Table 2. Characteristics of AML caused by C/EBPα mutants

	pMYs-IG/pMYs-IR (n = 8)	Myc-C^m/pMYs-IR (n = 6)	Myc-C^m/Flag-N^m (n = 8)
WBC (/μL)	9060 ± 1648	5816 ± 3128	36 675 ± 22 956
Hb (g/dL)	16.4 ± 3.2	12.4 ± 2.2	10.7 ± 2.3
Plt (× 10^4/μL)	79.4 ± 43.1	7.2 ± 4.5	19.8 ± 13.1
BM count (× 10^7)	3.34 ± 0.73	1.69 ± 0.30	2.83 ± 0.88
Leukemic cells (%)	-	60-92	72-94
Liver weight (mg)	1433 ± 153	2071 ± 1281	2441 ± 1315
Spleen weight (mg)	113 ± 24	476 ± 220	549 ± 239

Averages and standard deviations are shown. BM cells were isolated from both tibias and femurs.

WBC indicates white blood cell; Hb, hemoglobin; and Plt, platelets.

C/EBPα-C^m–induced leukemia (Table 2 and Figure 5B). Morphologies of the leukemic blasts are more immature in mice/Myc-C^m/Flag-N^m than mice/Myc-C^m/pMYs-IR (Figure 5B), consistent with the lower expression of Gr-1 in the former (Figure 5C and data not shown). Flow cytometric analysis delineated that most leukemic cells of mice/Myc-C^m/Flag-N^m expressed both GFP and dsRED (Figure 5C) and invariable markers: CD11b-inntermediate and Gr-1, B-220, c-kit-low (Figure 5C). Expression of both C/EBPα-C^m protein and p30 protein generated by C/EBPα-N^m in leukemic cells of mice/Myc-C^m/Flag-N^m was confirmed by Western blot analysis (Figure 5D). Expression levels of p30 protein generated by C/EBPα-N^m were not correlated with the disease latency in mice/Myc-C^m/Flag-N^m (Figure 5A,D). The morbid mice/Myc-C^m/Flag-N^m suffered from anemia and thrombocytopenia-like mice/Myc-C^m/pMYs-IR; however, it was of note that unlike mice/Myc-C^m/pMYs-IR, most mice/Myc-C^m/Flag-N^m exhibited marked leukocytosis (Figure 5E-F and Table 2). These results suggested that C/EBPα-N^m either confers a proliferative advantage on immature myeloid cells or collaborates with C/EBPα-C^m in blocking differentiation of myeloid cells in vivo. It is of note that this collaborative effect was induced by relatively low levels of p30 protein generated by C/EBPα-N^m.

C/EBPα-C^m, but not C/EBPα-N^m, collaborated with Flt3-ITD in inducing AML in a BMT model

Because C/EBPα-C^m possessed the potential to strongly suppress myeloid differentiation, this mutation could be categorized into class II mutations. We speculated that AML would be efficiently induced by combining C/EBPα-C^m with class I gene alterations. To test this, murine BM mononuclear cells, transduced with both Flt3-ITD and either C/EBPα-C^m or C/EBPα-N^m, were transplanted into the recipient mice. BM mononuclear cells expressing both mutants were recognized as GFP- and dsRED-double positive cells, 10%-20% of BM cells before the transplantation. As reported previously,[49] mice receiving transplants of BM cells expressing both Flt3-ITD-IRES-GFP and mock (pMYs-IR) (mice/FLT/pMYs-IR) developed myeloproliferative neoplasm (MPN) within 1.5-3 months after transplantation (Figure 6A). BM and spleen were occupied with increased numbers of mature myeloid cells expressing CD11b at high levels and Gr-1 at intermediate to high levels (Figure 6B-C). Intriguingly, mice transplanted with BM cells expressing both Flt3-ITD-IRES-GFP and C/EBPα-C^m-IRES-dsRED (mice/FLT/C^m) developed aggressive leukemia within 2-3 weeks after transplantation (Figure 6A). Histologic examination of mice/FLT/C^m showed that BM was occupied with the 2 populations: large and small blastlike cells (Figure 6B). However, flow cytometric analysis demonstrated that both populations, double positive for GFP and dsRED, similarly expressed B220, CD19, Gr-1, and CD11b and could not be differentiated (Figure 6C and data not shown). Thus, mice/FLT/C^m invariably developed biphenotypic leukemia. Western blot analysis demonstrated that both Flt3-ITD and C/EBPα-C^m proteins were expressed in spleen cells of mice/FLT/C^m (Figure 6D). On the other hand, mice that received transplants of BM cells expressing both Flt3-ITD-IRES-GFP and C/EBPα-N^m-IRES-dsRED (mice/FLT/N^m) developed MPN with latencies comparable with those of MPN developed by mice/FLT/pMYs-IR, although some lymphoid blast cells were observed in 2 mice/FLT/N^m (Figure 6A and data not shown). Thus, C/EBPα-N^m did not significantly collaborate with Flt3-ITD in leukemogenesis in the present BMT model. Finally, leukemic cells derived from mice/FLT/C^m proliferated independently of IL-3 in the culture, while those from mice/C^m still required IL-3 for their growth. Leukemic cells derived from mice/FLT or mice/FLT/N^m did not survive even in the presence of IL-3. Moreover, we found stronger activation of STAT5, STAT3, AKT, and ERK in leukemic cell lines derived from mice/C^m/FLT compared with those from mice/C^m (Figure 6E). These results indicated that Flt3-ITD conferred additional proliferative potentials as a class I mutation on the cells expressing C/EBPα-C^m alone, thereby inducing aggressive leukemia.

Discussion

The present results on *CEBPA* mutations of AML patients confirmed previous reports[20-28]; *CEBPA* mutations are found in 5%-14% of de novo AML, and most of them harbor 2 distinct mutations on different alleles and have good prognosis. In addition, our results suggested that mutations of *CEBPA* are found only in one allele in most cases of therapy-related AML or MDS, and AML progressed from MDS harboring CEBPA mutations (8/71 and 7/224). While we did not find additional mutations in other genes in de novo AML patients with double CEBPA mutations, we detected 3 additional mutations in 15 patients with therapy-related AML or MDS and MDS/AML. These results indicate that a *CEBPA* mutation collaborates with either a different type of CEBPA mutations or mutations in different genes in inducing leukemia.

