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ORIGINAL ARTICLE

C-terminal mutation of *RUNX1* attenuates the DNA-damage repair response in hematopoietic stem cells

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Loss-of-function mutations of *RUNX1* have been found in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDSs). Although several reports have suggested roles for RUNX1 as a tumor suppressor, its precise function remains unknown. Because gene alterations of *RUNX1* by themselves do not lead to the development of leukemia in mouse models, additional mutation(s) would be required for leukemia development. Here, we report that the C-terminal deletion mutant of *RUNX1*, RUNX1dC, attenuates DNA-damage repair responses in hematopoietic stem/progenitor cells. γH2AX foci, which indicate the presence of DNA double-strand breaks, were more abundantly accumulated in RUNX1dC-transduced lineage$^-$ Sca1$^+$c-kit$^+$ (LSK) cells than in mock-transduced LSK cells both in a steady state and after γ-ray treatment. Expression profiling by real-time -PCR array revealed RUNX1dC represses the expression of *Gadd45a*, a sensor of DNA stress. Furthermore, bone marrow cells from MDS/AML patients harboring the *RUNX1*-C-terminal mutation showed significantly lower levels of *GADD45A* expression compared with those from MDS/AML patients with wild-type *RUNX1*. As for this mechanism, we found that RUNX1 directly regulates the transcription of *GADD45A* and that RUNX1 and p53 synergistically activate the *GADD45A* transcription. Together, these results suggest Gadd45a dysfunction due to *RUNX1* mutations can cause additional mutation(s) required for multi-step leukemogenesis.
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Introduction

Myelodysplastic syndromes (MDSs) are clonal hematological disorders derived from gene alterations at the level of hematopoietic stem cells, which are characterized by ineffective hematopoiesis, dysplastic morphology of blood cells and a high possibility of transition to acute myeloid leukemia (AML). A number of genetic or epigenetic alterations involved in the pathogenesis of MDS have been identified: activating point mutations of signaling molecules such as N-RAS and FLT3;[1] deletion, point mutations and/or silencing of cell cycle inhibitory molecules such as p15 and p53;[2,3] deletion, point mutations and generation of chimeric genes from transcriptional factors such as EVI-1 and RUNX1;[4,5] and point mutations of nuclear proteins such as nucleophosmin and TET2.[6,7] Among these changes, point mutations of *RUNX1* have been detected in about 10–20% of patients classified as MDS/AML (high-risk MDS and AML following MDS).[5] The transcription factor RUNX1 and its heterodimeric partner core-binding factor (CBF)β (also known as phosphatidylethanolamine-binding protein2β) comprise CBFs. CBFs are the most frequent targets of gene rearrangement and mutation in human leukemias; leukemias harboring mutations in either subunit of a CBF are commonly called CBF leukemias.[8] Recently, Tang *et al.*[9] reported *RUNX1* mutations were detected in 13.2% of 470 adult patients with *de novo* AML. In addition, hereditary loss-of-function mutations of *RUNX1* cause familial platelet disorder with predisposition to AML, which is characterized by decreased platelet count and propensity to develop AML.[10] These findings suggest RUNX1 works as a tumor suppressor and impaired RUNX1 function promotes leukemia development. Nonetheless, *RUNX1* deletion or dominant-negative inhibition of RUNX1 by itself is not sufficient for leukemia development in several mouse models,[11,12] indicating that additional cooperating events are required. However, the mechanisms by which impaired RUNX1 functions lead to subsequent genetic alterations are not fully understood.

Cells in the human body are always exposed to DNA stresses, which induce damages to chromosomal DNA.[13] Physiological stresses such as hydrolytic reactions, non-enzymatic methylations and oxygen radicals generate DNA-base lesions.[14] Environmental agents such as ultraviolet (UV), ionizing radiation and a lot of genotoxic chemicals also induce DNA damages including single- and double-strand breaks (DSBs). These DNA lesions are repaired through damage-specific repair pathways. However, if these lesions are left unrepaired, these cells alone and/or in combination with additional mutations would have a higher risk of tumor development.[15] In a previous study, it was reported that *Runx1*-deficient mice had an increased incidence of hematological malignancy compared with wild-type (WT) mice after treatment with the mutagen, *N*-ethyl-*N*-nitrosourea.[16]

We speculated RUNX1 might have a role in the DNA-damage repair (DDR) response. Here, we show that a C-terminal deletion mutant of *RUNX1*, RUNX1dC, enhances DNA-damage accumulation in hematopoietic stem cell-enriched lineage$^-$Sca1$^+$c-kit$^+$ (LSK) cells. Furthermore, we found RUNX1dC attenuates the DDR response after exposure to DNA-damage agents. As for this mechanism, we found that RUNX1dC suppresses the transcription of a sensor of DNA stress, *Gadd45a*.[17] Moreover, bone marrow (BM) cells from MDS/AML patients harboring a *RUNX1*-C-terminal mutation showed significantly lower *GADD45A* expression than those from MDS/AML patients with WT *RUNX1*. These results suggest RUNX1

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regulates *Gadd45a* transcription and impaired RUNX1 function can cause additional mutation(s) required for multi-step leukemogenesis.

Materials and methods

Real-time (RT)-PCR array

Total cellular RNA was extracted from 4×10^6 cells using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). A total of 4 μg of RNA was reverse-transcribed into cDNA using the RT^2 First Strand Kit (SABiosciences, Frederick, MD, USA) and subjected to RT-PCR array analysis (RT^2Profiler PCR Array: Mouse DNA Damage Signaling, SABiosciences). Gene expression profiles of 32D-neo and 32D-RUNX1dC cells were analyzed by the ΔΔCt method.

Colony-forming assay

32D-neo and 32D-RUNX1dC cells (1×10^5 cells) were suspended in 500 μl of phosphate buffered saline and exposed to UV-B (800 or 1600 J/m^2). Then, cells were washed twice with PBS containing 2% FBS, plated into dishes (1×10^3 cells/dish) and cultured in the methylcellulose media M3231 (Stem Cell Technologies, Vancouver, British Colombia, Canada) containing mouse interleukin-3 (mIL-3). Colony numbers were counted after 7 days of culture. Also, retrovirus-transfected LSK cells were exposed to UV-B (2400 J/m^2), plated into dishes (2×10^3 cells/dish) and cultured in methylcellulose media M3434 (Stem Cell Technologies) containing mSCF, mIL-3, human IL-6 (hIL-6) and human erythropoietin. Colony numbers were counted after 10 days. All experiments were carried out in triplicate. DNA repair activities of the test cells are represented relative to those of untreated cells.

Quantitative PCR assays for the expression of GADD45A in MDS/AML patients

Total cellular RNA was extracted from BM mononuclear cells of 23 MDS/AML patients, 5 healthy donors and 5 non-Hodgkin

