In summary, our experiments identified direct regulation of NF-κB signaling by AML1. AML1 negatively regulates NF-κB signaling by inhibiting kinase activity of IKK. Furthermore, mutated forms of AML1 found in MDS or AML fail to inhibit NF-κB signaling. NF-κB signaling can be a promising molecular target for the treatment of AML1-related hematologic malignancies.

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Authorship

Contribution: M.N. conceived of and designed the research, performed experiments and analyses, and wrote the paper; M.S., N.W.-O., S.A., A.Y., A.S., N.N., K. Kataoka, and T.S. performed experiments; M.I., K. Kumano, Y.N., and Y.I. wrote the paper; and M.K. supervised the whole project and wrote the paper.

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Loss of AML1/Runx1 accelerates the development of MLL-ENL leukemia through down-regulation of p19^{ARF}

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Dysfunction of AML1/Runx1, a transcription factor, plays a crucial role in the development of many types of leukemia. Additional events are often required for AML1 dysfunction to induce full-blown leukemia; however, a mechanistic basis of their cooperation is still elusive. Here, we investigated the effect of AML1 deficiency on the development of MLL-ENL leukemia in mice. *Aml1* excised bone marrow cells lead to MLL-ENL leukemia with shorter duration than *Aml1* intact cells in vivo. Although the number of MLL-ENL leukemia-initiating cells is not affected by loss of AML1, the proliferation of leukemic cells is enhanced in *Aml1*-excised MLL-ENL leukemic mice. We found that the enhanced proliferation is the result of repression of p19^{ARF} that is directly regulated by AML1 in MLL-ENL leukemic cells. We also found that down-regulation of p19^{ARF} induces the accelerated onset of MLL-ENL leukemia, suggesting that p19^{ARF} is a major target of AML1 in MLL-ENL leukemia. These results provide a new insight into a role for AML1 in the progression of leukemia. (*Blood*. 2011; 118(9):2541-2550)

Introduction

AML1, also called RUNX1, CBFA2, or PEBP2αB, was found at the breakpoint on chromosome 21 from acute myeloid leukemia (AML) patients with t(8;21)(q22;q22).[1] AML1 is a transcription factor that belongs to RUNX family proteins. It heterodimerizes with CBFβ and binds to the specific DNA sequence (TGT/CGGT), called the PEBP2 binding site.[2-4] AML1 regulates transcription of various genes related to normal hematopoiesis, and targeted disruption of AML1 in mice revealed that it is essential for definitive hematopoiesis during embryogenesis.[5] Conventional knockout mice are embryonic lethal because of hemorrhage in the central nervous system. We generated conditional knockout mice of AML1 to study a role of AML1 in adult hematopoiesis after birth.[6] These mice showed thrombocytopenia because of maturation block of megakaryocytes, perturbed lymphocyte development, and increase in the number of hematopoietic stem/progenitor cells.

The disruption of AML1 functions is highly related to occurrence of myeloid malignancies through chromosomal translocation or point mutation.[7-9] Although introduction of AML1-ETO, the fusion protein generated in AML with t(8;21) chromosomal translocation, into mouse bone marrow (BM) cells leads to proliferation of myeloid cells, it is not sufficient to induce leukemia without providing alkylating agents for the mice.[10-14] AML1-ETO acts as a dominant negative effector for wild-type AML1, and it is supposed that function of AML1 is lost in AML1-ETO–expressing cells. These results suggest that the other genetic change in addition to the loss of AML1 function is necessary for the development of full-blown leukemia. In mice, *c-Kit* and *FLT3-ITD* mutations are reported to collaborate with gene alteration of AML1 in leukemogenesis.[15,16] Furthermore, positive correlation between *c-Kit* mutations and AML1-ETO is reported in human cases.[17,18] However, precise molecular mechanisms that underlie the development of AML1-related leukemia are still to be elucidated.

We previously demonstrated that hematopoietic stem/progenitor cells are expanded in *Aml1*-deficient mice.[19] Expansion of the hematopoietic stem/progenitor cells is also observed in the mouse models of AML with t(8;21), in which the chimeric protein AML1-ETO suppresses the normal function of AML1. Expansion of the hematopoietic stem/progenitor cells is supposed to predispose the animals to full-blown leukemia when additional mutations occur in the proliferating cells.

Mixed lineage leukemia (MLL) is located on band q23 of the chromosome 11 and is frequently translocated in human leukemias. In addition to formation of fusion genes with > 50 partners, partial tandem duplication of MLL (MLL-PTD) is also found in human leukemias. Interestingly, in human leukemias without 11q23 chromosomal translocation, point mutations of *AML1* and *MLL-PTD* are frequently observed in the same patients.[17] This prompted us to speculate that AML1 loss and *MLL* mutations may cooperate in the development of human leukemia. To test this, we evaluated the effect of AML1 loss on MLL-related leukemia using a mouse model and found that loss of AML1 significantly accelerated the development of MLL-leukemia. We also found that p19^{ARF}, a known target of AML1 and AML1-ETO,[20] plays a critical role in the leukemia acceleration caused by AML1 loss. These findings provide a novel mechanistic basis of cooperation between impaired AML1 function and other leukemia-related gene alteration.

Methods

Mouse strains

Aml1$^{flox/flox}$ Mx-Cre (+) mice and *Aml1*$^{flox/flox}$ Mx-Cre (−) mice were previously described.[6] To induce *Aml1* deletion in vivo, mice were

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intraperitoneally injected with 250 μg of polyinosinic-polycytidylic acid (pIpC; Sigma-Aldrich) 3 times every other day and were used for the experiments after 4 to 8 weeks.[6] The genotypes of the *lox*P-flanked *Aml1* (*Aml1*^{f}) and excised *Aml1* (*Aml1*$^{\Delta}$) loci were analyzed, using primers as described previously.[6] Eight- to 10-week-old female C57BL/6J mice were used as recipients in transplantation. Mice were kept at the Center for Disease Biology and Integrative Medicine, University of Tokyo, according to institutional guidelines. All animal experiments were approved by the University of Tokyo Institutional Animal Care and Use Committee.

Retrovirus infection

The cDNA of MLL-ENL (generous gift from Toshio Kitamura) was subcloned into the *Eco*RI site of pMSCV-neo (Clontech).[21] To produce MLL-ENL–expressing retrovirus, Plat-E packaging cells (generous gift from Toshio Kitamura) or Ecopack2-293 cells (Clontech) were transiently transfected with retroviral constructs, as described previously.[22,23] To produce green fluorescent protein (GFP)– or AML1-GFP–expressing retrovirus, we used cMP34 packaging cells.[24] Two retrovirus vectors expressing small hairpin RNAs were constructed for p19^{ARF}.[25] After transfection, puromycin-resistant cells were selected in medium (RPMI with 20% FCS, 10 ng/mL IL-3) containing 2 μg/mL puromycin for 3 days.

Colony replating assay

The cells infected with retrovirus were washed by PBS and resuspended in IMDM (with 2% FCS), and 1×10^5 cells were plated in the 35-mm plate with Methocult M3434 (StemCell Technologies) containing 10 ng/mL of murine GM-CSF and 0.8 mg/mL of G418. After 7 days, the cells were collected and washed by PBS twice. A total of 1×10^4 cells were plated in the same semisolid culture medium without G418. Colony counting and replating were performed every 7 days.

Transplantation assay

A total of 1×10^6 of cells infected with retrovirus were injected into sublethally irradiated (x-ray, 7.5 Gy) recipient mice via the tail vein. To transplant leukemic cells into recipient mice, mononuclear cells isolated from the spleen of leukemic mice were infected with retrovirus and injected into sublethally irradiated (7.5 Gy) recipient mice via the tail vein.

In vitro liquid culture

Leukemic or immortalized cells were cultured in RPMI medium containing 20% FCS and 10 ng/mL of IL-3. In apoptotic cell analyses, liquid culture medium without IL-3 was also used.

Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously.[26] mRNA expression levels of all genes, relative to those of normal BM mononuclear cells, were normalized to *Gapdh*. The primers used are as follows: *Aml1*: TaqMan Gene Expression Assay (Applied Biosystems; Assay ID Mm00486762_m1); *Gapdh*: forward, TGGTGAAGCAGGCATCTGAG; reverse, TGCTGTTGAAGTCGCAGGAG; *p19^{ARF}*: forward, CATGTTGTTGAGGCTAGAGAGG; reverse, TCGAATCTGCACCGTAGTTG; *p21^{CIP1}*: forward, CTGTTCCGCACAGGAGCAA; reverse, ACGGCGCAACTGCTCACT/TaqMan probe TGTGCCGTTGTCTCTTCGGTCCC (Applied Biosystems); *p53*: forward, CACAGCGTGGTGGTACCTTATG; reverse, TTCCAGTGTGATGATGGTAAGGA/TaqMan probe CCACCCGAGGCCGGCTCTG (Applied Biosystems); *p27^{KIP1}*: forward, GGCCCGGTCAATCATGAA; reverse, TTGCGCTGACTCGCTTCTTC; *p15^{INK4B}*: forward, TCAGAGACCAGGCTGTAGCAA; reverse, CCCCGGTCTG; *Bax*: forward, AAAATGGCCAGTGAAGAGCA; reverse, GTGAGCGGCTGCTTGTCT/TaqMan probe (Roche Universal Probe Library #83); *p16^{INK4A}*: forward, CCCAACGCCCCGAACT; reverse, GTGAACGTTGCCCATCATCA; *PU.1*: forward, GGAGAAGCTGATGGCTTGG; reverse, CAGGCGAATCTTTTTCTTGC TaqMan probe (Roche Universal Probe Library #94); *Bmi1*: forward, AAACCAGACCACTCCTGAACA; reverse, TCTTCTTCTCTTCATCTCATTTTTGA/TaqMan probe (Roche Universal Probe Library #20); *Hoxa5*: forward, GCAAGCTGCACATTAGTCAC; reverse, GCATGAGCTATTTCGATCCT; *Hoxa7*: forward, CTCTTTCTTCCACTTCATGCGCCGA; reverse, TGCGCCTCCTACGACCAAAACATC; *Hoxa9*: forward, TCCCTGACTGACTATGCTTGTG; reverse, GTTGGCAGCCGGGTTATT/TaqMan probe (Roche Universal Probe Library #25); *Hoxa10*: forward, GGAAGGAGCGAGTCCTAGA; reverse, TTCACTTGTCTGTCCGTGAG; *Meis1*: forward, TTGTAATGGACGGTCAGCAG; reverse, GCTACATACTCCCCTGGCATA/TaqMan probe (Roche Universal Probe Library #105); *Gapdh* promoter: forward, CACAAACAGGACCCAACATT; reverse, ATGAAGTGTCCCTCCTTGTC; *p19^{ARF}* AML1 binding site (distal): forward, AGTTAACCGGAGCGAAAGCC; reverse, CACCCATCGCGGTGACAG; *p19^{ARF}* AML1 binding site (proximal): forward, GGATTACAACTTACACCTGCGGTC; reverse, CCACAGATTCTATTTTTCACGCAC.

Flow cytometric analysis

Cells were sorted with FACSAria, and analysis was performed on an LSRII (BD Biosciences). To analyze the cell surface antigen, anti–Mac-1 (phycoerythrin-conjugated), Gr-1 (allophycocyanin), CD117 (allophycocyanin), and Sca-1 (phycoerythrin; BD Biosciences) were used. To analyze the cell-cycle status, cells were stained with propidium iodide (BD Biosciences) at room temperature for 30 minutes. Apoptosis was assayed by annexin V and propidium iodide staining. To analyze the intracellular protein levels, cells were fixed and permeabilized with fixation/permeabilization solution (BD Cytofix/Cytoperm Fixation/Permeabilization kit) following the manufacturer's protocol, before incubation with antibodies. Fixed cells were incubated with either anti-p53 (1C12) mouse monoclonal antibody (AlexaFluor-647–conjugated) or mouse (MOPC-21) monoclonal antibody IgG1 isotype control (AlexaFluor-647–conjugated; Cell Signaling Technology) at room temperature for 60 minutes. The geometric mean fluorescence intensity was calculated by the subtraction of that of the cells stained with isotype control IgG1 from that of the cells stained with anti-p53 antibodies.

