), WBC count (more than $100 \times 10^9/L$), and presence of the *FLT3* mutation were not associated with the CR rate. Multivariate logistic regression analysis showed that wild-type *NPM1* ($P < .001$), FAB subtypes other than M2 ($P = .008$), and cytogenetic findings other than good

Figure 1. Mutations in NPM1 exon 12. (A) The mutated nucleotide and the predicted amino acid sequence in *NPM1* exon 12 found in the present study are shown in comparison with the wild-type sequence. Type of mutation (mutations A, B, D) is designated according to a previous report. Four novel mutant variants are designated mutations G, H, I, and J. Red and blue indicate nucleotide insertions and termination codons, respectively. Green indicates the conserved residues in mutant NPM. Purple indicates putative residues of a consensus nuclear export signal (Lx[1-3]Vx[2-3]VxL; x indicates any residue). (B) Sequence results after cloning. Boxes indicate inserted 4-bp nucleotides. Arrowhead indicates the position of the insert at nucleotide 960 of the *NPM1* gene.



risk ($P = .039$) were independent unfavorable factors for achieving CR (Table 3).

Kaplan-Meier analyses according to *NPM1* mutation are shown in Figure 2A. Univariate analysis showed that the poor prognostic factors for overall survival were age 60 or older ($P = .001$), mutation of *TP53* ($P < .001$), FAB subtypes other than M2 ($P = .005$), *FLT3/ITD* ($P = .010$), high WBC count (more than $100 \times 10^9/L$) ($P = .019$), and cytogenetic findings (poor vs others) ($P = .024$). Multivariate Cox regression analysis with stepwise selection showed that the mutation of *TP53* (odds ratio, 4.002 [95% confidence interval (CI), 1.876-8.538]; $P < .001$), age 60 or older (odds ratio, 1.651 [95% CI, 1.131-2.410]; $P = .009$), and FAB subtypes other than M2 (odds ratio, 1.643 [95% CI, 1.127-2.396]; $P = .010$) were independent poor prognostic factors for overall survival (Table 4). When adjusted with age 60 years or older, *FLT3/ITD*, FAB subtype other than M2, high WBC count (more than $100 \times 10^9/L$), and mutation of *NRAS*, mutation of *NPM1* was an adverse prognostic factor (odds ratio, 1.949 [95% CI, 1.164-3.268]; $P = .011$). Although careful assessment and further investigation are needed, the mutation of *NPM1* may act as a prognostic factor for overall survival.

Because only 1 of 139 patients who achieved CR died during CR, we compared the probability of relapse between patients with and without *NPM1* mutations (Figure 2A). Univariate analysis showed that the unfavorable factors for relapse were mutation of *NPM1* ($P = .006$), *FLT3/ITD* ($P = .007$), cytogenetic findings (poor vs others) ($P = .007$), high WBC count (more than $100 \times 10^9/L$) ($P = .016$), FAB subtypes other than M2 ($P = .024$), and age 60 or older ($P = .024$). Multivariate Cox regression analysis with stepwise selection identified that cytogenetic findings (poor vs others) (odds ratio, 3.876 [95% CI, 1.718-8.772]; $P = .001$) and mutation of *NPM1* (odds ratio, 2.106 [95% CI, 1.324-3.350]; $P = .002$) were independent unfavorable factors for relapse (Table 5).

In addition, we analyzed the prognostic value of *NPM1* mutations in 79 patients with normal karyotype. *NPM1* mutations were found in 37 of 79 (46.8%) patients. Of these 79 patients, 59

(74.7%) achieved CR after induction chemotherapy. The CR rate was significantly higher in the patients with *NPM1* mutations (32 of 37; 86.4%) than in those without (27 of 42; 64.3%) ($P = .037$ by Fisher exact test). Multivariate logistic regression analysis, including wild-type *NPM1*, FAB subtypes (other than M2), presence of *FLT3*, *NRAS*, and *TP53* mutations, age (older than 60 years), and WBC count (more than $100 \times 10^9/L$) showed that wild-type *NPM1* was the only independent unfavorable factor for achieving CR (odds ratio, 4.908 [95% CI, 1.011-23.824]; $P = .048$). Kaplan-Meier curves according to *NPM1* mutation in this group are shown in Figure 2B. Multivariate Cox regression analysis with stepwise selection showed that age 60 or older was the only poor prognostic factor for overall survival (odds ratio, 2.068 [95% CI, 1.175-3.641]; $P = .012$). Mutation of *NPM1* was not a significant prognostic factor for overall survival regardless of whatever factors were used for adjustment by means of Cox analysis. The relapse risk was analyzed in 59 patients who achieved CR. Multivariate Cox regression analysis with stepwise selection identified that the *NPM1* mutation was the only independent unfavorable factor for relapse (odds ratio, 2.096 [95% CI, 1.050-4.186]; $P = .036$). However, because the patient number of this group was small, larger-scale analysis was required.