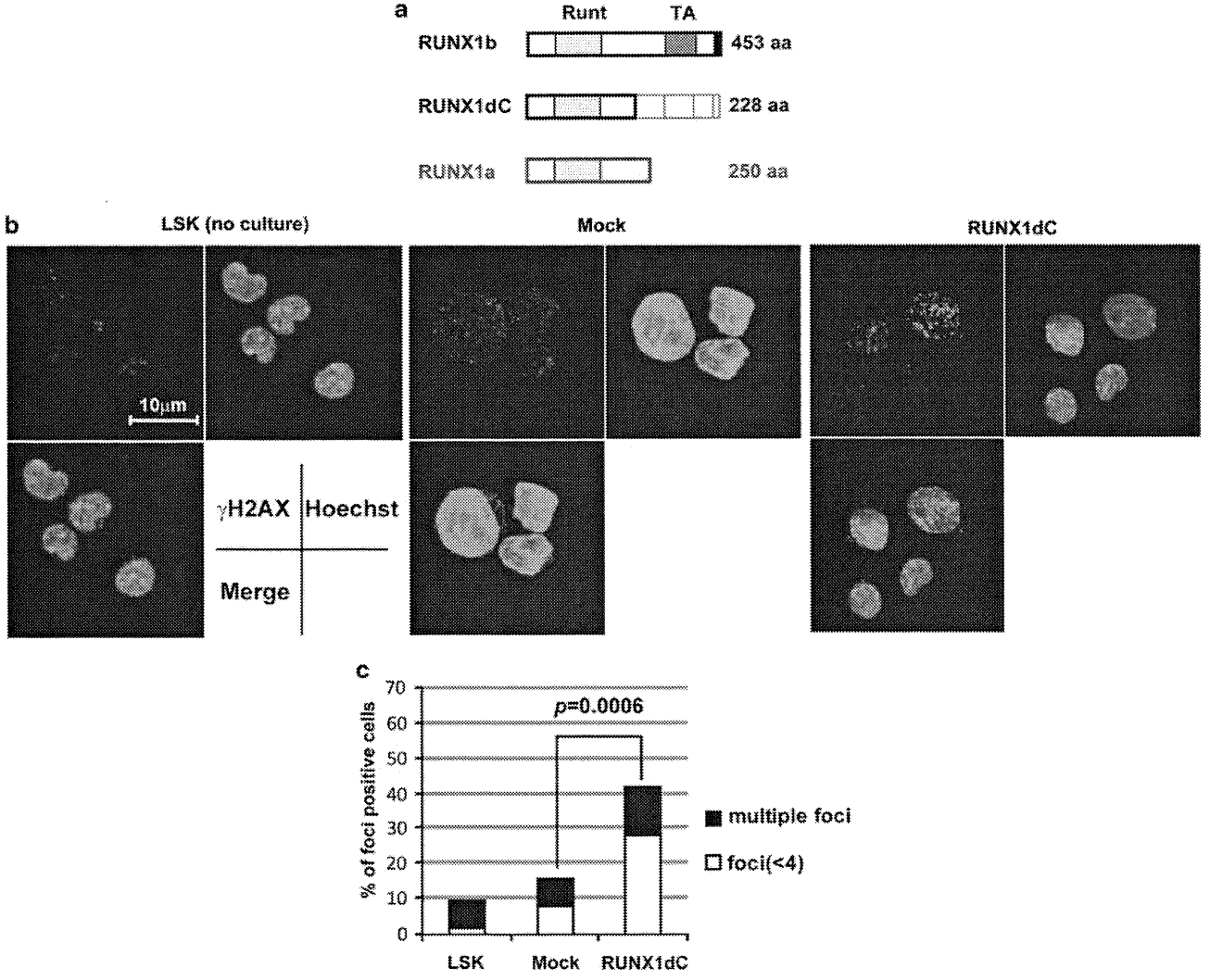

Figure 1 RUNX1dC induces DNA-damage accumulation in LSK cells. (**a**) Structures of WT RUNX1 (RUNX1b), RUNX1dC and RUNX1a. RUNX1dC lacks 225 C-terminal amino acids because of the insertion of ACCGT at 669–670 bp, which causes a frameshift mutation. (**b**) Accumulations of DSBs in Mock- and RUNX1dC-transduced LSK cells were detected by γH2AX antibody (2 days after gene transduction). LSK refers to LSK cells just after FACS sorting. Hoechst refers to Hoechst 33342. The scale bar (10 μm) applies to all images. (**c**) The percentage of γH2AX foci-positive cells in Mock- and RUNX1dC-transduced LSK cells. Results shown are the average of three experiments. Open square indicates % of γH2AX foci-positive cells (with fewer than four foci per cell), and closed square indicates % of multiple foci-positive cells (four or more foci per cell).

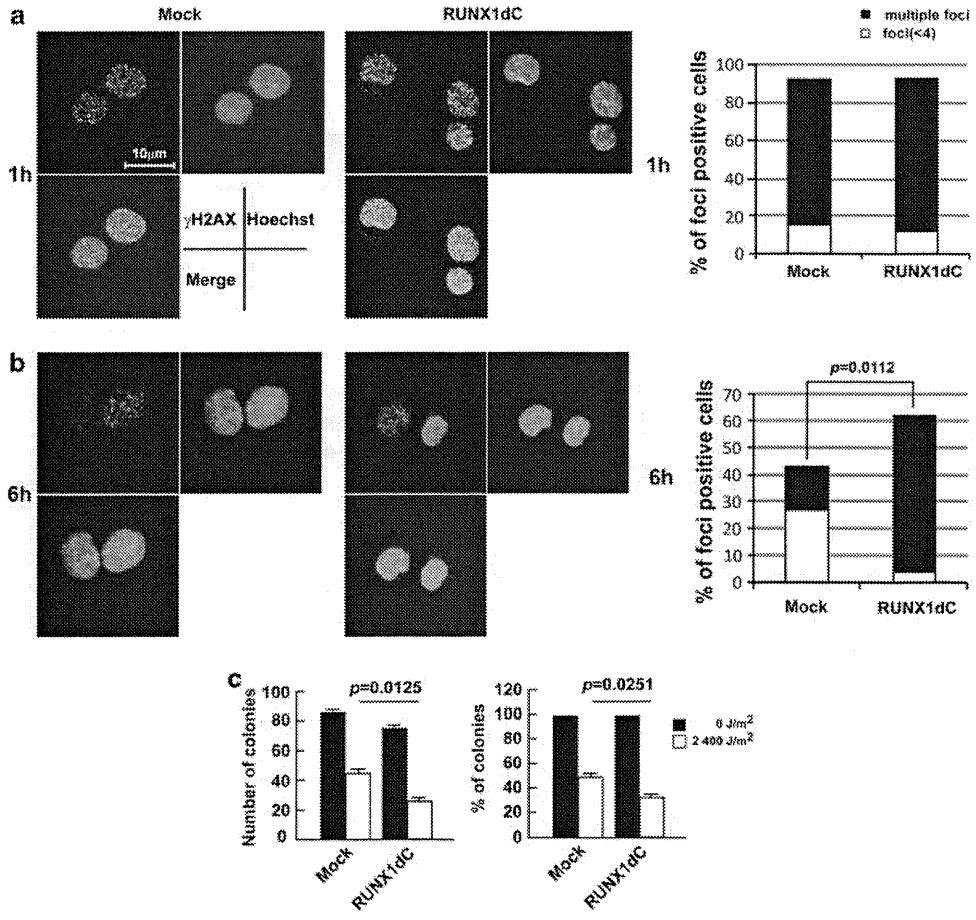

Figure 2 RUNX1dC-transduced cells attenuate the DDR response to double-strand breaks in LSK cells. (**a**) Accumulations of γH2AX foci in Mock- and RUNX1dC-transduced LSK cells 1 h after γ-ray treatment (left panel). Hoechst refers to Hoechst 33342. The scale bar (10 μm) applies to all images. The percentage of γH2AX foci-positive cells in Mock- and RUNX1dC-transduced LSK cells are shown (right panel). Results are the average of three experiments. Open square indicates % of γH2AX foci-positive cells (with fewer than four foci per cell), and closed square indicates % of multiple foci-positive cells (four or more foci per cell). (**b**) Accumulations of γH2AX foci in Mock- and RUNX1dC-transduced LSK cells 6 h after γ-ray treatment (left panel). The percentage of γH2AX foci-positive cells in Mock- and RUNX1dC-transduced LSK cells 6 h after γ-ray treatment are shown (right panel). (**c**) Colony-forming ability of Mock- and RUNX1dC-transduced LSK cells after UV-B treatment. Each bar shows absolute colony numbers (left panel) or the % of colonies relative to those of non-treated cells (right panel). Data are means ± s.e.m. of triplicate experiments.

lymphoma patients, who did not have BM involvement of the disease, as controls. All of the MDS/AML patients were diagnosed at Hiroshima University Hospital and its affiliated hospitals between 2000 and 2005. Diagnosis was made based on morphologic and immunophenotypic studies according to the French-American-British classification.[18] Clinical characteristics of MDS/AML patients are shown in Supplementary Table 2.[19] This project was approved by the Institutional Review Board at Hiroshima University, and BM samples were taken after obtaining informed consent. Probes and primers used in the quantitative PCR assay are listed in the Supplementary Methods.

Statistical analysis

All values are expressed as means ± s.e.m. We performed statistical analysis using Student's *t*-test. Values were considered statistically significant at $P<0.05$.

Results

RUNX1dC induces DNA-damage accumulation in LSK cells

DNA-damage accumulation and its repair pathways have important roles in tumor development.[13] Thus, we first evaluated DNA-damage accumulation in a C-terminal truncated RUNX1 mutant-transduced LSK cells. In this study, we utilized the RUNX1dC, which lacks 225 amino acids covering both transactivating and transrepressing domains of WT RUNX1 (Figure 1a). This mutant was originally identified in a patient with MDS, and we and others previously showed it functions as a dominant-negative mutant over WT RUNX1 (RUNX1b) by inhibiting its DNA-binding activity.[20,21] RUNX1dC resembles in size an isoform of RUNX1, RUNX1a, (Figure 1a), which also has an inhibitory effect against RUNX1b.[22] Two days after gene transduction, we evaluated accumulations of DSBs using an antibody recognizing the phosphorylated histone variant H2AX at serine 139 (γH2AX).[23] As shown in Figures 1b and c, more DSBs were detected in RUNX1dC-transduced LSK cells than in Mock-transduced LSK cells (42% foci-positive cells in RUNX1dC-transduced cells vs 15% foci-positive cells in Mock-transduced cells, $P=0.0006$).