ChIP assay

ChIP assays were performed as described earlier,[27] with minor modifications. A total of 1×10^7 of splenocytes from leukemic mice were crosslinked with 1% formaldehyde. Subsequently, chromatin was fragmented by sonication to obtain an average fragment length of 200-900 bp (Bronson Sonifier 250). After the chromatin fraction was incubated with normal rabbit IgG (Abcam) or polyclonal rabbit AML1/Runx1 antibody (Active Motif), immune complexes were bound to Dynabeads protein G (Invitrogen). Eluted DNA samples were then analyzed by quantitative real-time PCR using specific primer pairs listed in the primers for quantitative real-time PCR. PCR results were calculated validly using the $\Delta\Delta C_t$ method.

Luciferase reporter assay

The mouse *p19^{ARF}* promoter region was obtained by PCR with the following primers: forward, GCCGGTACCGTACCGCTAAGGGTTCAAAACGCCC; reverse, GCGAGATCTCTCACAGTGACCAAGAACCTGCGAC. This fragment was subcloned into luciferase reporter vector, pGL4.10. Mutations of the PEBP2 sites in the *p19^{ARF}* promoter construct were introduced by QuickChange site-directed mutagenesis kit (Stratagene) using the following primers: forward, CCGCGGCGCTGGCTGTCAAAAAAATGGGTGGCGAGCGAAGC; reverse, GCTTCGCTCGCCACCCATTTTTTTGACAGCCAGCGCCGCGG. For reporter assays, COS-7 cells were seeded in 12-well culture plates at a density of 1×10^5 cells per well. At 6 hours after seeding, the cells were transfected with 200 ng of each luciferase reporter construct, together with 200 ng of each appropriate expression plasmid (eg, pME18S vector [Mock], pME18S-AML1)

using FuGENE 6 (Roche Diagnostics). The cells were harvested 40 hours after transfection, and luciferase activities were analyzed. CMV β-gal expression vector was also cotransfected for normalization of transfection efficiency. Results are expressed as fold activation with SD.

Statistical analysis

To compare data between groups, unpaired Student *t* test was used when equal variance was met by the F test. When unequal variances were detected, the Welch *t* test was used. Differences were considered statistically significant at a P value $< .05$. Statistical analyses were performed using the statistical software package R Version 2.13.0.

Results

Proliferation of MLL-ENL–transduced hematopoietic precursors is enhanced on AML1 deletion

To examine the effect of AML1 deletion on proliferation of MLL leukemic cells, we retrovirally transduced BM progenitors from *Aml1*$^{flox/flox}$; Mx-Cre(−; *Aml1*$^{f/f}$) or *Aml1*$^{flox/flox}$; Mx-Cre (+) mice that had been injected with pIpC (*Aml1*$^{Δ/Δ}$), with MLL-ENL vector (Figure 1A). As shown in the previous study, MLL-ENL-infected BM progenitors were immortalized and proliferated in the methylcellulose culture.[21] Cre-mediated recombination was confirmed by genomic PCR in the BM cells harvested from the pIpC-treated *Aml1*$^{flox/flox}$; Mx-Cre (+) mice (Figure 1B). The absence of AML1 mRNA was additionally confirmed by quantitative reverse-transcribed PCR of *Aml1*-excised immortalized cells (Figure 1C). We transferred these immortalized cells into liquid cultures in the presence of IL-3. Although growth of the cells differed immediately after the initiation of liquid cultures, each type of cells showed exponential growth after several days (Figure 1D-E). After day 9, *Aml1*-excised immortalized cells proliferated significantly faster than the *Aml1* intact controls ($P < .001$, Figure 1E). These results indicate that the proliferation of MLL-ENL-transformed cells is enhanced in the absence of AML1. *Aml1*-excised transformed cells were Gr-1$^+$, Mac-1$^+$, Sca1$^-$, and c-Kit$^{low/-}$, which was not significantly different from control cells (Figure 1F). Morphologic changes were neither observed (data not shown).

Loss of AML1 accelerates leukemia onset in MLL-ENL mice

Our in vitro studies showed that loss of AML1 enhances the proliferation of MLL-ENL–transformed cells. These results suggest that loss of AML1 accelerates the onset of MLL-ENL leukemia. To test this, we retrovirally transduced MLL-ENL into *Aml1*-intact or -excised BM progenitors. Those cells were transplanted into sublethally irradiated recipient mice (Figure 2A). Mice transplanted with control MLL-ENL cells died of leukemia within 90 days, as is consistent with the previous report (Figure 2B).[24] Remarkably, leukemia onset was significantly earlier in mice transplanted with *Aml1*-excised MLL-ENL cells (*Aml1*$^{Δ/Δ}$; 49.5 ± 6 days vs *Aml1*$^{f/f}$; 75 ± 9 days, $P < .01$). Immunophenotyping of leukemic cells revealed infiltration of Mac-1$^+$/Sca-1$^-$ cells, and Wright-Giemsa–stained peripheral blood smears showed immature blasts in both types of leukemia (Figure 2C; and data not shown). Surface marker expression, including c-Kit, Sca-1, Mac-1, and Gr-1, was not significantly changed regardless of *Aml1* status (Figure 2C).

Induction of MLL-ENL leukemia after conditional deletion of AML1

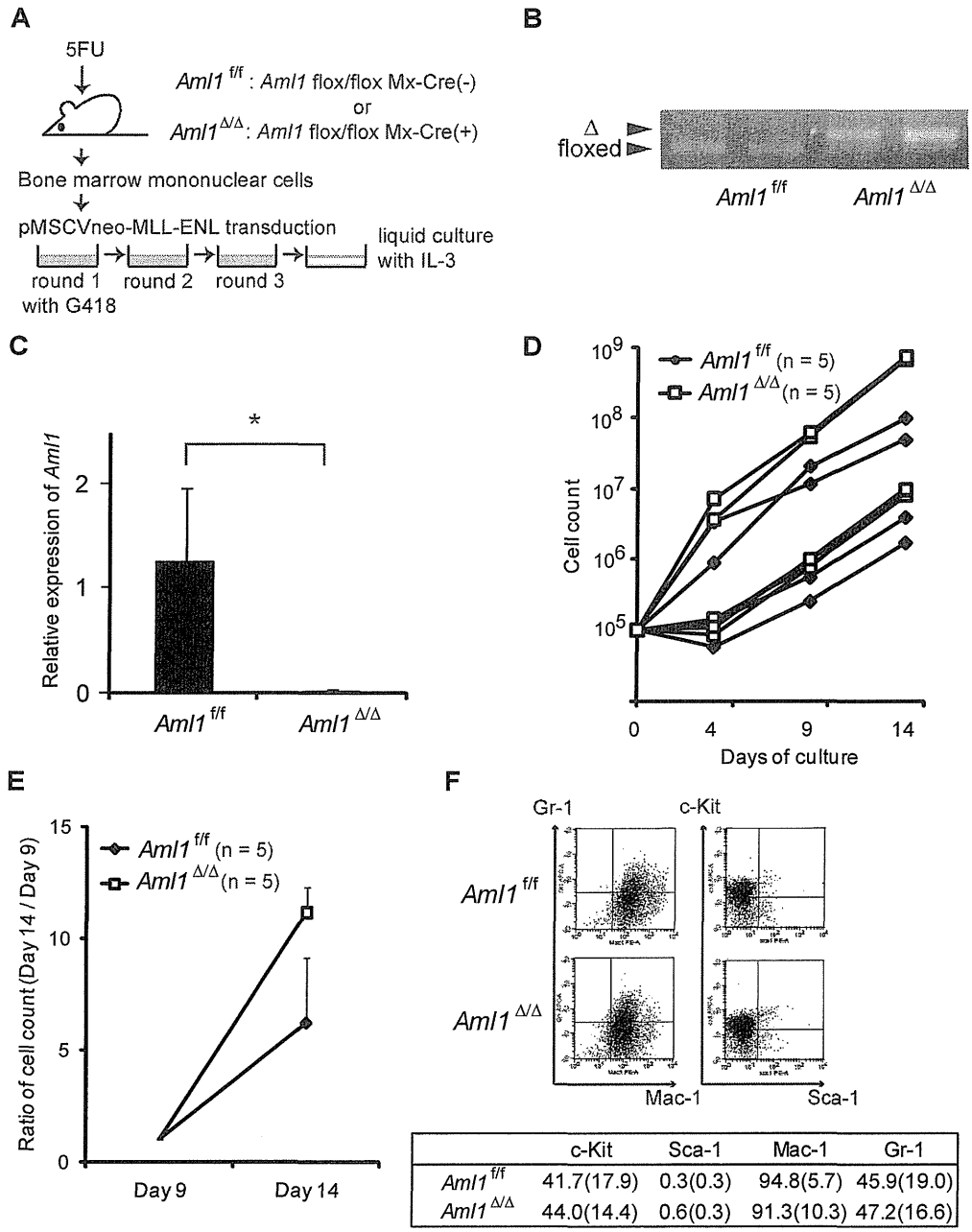
A previous study shows that MLL-ENL leukemia can arise from hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), and granulocyte-macrophage progenitors (GMPs), whereas transformation efficiency is higher in HSCs than in CMPs and GMPs.[28] In our previous experiment of MLL-ENL leukemia, we retrovirally introduced MLL-ENL into *Aml1*-excised BM cells. On the other hand, we have already reported that the number of HSCs is increased in *Aml1*-excised mice.[19] Because HSCs are increased in the *Aml1*-excised BM, there is one possibility that earlier onset of MLL-ENL leukemia from *Aml1*-excised BM cells is just the result of an increased number of MLL-ENL–transduced HSCs in the *Aml1*-excised BM. To explore this possibility, we transduced MLL-ENL into the BM cells from *Aml1*$^{flox/flox}$; Mx-Cre (−) or *Aml1*$^{flox/flox}$; Mx-Cre (+) before injection of pIpC. We transplanted those cells into sublethally irradiated recipient mice and injected pIpC one month after transplantation to delete *Aml1* in *Aml1*$^{flox/flox}$; Mx-Cre (+) cells (Figure 3A). We found that the onset of leukemia from *Aml1*-excised cells (*Aml1*$^{Δ/Δ}$) was earlier than that of control cells (*Aml1*$^{f/f}$; Figure 3B), indicating that loss of AML1 accelerates the development of leukemia even after introduction of MLL-ENL. These results suggest that the earlier onset of MLL-ENL leukemia from *Aml1*-excised hematopoietic progenitors is not simply the result of an increase in immature cells that can be efficiently transformed by MLL-ENL but potentially caused by an enhanced leukemogenic potential of MLL-ENL–transduced cells.

AML1 deletion in MLL-ENL mice does not increase leukemia-initiating cells

Next, we performed limiting dilution analysis to estimate the frequency of leukemia-initiating cells (LICs) of MLL-ENL leukemia in these murine models. Twenty to 500 000 MLL-ENL leukemic cells harvested from recipient mice were transplanted into sublethally irradiated secondary recipient mice (Figure 2A). As shown in Table 1, 500 leukemic cells were sufficient to induce leukemia in all secondary recipient mice, regardless of the status of *Aml1*. Moreover, the incidence of leukemia in the recipient mice injected with 20 *Aml1*-excised leukemic cells was not significantly changed. These results indicate that the frequency of LICs is not altered in *Aml1*-excised MLL-ENL leukemic cells and that the early onset of MLL-ENL leukemia is not the result of an increased number of LICs.