Analysis of CEBPA mutations in in vitro assays provided novel insights concerning the role of CEBPα in blood cells. C/EBPα-N^m and p30, but not C/EBPα-C^m, suppressed transcriptional activation of C/EBPα-WT in a luciferase assay using 293T cells (Figure 2A) as reported previously.[20] Curiously, expression of G-CSF-R, a major target of C/EBPα, was profoundly suppressed by C/EBPα-C^m but not by C/EBPα-N^m in 32Dcl3 cells (Figure 1F). C/EBPα-C^m suppressed G-CSF–induced granulocytic differentiation of 32Dcl3 cells more efficiently than C/EBPα-N^m (Figure 1D-E). It is possible that insufficient suppression of G-CSF–induced differentiation of 32Dcl3 cells by C/EBPα-N^m despite its inhibitory activity on transcriptional activation of C/EBPα-WT in 293T cells may be because of the low expression of C/EBPα-N^m in 32Dcl3 cells (Figure 1B). In fact, C/EBPα-p30 moderately suppressed the expression of G-CSF-R and inhibited G-CSF–induced differentiation of 32Dcl3 cells (Figure 1D-F). However, this does not explain why C/EBPα-C^m efficiently blocks the differentiation of 32Dcl3 cells despite its inability to suppress C/EBPα activation in the luciferase assay. Therefore, we speculated that C/EBPα mutants behave differently in epithelial 293T cells and hematopoietic 32Dcl3 cells and tested whether hemotopoietic cell–specific transcription factors play some role in 32Dcl3 cells. Because it was

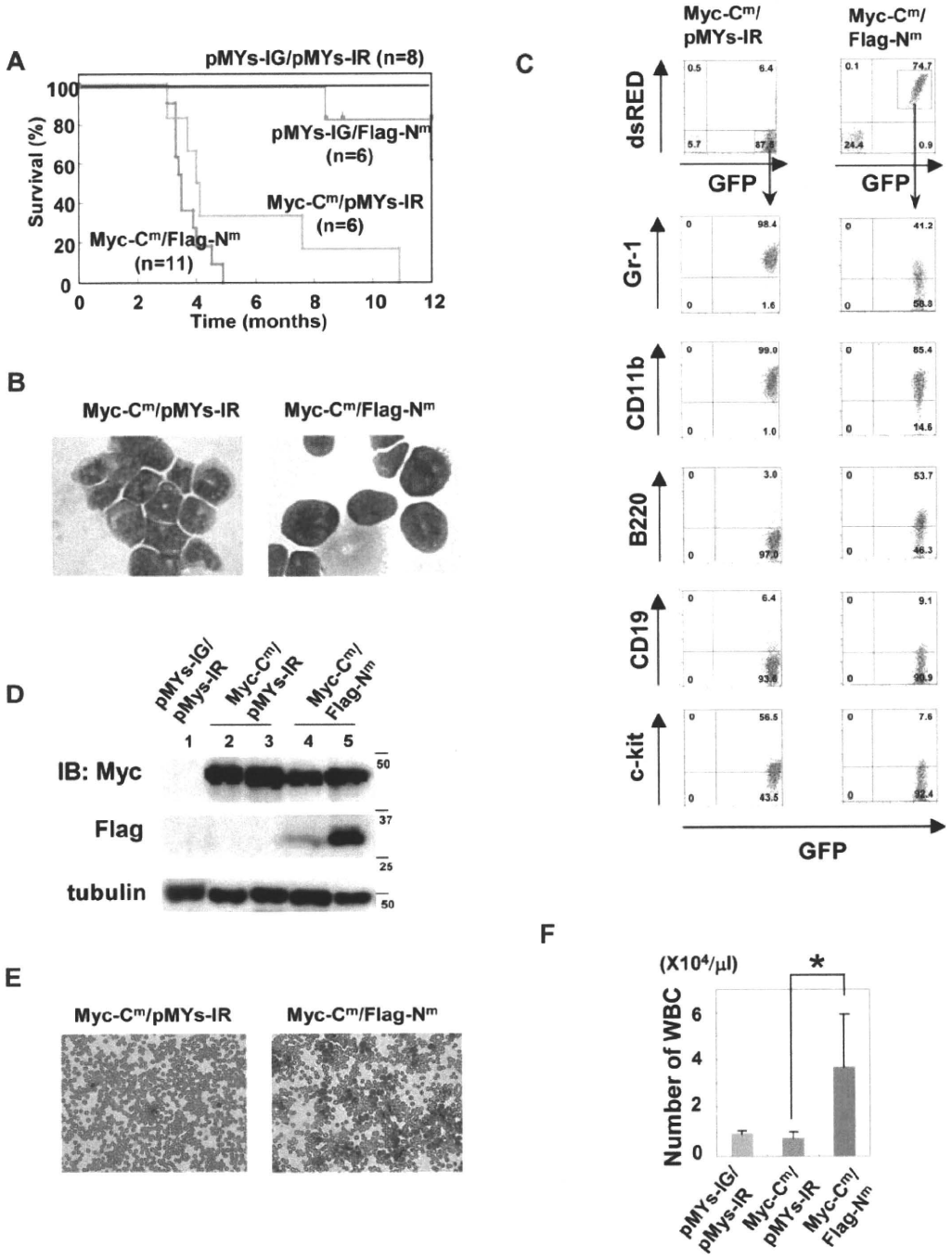

Figure 5. Coexpression of both C/EBPα-C^m and C/EBPα-N^m led to AML with leukocytosis with shorter latencies. (A) Kaplan-Meier analysis for the survival of mice that received transplants of BM cells transduced with both Myc-tagged C/EBPα-C^m-IG and pMYs-IR (Myc-C^m/pMYs-IR, n = 6), both pMYs-IG and Flag-tagged C/EBPα-N^m-IR (pMYs-IG/Flag-N^m, n = 6), both Myc-tagged C/EBPα-C^m-IG and Flag-tagged C/EBPα-N^m-IR (Myc-C^m/Flag-N^m, n = 11), or mock (pMYs-IG/pMYs-IR, n = 8). (B) Cytospin preparations of BM cells derived from mice/My-C^m/pMYs-IR or mice/Myc-C^m/Flag-N^m were stained with Giemsa. A representative photograph is shown. Images were obtained with a BX51 microscope and a DP12 camera (Olympus); objective lens, UplanFl (Olympus); original magnification ×100. (C) Flow cytometric analysis of BM cells derived from mice/Myc-C^m/pMYs-IR (left) or mice/Myc-C^m/Flag-N^m (right). The dot plots show expression of dsRED versus expression of GFP (1st panel). In the indicated gating, the dot plots show expression of Gr-1, CD11b, B220, CD19, or c-kit labeled with phycoerythrin-Cy5–conjugated streptavidin versus expression of GFP. (D) Expression of Myc-tagged C/EBPα-C^m protein and p30 protein generated by Flag-tagged C/EBPα-N^m in BM cells derived from mice/pMYs-IG/pMYs-IR (lane 1), mice/Myc-C^m/pMYs-IR (lanes 2-3), or mice/Myc-C^m/Flag-N^m (lanes 4-5) was detected by using anti-Myc monoclonal Ab (top) and ant-Flag mAb (middle), respectively, in Western blot analysis. Equal loading was evaluated by probing the immunoblots with anti-tubulin Ab (bottom). Data are representative of 3 independent experiments. (E) Peripheral blood smears obtained from mice/Myc-C^m/pMYs-IR (left) or mice/Myc-C^m/Flag-N^m (right) were stained with Giemsa. Images were obtained with a BX51 microscope and a DP12 camera (Olympus); objective lens, UplanFl (Olympus); original magnification ×20. (F) Counts of white blood cells (WBC) obtained from mice/Myc-C^m/pMYs-IR (n = 6), mice/Myc-C^m/Flag-N^m (n = 8), or mice/pMYs-IG/pMYs-IR (n = 8). All data points correspond to the mean and the standard deviation (SD). Statistically significant differences are shown. *$P < .05$.