RUNX1dC transduction results in the attenuation of the DDR response to DSBs in LSK cells

We next examined the DDR response in RUNX1dC-transduced LSK cells after exposure to environmental stressors. γH2AX foci, which indicate the immediate chromatin modification in

response to the formation of DSBs, reached a peak 30–60 min after irradiation (Figure 2a). Thereafter, the number of foci decreased with a half-life of several hours due to the DNA-repair response to DSBs.[24] Therefore, sustained accumulation of γH2AX foci indicates attenuation of the DDR response to DSBs. A total of 6 h after γ-ray treatment, RUNX1dC-transduced cells showed significantly higher accumulation of γH2AX foci than Mock-transduced LSK cells ($P=0.0112$; Figure 2b). Moreover, although the percentage of multiple foci-positive cells carrying more severe accumulations of DSBs was only 37.6% among γH2AX-positive cells in Mock transduction, most (93.5%) γH2AX-positive cells had multiple foci in RUNX1dC-transduced LSK cells (Figure 2b right panel). In addition, we analyzed the colony-forming ability of Mock- or RUNX1dC-transduced LSK cells after treatment with UV-B. In this assay, the DDR response of tested cells can be measured by relative colony numbers because only cells that recovered from DNA damage can make colonies.[25] At steady state, there was no significant difference in colony-forming ability between Mock- and RUNX1dC-transduced LSK cells (Figure 2c left panel). On the other hand, analyses of relative colony numbers revealed RUNX1dC-transduced LSK cells showed significantly lower colony-forming ability than Mock-transduced LSK cells after UV-B exposure (32.2% in RUNX1dC-transduced LSK vs 48.8% in Mock-transduced LSK, $P=0.0251$; Figure 2c right panel).

RUNX1dC suppresses Gadd45a expression in LSK cells

As DSBs were more prominent in RUNX1dC-transduced LSK cells than in Mock-transduced LSK cells, we speculated that the defect in the DDR response gene may be a cause of enhanced DNA-damage accumulation in RUNX1dC-transduced LSK cells. Therefore, we next examined whether RUNX1dC alters expressions of DNA-damage signaling molecules. We prepared 32Dcl3 cells expressing RUNX1dC and confirmed expression by immunoblot (Supplementary Figure 1). We profiled the expression of 84 genes involved in DNA-damage signaling by RT-PCR array using this subclone. Before performing the RT-PCR array experiment, we confirmed 32D-RUNX1dC showed significantly lower colony-forming ability than 32D-neo cells after UV-B exposure, just like RUNX1dC-transduced LSK cells (at 1600 J/m^2: 38.2% in 32D-RUNX1dC vs 67.2% in 32D-neo, $P<0.0234$; Figure 3a right panel). Furthermore, we evaluated the accumulation of cyclobutane pyrimidine dimers, which are major products of DNA damage induced by UV-B, in 32D-neo and 32D-RUNX1dC cells.[26] There was no distinct difference in cyclobutane pyrimidine dimer accumulation between 32D-neo and 32D-RUNX1dC cells before UV-B treatment (Figure 3b). Although 1 h after UV-B treatment the levels of cyclobutane pyrimidine dimer accumulation were the same in 32D-neo and 32D-RUNX1dC cells, the removal of cyclobutane pyrimidine dimer was significantly impaired in 32D-RUNX1dC cells compared with 32D-neo (Figure 3b). In addition, as with RUNX1dC-transduced LSK cells, 32D-RUNX1dC cells showed sustained accumulation of γH2AX foci after γ-ray irradiation (Figure 4b). After correction using five housekeeping genes (*Gusb*, *Hprt1*, *Hsp90ab1*, *Gapdh* and *Actb*), we obtained four candidate genes, in which expression was repressed in 32D-RUNX1dC cells compared with 32D-neo cells with a fold-difference cutoff of <0.5 (Figure 3c and Supplementary Table 1). Among these candidate genes, the expression of *Gadd45a* was particularly decreased in 32D-RUNX1dC cells compared with 32D-neo cells (Figure 3c and Supplementary Figure 2a). Quantitative RT-PCR using 32D-RUNX1dC and 32D-neo cells confirmed the expression of *Gadd45a* was

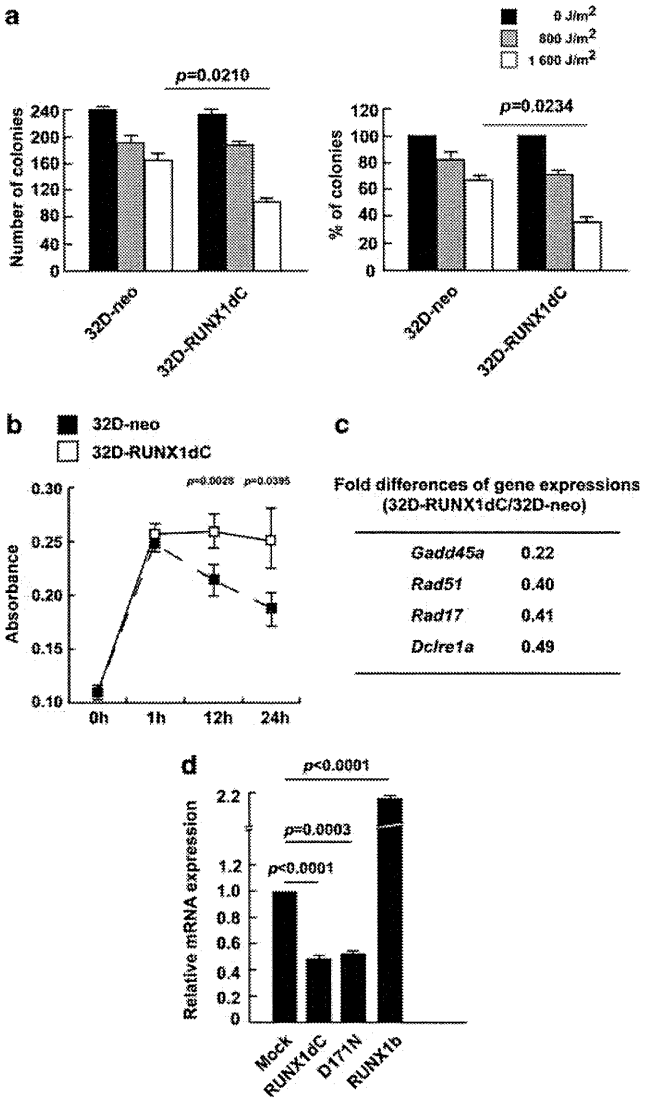

Figure 3 RUNX1dC suppresses *Gadd45a* expression in LSK cells. (**a**) Colony-forming assays for 32D-neo and 32D-RUNX1dC cells after treatment with UV-B. Test cells (1×10^3) were plated onto methylcellulose medium and cultured with IL-3. Each bar shows absolute colony numbers (left panel) or the % of colonies relative to that of non-treated cells (right panel). Data are means ± s.e.m. of triplicate experiments. (**b**) Determination of UV-B induced DNA damage by enzyme linked immunosorbant assay using anti-cyclobutane pyrimidine dimers antibody. Data are means ± s.e.m. of quadruplicate experiments. (**c**) Expression profiling of 84 genes involved in DNA-damage signaling by RT-PCR array. Fold differences of gene expressions with a cutoff of <0.5 (32D-RUNX1dC/32D-neo) are shown. (**d**) Relative mRNA expression levels of *Gadd45a* in Mock-, RUNX1dC-, D171N- and RUNX1b-transduced murine LSK cells (normalized to *Gapdh*). Data are means ± s.e.m. of triplicate experiments.

significantly lower (42%) in 32D-RUNX1dC cells than in 32D-neo cells (Supplementary Figure 2b). To examine if this result was relevant to normal LSK cells, we also examined the expression of *Gadd45a* in RUNX1dC-transduced LSK cells. RUNX1dC-transduced LSK cells showed significantly lower expression (44%) of *Gadd45a* than Mock-transduced LSK cells (Figure 3d). LSK cells, which were transduced with a RUNX1 mutant harboring a point mutation in the Runt homology domain (D171N)[19,21] also showed significantly lower (53%) expression of *Gadd45a* compared with Mock-transduced LSK