Decreased expression of cell cycle– and apoptosis-related genes in *Aml1*-excised leukemic cells

Given that the number of LICs is not significantly altered in *Aml1*-excised MLL-ENL leukemic cells, we evaluated the correlation between *Aml1* status and proliferative potentials of MLL-ENL leukemic cells. As shown in Figure 4A, the growth rate of *Aml1*-excised MLL-ENL cells obtained from the leukemic mice culture was enhanced in liquid compared with that of *Aml1*-intact MLL-ENL cells, as is consistent with the proliferation of the in vitro transformed cells (Figure 1E). To explore the mechanism of the growth advantage of MLL-ENL leukemic cells in the absence of AML1, we analyzed cell-cycle status and apoptotic rate of *Aml1*-excised MLL-ENL leukemic cells. Cell-cycle analyses revealed a significant increase of S/G_2/M phase cells in *Aml1*-excised MLL-ENL leukemic cells (Figure 4B-C). Moreover, the rate of annexin V-positive apoptotic cells was reduced in *Aml1*-excised MLL-ENL leukemic cells in liquid culture with and without IL-3 (Figure 4D). These results suggest that

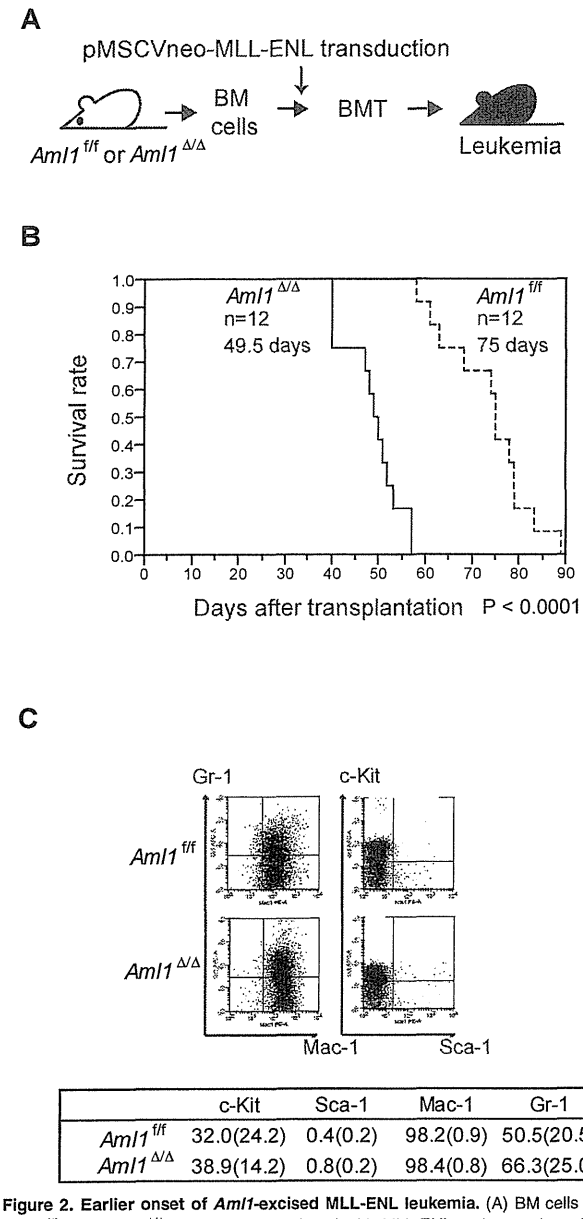


	c-Kit	Sca-1	Mac-1	Gr-1
Aml1 f/f	41.7(17.9)	0.3(0.3)	94.8(5.7)	45.9(19.0)
Aml1 Δ/Δ	44.0(14.4)	0.6(0.3)	91.3(10.3)	47.2(16.6)

Figure 1. Proliferation of *Aml1*-excised immortalized BM cells is enhanced in vitro. (A) MLL-ENL was retrovirally transduced into *Aml1* intact (*Aml1* f/f) and excised (*Aml1* Δ/Δ) BM cells, and replating assay was performed using these cells. (B) Genotyping of *Aml1* floxed and Δ alleles by PCR from *Aml1* f/f and *Aml1* Δ/Δ immortalized cells. Each lane indicates the PCR products of an independent case. (C) mRNA levels of *Aml1* in *Aml1* f/f and *Aml1* Δ/Δ immortalized cells were measured. *$P < .05$. (D) Growth of *Aml1* f/f (n = 5; ♦) or *Aml1* Δ/Δ (n = 5; □) immortalized cells in liquid medium. Data are on a semilogarithmic plot of cell counts versus time. (E) Growth of the immortalized cells as in Figure 1D after day 9. Cell counts at day 14 relative to those at day 9 are shown as mean ± SD on a linear plot (n = 5 from each group). Day 14 proliferation was significantly different between groups (*t* test, $P < .001$). (F) Flow cytometric analyses of the colony-forming cells after 3 rounds of replating. (Top) Representative fluorescence-activated cell sorter plots. (Bottom) Percentages of cells expressing indicated surface markers in each group (mean ± SD).

growth advantage of *Aml1*-excised MLL-ENL leukemic cells depends on both acceleration of cell-cycle progression and inhibition of apoptosis. Consistently, expression of cell cycle–regulating genes, such as $p19^{ARF}$ and $p21^{CIP1}$, decreased in *Aml1*-excised leukemic cells (Figure 5A). Expression of apoptosis-related genes, such as *p53* and *Bax*, also decreased in those cells (Figure 5A). On flow cytometric analysis, we observed that *Aml1*-excised leukemic cells expressed lesser amount of p53 protein (Figure 5B). The geometric mean fluorescence intensity and SD of p53–AlexaFluor-647 was 9.7 ± 1.3 for *Aml1*-excised cells and 14.2 ± 2.0 for controls ($P = .030$). In contrast, expression of *Meis1* and *Hoxa* (*Hoxa5*, *Hoxa7*, *Hoxa9*, and *Hoxa10*), which are direct target genes of MLL-ENL,[29] was not changed in *Aml1*-excised MLL-ENL cells compared with controls (Figure 5C).

It was reported that loss of AML1 in mouse BM cells induces the enhanced expression of the Polycomb gene *Bmi-1* and an increase in the stem/progenitor cells because of suppression of



	c-Kit	Sca-1	Mac-1	Gr-1
Aml1 f/f	32.0(24.2)	0.4(0.2)	98.2(0.9)	50.5(20.5)
Aml1 Δ/Δ	38.9(14.2)	0.8(0.2)	98.4(0.8)	66.3(25.0)

Figure 2. Earlier onset of *Aml1*-excised MLL-ENL leukemia. (A) BM cells from *Aml1* f/f and *Aml1* Δ/Δ mice were transduced with MLL-ENL and transplanted into congenic mice. (B) Survival curves of the mice transplanted with *Aml1* f/f and *Aml1* Δ/Δ cells transduced with MLL-ENL. Data from 12 mice for each group are shown. Comparison of survival curve was performed using log-rank test. (C) Flow cytometric analyses of the leukemic cells from transplanted mice. (Top) Representative fluorescence-activated cell sorter plots. (Bottom) Percentages of cells expressing indicated surface markers in each group (mean ± SD).

apoptosis.[30] Bmi-1, which is highly expressed in HSCs, critically suppresses the expression of $p19^{ARF}$ and $p16^{Ink4a}$ in the regulation of hematopoietic cell proliferation.[31] Therefore, down-regulation of $p19^{ARF}$ may be a consequence of Bmi-1 up-regulation by loss of AML1. To explore this possibility, we determined the expression of *Bmi-1* in *Aml1*-excised MLL-ENL–transduced cells and found that there was no significant change in *Bmi-1* expression, suggesting that $p19^{ARF}$ down-regulation is independent of Bmi-1. Consistently, the expression of $p16^{Ink4a}$, another target gene of Bmi-1, is also unaffected by loss of AML1 (Figure 5D).

Transcriptional regulation of $p19^{ARF}$ by AML1 in MLL-ENL leukemic cells

It has been reported that overexpression of AML1 up-regulates the expression of $P14^{ARF}$ (human homolog of murine $p19^{ARF}$), whereas AML1-ETO, a chimeric protein that exerts a dominant-negative effect over normal AML1, down-regulates its expression by directly binding to the promoter.[20] Because $p19^{ARF}$ affects cell cycle and apoptosis by regulating p53, Bax, and $p21^{CIP1}$, we hypothesized that $p19^{ARF}$ is a critical effector in the AML1-mediated regulation of MLL-ENL leukemia. To test this, we expressed AML1 in *Aml1*-excised MLL-ENL leukemic cells and evaluated whether $p19^{ARF}$ is expressed in an AML1 dose-dependent manner. We collected MLL-ENL leukemic cells and transduced them with AML1. Forty-eight hours after transduction, we assessed the expression of $p19^{ARF}$ in these cells by real-time reverse-transcribed PCR. As shown in Figure 6A, overexpression of AML1 enhanced $p19^{ARF}$ expression in *Aml1* intact MLL-ENL–transformed cells. Expression of $p19^{ARF}$ in *Aml1*-excised MLL-ENL cells is decreased compared with controls, and this reduction was rescued by restoration of AML1 to the level observed in *Aml1* intact cells transduced with GFP (Figure 6A). $p19^{ARF}$ expression levels in this setting were higher than the results of primary leukemic cells (Figure 5A), probably because of additional retroviral transduction and in vitro culture. These results are consistent with the hypothesis that AML1 up-regulates the expression of $p19^{ARF}$ also in MLL-ENL leukemia.

To determine a role of $p19^{ARF}$ in MLL-ENL leukemia in vivo, we tested whether down-regulation of $p19^{ARF}$ can accelerate the onset of MLL-ENL leukemia. We used short hairpin RNA (shRNA) to knock down the expression of $p19^{ARF}$.[25] We retrovirally transduced 3000 MLL-ENL leukemic cells with 2 types of $p19^{ARF}$-directed shRNAs and injected them into sublethally irradiated secondary recipient mice. As shown in Figure 6B, only 42% of mice injected with leukemic cells transduced with control shRNAs developed leukemia. On the other hand, nearly all mice injected with leukemic cells transduced with $p19^{ARF}$ shRNAs developed leukemia in shorter latencies. $p19^{ARF}$ expression levels in cells of secondary leukemic mice were lower than those in primary leukemic mice (Figures 5A, 6C), possibly because of the development of secondary leukemia in vivo. We confirmed that expression of $p19^{ARF}$ was efficiently suppressed in leukemic cells obtained from $p19^{ARF}$ shRNAs-transduced MLL-ENL mice (Figure 6C). These results indicate that $p19^{ARF}$ down-regulation promotes the development of MLL-ENL leukemia, which supports the notion that $p19^{ARF}$ plays a critical role in the acceleration of MLL-ENL leukemia induced by loss of AML1.