reported that PU.1 plays important roles in macrophage differentiation, which is hampered by its interaction with C/EBPα through its C-terminal bZIP domain,[50] we investigated whether PU.1 plays some role in C/EBPα-C^m–mediated transcription. The present

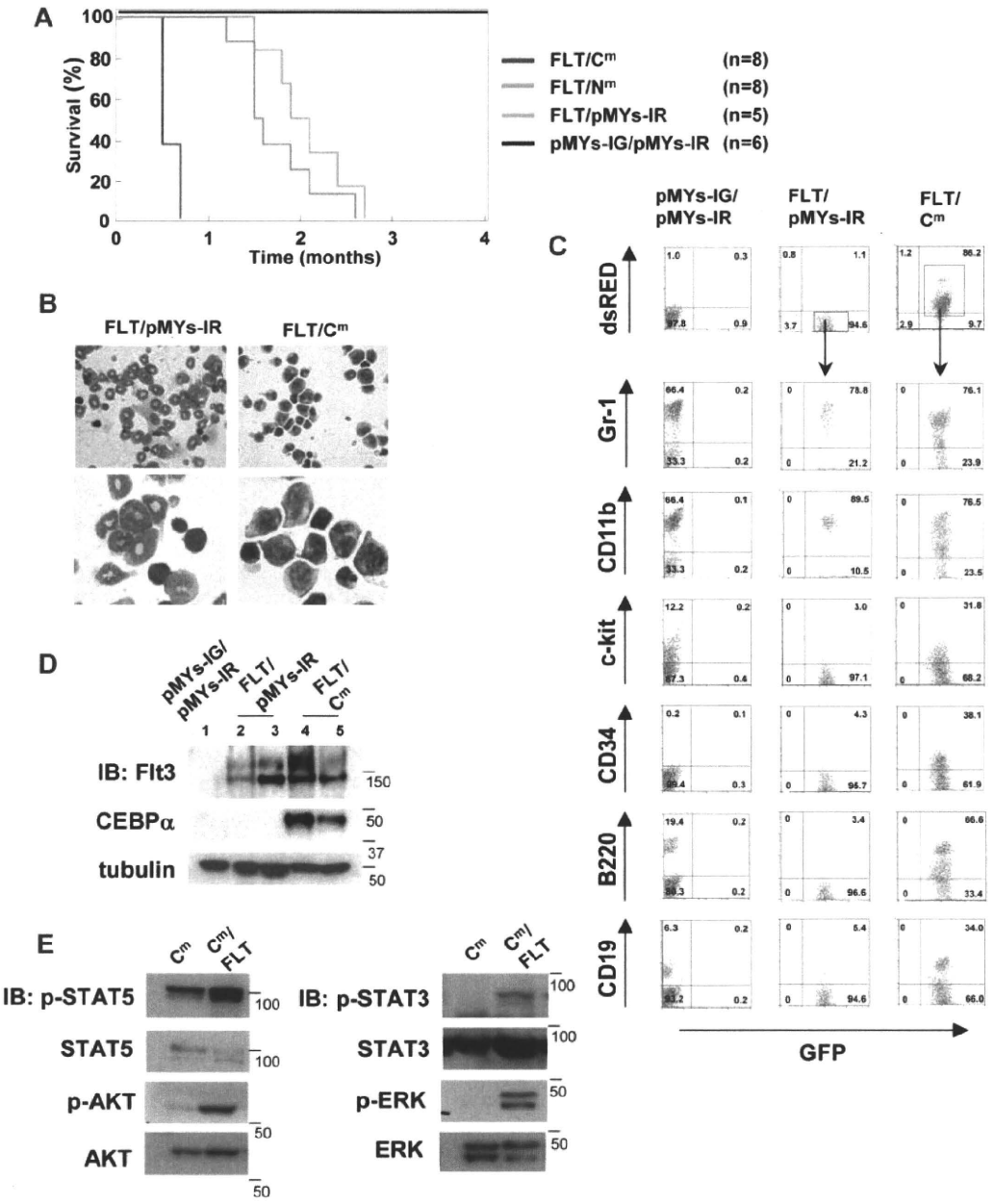

Figure 6. C/EBPα-C^m, but not C/EBPα-N^m, collaborated with Flt3-ITD in inducing aggressive AML. (A) Kaplan-Meier analysis for the survival of mice that received transplants of BM cells transduced with both Flt3-ITD-IG and pMYs-IR (FLT/pMYs-IR, n = 5), both Flt3-ITD-IG and C/EBPα-C^m-IR (FLT/C^m, n = 8), both Flt3-ITD-IG and C/EBPα-N^m-IR (FLT/N^m, n = 8), or mock (pMYs-IG/pMYs-IR, n = 8). (B) Cytospin preparations of BM cells derived from mice/FLT/pMYs-IR or mice/FLT/C^m were stained with Giemsa. Images were obtained with a BX51 microscope and a DP12 camera (Olympus); objective lens, UplanFl (Olympus); original magnification ×40 (top), ×100 (bottom). (C) Flow cytometric analysis of BM cells derived from mice/pMYs-IG/pMYs-IR, mice/FLT/pMYs-IR, or mice/FLT/C^m. The dot plots show expression of dsRED versus expression of GFP (first panel). In the indicated gating, the dot plots show expression of Gr-1, CD11b, c-kit, CD34, B220, or CD19 labeled with phycoerythrin-Cy5–conjugated streptavidin versus expression of GFP. (D) Expression of C/EBPα-C^m or Flt3-ITD in spleen cells of mice/pMYs-IG/pMYs-IR (lane 1), mice/FLT/pMYs-IG (lanes 2-3), or mice/FLT/C^m (lanes 4-5) was detected by using anti-C/EBPα (14AA) Ab (middle) or anti-Flt3 Ab (top), respectively, in Western blot analysis. Equal loading was evaluated by probing the immunoblots with anti-tubulin Ab. Data are representative of 3 independent experiments. (E) Immortalized leukemic cells derived from mice/FLT/C^m had increased phosphorylation of STAT5, AKT, STAT3, and ERK compared with those from mice/C^m. Whole-cell extracts of the former cells (immortalized in the absence of IL-3) or the latter (immortalized in the presence of IL-3) were immunoblotted with phospho-specific Abs as described in the Methods. Equal loading was evaluated by reprobing the immunoblots with anti-STAT5, anti-AKT, anti-STAT3, or anti-ERK Abs. Data are representative of 3 independent experiments.

result indicated that C/EBPα synergized with exogenously expressed PU.1 in stimulating transcription of the target genes in 293T cells, which was profoundly inhibited by C/EBPα-C^m but not by C/EBPα-N^m (Figure 2B), and suggested that C/EBPα-C^m hampers PU.1 from interacting with other molecules including C/EBPα in hemopoietic cells, leading to inhibition of granulocytic differentiation.