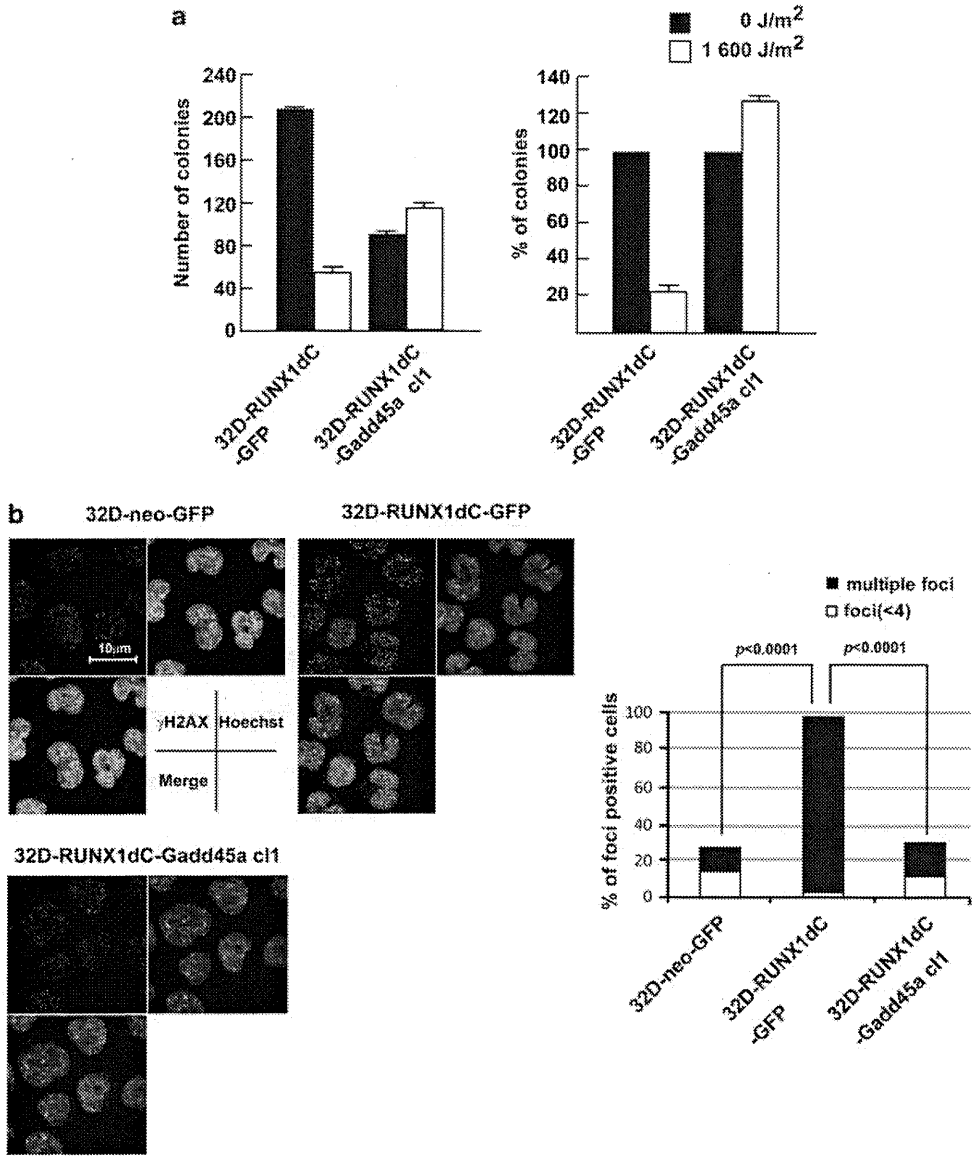

Figure 4 Exogenously expressed Gadd45a reduced γH2AX-positive foci in 32D-RUNX1dC. (**a**) Colony-forming ability of 32D-RUNX1dC-GFP and 32D-RUNX1dC-Gadd45a cl1 cells after UV-B treatment. Each bar shows absolute colony numbers (left panel) or the % colonies relative to those of non-treated cells (right panel). Data are means ± s.e.m. of triplicate experiments. (**b**) Accumulations of γH2AX foci in 32D-neo-GFP, 32D-RUNX1dC-GFP and 32D-RUNX1dC-Gadd45a cl1 cells 6 h after γ-ray treatment (left panel). The percentage of γH2AX foci-positive cells in 32D-neo-GFP, 32D-RUNX1dC-GFP and 32D-RUNX1dC-Gadd45a cl1 cells 6 h after γ-ray treatment are shown (right panel). Results are the average of three experiments. Open square indicates % of γH2AX foci-positive cells (with fewer than four foci per cell), and closed square indicates % of multiple foci-positive cells (four or more foci per cell).

cells (Figure 3d). Moreover, WT RUNX1-transduced LSK cells expressed more *Gadd45a*, an increase of 108% relative to Mock-transduced LSK cells (Figure 3d). To examine whether the impaired colony-forming ability of 32D-RUNX1dC cells attributed to the repressed expression of *Gadd45a* by RUNX1dC, we introduced the expression vector for Gadd45a into 32D-RUNX1dC cells, which was named 32D-RUNX1dC-Gadd45a (Supplementary Figure 3a). Consistent with the report from Perugini *et al.*,[27] Gadd45a overexpression reduced the cell proliferation ability at steady state (Figure 4a left panel). After UV-B treatment, exogenously expressed Gadd45a restored the colony-forming ability in 32D-RUNX1dC-Gadd45a cl1 cells (32D-RUNX1dC-GFP, 21.8%; 32D-RUNX1dC-Gadd45a cl1, 132%; Figure 4a right panel). Similar results were obtained from two other clones (Supplementary Figure 3b). Furthermore, we found exogenously expressed Gadd45a reduced γH2AX-positive foci in 32D-RUNX1dC-Gadd45a cl1 cells compared with 32D-RUNX1dC-GFP cells after UV-B treatment (Figure 4b). In addition, the reduction of γH2AX-positive foci in 32D-RUNX1dC-Gadd45a cl1 cells was also observed at steady state (Supplementary Figure 3c).

RUNX1 transcriptionally regulates Gadd45a expression

To analyze the mechanism by which RUNX1 regulates *Gadd45a* expression, we utilized luciferase assays using a human myeloid leukemia cell line UT-7/GM. Although there was no consensus sequence for RUNX binding in the 5′-flanking region of the human *GADD45A* gene, we found two RUNX-binding sequences neighboring the p53-binding site in