To examine the direct binding of AML1 to the $p19^{ARF}$ promoter, we performed chromatin immunoprecipitation assays. Two consensus AML1 binding sites (PEBP2 sites) were found in the $p19^{ARF}$ promoter (Figure 6D). The splenocytes from MLL-ENL leukemic mice were lysed after crosslinking by formaldehyde and eluted DNA was broken by sonication. Protein-DNA complexes were immunoprecipitated by the anti-AML1 antibody or normal rabbit IgG. Then we amplified the genomic DNA from this solution using the primers for the sequence containing the PEBP2 sites and found that the distal PEBP2 site of $p19^{ARF}$ promoter was significantly coprecipitated with AML1, suggesting that AML1 binds to the $p19^{ARF}$ promoter (Figure 6E). To ascertain whether the distal PEBP2 site contributes to AML1-dependent transactivation of the $p19^{ARF}$ promoter, we performed a luciferase reporter assay. We

A

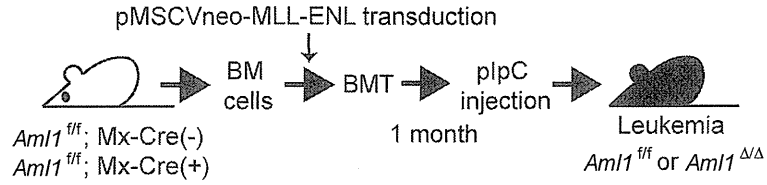
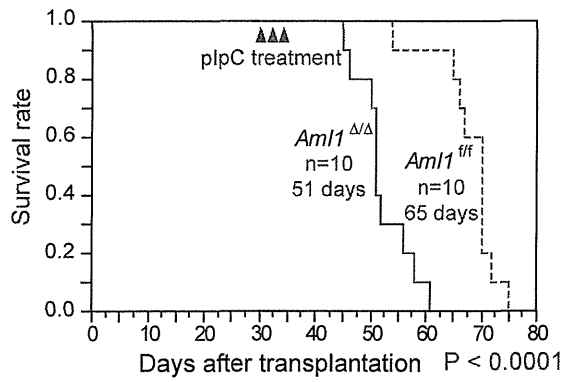


Figure 3. Deletion of the AML1 gene after MLL-ENL induction accelerates the onset of leukemia in transplanted mice. (A) BM cells from *Aml1* $^{f/f}$; Mx-Cre (+) or *Aml1* $^{f/f}$; Mx-Cre (−) were transduced with MLL-ENL and transplanted into congenic mice. Injection with pIpC was performed 3 to 4 weeks after transplantation, so that the *Aml1* gene was excised in transplanted *Aml1* $^{f/f}$; Mx-Cre (+) cells (*Aml1* $^{\Delta/\Delta}$). (B) Survival curves of the recipient mice. Data from 10 mice for each group are shown. Arrowheads indicate pIpC injection. Comparison of survival curve was performed using log-rank test.

B



constructed a luciferase reporter containing the 0.6-kb fragment of the *p19*^{ARF} promoter, and a mutant reporter containing the same promoter fragment in which the distal PEBP2 site (CGCGGT) was mutated (TTTTTT). COS-7 cells were cotransfected with an AML1 expression plasmid and these luciferase reporter plasmids. As shown in Figure 6F, AML1 activated the *p19*^{ARF} promoter > 2-fold, whereas *p19*^{ARF}-mutated promoter was not activated by AML1. These results indicate that AML1 regulates p19^{ARF} transcription through binding to the distal PEBP2 site.

Discussion

The results presented here provide direct evidence that loss of AML1 induces the accelerated onset of MLL-ENL leukemia in mice because of enhanced proliferation of leukemic cells. Because additional mutations are required for the development of full-blown leukemia along with loss of AML1 function, their cooperation in leukemogenesis is of interest in understanding the molecular mechanisms of human leukemia. Recently, coexistence of *MLL-PTD* mutation and *AML1* point mutation in AML with normal karyotype was reported,[17] and the significance of this correlation in leukemogenesis is to be elucidated. In this regard, to understand the interaction between *AML1* mutation and MLL leukemia, we explored accelerated leukemogenesis in *Aml1*-excised cells in vitro and in vivo using MLL-ENL fusion-induced mouse AML model. Our results indicate that AML1 acts as a tumor suppressor against the *MLL-ENL* oncogene, and loss of AML1 supports the development of leukemia via down-regulation of the genes related to the cell-cycle inhibition and apoptosis. Among them is p19^{ARF}, which acts as a negative regulator of cellular proliferation upstream of the cascade, including p53 and p21^{CIP1}. We found that AML1 activates transcription of p19^{ARF} in MLL-ENL leukemic cells mainly through binding to distal consensus AML1 binding site (PEBP2 site) in the *p19*^{ARF} promoter (Figure 6D) and that down-regulation of p19^{ARF} induces the early onset of MLL-ENL leukemia, suggesting that p19^{ARF} is a major target of AML1 in MLL-ENL leukemia (Figure 6A-B). These results suggest the function of AML1 as a tumor suppressor. Supporting our observation with the mouse model, down-regulation of p14^{ARF}, a human homolog of mouse p19^{ARF}, has also been reported in patients with human AML1-ETO leukemia.[20] Several other mutations, such as *ASXL1*[32] and *FLT3*[33] mutations, are reported in AML with point mutations of *AML1*, and our results suggest a novel mechanistic basis also for these leukemias.

Motoda et al reported that loss of AML1 in mouse BM cells induces the enhanced expression of Bmi-1 and an increase in hematopoietic stem/progenitor cells because of suppression of apoptosis.[30] They demonstrated that p19^{ARF} expression was decreased in AML1-deficient BM cells and that the enhanced expression of p19^{ARF} by *N-Ras* mutation was also decreased by

Table 1. Quantification of leukemia initiating cells

	No. of leukemic mice/no. of transplanted mice	
No. of transplanted cells	*Aml1* $^{f/f}$ MLL-ENL cells	*Aml1* $^{\Delta/\Delta}$ MLL-ENL cells
500 000	8/8	8/8
50 000	8/8	8/8
5000	8/8	8/8
500	8/8	8/8
20	5/8	4/8

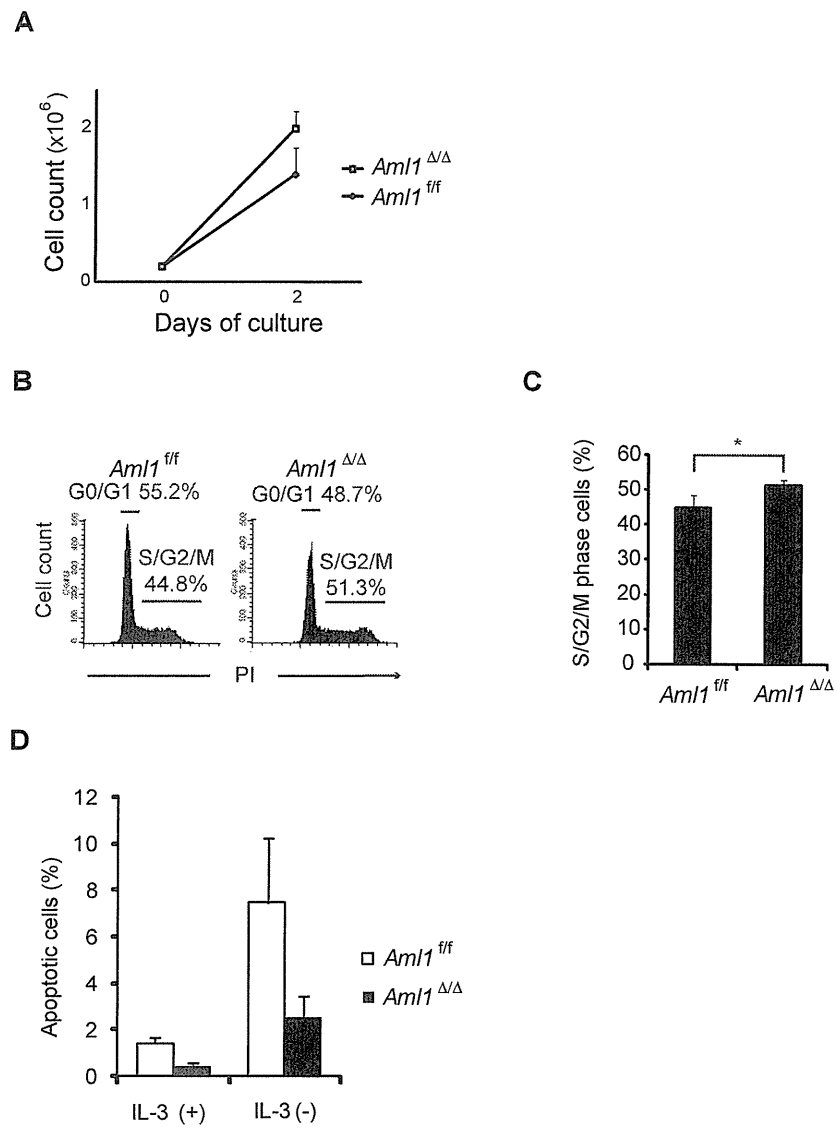


Figure 4. Aml1-excised MLL-ENL leukemic cells revealed accelerated growth rate because of enhanced cell-cycle progression and inhibition of apoptosis. (A) Growth rate of *Aml1*-excised leukemic cells in liquid medium was compared with controls. Data are mean ± SD. (B-C) Cell-cycle analyses of leukemic cells by PI. (B) Representative histograms are shown. (C) Percentages of cells in S/G_2/M phase (mean ± SD). (D) Percentages of apoptotic cells in each liquid medium (mean ± SD). *$P < .05$. We performed 3 independent experiments and confirmed that similar results were reproduced. Statistical significance was evaluated by unpaired *t* test.

loss of AML1. In their study, it is suggested that *N-Ras* mutation directly activates $p19^{ARF}$ and that AML1 indirectly induces the expression of $p19^{ARF}$ via down-regulation of Bmi-1. However, we found that the expression level of Bmi-1 is not changed by loss of AML1 in MLL-ENL leukemic cells and that $p19^{ARF}$ is directly regulated by AML1 (Figures 5D, 6E-F). Therefore, different molecular mechanisms that cooperate with loss of AML1 may exist in MLL-ENL and N-Ras leukemias, which remain to be elucidated.

In our study, we found that the expression level of *p53* gene is also decreased in *Aml1*-excised MLL-ENL leukemic cells (Figure 5A). These cells expressed significantly less amount of p53 protein (Figure 5B). Therefore, loss of p53 is probably involved in the enhanced proliferation of AML1-deficient MLL-ENL leukemic cells, supporting our notion that the $p19^{ARF}$-MDM2-p53 pathway may play a critical role in the acceleration of MLL-ENL leukemia induced by loss of AML1. However, it is well known that $p19^{ARF}$ blocks p53 degradation by binding to MDM2,[34,35] and regulation of p53 transcription by $p19^{ARF}$ has not been reported. We confirmed that the expression levels of p53 did not increase in MLL-ENL leukemic cells by overexpression of $p19^{ARF}$ (data not shown). Therefore, it remains to be elucidated which of transcriptional regulation and posttranslational regulation is more important than the other for the reduction of p53 protein.

HSCs and LICs share several biologic properties, such as self-renewal capacities and an ability to differentiate into more differentiated cells. These similarities have led us to hypothesize that the number of LICs may be increased in *Aml1*-excised MLL-ENL leukemic cells as a consequence of HSC expansion by loss of AML1.[36-42] However, the number of LICs was not affected by loss of AML1 in MLL-ENL mice, suggesting that promotion of MLL-related leukemia by loss of AML1 is not the result of the expansion of target population for leukemic transformation but mainly derived from the enhanced proliferation of MLL-ENL leukemic cells (Table 1). Given that MLL-ENL provides self-renewal capacities to the myeloid progenitors, including CMPs and GMPs, which are normally incapable of self-renewal,[28] HSC expansion caused by loss of AML1 may not influence MLL-related leukemogenesis.