As for cooperation between C/EBPα-N^m and C/EBPα-C^m in leukemogenesis, we demonstrated using BMT models that C/EBPα-N^m and C/EBPα-C^m in combination induced AML with shorter latencies compared with transplantation of C/EBPα-C^m–transduced BM cells alone. In addition, combining both mutations resulted in increased number of leukemic cells, implicating C/EBPα-N^m in expansion of the cells whose differentiation was

blocked by C/EBPα-C^m. Thus, these results suggested that C/EBPα-C^m works as a class II mutation while C/EBPα-N^m works as a class I mutation in inducing leukemia.[31-36] To test this hypothesis, we built another BMT model where BM cells transduced with Flt3-ITD and either C/EBPα-C^m or C/EBPα-N^m were transplanted to lethally irradiated mice. Flt3-ITD dramatically shortened the latency of leukemia induced by C/EBPα-C^m but not by C/EBPα-N^m, indicating that C/EBPα-C^m worked as a class II mutation in inducing leukemia. Transplantation of BM cells transduced with both C/EBPα-C^m and Flt3-ITD quickly induced leukemia in just 2 weeks after transplantation. Most of the transplanted mice seemed to develop biphenotypic leukemia as assessed on the morphology and surface marker expressions (Figure 6B-C). In our hands, BM cells transduced with Flt3-ITD sometimes induce lymphoid malignancies in addition to myeloproliferative disease,[49] bringing some complexity to the experiment. Nonetheless, dramatically shortened latencies with the combination of C/EBPα-C^m and Flt3-ITD strongly indicated that C/EBPα-C^m works as a class II mutation in inducing leukemia. On the other hand, because the expression levels of C/EBPα-N^m was low in our experiments, further experiments will be required to firmly demonstrate that C/EBPα-N^m plays a class I-like role. One possible experiment is to test a combination between C/EBPα-N^m and a known class II mutation. Nonetheless, a class I-like role of C/EBPα-N^m was suggested by the marked increase in the number of leukemic cells in mice/Myc-C^m/Flag-N^m compared with mice/Myc-C^m/pMYs-IR. In relation to this, although the "2-hit theory" well explain many clinical observations, additional classes of mutations may be required for the comprehensive understanding of leukemogenesis as proposed by Renneville et al[32] In fact, we detected more than 3 mutations including mutations, chromosomal translocations, or deletions in 5 of 20 patients with leukemia and MDS (Table 1).

Concerning the in vivo effects of *CEBPA* mutations, several different results were reported.[29,30,51-54] Bereshchenko et al[30] have recently published a report using knock-in mice that C/EBPα-p30 and a C-terminal mutation collaborated in inducing leukemia. Our results basically agreed with those by Bereshchenko et al. However, while our results implicated C-terminal mutations of C/EBPα in differentiation, leading to leukemia with relatively long latencies, Bereshchenko et al[30] suggested premalignant HSC expansion by C-terminal mutations. The reason for the disparity is not clear, but was partly caused by the difference in the strength or functions of different C-terminal mutants or in the expression levels of mutants in knock-in mice and BMT models. Concerning the experimental systems, knock-in mice are superior to mouse BMT models in several aspects as indicated previously.[29,30] Most importantly, expression of the mutant C/EBPα is driven by the authentic promoter in knock-in mice while it is over-driven by an external promoter in BMT models. Moreover, replacement of both alleles with different C/EBPα mutations closely mimics human leukemia, as it lacks the WT C/EBPα unlike the BMT model. In addition, in BMT models, retrovirus integration sites sometimes modify the phenotype of the disease. However, BMT models do have some advantages. First, in contrast to knock-in mice where all hemopoietic cells express the mutant allele, only some cells can be of a leukemia origin, which would more faithfully mimic human pathologic situations. In addition, various mutants can be readily tested in vivo. Bereshchenko et al[30] used K313dup as a C-terminal *CEBPA* mutation, demonstrating that mice$^{K313dup/+}$ did not develop leukemia. K313dup was a weak inducer of leukemia in our BMT model, where only 1 of the 4 transplanted mice developed myeloid leukemia in 10 months (data not shown). On the other hand, C/EBPα-C^m, a C-terminal mutation with 304-323dup that we used in the present study, induced leukemia in most transplanted mice (Figure 4A). Thus, knock-in mice models and BMT models can complement with each other in investigating in vivo leukemogenesis.

To summarize, we have presented a series of evidence, including clinical data, in vitro experiments, and mouse BMT models, showing that 2 different mutations of *CEBPA*, C/EBPα-N^m and C/EBPα-C^m, play distinct roles in leukemogenesis. Moreover, our results strongly indicated that C/EBPα-C^m is able to play as a class II mutation in concert with Flt3-ITD in inducing leukemia. Further elucidation of the molecular mechanism of *CEBPA* mutations–induced leukemia may pave a novel way to treating patients with leukemia.

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Authorship

Contribution: N.K. did all the experiments and participated in writing the manuscript; J.K. oversaw all the experiments and actively participated in manuscript writing; N.D., Y.K., N.W.O., K.T., F.N., T.O., and Y.E. technically supported BMT; Y.F. and H.N. provided plasmids and reagents; Y.H. and H.H. provided and analyzed human samples; and T.K. conceived the project, secured funding, and actively participated in manuscript writing.

Conflict-of-interest disclosure: T.K. serves as a consultant for R&D Systems. The remaining authors declare no competing financial interests.

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PROSPECT

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Molecular Mechanisms That Produce Secondary MDS/AML by *RUNX1/AML1* Point Mutations

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ABSTRACT

RUNX1/AML1 point mutations have been identified in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) patients. A heterozygous germline mutation of the *RUNX1* gene causes a familial platelet disorder with a predisposition to AML. *RUNX1* mutations have also been detected with high frequency in minimally differentiated AML M0 subtypes and myelodysplastic/myeloproliferative neoplasms. Here we propose a new disease category of myelodysplastic neoplasms (MDN) consisting of MDS refractory anemia with excess blasts and AML with myelodysplasia-related changes, including therapy-related cases. *RUNX1* mutations have been detected in about 20% of patients with "MDN". Among the MDN cases, histories of radiation exposure, therapy-related myeloid neoplasms after successful treatment for acute promyelocytic leukemia, and leukemic transformation of myeloproliferative neoplasms have been reported to have a strong association with *RUNX1* mutations. The mutations occur in a normal, a receptive, or a disease-committed hematopoietic stem cell. It is suspected that the "MDN" phenotypes are defined by the *RUNX1* mutations in addition to some other abnormalities. J. Cell. Biochem. 112: 425–432, 2011.