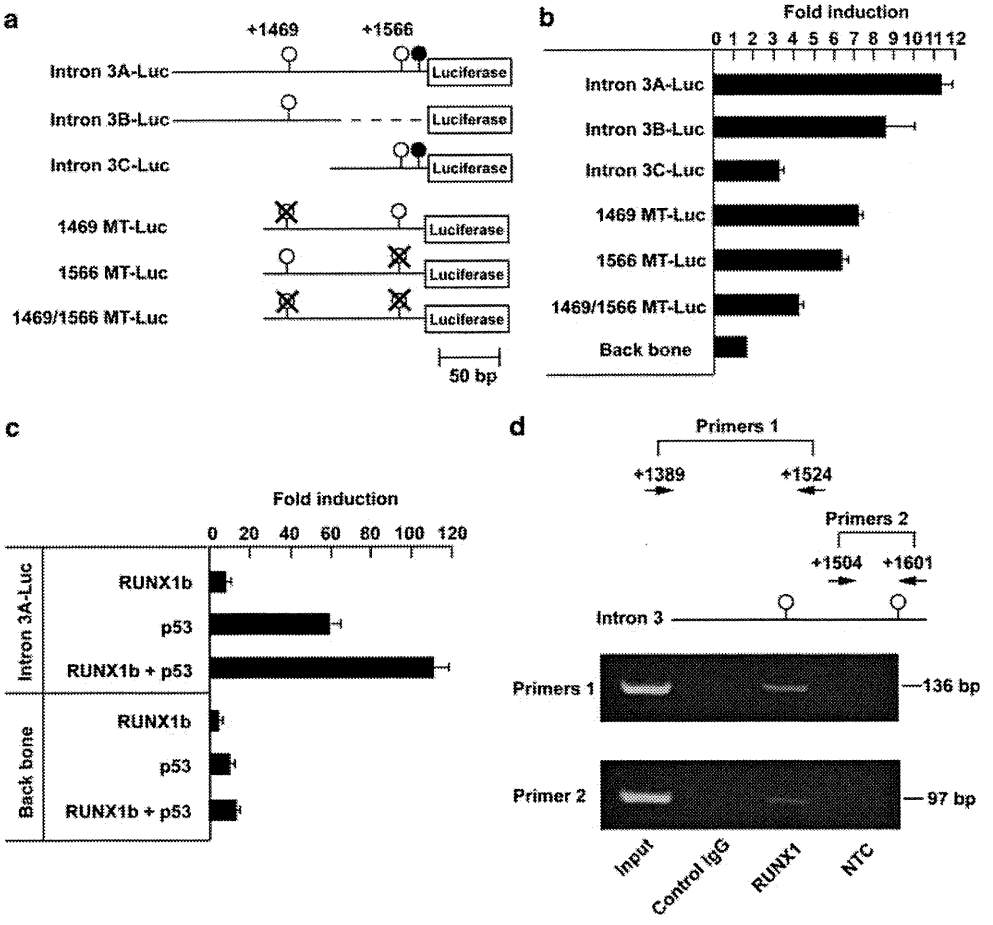

Figure 5 Analyses of transcriptional regulation of *GADD45A* by RUNX1. (**a**) Structures of human *GADD45A* luciferase reporter genes. Open circle indicates RUNX-binding sequences (at +1469 and +1566) and the closed circle indicates the reported p53-binding site in intron 3 of *GADD45A*. Crossed open circle indicates a mutation of the RUNX-binding sequence (TGTGGT changed into TGTTAG). (**b**) Reporter assays using human *GADD45A* luciferase reporter genes. Each bar indicates the fold induction of reporter activity induced by RUNX1b (relative to pRC-CMV control vector). UT-7/GM cells were transfected with 2 μg of each reporter gene (intron 3A-Luc (containing +1389–1601), intron 3B-Luc (+1389–1524), intron 3C-Luc (+1505–1601), 1469 MT-Luc, 1566 MT-Luc and 1469/1566 MT-Luc) or back bone plasmid, 0.5 μg of pRL-CMV-Rluc, 1 μg of CBFβ, and 2 μg of RUNX1b or pRC-CMV. Results shown are the means ± s.e.m. of triplicate experiments. (**c**) Co-expression experiment of RUNX1b and p53 using the intron 3A-Luc construct. Each bar indicates the fold induction of reporter activity induced by RUNX1b and/or p53 (relative to pRC-CMV control vector). Cells were transfected with 2 μg each of RUNX1b and/or p53 with 2 μg of intron 3A-Luc or Back bone plasmid, 0.5 μg of pRL-CMV-Rluc and 1 μg of CBFβ. Results shown are the means ± s.e.m. of triplicate experiments. (**d**) ChIP assays using the nuclear extract of UT-7/GM cells with the anti-RUNX1 Ab or control goat IgG. Two sets of closed arrows indicated primer positions in intron 3 of the *GADD45A* gene. Open circle indicates the RUNX-binding sequences and NTC means non-template control.

intron 3 of the *GADD45A* gene. Intron 3 is highly conserved between humans and rodents, and p53 transcriptionally activates *GADD45A* expression through this p53-binding site.[28,29] Therefore, we generated three types of luciferase reporter genes covering this region. Intron 3A-Luc contained the two RUNX-binding sequences (at +1469 and +1566) of the human *GADD45A* gene. Intron 3B-Luc contained only the upstream binding sequence (+1469) and intron 3C-Luc contained only the downstream binding sequence (+1566; Figure 5a). RUNX1b together with its heterodimerization partner CBFβ activated intron 3A-Luc, intron 3B-Luc and intron 3C-Luc 11.2-fold, 8.6-fold, and 3.2-fold, respectively (Figure 5b). The 1469 MT-Luc and 1566 MT-Luc constructs, which have one mutated RUNX-binding sequence (TGTGGT changed to TGTTAG), showed reduced reporter activity by RUNX1b compared with intron 3A-Luc (7.3-fold activation in 1469 MT-Luc and 6.1-fold activation in 1566 MT-Luc, Figures 5a and b). Furthermore, the 1469/1566 MT-Luc construct, which has two mutated RUNX-binding sequences, showed a 63% reduction of reporter activity by RUNX1 compared with intron 3A-Luc (4.1-fold activation). In addition, the co-expression experiment with RUNX1b and p53 revealed these two genes synergistically activate the intron 3A-Luc construct (only p53: 59.6-fold activation and p53 with RUNX1: 108.4-fold activation; Figure 5c). We also examined the change in intron 3A-Luc activation by RUNX1b and/or p53 after γ-ray irradiation; yet, there was no difference in reporter activities between γ-ray irradiated and non-irradiated UT-7/GM cells (data not shown). Next, we examined the co-expression effects of RUNX1 mutants on *GADD45A*-reporter activation with RUNX1b. We had to transfect high amount of DNA into test cells; therefore we utilized the adherent cell line, 293T, to perform the luciferase assay.[20] Single transduction experiment of RUNX1dC revealed RUNX1dC showed reduced *GADD45A*-reporter activity compared with the transduction of RUNX1b (10-fold activation in RUNX1b and 5-fold activation in RUNX1dC). In the case of the D171N mutant, the D171N mutant did not show the significant *GADD45A*-reporter activity (3.3-fold) compared with back-bone

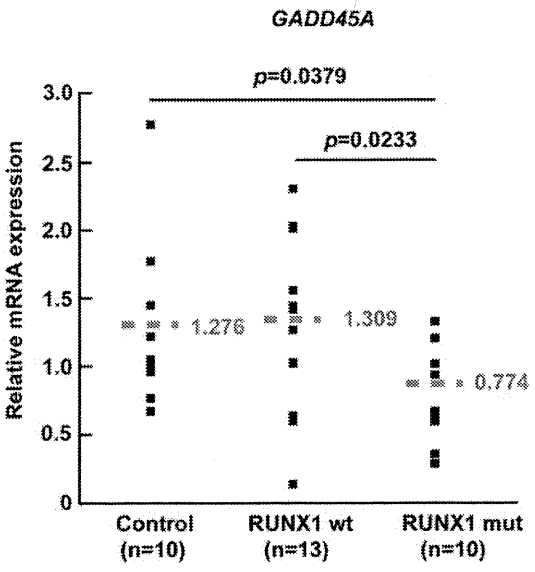

Figure 6 Expression levels of *GADD45A* in MDS/AML patients. Relative expression levels of *GADD45A* in BMMNCs from controls and MDS/AML patients with WT *RUNX1* (RUNX1 wt) or C-terminal mutations (RUNX1 mut) are shown. Horizontal bar and value denotes mean *GADD45A* expressions (normalized to *GAPDH*). The patients' characteristics are shown in Supplementary Table 2.

reporter activity (2.9-fold). In addition, we found RUNX1b-induced intron 3A-Luc activity was reduced to 55% by RUNX1dC and to 37% by the D171N mutant (Supplementary Figure 4). To test whether endogenous RUNX1 binds to the *GADD45A* gene *in vivo*, we conducted ChIP assays using the nuclear extract of UT-7/GM cells. As shown in Figure 5d, the two RUNX-binding sequences were immunoprecipitated with the anti-RUNX1 antibody but not by control IgG. Together, these data indicate that endogenous RUNX1 binds to two RUNX1-binding sites in intron 3 of the *GADD45A* gene, thereby regulating its transcription.

GADD45A expression is significantly decreased in MDS/AML patients harboring RUNX1-C-terminal mutations compared with those with WT RUNX1

Finally, we evaluated *GADD45A* expression in BM mononuclear cells from 23 MDS/AML patients with or without *RUNX1* C-terminal mutations and from 10 controls.[19] We found BM mononuclear cells from MDS/AML patients harboring *RUNX1*-C-terminal mutations had significantly lower *GADD45A* expression compared with those from MDS/AML patients with WT *RUNX1* ($P=0.0233$; Figure 6).