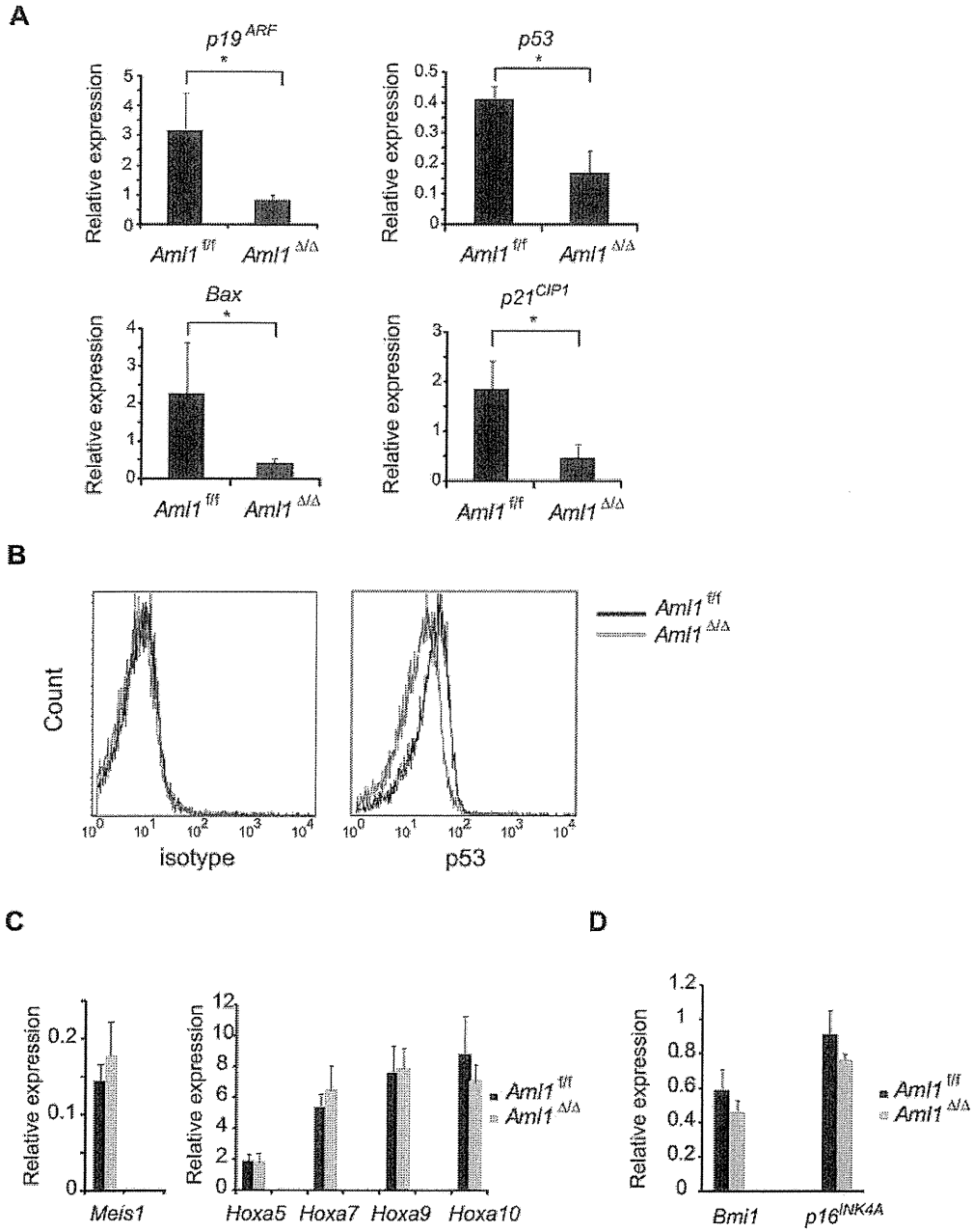


Figure 5. Decreased expression of cell cycle– and apoptosis-related genes in *Aml1*-excised leukemia cell. (A) Expression analyses of *p19ARF*, *p53*, *Bax*, and *p21CIP1* by quantitative real-time PCR. The expression of each mRNA, normalized to that of *Gapdh*, is shown as the ratio to that of the normal BM mononuclear cells. (B) Intracellular staining of p53 in MLL-ENL leukemic cells was detected by flow cytometry. Representative histograms are shown. (Left) Staining with AlexaFluor-647–conjugated IgG1 isotype control antibodies. (Right) Staining with AlexaFluor-647–conjugated anti-p53 antibodies. Expression analyses of (C) *Meis1* and *Hoxa* genes and (D) *Bmi1* and *p16INK4A* by quantitative real-time PCR. Error bars represent SD. $^*P < .05$. We performed 3 independent experiments and confirmed that similar results were reproduced. Statistical significance was evaluated by unpaired *t* test.

Aml1-excised transformed cells developed MLL-ENL leukemia earlier than *Aml1* intact cells (Figures 2B, 3B). When *Aml1* was excised from the MLL-ENL–transduced cells after engraftment in the individual mice, transplanted mice developed leukemia in as early as 21 days (Figure 3B); in contrast, the mice transplanted with MLL-ENL–transduced, *Aml1*-excised cells developed leukemia in 49.5 days after transplantation (Figure 2B). This may be because the transplanted cells in Figure 3B had already expanded and progressed to the leukemic or preleukemic state by MLL-ENL at the time of *Aml1* excision and the *Aml1* excision caused enhanced proliferation of the leukemic cells to shorten the latency to develop leukemia (Figure 3B).

Our study is the first report to reveal the molecular mechanism of leukemia acceleration caused by loss of AML1. Our results demonstrate that $p19^{ARF}$ is a key molecule for the proliferation of leukemic cells in AML1-related leukemia. Targeted therapy for aberrant $p19^{ARF}$ signaling pathway may be a novel therapeutic strategy against AML1-related leukemia.

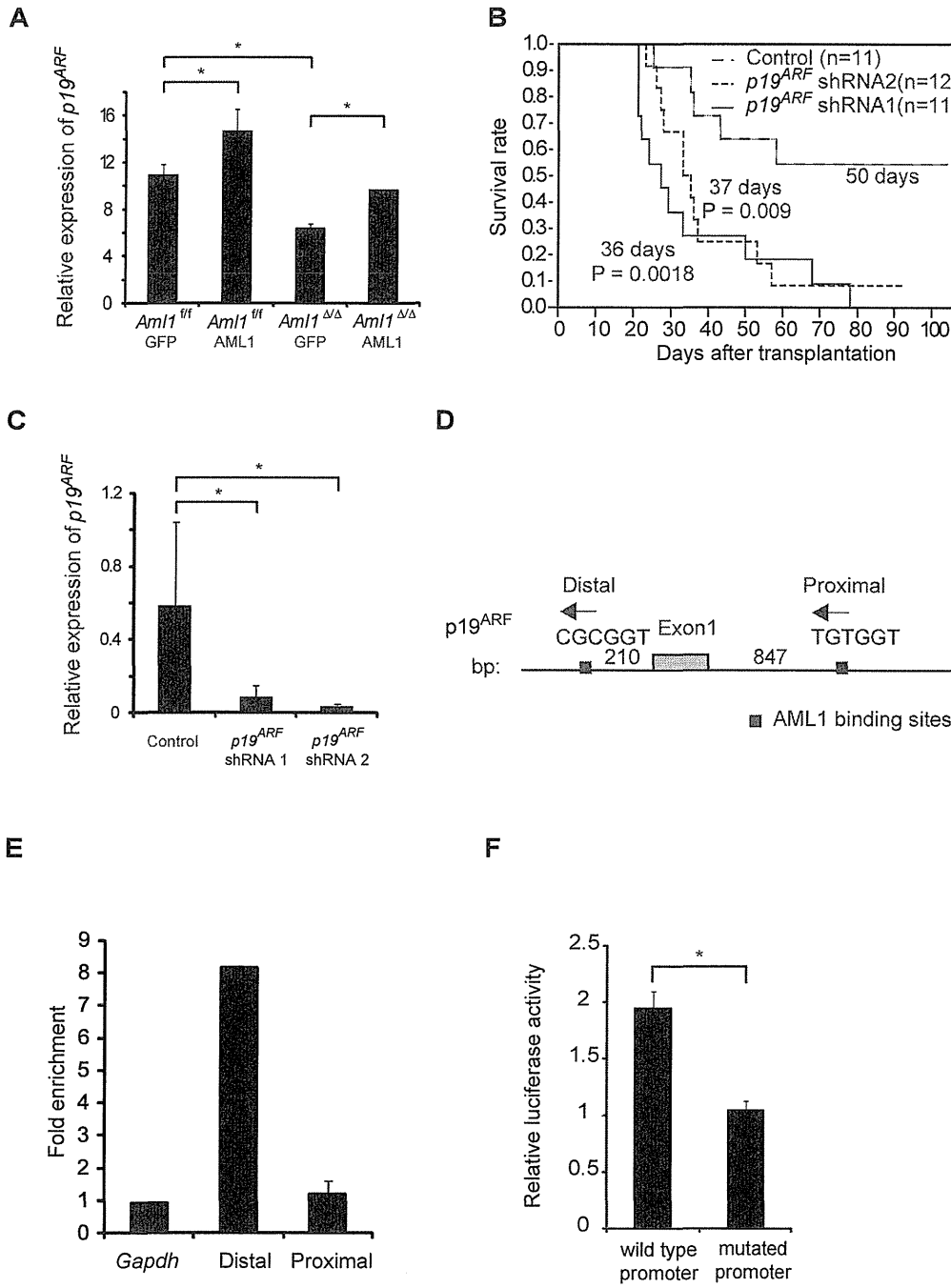


Figure 6. Expression of *p19ARF* is regulated by AML1 in MLL-ENL leukemia. (A) Expression analyses of *p19ARF* in MLL-ENL leukemic cells. *Aml1* intact or *Aml1*-excised MLL-ENL leukemic cells harvested from each spleen were transduced with GFP or AML1-GFP. Forty-eight hours later, expression levels of *p19ARF* were measured by quantitative real-time PCR. (B) *Aml1* intact MLL-ENL leukemic cells were transduced with *p19ARF* shRNA or control shRNA and transplanted into secondary recipient mice. Survival curves of 12 mice from each group are shown. Comparison of survival curve was performed using log-rank test. (C) Expression levels of *p19ARF* in leukemic cells from secondary recipient mice were measured by quantitative real-time PCR. (D) Two AML1 binding sites located in the promoter of *p19ARF* are shown as indicated. (E) Chromatin immunoprecipitation analyses of AML1 for *p19ARF* promoter region in MLL-ENL leukemic cells. Fold enrichment normalized to the control locus *Gapdh* was shown. (F) COS7 cells were cotransfected with expression vector for AML1 and wild-type or PEBP2-site mutated *p19ARF* promoter vector. The relative luciferase activity was calculated as the ratio of luciferase activity with AML1 expression to that without AML1 expression. All luciferase reporter assays were performed in duplicate in 2 independent experiments. Values and error bars represent the mean and the SD, respectively. *$P < .05$. We performed 3 independent experiments and confirmed that similar results were reproduced, except for panel B. Statistical significance was evaluated by unpaired *t* test.