KEY WORDS: RUNX1/AML1; MYELODYSPLASTIC NEOPLASMS; MOLECULAR MECHANISM; THERAPY-RELATED MYELOID NEOPLASMS

RUNX1/AML1 point mutations have been identified in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) since the first report in 1999 [Osato et al., 1999]. Most of the mutants lose *trans*-activation potential leading to a loss of normal function, indicating that RUNX1 dysfunction is one of the major pathogenic mechanisms of MDS and AML [Harada et al., 2003, 2004]. Some types of RUNX1 mutants show a dominant-negative effect on the *trans*-activation activity, suggesting that they may have some oncogenic potential in addition to the loss of normal function. Biological analysis using a mouse bone marrow transplantation model and human $CD34^+$ cells transduced with RUNX1 mutants has confirmed the oncogenic ability of RUNX1 mutants [Watanabe-Okochi et al., 2008; Harada and Harada, 2009]. These data suggest that RUNX1 mutants are factors that initiate MDS-genesis by inhibiting differentiation of hematopoietic stem cells (HSC). One type of RUNX1 mutants requires that cells acquire the ability to proliferate, while another type may induce proliferation directly. Thus, RUNX1 mutants play a central role in the pathogenesis of MDS and AML.

To what disease category do *RUNX1* mutations contribute? In this prospect, we focus on *RUNX1* mutations in patients with "secondary" (i.e., radiation-induced, therapy-related, and blastic crisis from chronic phase [CP]) MDS and AML, in which we can assume the onset of the mutations, and we attempt to describe the relationship between *RUNX1* mutations and secondary MDS and AML.

MYELOID NEOPLASMS CAUSED BY *RUNX1/AML1* POINT MUTATIONS

RUNX1/AML1 point mutations have been reported in various myeloid neoplasms (Fig. 1). A heterozygous germline mutation of the *RUNX1* gene is known to cause familial platelet disorder with a predisposition to AML (FPD/AML) [Song et al., 1999; Osato, 2004], an autosomal dominant disorder characterized by congenital qualitative and quantitative platelet defects and the propensity to develop MDS or AML at a high incidence (20–50%). The affected

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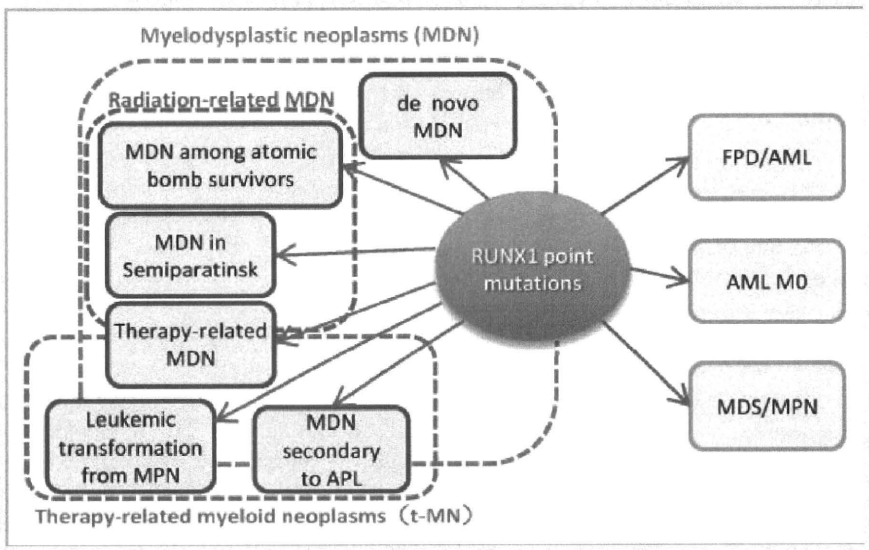

Fig. 1. *RUNX1* mutations in MDN.

individuals may develop leukemia at various times throughout their life span, suggesting that the acquisition of additional mutations is needed to cause leukemia during this long latency period.

It has been demonstrated that *RUNX1* mutations occur at low frequency in de novo AML without myelodysplastic features, but they have been detected in 15–35% of cases of minimally differentiated AML M0 subtypes [Osato, 2004]. Moreover, half of the *RUNX1*-mutated AML M0 cases lost the wild-type *RUNX1*, and de novo AML with *RUNX1* mutation is associated with acquired but not congenital trisomy 21 [Preudhomme et al., 2000]. A significant association between *RUNX1* mutations and activating *FLT3* mutations [Matsuno et al., 2003] and trisomy 13 with increasing *FLT3* expression levels [Dicker et al., 2007] was detected. Furthermore, *RUNX1*-mutated AML M0 is a distinct entity, with expression of both myeloid and B-lymphoid genes [Silva et al., 2009]. Recently, *RUNX1* mutations were identified in 13.2% of de novo non-M3 AML cases [Tang et al., 2009], however, no information was given about their myelodysplasia.

RUNX1 mutations were also reported in 37% of chronic myelomonocytic leukemia (CMML) [Kuo et al., 2009] and 14% of myelodysplastic/myeloproliferative neoplasms (MDS/MPN) [Ernst et al., 2010]. MDS/MPN, including CMML, atypical chronic myeloid leukemia (aCML) and MDS/MPN unclassifiable, are clonal myeloid neoplasms characterized by the simultaneous presence of both myelodysplastic and myeloproliferative features at the time of their initial presentation [Orazi and Germing, 2008]. The molecular pathogenesis of MDS/MPN is only partially understood in the "myeloproliferative" side, but *RUNX1* mutations may explain the molecular mechanism of the "myelodysplastic" side of MDS/MPN. However, the discrimination criteria between MDS/MPN and MDS refractory anemia with excess blasts (RAEB) is unclear.

On the other hand, the frequency of *RUNX1* point mutations in MDS was initially reported to be low [Osato et al., 1999; Song et al., 1999; Imai et al., 2000; Preudhomme et al., 2000], mainly because the MDS patients in those reports comprised many cases with a low blast percentage (<5%). However, subsequent analyses of *RUNX1* gene mutations have indicated that they occur in about 10–20% of patients classified as MDS-RAEB and AML following MDS, and their frequency is substantially higher among radiation-associated (including atomic-bomb survivors) and therapy-related cases [Harada et al., 2003, 2004; Christiansen et al., 2004; Zharlyganova et al., 2008].

THE CONCEPT OF "MYELODYSPLASTIC NEOPLASMS"

MDS is distinguished from AML by the blast threshold, defined as 20% blasts in the blood or bone marrow, according to the World Health Organization (WHO) classification system that takes advantage of morphological, genetic, immunophenotypic, biological, and clinical features to define specific disease entities [Swerdlow et al., 2008]. Unlike the classification for AML, which is based on cytogenetic and genetic abnormalities, the classification for MDS still relies on morphological findings alone, due to unsatisfactory insights into the molecular pathogenesis. Only one category of 5q− syndrome in MDS is well defined, and its molecular mechanisms and appropriate therapies have been investigated in recent years [Boultwood et al., 2010], however, other types of MDS are not established as definite disease categories based on molecular mechanisms. It is necessary to clarify the molecular mechanisms of MDS in order to establish a new classification scheme that would include a characteristic constellation of clinical, genetic, and pathologic findings, similar to AML.