Discussion

We found RUNX1 transcriptionally regulates *Gadd45a*. In our experiment using LSK cells, the expression of *Gadd45a* was reduced to 44% of that of control cells by RUNX1dC (Figure 3d). This level of *Gadd45a* expression is roughly the same as that observed in *Gadd45a*$^{+/-}$ mice, which do not develop spontaneous tumors but are susceptible to tumor development in response to mutagens because of genomic instability.[30] Like *Gadd45a*$^{+/-}$ mice, heterozygous deletion of *RUNX1* predisposes mice to AML when accompanied by other gene alterations.[31] The similar phenotypes of *Gadd45a*$^{+/-}$ and *RUNX1*$^{+/-}$ mice raise the possibility that both molecules have closely related roles in maintaining genome stability. As for the relationship between RUNX1 and Gadd45a, Perugini *et al.*[27] recently reported that *GADD45A* expression was significantly downregulated in RUNX1-ETO-positive AML cells compared with normal controls. Although they demonstrated ERK1/2 signaling was involved in the repression of *GADD45A* in several AML cell lines, its mechanism was not analyzed in their study. In previous papers, RUNX1-ETO was shown to decrease the expression of several genes involved in the base excision repair pathway, such as oxoguanine DNA glycosylase and polymerase epsilon, thereby DNA damage accumulated.[32,33] Gadd45a functions in the nucleotide excision repair pathway through its interaction with proliferating cell nuclear antigen;[34–36] therefore, both base excision repair and nucleotide excision repair might be impaired in hematopoietic cells carrying RUNX1 mutations. Gadd45a also functions as a stress sensor, which is mediated by a complex interplay of physical interactions with other cellular proteins that are implicated in cell-cycle regulation and the response of cells to stress.[17] Gupta *et al.*[37] reported that Gadd45a-deficient hematopoietic cells demonstrated an impaired DDR response. They found myeloid-enriched BM cells from Gadd45a- or Gadd45b-deficient mice were defective in G2/M arrest following exposure to UV and VP-16 and these cells were sensitized to genotoxic-stress-induced apoptosis.[37] Similarly, we found that RUNX1dC-transduced LSK cells showed lower colony-forming ability than Mock-transduced LSK cells after UV-B exposure. Like RUNX1dC-transduced cells, *Gadd45a* expression was reduced in D171N-transduced LSK cells compared with Mock-transduced LSK cells (Figure 3d). The D171N mutant does not have DNA-binding ability, whereas it still maintains the ability to bind to CBFβ.[5] The co-expression experiment with D171N inhibited *GADD45A* reporter activation induced by RUNX1b (Supplementary Figure 4); therefore, it is possible that D171N inhibits the function of WT RUNX1 by trapping CBFβ. Differences in the DDR response between N-terminal in-frame type mutations (such as the D171N mutant) and C-terminal truncated mutations (such as the RUNX1dC) of *RUNX1* should be explored in a future study.

Gadd45a is a well known transcriptional target of p53.[29] p53 binds to the conserved sequence within intron 3 of the *GADD45A* gene in response to ionizing radiation.[29] Here, we showed that RUNX1 activates transcription of *GADD45A* through binding to two RUNX-binding sites neighboring the p53-binding site within intron 3 (Figure 5b). RUNX1 and p53 showed synergistic effects on intron3A-Luc activation (Figure 5c); therefore, these two molecules may cooperatively regulate *GADD45A* expression. In our experiment, however, γ-ray irradiation did not influence intron 3A-Luc activation by RUNX1 and/or p53. We are now trying to examine the cooperating effect of RUNX1 and p53 on DDR response, including *GADD45A* regulation, using a more physiological setting.

MDS patients with a *RUNX1* mutation frequently present with more advanced diseases and *RUNX1* mutations are considered to be markers of a poor prognosis.[19,38,39] We speculate that a RUNX1 mutation may be a cause of additional mutation(s) through the impaired DDR response. In future studies, whole genome analysis of MDS/AML samples harboring RUNX1 mutations would clarify the role of RUNX1 mutations in the occurrence of genetic abnormalities in these patients.

In conclusion, we demonstrated that a *RUNX1* C-terminal deletion mutant attenuates the DDR response to environmental and physiological stresses in LSK cells. As a possible explanation

for this mechanism, we found a novel role for RUNX1 in the transcriptional regulation of *Gadd45a*. The impaired Gadd45a function leads to genomic instability; therefore, Gadd45a dysfunction induced by *RUNX1* mutations can cause the additional mutation(s) required for multi-step leukemogenesis. Further studies on RUNX1/Gadd45a would be useful to understand the pathophysiology of MDS/AML patients harboring RUNX1 abnormalities and to prevent disease progression.

Conflict of interest

The authors declare no conflict of interest.

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Increased natural killer cells and decreased $CD3^+CD8^+CD62L^+$ T cells in CML patients who sustained complete molecular remission after discontinuation of imatinib

The recent study by Mahon *et al* (2010) demonstrated that approximately 60% of chronic myeloid leukaemia (CML) patients who stopped imatinib therapy after a period of stable complete molecular remission (CMR) relapsed molecularly within the next 6 months, whereas nearly all the remaining patients sustained a stable CMR beyond 18 months of follow-up. The risk factors for molecular relapse were female gender, high Sokal score at the time of CML diagnosis, and treatment with imatinib for <50 months (Mahon *et al*, 2010). In a similar study, patients who had maintained a stable CMR for at least 2 years on imatinib therapy were asked to stop treatment (STOP-IM), but DNA polymerase chain reaction (PCR) showed that CML cells persisted (Ross *et al*, 2010), suggesting that the existence of minimum residual disease might not always result in relapse of CML. On the other hand, Mustjoki *et al* (2009) reported that treatment with dasatinib, a second-generation tyrosine kinase inhibitor, resulted in a favourable outcome in Ph^+ leukaemia with clonal expansion of natural killer (NK) or T-cells. These findings suggest that, although CML stem cells may remain, even in CMR (Sloma *et al*, 2010), some CML patients could discontinue imatinib therapy and maintain a stable condition, possibly owing to immune surveillance. However, the safety profile for stopping imatinib treatment is still under the discussion (Deininger, 2011).

To elucidate the immunological diversity of CML patients, we assessed subsets of circulating lymphocytes obtained from 30 CML patients: 16 CML patients who had discontinued imatinib treatment for more than 6 months (STOP-IM), and 14 CML patients who were receiving imatinib with CMR for more than 2 consecutive years. Remission criteria were similar

to those used in the study by Mahon *et al* (2010): patients with CMR showed a 4-log or more reduction in *BCR-ABL1* transcripts by quantitative reverse transcription (RT)-PCR, and 4 of these CMR had no evidence of *BCR-ABL1* transcripts by qualitative RT-PCR.

One-way analysis of variance revealed that certain cell fractions of lymphocyte subsets were significantly altered in STOP-IM patients compared with healthy volunteers and CML-CMR patients (Table S1). We focused on the $CD3^+CD8^+CD62L^+$ and $CD3^-CD56^+$ cell fractions, because other cell fractions, except $CD19^+CD20^+$, were not particularly different between STOP-IM and CML-CMR patients. The $CD3^+CD8^+CD62L^+$ cell fractions in STOP-IM patients tended to down-regulate (P = 0·0658) (Fig 1A) and the $CD3^-CD56^+$ cell fraction increased significantly compared with that in CML-CMR (P = 0·0214) (Fig 1B). Our observations indicate that some lymphocyte subsets may be affected by imatinib therapy, even in CMR. The differences in subset cell fractions between CML-CMR and STOP-IM patients therefore may have some bearing on discontinuation of imatinib therapy.

The CD62L antigen (L-selectin) is the important surface molecule for homing of lymphocyte and is highly expressed on naïve T cells and subsets of memory T cells. In contrast, Usuki *et al* (2009) demonstrated that CD8+ memory T cells were significantly higher in therapy-free patients with sustained major molecular remission. Rohon *et al* (2010) reported immunophenotype profiling of CML patients during imatinib therapy and found that the immunoprofile resembled healthy controls, while some CML patients during dasatinib therapy showed elevations of $CD8^+$, NK- and NKT-like cells with elevation of late memory cytotoxic lymphocytes (expression of $CD57^+$, HLA-DR and CD45RO but low CD62L expression) in peripheral blood. In our study, CML-CMR patients did not show significant elevation of terminal effector cytotoxic T cells ($CD3^+CD8^+CD57^+$). An increased number of NK cells, in combination with reduction of $CD3^+CD8^+CD62L^+$ cell fraction, in STOP-IM patients, may therefore be an important indicator that imatinib treatment could be discontinued. The $CD19^+CD20^+$ cell fraction decreased significantly in CML-CMR patients compared with STOP-IM (P = 0·0083), possibly due to imatinib therapy (Table S1) and links to low levels of immunoglobulin during imatinib therapy.

Of note, in the STOP-IM patients in our study, an elevated cell fraction was detected only in $CD3^-CD56^+$ NK cells, and others were down-regulated. We were able to determine NK cell activity in 11 STOP-IM patients. This activity ranged from 24% to 69% (normal range, 18–40%); 7 of the 11 patients had elevated NK cell activity, and a positive correlation was found between NK cell activity and NK cell percentages in the 11 STOP-IM patients (data not shown). Two CMR patients satisfied criteria for both up-regulated $CD3^-CD56^+$ NK cells and down-regulated $CD3^+CD8^+CD62L^+$ cells compared with those of healthy subjects: currently, one patient has sustained CMR for more than 7 months after discontinuation of imatinib therapy.