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Authorship

Contribution: N.N., Y.I., M.N., M.I., and M.K. designed the experiments and the study; N.N., S.A., Y.I., M.N., S.G., K.K., T.T., Y.K., M.I., and M.K. wrote the manuscript; N.N., S.A., Y.I., M.N., and M.I. performed experiments and collected and analyzed data; S.G., K.K., and T.T. provided technical advice and support; and M.K. supervised all of the experiments and data interpretation.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Comparison of Allogeneic Hematopoietic Cell Transplantation and Chemotherapy in Elderly Patients with Non-M3 Acute Myelogenous Leukemia in First Complete Remission

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The benefits of allogeneic hematopoietic cell transplantation (allo-HCT) for patients with acute myelogenous leukemia (AML) in first complete remission (CR1) have mostly been evaluated in younger patients. Although favorable outcomes of allo-HCT over chemotherapy have been reported with the use of reduced-intensity conditioning (RIC) regimens in elderly patients with AML in CR1, information is still limited, especially on the effects of cytogenetic risks and donor sources. We collected data from AML patients aged 50 to 70 years who achieved CR1, and compared the outcome in 152 patients who underwent allo-HCT in CR1 (HCT group) to that in 884 patients who were treated with chemotherapy (CTx group). The cumulative incidence of relapse in the HCT group was significantly lower than that in the CTx group (22% versus 62%). Both overall survival (OS) and relapse-free survival (RFS) were significantly improved in the HCT group (OS: 62% versus 51%, $P = .012$), not only in the whole population, but also in the intermediate-risk group. Among patients who had a suitable related donor, the outcomes in the HCT group were significantly better than those in the CTx group. The introduction of appropriate treatment strategies that include allo-HCT may improve the outcome in elderly patients with AML in CR1.

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KEY WORDS: Acute myelogenous leukemia, Elderly patients, Allogeneic hematopoietic cell transplantation, First complete remission

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INTRODUCTION

The biologic characteristics of acute myelogenous leukemia (AML) change as the patient becomes older, because such patients are more often associated with unfavorable profiles such as antecedent hematologic disorder (AHD), expression of P-glycoprotein in blasts, and unfavorable-risk cytogenetic abnormalities [1-4]. In addition, elderly patients are more likely to have a worse performance status and an increased risk of comorbidities, which makes it difficult for them to undergo aggressive therapies [5,6]. Consequently, the reported probability of achieving a first complete remission (CR1) is lower than that in younger patients. In most previous studies, the duration of remission has been reported to be 6 to 8 months, with a 3-year survival rate of <20% [7-10].

Although allogeneic hematopoietic cell transplantation (allo-HCT) is an effective strategy for decreasing the risk of relapse in younger patients, an increase in the risk of treatment-related toxicity is inevitable. Although >50% of the reported AML patients are 50

years of age or older, most previous studies have investigated treatment strategies that include allo-HCT in related younger donor/patient pairs by allocating treatment options based on donor availability. Over the past decade, several studies showed that allo-HCT with reduced-intensity conditioning (RIC) is acceptably safe and effective in elderly patients [11-18]. Allo-HCT with RIC has also been reported to be superior to conventional chemotherapy in elderly AML patients in CR1, particularly when they have a matched related donor [19,20]. However, most of these studies included small numbers of patients, and there is still limited information available on the effects of risk factors of AML, differences in donor sources, and conditioning regimens. To address these critical questions, we performed a nationwide retrospective survey.

PATIENTS AND METHODS

Data Source

The study protocol was approved by the institutional review board at the National Cancer Center Hospital. The targeted population was adult patients who were diagnosed with AML between 1999 and 2006, aged 50 to 70 years, and who had achieved CR1 after 1 or 2 courses of induction chemotherapy. The diagnosis of AML was determined by the WHO classification and included myelodysplastic syndrome with 20% or more bone marrow (BM) blasts. CR was evaluated according to standard criteria for hematologic CR, which was defined as a normocellular BM aspirate containing 5% or less blasts with normal maturation. The presence of minimal residual disease was not molecularly examined in this study. Among them, patients with acute biphenotypic leukemia who were treated with chemotherapy for acute lymphoblastic leukemia, those who had extramedullary AML without BM invasion or extramedullary lesion that did not totally disappear after remission induction chemotherapy, those with acute promyelocytic leukemia, and those who received autologous HCT in CR1 were excluded from the analysis. Information about the disease risks at diagnosis, clinical course, HLA typing and donor availability during CR1, conditioning regimen, and donor source of allo-HCT were collected. Related donors included an HLA-matched or 1-antigen (Ag)-mismatched related donor. A haploidentical related donor who had 2 or more Ag mismatches was considered as an alternative donor. Unrelated donors included volunteer BM donors with 0 or 1-Ag mismatches and unrelated cord blood with three or less-Ag mismatches. As HLA typing for unrelated BM donors was predominantly performed by matches at serum levels in this era, detailed information on allele-level matches was not completely available.

Statistical Analysis

Data were retrospectively reviewed and analyzed as of December 2009. Background differences between the 2 groups was examined with the chi-square test for categoric variables, and with t-test for metric variables. The primary endpoints of the study were relapse-free survival (RFS) and overall survival (OS) from when CR1 was achieved. The unadjusted probabilities of RFS and OS were estimated using the Kaplan-Meier product limit method according to the treatment group, and 95% confidence intervals (CIs) were calculated using the Greenwood formula. To compare RFS and OS between the treatment groups, the log-rank test was used. We performed landmark analyses by excluding patients who died or relapsed within 60 days from CR1 for those who were treated with chemotherapy alone. Cumulative incidences were estimated for relapse and nonrelapse mortality (NRM) to take into account competing risks. The Pepe and Mori's test was used to evaluate the differences between groups. RFS, OS, incidences of relapse, and NRM were estimated as probabilities at 3 years from CR1. Associations between treatment groups and outcome were evaluated using Cox proportional hazard regression models. In addition to whether allo-HCT in CR1 was performed or not, the following factors were considered as covariates: cytogenetic classification according to the Southwest Oncology Group (SWOG), FAB classification, the number of courses of chemotherapy required to achieve CR1, initial white blood cell (WBC) count, and dysplasia at diagnosis. We considered 2-sided P-values of $<.05$ to be statistically significant. Statistical analyses were performed with the SPSS software package and SAS version 9.1.3 (SAS, Cary, NC, USA).

RESULTS

Patients

Clinical data for around 1300 patients were collected from 67 institutions. After excluding 45 patients who received autologous HCT in CR1 or other ineligible patients as described in Patients and Methods, 1036 were eligible for this study (Table 1). The median follow-up of the surviving patients was 44 months. As a remission induction therapy, 89% of elderly patients had received cytarabine- and anthracycline (daunorubicin or idarubicin)-based regimens. Low-dose cyatarabine-based regimens were performed in 8% of the elderly patients. Consolidation therapy was continued with cytarabine-based regimens with or without maintenaice therapy at the discretion of physicians.

Donor Availability and Consideration of allo-HCT in CR1

Information on HLA typing during CR1 and the availability of related donors was obtained in 953

Table 1. Patient Characteristics

Characteristics	All Patients n = 1036	Allo-HCT in CR1 n = 152 (%)	No HCT in CR1 n = 884 (%)	*P*
Median age				
years, (range)	60 (50-70)	55 (50-70)	61 (50-70)	<.001
Median time from diagnosis to CR1				
days, (range)	40 (26-283)	48 (26-242)	39 (13-283)	<.001
Disease				
M0, 6, 7	102	24 (16)	78 (9)	<.001
AHD	37	19 (13)	18 (2)	<.001
Cytogenetic risks (SWOG)				<.001
Favorable	164	5 (3)	159 (18)	
Intermediate	589	93 (61)	496 (56)	
Unfavorable	166	27 (18)	139 (16)	
Unknown	99	25 (16)	74 (8)	
Remission induction				0.13
2 courses	199	36 (24)	163 (18)	
WBC (/μL)				<.001
Higher than 20,000	335	28 (18)	307 (35)	
Dysplasia				<.001
Yes	268	74 (49)	194 (22)	

Allo-HCT indicates allogeneic hematopoietic cell transplantation; CR1, first complete remission; AHD, antecedent hematologic disorder; WBC, white blood cell; SWOG, Southwest Oncology Group.

elderly patients. Among these patients, HLA typing was performed in 331 patients in CR1 (35%) and these patients were younger than those who did not have their HLA typed during CR1 (median, 56 years versus 62 years) (Table 2 and Figure 1). Patients who had their HLA typed were associated with more unfavorable features, such as unfavorable FAB types, AHD, a requirement of 2 courses of remission induction therapy, dysplasia at diagnosis, and a lower frequency of favorable-risk AML by the SWOG classification. Related donors (HLA-matched and 1-Ag-mismatched related donors) were found in 134 patients (40%). No significant difference was found in the distribution of age and risk factors between patients who found a related donor and those who did not after HLA typing (Table 2). Among the patients who had a related donor, 76 (57%) actually underwent allo-HCT during CR1. Among the 197 patients who did not find a related donor, 76 (39%) received allo-HCT from an alternative donor in CR1 (Figure 1).

Patients Who Received allo-HCT in CR1

Of the total 1036 patients, 152 underwent allo-HCT in CR1 (15%). Patients who received allo-HCT in CR1 were younger and associated with more unfavorable characteristics than those who did not (Table 1). As shown in Table 3, 49% of the patients

Table 2. Donor Search and Transplantation

		HLA Check in CR1, n = 331				Statistical Differences		
Characteristics	No HLA Check in CR1 N = 622 (%)	Related Donor Available/HCT+[*a] n = 76 (%)	Related Donor Available/HCT−[b] n = 58 (%)	Related Donor not Available/HCT+[c] n =76 (%)	Related Donor not Available/HCT−[d] n =121 (%)	*P**	*P*†	*P*‡
Age, median, years	62	55	55	55	57	<.001	.396	.906
Disease								
M0, 6, 7	47 (8)	17 (22)	5 (9)	7 (9)	13 (11)	0.008	.170	.160
AHD	11 (2)	4 (5)	2 (3)	15 (20)	2 (2)	<.001	186	.450
Cytogenetic risks (SWOG)						<.001	.561	.045
Favorable	118 (19)	4 (5)	12 (21)	1 (1)	19 (16)			
Intermediate	354 (57)	43 (57)	28 (48)	50 (66)	69 (57)			
Unfavorable	92 (15)	13 (17)	9 (16)	14 (18)	17 (14)			
Unknown	48 (8)	16 (21)	9 (16)	11 (14)	14 (12)			
Remission induction						.009	.541	.871
2 courses	103 (17)	19 (25)	14 (24)	17 (22)	29 (24)			
WBC (/μL)						.021	.178	.004
Higher than 20,000	223 (36)	11 (14)	19 (33)	17 (22)	39 (32)			
Dysplasia						<.001	.991	.117
Yes	127 (20)	31 (41)	16 (28)	43 (57)	26 (21)			

CR indicates complete remission; HCT, allogeneic hematopoietic cell transplantation; AHD, antecedent hematologic disorder; WBC, white blood cell; SWOG, Southwest Oncology Group.

*P-value of comparing "No HLA check in CR1" versus "HLA check in CR1."

†P-value of comparing "Related donor available[a+b]" versus "Related donor not available[c+d]".

‡P-value of comparing "HCT+[a]" versus "HCT-[b]" among those who had a related donor.

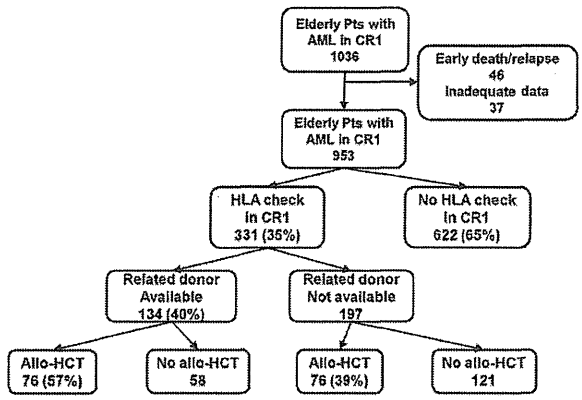

Figure 1. Patient flow. Among 953 patients for whom information was available, HLA typing was performed in 331 patients in CR1 (35%). Related donors were found in 134 patients (40%). Among the patients who had a related donor, 76 (57%) actually underwent allo-HCT in CR1. Among the 197 patients without a related donor, 76 (39%) received allo-HCT from an alternative donor in CR1.

received allo-HCT in CR1 from an HLA-matched or 1-Ag-mismatched related donor. The median interval from CR1 to allo-HCT was 139 days. An RIC regimen was given to 93 patients (61%) with a higher median age of 58 years compared to those who received a myeloablative (MA) regimen, 52 years. Extensive chronic graft-versus-host disease (cGVHD) developed in 61 patients (45%) among 135 who lived and had a follow-up period of longer than 100 days.