In Japan, most people have a medical examination including blood cell counts every year, as required by their employers, and it is easy for everybody to undergo a blood test because of the full-cover obligatory health insurance system of Japan. Thus, many patients with hematological diseases are diagnosed before they develop apparent subjective symptoms. Patients whose disorders are

diagnosed early usually have fewer blast cells, compared with symptomatic patients, and therefore they are frequently diagnosed as MDS. Because most patients with *RUNX1*-mutated MDS progress to AML, it is suspected that we may analyze a patient in the MDS phase and others may analyze the AML phase of the same myeloid neoplasm.

Among the disease categories of AML according to the WHO classification, a category of "AML with myelodysplasia-related changes (AML/MLC)" calls our attention to the biological and clinical importance of MDS-related AML, which is associated with multilineage dysplasia, poor-risk cytogenetic findings, age-dependent increased incidence, and a poor response to therapy [Nimer, 2008]. In contrast to de novo AML without significant myelodysplastic features, MDS-related AML is generally considered similar to MDS-RAEB, which develops as a result of accumulated genetic abnormalities in HSC [Nolte and Hofmann, 2008]. It is suspected that MDS-related AML and MDS-RAEB probably, at least in part, develop via identical molecular mechanisms. Furthermore, a category of "therapy-related myeloid neoplasms (t-MN)" is also classified independently. However, gene abnormalities found in therapy-related MDS and AML are also found in sporadic MDS and AML. The frequency of some abnormalities that are sensitive to chemicals is higher in therapy-related cases than in sporadic cases. It seems that the difference between therapy-related cases and spontaneous cases is only that therapy-related cases progress a few steps ahead of spontaneous cases during the stepwise mechanism of myeloid neoplasms. Thus, we think that it is not necessary to consider the molecular mechanisms of these two categories separately, and the molecular mechanism of the therapy-related cases can be applicable to spontaneous cases.

On the basis of these genetic findings, we propose a disease category of "MDN" consisting of MDS-RAEB and AML/MLC, including therapy-related cases. *RUNX1* mutations have been detected in about 20% of patients with "MDN" in our analysis.

RUNX1 MUTATIONS IN RADIATION-EXPOSED PATIENTS WITH MDN

Hematological diseases among the atomic-bomb survivors in Hiroshima and Nagasaki have been well analyzed [Preston et al., 1994]. Acute and chronic leukemias among atomic-bomb survivors appeared after a minimum latency period of 2–3 years, reached a maximum after 6–7 years, decreased slowly with time and then returned to the background level after 30 years [Kato and Shimizu, 1995]. However, MDS incidence increased after long minimum latency periods of 10 or more years, then continued to increase with time, and is still high, even now, more than 60 years after exposure, having similar kinetics to cancers.

The *RUNX1* gene was reported as a target of gene alteration by ionizing radiation and anticancer drugs in experimental systems [Stanulla et al., 1997; Deininger et al., 1998]. Moreover, human leukemias associated with *RUNX1* gene translocations after anticancer therapy or low-dose radiation have been reported [Roulston et al., 1998; Hromas et al., 2000]. These data prompted us to test the frequency of point mutations in the *RUNX1* gene in patients with hematological malignancies, including atomic-bomb survivors in Hiroshima. We found that the *RUNX1* gene was frequently mutated in MDN patients among atomic-bomb survivors and radiation therapy-related MDN patients [Pedersen-Bjergaard et al., 2002; Harada et al., 2003, 2004]. These studies indicate that exposure to radiation may have an effect on the development of MDN through mutations of the *RUNX1* gene.

We also analyzed gene mutations in MDS patients among nuclear victims around the world. The former Soviet Union's first nuclear bomb test was conducted at the Semipalatinsk Nuclear Test Site (SNTS) in the Republic of Kazakhstan, on August 29, 1949. During the following 40 years, there were 456 nuclear explosions including atmospheric and surface events between 1949 and 1962 [Mikhailov, 1996]. As a result, it is suspected that several hundreds of thousands of residents near the SNTS in Kazakhstan were exposed to radiation due to extensive radioactive contamination from the test site. Considerable efforts have been made to assess the radiation doses and the effect of ionizing radiation on populations residing around the SNTS [Gordeev et al., 2002; Stepanenko et al., 2006], and it is well known that solid cancers and leukemias occur more frequently among residents near the SNTS than in the general population [Abylkassimova et al., 2000; Bauer et al., 2005].

The number of patients with MDN in this area is increasing. Morphology of the bone marrow cells from patients with leukemia in the radiation-affected area is quite strange with strong myelodysplasia. The frequency of *RUNX1* mutations in radiation-exposed patients with MDN among the residents near the SNTS was significantly higher compared with unexposed patients. Furthermore, a significant association between *RUNX1* mutations in MDN patients and individual radiation doses was detected [Zharlyganova et al., 2008]. These results suggested that radiation might contribute to the development of MDS/AML through *RUNX1* mutations among the residents near the SNTS. Considering these results, *RUNX1* point mutations might be a specific biomarker that differentiates radio-induced MDN from spontaneous MDN.

In general, the apparent difference in the pattern of onset between leukemia and MDS may be explained by different molecular mechanisms. Chromosomal translocations caused by double-strand DNA breaks resulting from high-dose radiation are likely to contribute to the development of leukemia after a short latency time, whereas point mutations of genes, especially *RUNX1*, induced by low-dose radiation may contribute to the development of MDS decades later. The reason why atomic-bomb survivors have increased risks of various cancers even 60 years after a single radiation exposure is because radiation-induced mutations required for the initiation of carcinogenesis were presumably recorded in long-lived stem cells in various organs with self-renewal capacity [Langlois et al., 1987; Kyoizumi et al., 1996]. Cytogenetic and molecular findings provide evidence that a model of stepwise genetic progression may explain the development and evolution of MDN [Rosenfeld and List, 2000]. In this model, a primary genetic event incites the initial DNA damage and subsequently increases its susceptibility to further damage. Secondary genetic events promote acquisition of the cytogenetic or molecular-genetic abnormalities common to MDN and precipitate additional abnormalities. Thus, one HSC that acquired a *RUNX1* gene mutation due to radiation