In the current study, we noticed deviation in some fractions of circulating lymphocytes in STOP-IM patients with CML, in comparison with CML patients who were in CMR but were still taking imatinib. Our results therefore may aid in the selection of possible candidates for discontinuation of imatinib therapy in patients with CMR. The minimal requirements for discontinuation of imatinib treatment might be a CMR duration of more than 2 years and low to intermediate Sokal scores at the time of CML diagnosis, as presented by Mahon *et al* (2010). In addition, increasing number of NK cells and

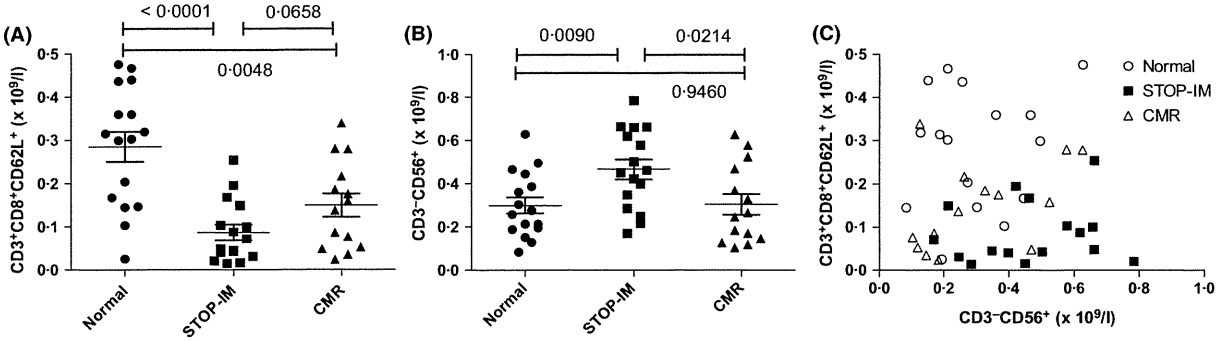

Fig 1. Comparison of lymphocyte subsets in healthy volunteers (healthy volunteers, n = 16), CML patients who discontinued imatinib therapy (STOP-IM, n = 16), and CML patients receiving imatinib therapy who were in complete molecular remission (CMR, n = 14). Heparinized whole blood (10 ml) was obtained and mononuclear cells were separated by gradient method. Immunophenotyping was done with flow cytometry using antibodies against the following antigens: CD3, CD4, CD8, CD16, CD45, CD56, CD57, HLA-DR, CD27, CD62L, CD45RO, CD19, CD20, T-cell receptor (TCR) -α/β and TCR-γ/δ (Beckman Coulter, Miami, FL, USA). The results are shown as cell number of each cell fraction. The analyses were performed with three-color flow cytometer (EPICS XL, Beckman Coulter). This study was approved by the institutional review board of Tokyo Medical University (no. 930: approved on June 24, 2008). (A) The $CD3^+CD8^+CD62L^+$ fraction in STOP-IM patients significantly decreased, compared with healthy volunteers (P < 0·0001), and tended to be lower than in CMR patients (P = 0·0658). (B) The CD45 gating $CD3^-CD56^+$ fraction in STOP-IM patients significantly increased compared with healthy volunteers (P = 0·0090) and CMR patients (P = 0·0214). Bars indicate mean ± standard error of the mean. (C) Schematic diagram of natural killer (NK) cells (x axis) and $CD3^+CD8^+CD62L^+$ cells (y axis). Two CMR patients fall inside the area of NK cells > 0·38 × 10^9/l and $CD3^+CD8^+CD62L^+$ cells < 0·21 × 10^9/l.

decreased $CD3^+CD8^+CD62L^+$ cell fractions may also indicate that imatinib therapy can be stopped (Fig 1C). Further study in details of time to relapse after imatinib discontinuation will be needed in order to clarify the immunological background in CML patients who are taking imatinib.

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Conflict of Interest

No potential conflicts of interest were disclosed.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table SI. Lymphocyte subset in normal subjects and chronic myeloid leukaemia (mean ± standard error).

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Romiplostim for the early management of severe immune thrombocytopenia unresponsive to conventional treatment

Primary immune thrombocytopenia (ITP) is an autoimmune disorder that occurs both in children and in adults, and is characterized by a peripheral blood platelet count < 100 × 10^9/l in the absence of a clear predisposing aetiology. The thrombocytopenia is due to both increased platelet destruction and suboptimal platelet production. Clinical bleeding does not

MPNのリスク分類（予後因子）

臼杵憲祐*

要　旨

本態性血小板血症（ET）と真性赤血球増加症（PV）の生命予後を規定する血栓症の主なリスク因子は年齢60歳以上と血栓症の既往であり，最近，白血球増加が報告されている．またJAK2 V617F allele burdenは，ETでは血栓症のリスクであり，PVでは骨髄線維症への進展のリスクである．白血病化のリスク因子はETでは貧血と血小板増加，PVでは高齢であり，ヒドロキシ尿素の投与はリスク因子とはならない．原発性骨髄線維症（PMF）では，IPSS，DIPSS，DIPSS Plusなどの新しい予後スコアが報告されている．

はじめに

生存期間に関して，骨髄増殖性新生物のうち原発性骨髄線維症（PMF）の生存期間中央値は4～5.5年で，予後が最も不良である．一方，真性赤血球増加症（PV）の生存期間中央値は9.1～12.6年，本態性血小板血症（ET）では生存期間中央値18.9年で，PMFと比べると経過が長い疾患である．そのため，生命予後の予測のためのリスク分類は主にPMFで研究され，ETやPVでは主にその生命予後を規定する血栓症のリスクが研究されている[1-3]．

ETとPVの予後因子

これまでの多くの研究から，年齢60歳以上と血栓症の既往の2つが主たる予後因子とされている（表1）．血小板増加は血栓症のリスクではなく，血小板数150万/μl以上では出血のリスクである．これまでに報告されている血栓症のリスクを表2に示す．

1．白血球増加

血栓症のリスクとして，白血球増加が最近報告されている．PVで血栓症予防のための低用量のアスピリンの効果を調べるヨーロッパの多施設共同の二重盲検ランダム化比較研究（European Collaboration on Low-dose Aspirin in Polycythemia Vera：ECLAP）で，Landolfiら[4]が血栓症リスクの解析を報告している．PV 1,638例の観察期間2.7年で，血栓症は205件であった．15,000/μl以上の白血球増加では血栓症のリスクが高く，主に心筋梗塞のリスクが高かった．動脈血栓のリスクは高血圧でやや増加の傾向が見られたが，喫煙で有意に増加したことを報告している．

イタリアのBergamo統合病院のCarobbioら[5]は，年齢と血栓症の既往によるETの血栓症のリスク層別化に診断時の白血球増加を加えることを検討している．ET 657例の観察期間中央値4.5年，血栓症72件の臨床データを解析した．その結果，年齢と血栓症の既往が血栓症の独立したリスク因子であることが確認され，また診断時の白血球増加で血栓症が多い傾向が見られた．血小板数などのその他の検査結果と血栓症との関連はなかった．単に診断時の白血球増加をリスク因子として加えても，層別化された各群の変化は小さいが，若年で血栓症の既往なしの例（低リスク群）では白血球増加があると血栓症の高リスクであり，そのリスクは通常の高リスク群と同様であった（図1）．白血球数の最適なカットオフ値は9,400/μlであった．

Carobbioら[6]はさらに，ETの血栓症のリスク因子として，標準的なリスク因子（年齢，血栓症の既往）に加えて，血小板増加と新たなリスク因子（白血球増加，JAK2遺伝子のV617F変異）を1,063例のETで解析している．多変量解析では，血小板数＞100万/μlは白血球数＜11,000/μlであれば低リスクであり，血栓症の頻度は1.59%/人/年であった（表3）．逆に，最も高リスク（血栓症頻度2.95%/人/年）は白血球増加かつ血小板減少であり，ほとんどの症例はJAK2 V617F変異陽性であった（77%）．また，標準的なリス

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キーワード：白血球増加，JAK2 V617F allele burden，DIPSS，IPSS