FLT3/ITD has been identified as an unfavorable prognostic factor in patients with AML, although the prognostic implications of *FLT3/D835Mt* remain unclear. Given that the *NPM1* mutation was associated with *FLT3/ITD*, it was important to define the role of *NPM1* mutations alone or in combination with *FLT3/ITD* for long-term prognosis. Therefore, we compared the clinical impact of *NPM1* mutations in patients with and without *FLT3/ITD*. Interestingly, mutation of *NPM1* was an independent favorable prognostic factor for CR in patients with *FLT3/ITD* (odds ratio, 20.8 [95% CI, 2.0-200]; $P = .011$ by multivariate logistic regression analysis), but it did not affect the CR rate in patients without *FLT3/ITD*. In addition, mutation of *NPM1* was a favorable prognostic factor for overall survival in patients with *FLT3/ITD* ($P = .045$), but not in those without *FLT3/ITD*. However, mutation

Table 2. Characteristics of 190 patients with AML, excluding patients with the M3 subtype, treated according to JALSG protocols

	Total N = 190	NPM1		P
		Mutation, n = 49	Wild type, n = 141	
Age, y	50 (15-85)	58 (15-77)	47 (15-85)	.003
WBC count, × 10 ⁹ /L	24.5 (0.9-372)	52.2 (1.0-372)	23.3 (0.9-337.6)	.002
PB blast count, × 10 ⁹ /L	14.9 (0.017-357)	29.6 (0.093-357)	12.6 (0.017-324)	.029
FAB subtype				.004
M0	3	1	2	
M1	44	12	32	
M2	78	10	68	
M4	46	16	30	
M5	14	8	6	
M6	4	1	3	
M7	1	1	0	
Cytogenetics				< .001
Favorable	33	1	32	
t(8;21)	27	0	27	
inv(16)	6	1	5	
Intermediate	119	42	77	
Normal	79	37	42	
Others	40	5	35	
Poor	14	1	13	
t(9;22)	3	1	2	
del(5) or del(7)	11	0	11	
Unknown	24	5	19	
Outcome				.025
CR	139	42	97	
Failure	51	7	44	
FLT3				
Mutation, total	51	31	20	< .001
ITD mutation	43	27	16	< .001*
D835 mutation	8	4	4	.018*
Wild type	139	18	121	
P53				NS
Mutation	8	1	7	
Wild type	182	48	134	
NRAS				NS
Mutation	26	8	18	
Wild type	164	41	123	

Clinical characteristics of the 190 patients with AML, excluding those with the M3 subtype, who were treated according to the JALSG protocols, are shown. Median values (with range in parentheses) are indicated for age, WBC count, and PB blast count. Numbers of patients are shown by FAB type, cytogenetics, outcome, and gene mutation. The favorable risk group was defined by t(8;21) or inv(16); the poor risk group by t(9;22), del(5), or del(7); and the intermediate risk group by normal or other karyotypes.

PB indicates peripheral blood; NS, not significant.
*Compared with wild-type variables.

of *NPM1* was not associated with the high relapse rate in patients with *FLT3/ITD*, whereas it tended to be a worse factor for relapse in those without *FLT3/ITD* ($P = .084$) (Figure 3).

Comparison of mutational status of the *NPM1* gene at diagnosis and relapse

Mutational status of the *NPM1* and *FLT3* genes was compared between diagnosis and relapse in 39 AML patients. *NRAS* and *TP53*

Table 3. Logistic regression analysis results of the effect of unfavorable factors for CR in 190 patients, excluding those with the M3 subtype, treated according to JALSG protocols

Variable	P	Odds ratio	95% CI
<i>NPM1</i> , wild type	< .001	8.434	2.562-27.77
FAB, other than M2	.008	3.247	1.353-7.813
Cytogenetic, other than good risk	.039	5.240	1.091-25.16
<i>NRAS</i> , mutation	.056	2.667	0.978-7.299
<i>TP53</i> , mutation	.058	6.024	0.941-38.46
WBC count, more than 100 × 10 ⁹ /L	.090	2.427	0.872-6.757
<i>FLT3</i> , mutation	.279	1.719	0.645-4.583
Age, older than 60 y	.960	2.427	0.446-2.340