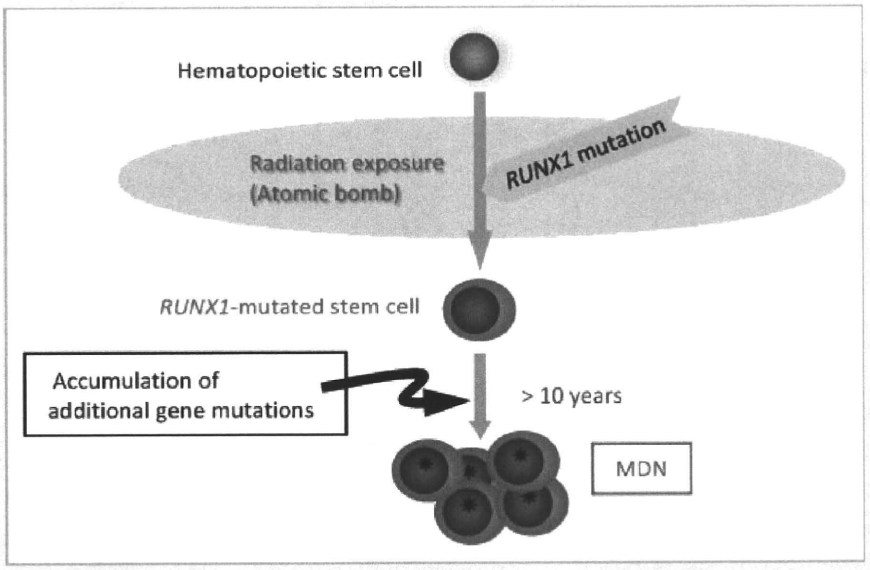

Fig. 2. Molecular mechanisms of radiation-associated MDN.

exposure took decades to be transformed by the accumulation of additional gene alterations, which then led to the development of MDN (Fig. 2).

RUNX1 MUTATIONS IN THERAPY-RELATED MYELOID NEOPLASMS AFTER SUCCESSFUL TREATMENTS FOR ACUTE PROMYELOCYTIC LEUKEMIA

Acute promyelocytic leukemia (APL) is a distinct subtype of AML characterized by a t(15;17) translocation leading to a *PML-RARA* fusion gene. APL is a highly curable disease with excellent complete remission (CR) and long-term survival rates. All-*trans* retinoic acid combined with anthracycline-based chemotherapy yields a CR rate of approximately 90% for newly diagnosed APLs. The relapse rate is approximately 20%, and with the development of new molecular target therapies such as arsenic trioxide, a cure can now be expected even for relapsed patients. However, the development of t-MN is being reported with an increasing frequency of 0.97–6.5% in patients successfully treated for APL [Latagliata et al., 2002; Lobe et al., 2003] and may be more popular than AML other than APL. The t-MN secondary to APL is usually difficult to treat, and it is one of the prognosis-limiting factors for the curable APL disease.

We clarified the different clinical features and hematological findings between t-MN and relapsed APL cases and found that *RUNX1* gene alterations were associated with t-MN [Imagawa et al., 2010]. Among 108 patients during their first CR from APL, 10 patients (9.3%) relapsed and 11 patients (10.2%) developed t-MN after a median follow-up of 8.6 years. It seems that inclusion of VP16 in chemotherapy and the accumulation of chemotherapeutic agents in the maintenance phase may increase the risk of t-MN [Lobe et al., 2003; Asou et al., 2007]. All of the relapse patients had the *PML-RARA* gene, whereas none of the patients with t-MN had *PML-RARA*. Instead, translocations involving 21q22 of *RUNX1* (*RUNX1-MTG16*) or 11q23 of *MLL* (*MLL-FOXO3* and *MLL-CBP*), four *RUNX1* mutations and one *CEBPA* mutation were detected. These abnormalities were not detected at the primary APL diagnosis or in the relapsed patients with APL.

It is assumed that *RUNX1* or other abnormalities may be induced in $CD34^+$ cells during chemotherapy resulting in t-MN after successful treatment of APL. *PML-RARA*-negative t-MN may develop from a "receptive" HSC or from a normal HSC, which is a myeloid committed progenitor, by the accumulation of chemotherapy-induced gene abnormalities, including *RUNX1* mutations (Fig. 3).

RUNX1 MUTATIONS IN LEUKEMIC TRANSFORMATION OF MYELOPROLIFERATIVE NEOPLASMS

The mechanisms that produce MDN from MPN are more complicated, as they involve *JAK2*V617F mutations. MPN including polycythemia vera, essential thrombocythemia, and primary myelofibrosis, are clonal HSC disorders characterized by proliferation of one or more myeloid cell lineages, and they are associated with the *JAK2*V617F mutation [James et al., 2005; Kralovics et al., 2005], whose detection is used in the differential diagnosis of MPN [Jones et al., 2005]. Some patients with MPN exhibit leukemic transformation (LT) after several years of disease, and treatment with alkylating agents, hydroxycarbamide, or their combination may increase the risk of LT [Kiladjian et al., 2006]. Recently, gene alterations involved in LT from patients in the CP of MPN have been identified [Ding et al., 2009; Beer et al., 2010]. Among these gene alterations, including translocations and mutations, a high frequency of *RUNX1* mutations was detected in patients at the LT, whereas no mutation was detected in patients at CP.

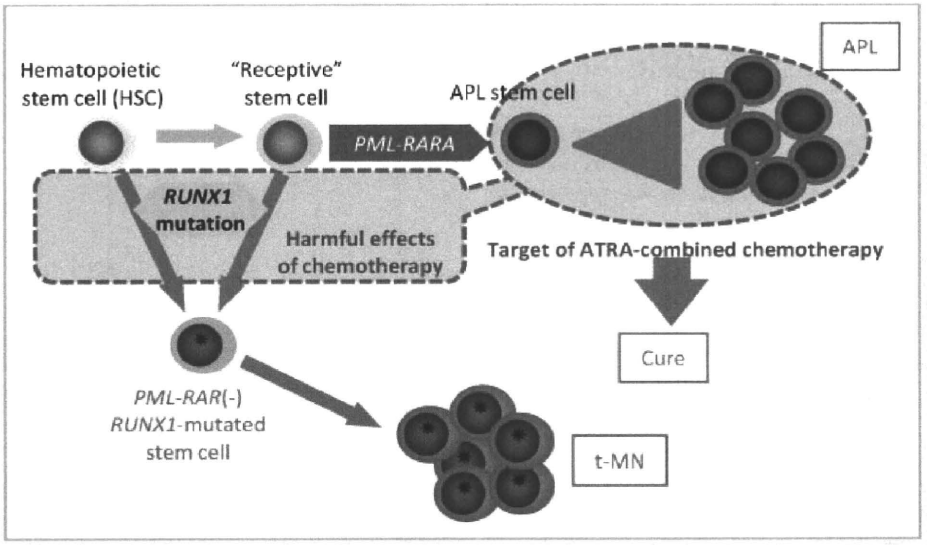

Fig. 3. Molecular mechanisms of t-MN after successful treatment for APL.