Comparison of the Outcomes of allo-HCT versus Chemotherapy in CR1

The outcome in patients who received allo-HCT in CR1 (HCT group) was compared to that in patients who did not receive allo-HCT in CR1 (CTx group). Landmark analyses were performed in all subgroups by excluding 46 patients from the CTx group who relapsed or died within 60 days after achieving CR1. In the CTx group, 183 patients ultimately received salvage allo-HCT after relapse (33% of relapsed patients). The cumulative incidence of relapse in the HCT group was significantly lower than that in the CTx group (22% versus 62% at 3 years from CR1, $P < .001$) (Figure 2). The cumulative incidence of NRM in the HCT group was higher than that in the CTx group (21% versus 3%, $P < .001$). The 3-year RFS in the HCT group was significantly higher than that in the CTx group (56% versus 29%, $P < .001$). Although the difference between the HCT and CTx groups decreased, the 3-year OS in the HCT group was also significantly higher than that in the CTx group (62% versus 51%, $P = .012$). Multivariate analyses for survival showed that performance of allo-HCT, a single course of induction therapy to achieve CR1, lack of dysplasia, WBC below 20,000/μL at diagnosis, and a more favorable cytogenetic risk were significantly associated with better RFS and OS (Table 4). We also used the Cox proportional hazards model with time-dependent variables after taking into account the time from CR1 to allogeneic HCT. By adjusting the influence of waiting time to allogeneic HCT in this analysis, we found that allogeneic HCT in CR1 was also independently associated with better OS.

In a subset analysis according to the cytogenetic risk, patients with intermediate-risk AML showed the similar trends in relapse, NRM, RFS, and OS to the entire patient population (OS: 67% versus 54%, $P = .024$) (Figure 3A). Among patients with unfavorable-risk AML, 27 received allo-HCT in CR1 and 125 did not. In this group of patients, relapse incidence in the HCT group was also substantial (Figure 3B) (41% at 3 years; 95% CI, 21%-61%), which led to OS that did not differ significantly compared to that in the CTx group (OS: 47% versus 35%, $P = .206$).

We also evaluated the outcome in relation to donor availability (Figure 4). Among 134 patients

Table 3. Characteristics of Transplantation in CR1

Characteristics	Allo HCT in CR1 n = 152 (%)	Median Age, Years (Range)	Median Interval from CR1 to HCT, Days (Range)
Total		55 (50-70)	139 (14-981)
Donor			
Matched related	64 (42)	55 (50-70)	121 (14-574)
1-Ag-mismatched related	10 (7)	57 (50-60)	99 (15-436)
Haplo-identical	3 (2)	51 (50-54)	144 (21-147)
Unrelated bone marrow	52 (34)	55 (50-64)	177 (40-981)
Cord blood	23 (15)	55 (50-67)	127 (14-650)
Conditioning			
Myeloablative			
TBI regimen	16 (11)	52 (50-58)	167 (52-436)
Non-TBI regimen	40 (26)	52 (50-59)	141 (14-361)
Reduced-intensity			
Flu/Bu-based	48 (32)	58 (50-70)	147 (15-574)
Flu/Mel-based	29 (19)	58 (50-66)	126 (14-981)
Others	16 (11)	58 (50-69)	99 (23-304)

Allo-HCT indicates allogeneic hematopoietic cell transplantation; CR, complete remission; Ag, antigen; TBI, total body irradiation; Flu, fludarabine; Bu, busulfan; Mel, melphalan.

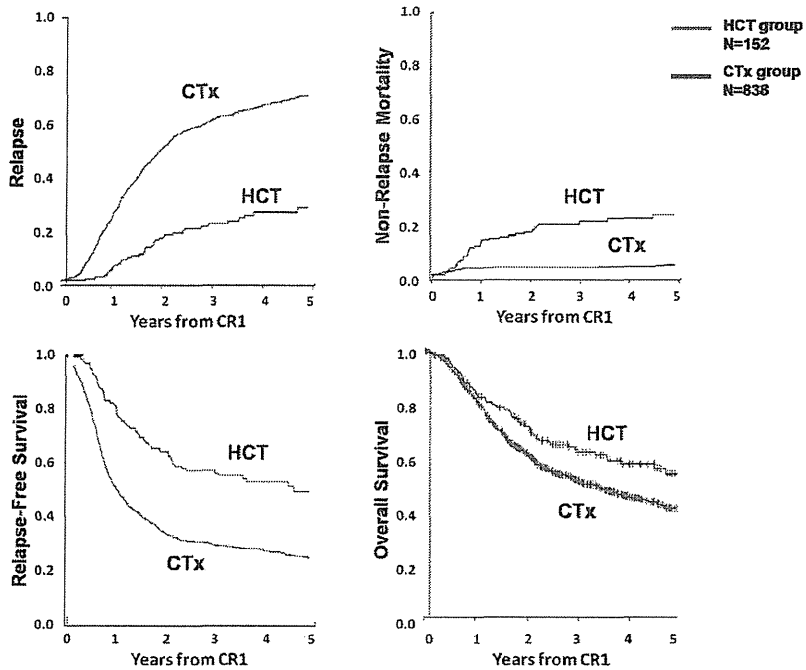

Figure 2. Outcomes according to treatment in CR1 (total elderly patients). Relapse (upper left), nonrelapse mortality (upper right), relapse-free survival (bottom left), and overall survival (OS) (bottom right) of patients who underwent allogeneic hematopoietic cell transplantation in CR1 and those who did not are shown. Forty-six patients who died or relapsed within 60 days from CR1 were excluded as described in the Statistical Analysis. OS was significantly improved in the HCT group ($P = .012$).

who had a related donor, 76 underwent allo-HCT in CR1. The incidence of NRM among the patients who received allo-HCT from a related donor was 14%, which was significantly lower compared to that observed in the whole HCT group. On the other hand, patients who found a related donor but did not undergo allo-HCT in CR1 had a substantial incidence of relapse (80%; 95% CI, 70%-90%). These results led to significant differences in RFS and OS between the HCT and CTx groups (RFS: 64% versus 11%, $P < .001$, OS: 66% versus 43%, $P = .001$) (Figure 4A). These results did not change when 622 patients who did not have their HLA typed (those who were not known to have a suitable related donor) were included in the CTx group (66% versus 54%, $P = .011$) (Appendix 1-A) or when landmark was extended to 5 months from CR1 for the patients in the CTx group who had a related donor (66% versus 54%, $P = .068$) (Appendix 1-B). We also performed the same comparison limited to intermediate-risk AML patients who had a related donor, and found significant differences between the HCT and CTx groups (RFS: 78% versus

Table 4. Multivariate Analysis

	RFS		OS	
Variables	HR (95% CI)	P	HR (95% CI)	P
Allo HCT in CR1 (versus Yes)				
No	2.58 (1.97-3.37)	<.001	1.81 (1.35-2.42)	<.001
Cytogenetic Risk (versus Favorable)				
Intermediate	1.14 (0.90-1.44)	.283	1.10 (0.84-1.45)	.487
Unfavorable	1.70 (1.28-2.24)	<.001	1.89 (1.37-2.59)	<.001
Unknown	1.62 (1.18-2.23)	.003	1.34 (0.92-1.95)	.132
FAB (versus M1, 2, 4, 5)				
M0, 6, 7	1.25 (1.00-1.57)	.052	1.38 (1.07-1.77)	.014
Remission Induction (versus 1 course)				
2 courses	1.52 (1.26-1.84)	<.001	1.61 (1.31-1.99)	<.001
Dysplasia (versus No)				
Yes	1.21 (0.98-1.48)	.075	1.29 (1.02-1.63)	.033
WBC (versus 20,000 or lower)				
Higher than 20,000	1.29 (1.09-1.54)	.004	1.24 (1.01-1.51)	.038

HR indicates hazard ratio; RFS, relapse-free survival; CI, confidence interval; OS, overall survival; allo-HCT, allogeneic hematopoietic cell transplantation; CR, complete remission; WBC, white blood cell count.

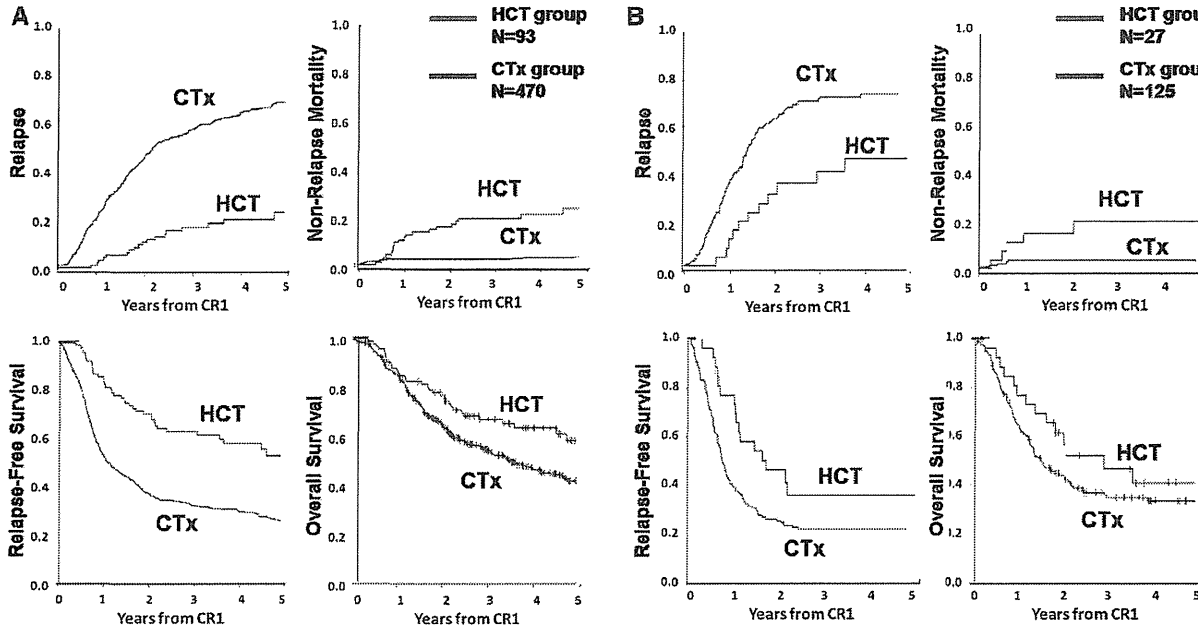

Figure 3. Outcomes according to treatment in CR1 (cytogenetic risks). Relapse (upper left), nonrelapse mortality (upper right), relapse-free survival (bottom left), and overall survival (OS) (bottom right) of patients who underwent allogeneic hematopoietic cell transplantation in CR1 and those who did not are shown among (A) intermediate-risk AML and (B) unfavorable-risk AML. (A) OS was significantly improved in the HCT group among patients with intermediate-risk AML. (B) Relapse incidence was high even after HCT, and OS in the HCT group did not significantly differ from that in the CTx group.

13%, $P < .001$, OS: 78% versus 63%, $P = .048$) (Appendix 1-C).