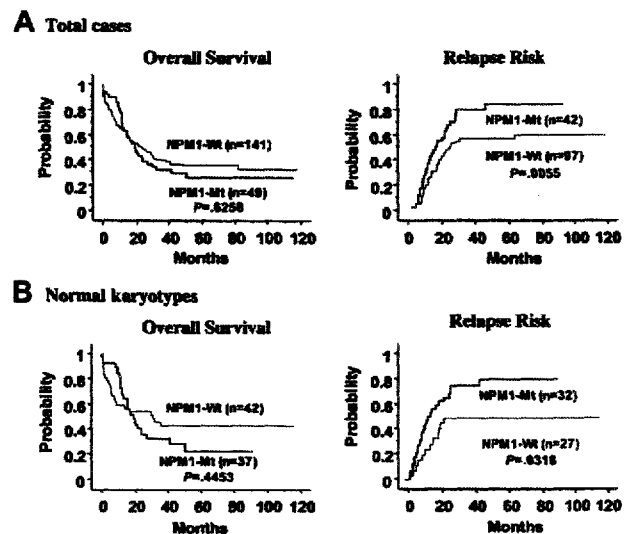


Figure 2. Kaplan-Meier curves according to *NPM1* mutation. (A) Overall survival and relapse risk in all patients. (B) Overall survival and relapse risk in patients with normal karyotype. Statistical difference was evaluated with the log-rank test.

Table 4. Unfavorable prognostic factors for overall survival in 190 patients, excluding those with the M3 subtype, treated according to JALSG protocols

Prognostic factors	P	Odds ratio	95% CI
<i>TP53</i> mutation	.003	4.002	1.876-8.538
Age, older than 60 y	.009	1.651	1.131-2.410
FAB, other than M2	.010	1.643	1.127-2.396

mutations were also analyzed in 20 and 29 patients, respectively (Table 6). An *NPM1* mutation was found in 17 of 39 patients at diagnosis. Of the 17 patients with *NPM1* mutations at diagnosis, 15 carried the same mutation at relapse, although 2 patients (unique patient number [UPN] 16 and UPN 17) lost the mutation at relapse. In UPN 16, the karyotype of leukemia cells was normal (46XY) at diagnosis, although it changed to 46XYdel(20)(q1?) at relapse. In contrast, *FLT3*, *NRAS*, and *TP53* were of the wild type at both stages. In UPN 17, leukemia cells showed normal karyotype (46XX), *FLT3/ITD*, and wild-type *NRAS* and *TP53* mutations at diagnosis, and these were retained at relapse.

An *FLT3* mutation was found in 11 of 39 patients at diagnosis, and all mutations were *FLT3/ITD*. The *FLT3* mutation was lost at relapse in 1 patient, but it emerged at relapse in 4 patients who did not have the *FLT3* mutation at diagnosis. An *NRAS* mutation was found in 5 of 20 patients at diagnosis. The *NRAS* mutation was lost at relapse in 2 patients, but it emerged at relapse in 1 patient. A *TP53* mutation was found in 3 of 28 patients at diagnosis. The *TP53* mutation was lost at relapse in 1 patient, but it emerged at relapse in 1 patient. Taken together, in 11 of 39 (28.2%) patients, the gene status of *NPM1*, *FLT3*, *NRAS*, and *TP53* was different at diagnosis than it was at relapse (Table 6). However, *NPM1* gene status was relatively stable, in contrast to that of *FLT3*, and was not related to inducing a genotypic change in leukemia cells at relapse. In addition, age and CR duration were not associated with the genotypic change of leukemia cells at relapse. Importantly, the *NPM1* mutation was not associated with achieving second CR after first CR.

Discussion

In this study, we found *NPM1* mutations in 64 of 257 (24.9%) adult patients with de novo AML. Of the 64 *NPM1* mutations, 60 were variants reported previously, and 4 were novel. All the novel variants had a 4-bp insertion at position 960, resulting in the same frameshift as previously reported. The C-terminal of NPM is important to the nuclear localization of NPM,³⁶ and tryptophan residues at positions 288 and 290 are reportedly associated with the nucleolar localization of NPM.³⁷ Interestingly, predicted mutant proteins contain a nuclear export signal (NES) motif at the C-terminal, which may be a reason for the cytoplasmic dislocation of mutant NPM.^{38,39} All novel variants found in the present study also contained the NES motif. Therefore, we believe that the generation of the NES motif by the insertion mutation is strongly associated with the cytoplasmic dislocation of mutant NPM.