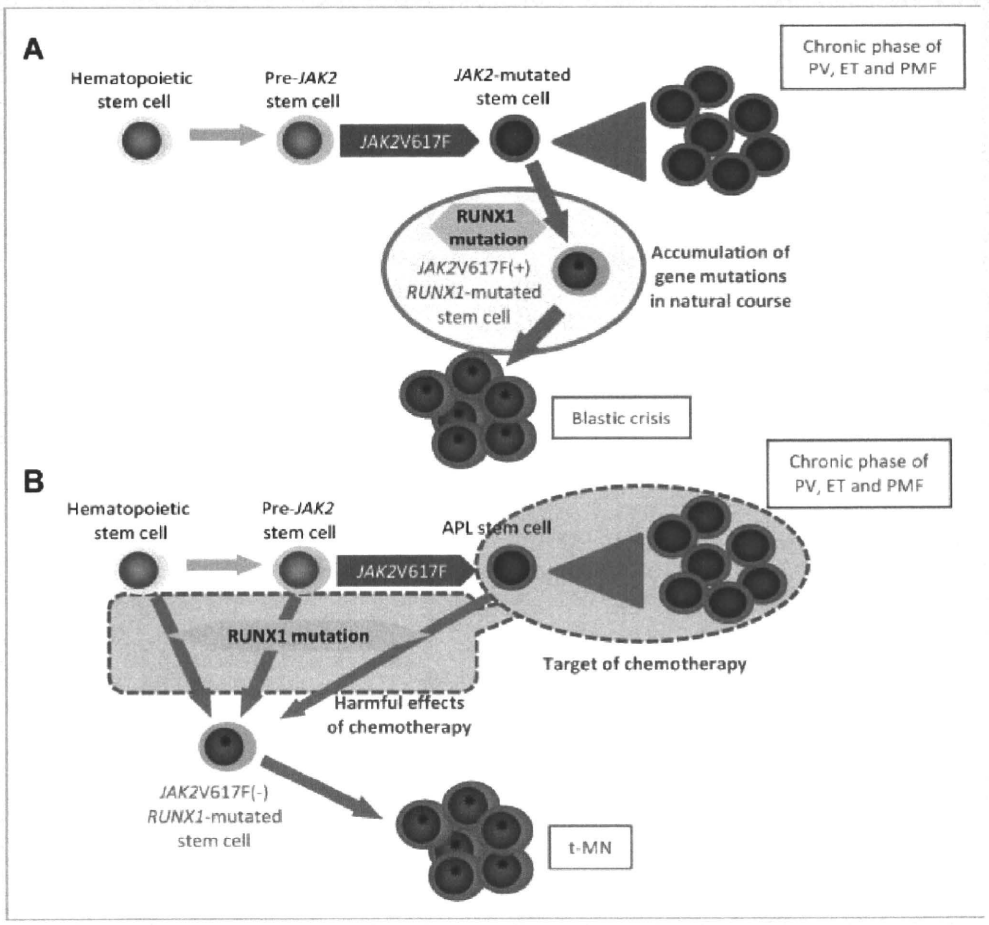

Fig. 4. Molecular mechanisms of LT of myeloproliferative neoplasms (A) blastic crisis-like pathway (B) t-MN pathway.

In contrast to the fact that the *BCR-ABL* fusion gene is retained in all cases of chronic myeloid leukemia (CML) in blast crisis (BC), half of the patients with *JAK2*V617F-positive MPN were reported to transform to *JAK2*V617F-negative AML, suggesting that leukemia developed from a *JAK2*V617F-negative (pre-*JAK2*) HSC [Campbell et al., 2006; Levine and Gilliland, 2008] or normal HSC [Beer et al., 2010]. However, other *JAK2*V617F-positive patients did not lose their *JAK2*V617F mutation during CP to LT, indicating that their leukemia probably arose in *JAK2*V617F-positive HSCs [Ding et al., 2009; Beer et al., 2010]. This hypothesis about origins of LT cells was supported by the recent report of chromosomal abnormalities analyzed by high-resolution single nucleotide polymorphism array [Thoennissen et al., 2010]. Patients with *JAK2*V617F-positive AML showed the same chromosomal alterations, with some additional changes, as those in CP, suggesting that the origin of the LT cells was *JAK2*V617F-positive HSCs. Patients with *JAK2*V617F-negative AML from *JAK2*V617F-positive MPN revealed quite different patterns from their CP, indicating their different origin, but a few common genetic abnormalities were detected between CP and LT, supporting the origin of the pre-*JAK2* HSCs.

RUNX1 mutations were detected at LT in both *JAK2*V617F-positive and -negative MPN patients, raising the possibility that the HSCs may have been transformed into leukemic blasts as a result of *RUNX1* mutations. Furthermore, most of the patients had undergone chemotherapy, suggesting that the LT in patients with MPN may be caused in part by gene abnormalities acquired due to chemotherapy. However, a few patients with *JAK2*V617F-positive MPN who were not treated with chemotherapeutic reagents also transformed to *JAK2*V617F-positive leukemia with *RUNX1* mutation. Thus, there may be another LT pathway that acquires a *RUNX1* mutation in the natural course of MPN. This mechanism is similar to the BC of CML (CML-BC). *RUNX1* rearrangements such as t(3;21) are frequently seen in CML-BC, and a *RUNX1* mutation in CML-BC was also reported [Osato et al., 1999].

To clarify the leukemogenic effect of RUNX1 mutants, the RUNX1 D171N mutant was transduced into $CD34^+$ cells from patients in the CP of MPN [Ding et al., 2009]. The effect of this mutant on cell differentiation/proliferation was assessed by colony-forming cells re-plating assays. The D171N-transduced cells formed fewer erythroid colonies and more myeloid colonies, retained more $CD34^+$ cells, proliferated more strongly than the control, and formed colonies after a third plating. Furthermore, long-term culture-initiating cells, a small minority of more primitive progenitors/stem cells among the $CD34^+$ cells, that have capability of self-renewal and clonogenic capacity after prolonged in vitro culture, increased significantly in the cells transduced with D171N. Thus, the RUNX1 mutant transduced into $CD34^+$ cells from MPN patients promoted proliferation of primitive progenitors, i.e., leukemic stem cells. These results indicate that *RUNX1* mutations may have a leukemogenic potential in a *JAK2*V617-positive HSC, in a pre-*JAK2* HSC, or in a normal HSC, and they may promote LT in MPN (Fig. 4).

CONCLUSION

Our recent study showed that *RUNX1* mutations define the molecular mechanisms of MDN. Once a *RUNX1* mutation occurs in a normal HSC, spontaneous additional gene abnormalities, which may be induced by the *RUNX1* mutation in part, are accumulated in the cell during a long latency period, MDN may then develop. Meanwhile, if a *RUNX1* mutation occurs in a "receptive" HSC that has already accumulated other gene abnormalities, the cell develops MDN over a short period. Thus, the *RUNX1* mutation is considered to be one of the disease-deciding factors of MDN, and we strongly propose that *RUNX1* mutations could be one of the genetic classification categories of MDS and AML.

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