Among 197 patients who did not have a related donor, 76 underwent allo-HCT from an alternative donor in CR1. Alternative donors included 51 unrelated BM, 22 unrelated CB, and 3 haploidentical related donors. Patients who received allo-HCT in CR1 from an alternative donor had a higher incidence of NRM than those who received allo-HCT from a related donor (28% versus 14% at 3 years, $P = .029$). Additionally, incidence of relapse in allo-HCT from an alternative donor was not reduced compared to that in a related donor transplant setting (22% versus 22%, $P = .743$). Consequently, if we compare the outcomes of the HCT and CTx groups among patients who did not have a related donor, OS did not significantly differ between the two groups (57% versus 47%, $P = .388$) (Figure 4B).

As shown in Table 3, 39% of the patients in the HCT group received an MA regimen. Except for the younger age in those who received an MA regimen, there was no difference in the disease risk between the MA and RIC groups. Additionally, the OS did not significantly differ between the two groups (3-year OS from CR1: 63% versus 61%, $P = .571$) (Appendix 2-A). We also found that OS was not significantly different according to the application of total body irradiation (TBI) (TBI regimen versus non-TBI: 67% versus 61%, $P = .932$) (Appendix 2-B) or among different RIC regimens (fludarabine + busulfan-based, 56%; fludarabine + melphalan-based, 67%; others, 68%, $P = .862$) (Appendix 2-C).

DISCUSSION

We performed retrospective analyses with a 60-day landmark to compare allo-HCT and CTx in 1036 patients aged 50 to 70 years with non-M3 AML in CR1. The results of this study revealed that, overall, elderly patients with AML who received allo-HCT in CR1 had improved outcomes compared to those who were treated with conventional chemotherapy alone. Based on cytogenetic subgroup analyses, patients with intermediate-risk AML had a significantly better OS when they received allo-HCT in CR1. On the other hand, patients with unfavorable-risk AML had a higher risk of relapse even after allo-HCT in CR1, which diminished the benefit of allo-HCT. We also observed that patients who had a related donor had a significantly improved outcome when they received allo-HCT in CR1.

Our results that allo-HCT in CR1 provided an improved OS agree with previously reported comparisons of allo-HCT versus chemotherapy in elderly patients with AML in CR1. Mohty et al. [20] performed a retrospective comparison of "donor" versus "no donor" based on their consistent policy of considering allo-HCT with RIC in CR1 when a patient with high-risk AML had an HLA-matched sibling. They reported superior survival rates not only in the "transplant group" compared to the "no transplant group," but also in the "donor group" compared to the "no donor group." Furthermore, Estey et al. [19] reported the first prospective

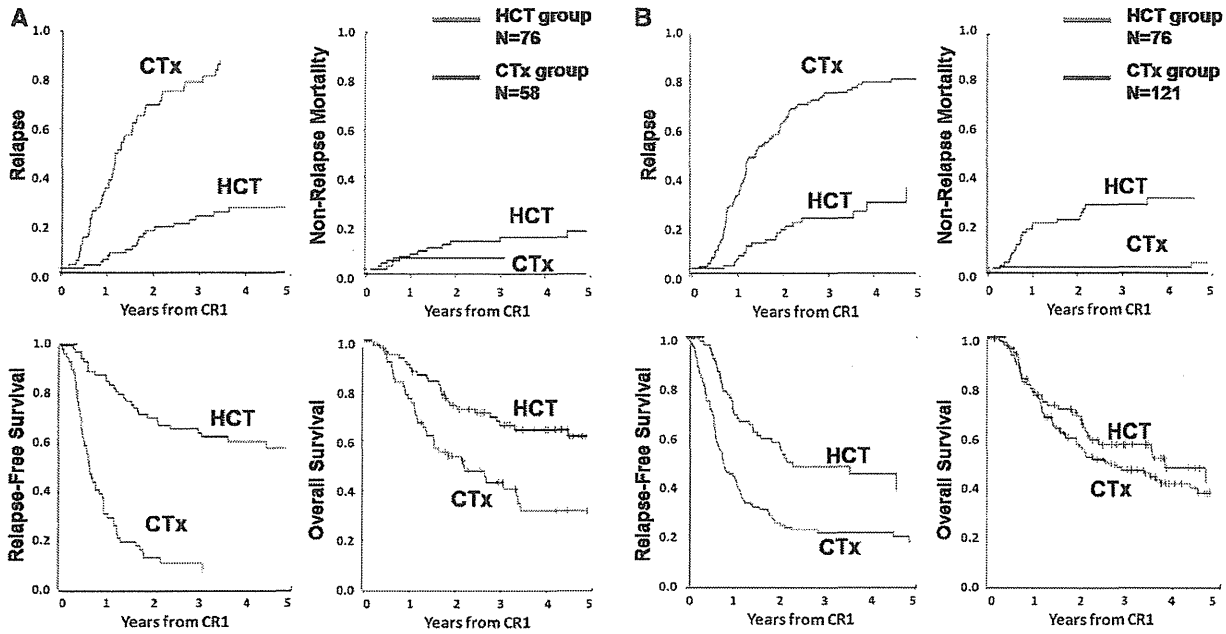

Figure 4. Outcomes according to treatment in CR1 (donor availability). Relapse (upper left), nonrelapse mortality (NRM) (upper right), relapse-free survival (bottom left), and overall survival (OS) (bottom right) of patients who underwent allogeneic hematopoietic cell transplantation in CR1 and those who did not are shown among (A) patients who had a suitable related donor and (B) patients who did not have a suitable related donor. (A) NRM was reduced in related donor transplant and survival probabilities were significantly improved in the HCT group. (B) OS in alternative donor transplant did not significantly differ from that in the CTx group.

observation of allo-HCT with RIC versus chemotherapy in elderly patients. Although the proportions of patients who were referred for transplantation (54%) and those who actually underwent allo-HCT in CR1 (14%) were relatively small, they presented an encouraging outcome that supported the benefit of allo-HCT.

In elderly patients with intermediate-risk AML, we also found improved OS when they received allo-HCT in CR1. This finding is consistent with the result indicated by a meta-analysis by Koreth et al. [21], although their report mostly included prospective studies that targeted younger patients. No previous studies have reported the effects of cytogenetic risks in the transplant setting for elderly patients. In the intermediate-risk group, we found a 60% relapse incidence at 3 years from CR1 when the patients were treated with chemotherapy alone. We also revealed that the incidence of relapse was reduced by 40% with the use of allo-HCT in CR1 without a significant increase in NRM compared to younger patients, which led to a significant improvement of OS.

Our current study did not show a significant benefit of allo-HCT among patients with unfavorable-risk AML. Although fewer patients were analyzed in this subgroup, which may have led to the unlikelihood of yielding a statistical significance, this result may also be explained by the fact that elderly patients tend to be given less-aggressive chemotherapy before allo-HCT because of concerns about toxicity [7,9]. Because no other realistic option can offer a chance of cure for patients with unfavorable-risk AML, many physicians would consider that allo-HCT is optimal for these patients. However, we clearly need to seek novel strategies to reduce the risk of relapse, for example, by reducing the tumor burden before allo-HCT with more intensified chemotherapy or conditioning regimen, or by prevention of recurrence after allo-HCT by vaccination strategy [22-27]. The role of new drugs such as clofarabine or hypomethylating agents should also be estimated for elderly patients with poor-risk AML who are vulnerable to intensive treatments [28,29].

We observed a markedly reduced incidence of NRM after transplantation from a related donor, which improved the outcome of patients who received allo-HCT in CR1 from a related donor. Among 134 patients who had a suitable related donor, 40% did not undergo allo-HCT during CR1. Unfortunately, the exact reason was not available from our retrospectively collected database. Possible reasons include disease relapse before the anticipated timing for allo-HCT, or failure to receive appropriate therapy because of being too ill. However, an analysis with a landmark extended to 5 months still proved that OS in the HCT group was significantly better compared to that in the CTx group among those who had a related donor.

In contrast to the favorable outcome in the setting of allo-HCT from a related donor, the outcome of allo-HCT from an alternative donor in CR1 was not significantly superior to that of chemotherapy alone. In addition to the significantly higher NRM after alternative

donor transplant, the incidence of relapse was not reduced in the alternative donor transplant compared to that in related donor transplant despite our expectation that a graft-versus-leukemia (GVL) effect would be more potent after allo-HCT from alternative donors. Several reports have indicated that the outcomes of allo-HCT from HLA allele-matched unrelated donors are comparable to those from related donors [14,27]. One possible explanation for this disparity is that patients who received allo-HCT from an alternative donor in our database were significantly more likely to have high-risk AML than those who received allo-HCT from a related donor. Second, HLA typing was predominantly performed serologically in the period of our study. About a third of the patient/donor pairs who are considered to be matched unrelated pairs by a serologic examination have been reported to have an allele-mismatch [30]. In addition, voluntary unrelated donors consisted only of BM donors because peripheral blood harvest is not yet allowed in our country, and unrelated CB accounted for one-third of the alternative donors in our study. Although allo-HCT from an alternative donor was not shown to have a benefit in elderly patients in our study, we may expect a better outcome with a smooth access to an allele-matched unrelated donor.

Whereas prior reports that have compared allo-HCT and chemotherapy in elderly patients targeted only allo-HCT with RIC [19,20], one-third of the HCT group patients in our study received an MA conditioning regimen. However, except for patient age, there were no significant differences in the disease risks between the MA and RIC groups, and OS was similar between the two groups. As has been previously pointed out, there were no significant differences in OS among different RIC regimens [31].

Because our database consists of retrospectively collected clinical data, this cohort of patients may have several inherent selection biases. Although we performed a landmark analysis to eliminate the biases by the patients who did not have a chance to receive allo-HCT in CR1 because of earlier relapse or comorbidity, patients in the HCT group may still have had favorable features that enabled them to successfully reach the point of allo-HCT in CR1. Furthermore, our database did not provide detailed information on consolidation chemotherapy after achievement of CR1 or the reasons why patients did not undergo allo-HCT such as the presence of comorbid conditions. Although the number of the elderly patients who received autologous HCT in CR1 was small, the exclusion of these patients may have made the non-HCT group have even more inherent selection bias, Nevertheless, the results drawn from our database, which includes 850 patients in the CTx group and 150 patients in the HCT group, may allow us to suggest optimal strategies for elderly patients with AML especially stratified by cytogenetic subgroups.

In conclusion, our study indicated that elderly patients with AML who underwent allo-HCT in CR1 had improved outcomes compared to those who were treated with conventional chemotherapy alone, and also revealed that intermediate-risk AML patients had an improved OS when they underwent allo-HCT in CR1. Because OS was better in elderly patients when they have a matched related donor and successfully undergo allo-HCT in CR1, they should be encouraged to seek the opportunity of allo-HCT in CR1 by performing HLA typing and donor search in the early period after achievement of CR1. Novel strategies to reduce the risk of relapse and better access to allele-matched unrelated donors should further improve the prognosis of elderly patients with AML.

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AUTHORSHIP STATEMENT

Contribution: S.K. designed the study, prepared the data file, performed the analysis, interpreted data, and wrote the manuscript; T.Yamaguchi was primarily responsible for designing the study, data analysis and interpretation of the data; N. Uchida., S.M., K.U., M.W., T. Yamashita., H.K., J. Tomiyama., Y. Nawa., S.Y., J. Takeuchi., K.Y., F.S., N. Uoshima., T. Yano., Y. Nannya, and Y.M. obtained the patients' data and interpreted data; I.M. reviewed the cytogenetic reports and interpreted data; Y.T. interpreted data and helped to write the paper; T.F. was primarily responsible for the entire paper as an accurate and verifiable report.

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