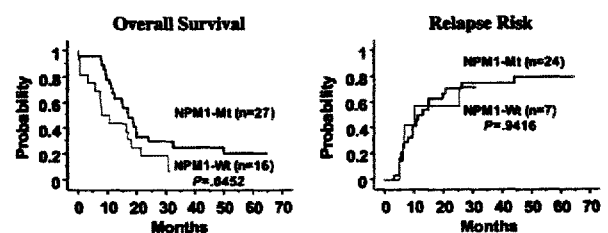
Table 5. Unfavorable prognostic factors for relapse in 139 patients with AML, excluding those with the M3 subtype, who achieved CR

Prognostic factors	P	Odds ratio	95% CI
Poor cytogenetics	.001	3.876	1.718-8.772
<i>NPM1</i> mutation	.002	2.106	1.324-3.350

FLT3, *TP53*, and *NRAS* mutations are the most frequent genetic alterations involved in the pathogenesis of AML. It was reported that the *NPM1* mutation is closely associated with *FLT3/ITD*. We found that it is also associated with *FLT3/D835Mt*, but a large-scale study is necessary to confirm this association because of the small number of patients with *FLT3/D835Mt* in this study. It is noteworthy that the *NPM1* mutation was not associated with *TP53*, *NRAS*, and *MLL-TD* mutations. In fact, the *NPM1* mutation was found in only 2 of 16 patients with the *TP53* mutation, although this was not statistically significant. Because the *TP53* mutation is known to be present in patients with karyotypic abnormalities,^{40,41} the *NPM1* mutation seems to be infrequent in this group. The *NRAS* mutation is negatively related to *FLT3/ITD* but is found in all patients with AML, regardless of karyotypic abnormalities.^{6,42} In contrast, a possible association between *MLL-TD* and *FLT3/ITD* was reported.⁴³ Therefore, to identify factors associated with *NPM1* mutations, we performed multivariate logistic regression analysis that included the presence or absence of *FLT3*, *TP53*, *NRAS*, and *MLL-TD* mutations and karyotypic abnormality in 119 patients in whom all these genetic alterations were determined. The analysis showed that the normal karyotype ($P < .001$; odds ratio, 26.0 [95% CI, 6.231-108.73]) and the *FLT3* mutation ($P < .001$; odds ratio, 15.2 [95% CI, 3.690-62.50]) were independently associated with *NPM1* mutation. These results suggested that the *NPM1* mutation might be involved in the pathogenesis of AML with and without *FLT3* mutations.

The most important finding in the present study is that in patients with AML, excluding those with the M3 subtype, the *NPM1* mutation is a favorable factor for achieving CR after induction chemotherapy, but it implicates a high relapse rate. *FLT3/ITD* is clinically demonstrated to be a poor prognostic factor in patients with AML, especially those in the intermediate-risk group. *FLT3/ITD* confers autonomous proliferation to hematopoietic progenitors through its constitutive kinase activity, though alone it is not sufficient for the development of AML. In the murine APL model, the transduction of *FLT3/ITD* into *PML-RARA* transgenic bone marrow cells resulted in a shortened latency with

A FLT3/ITD-positive cases



B FLT3/ITD-negative cases

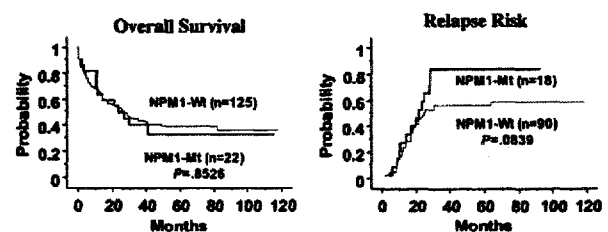


Figure 3. Kaplan-Meier curves according to *NPM1* mutation in the *FLT3/ITD*-positive and -negative patients. (A) In the *FLT3/ITD*-positive group, the *NPM1* mutation was a favorable prognostic factor for overall survival, but did not affect the relapse risk. (B) In the *FLT3/ITD*-negative group, the *NPM1* mutation did not affect overall survival. The *NPM1* mutation tended to be a worse factor for relapse in this group, although it was not statistically significant. Statistical difference was evaluated with the log-rank test.