表1　本態性血小板血症（ET）と真性赤血球増加症（PV）のリスク分類

（文献[2]より改変引用）

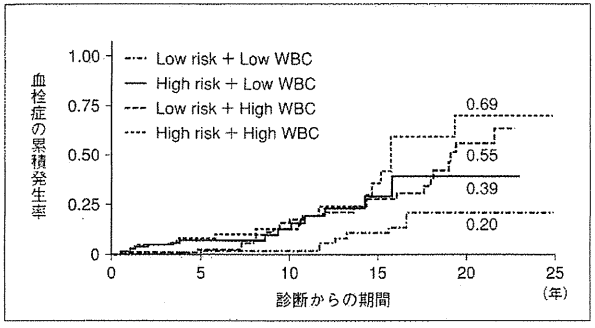
PV
高リスク：下記のうち1つ以上 　年齢＞60歳 　血栓症の既往，アスピリンに抵抗性の肢端紅痛症 　血小板数＞100万/μl 　薬物治療を要する糖尿病，高血圧 　肋骨弓下5 cm以上触知するか，あるいは疼痛，早期満腹感の症状のある脾腫 低リスク：上記のリスク因子なし
ET
高リスク：下記のうち1つ以上 　年齢＞60歳 　血栓症の既往，アスピリンに抵抗性の肢端紅痛症 　血小板数＞150万/μl 　ETに関連する出血の既往 　薬物治療を要する糖尿病，高血圧 低リスク 　上記のリスク因子を持たない40歳未満 中間リスク* 　上記のリスク因子を持たない40～60歳

* 中間リスク群については議論のあるところであり，低リスク群と高リスク群の2つに分けることを勧めるグループもあれば，心血管性疾患リスクを有する例を中間リスク群とすることを勧めるグループもある．

表2 本態性血小板血症（ET）における血栓症のリスク因子

- 高齢
- 血栓症の既往
- 心血管性疾患のリスク：喫煙・糖尿病・高血圧・高脂血症
- 造血細胞のクローナリティ：モノクローナリティ
- エリスロポエチン血中濃度：低下
- 骨髄巨核球の c-mpl 遺伝子発現：低下
- JAK2 遺伝子：V617F 変異
- PRV-1 遺伝子発現：増加
- 血栓性素因：凝固因子V Leiden，アンチトロンビンⅢ欠損症，プロテインC欠損症，プロテインS欠損症，抗リン脂質抗体症候群
- 外科手術
- 網状血小板増加
- 白血球増加

図1 本態性血小板血症（ET）の診断時の白血球数と標準的リスク因子別の血栓症の累積（文献[5]より改変引用）



比率は診断後 20 年の時点のもの．
Low risk：60 歳未満で血栓症の既往なし
High risk：60 歳以上あるいは血栓症の既往あり
Low WBC：診断時の白血球数＜8,700/μl
High WBC：診断時の白血球数≧8,700/μl

ク因子とは独立していた．ET では血小板数の増加で血栓症のリスクが増加するという考えに，異議をとなえる結果であった．

2．JAK2 遺伝子の V617F 変異

ET 症例において JAK2 遺伝子の V617F 変異が血栓症のリスクであるか否かについて多くの報告があるが，その結果は一定していない．Lussana ら[7]は，ET と PMF でメタ解析を行った（図2）．ET の 21 研究 3,150 例と PMF の 6 研究 442 例のメタ解析で，ET では JAK2 V617F 変異で血栓症のリスクが増加し，静脈血栓症と動脈血栓症のいずれのリスクも増加した．PMF では増加の傾向が見られたが，統計学的に有意ではなかったことを報告している．

表3 本態性血小板血症（ET）の診断時の白血球数と血小板数の関係（多変量解析，文献[6]より改変引用）

(万/μl)	n (%)	血管イベント比率*1	低リスク*2 (%)	JAK2 V617F*3 (%)	相対リスク (p)	(95% 信頼区間)
白血球数＜1.1＋血小板数＞100	170 (16)	1.59	95 (56)	35 (26)	1	—
白血球数＜1.1＋血小板数＜100	644 (62)	2.26	317 (49)	304 (56)	1.92 (0.034)	(1.07～2.87)
白血球数＞1.1＋血小板数＞100	99 (9)	2.88	41 (41)	40 (61)	2.38 (0.026)	(1.11～3.51)
白血球数＞1.1＋血小板数＜100	130 (12)	2.95	52 (40)	75 (77)	2.43 (0.017)	(1.25～2.94)

*1 %/人/年
*2 年齢 60 歳未満で血栓症の既往なし（未治療例が 90% を占める）
*3 JAK2 遺伝子の V617F 変異を調べた 860 例における比率

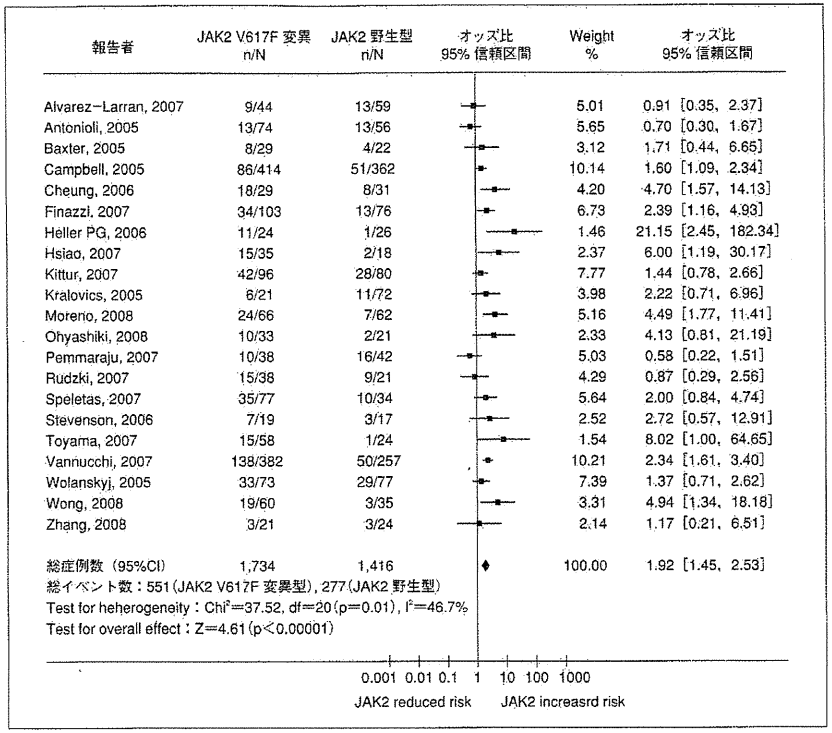
JAK2 遺伝子の V617F 変異が homozygous である頻度は，PV では 25～30%，ET では 2～4%である．GIMEMA の MPN ワーキンググループでは，JAK2 V617F homozygosity と臨床表現型の関連について，JAK2 V617F ホモ 118 例（PV 104 例，ET 14 例）とヘテロ 587 例，野生型 257 例を後方視的に解析し，フィレンツェ大学の Vannucchi ら[8]が報告している．臨床診断名に関係なく，ホモの例は高齢で，診断時の白血球増加とヘマトクリット高値があり，脾腫のサイズが大きかった．掻痒症はホモの PV により多かった．JAK2 V617F ホモは PV と ET の両方で二次性骨髄線維症への進展が多かった．多変量解析では，ホモの ET は野生型やヘテロよりも心血管系イベントのリスクが高かった（図3）．PV ではホモと血栓症のリスクに関連は見られなかった．JAK2 V617F ホモの例は疾患のコントロールに化学療法を受けている傾向があった．JAK2 V617F ホモの ET の症例は心血管系イベントのリスクが高く，症候性 ET と言える．

PV における JAK2 V617F allele burden と臨床像，病期進行，生存期間との関係を明らかにするために，イタリアの Pavia 大学の Passamonti ら[9]は 338 例の PV 症例（うち 320 例は JAK2 V617F 変異あり）を前方視的に解析した．mutant allele burden は，ヘモグロビン（Hb）濃度や白血球数，脾のサイズ，骨髄細胞密度と正の相関を呈し，血小板数と負の相関関係があった．8 例が二次性骨髄線維症に進展し，いずれの例も mutant allele は 50% 以上であった．10 例が白血病化した．mutant allele burden は骨髄線維症への進展と関連したが（表4），白血病化および血栓症とは関連しなかった．白血球増加は血栓症，白血病，骨髄線維症，生存期間には影響しなかった．JAK2 V617F mutant allele burden 50% 以上は骨髄線維症への進展のリスクであると結論している．

3．白血病化のリスク因子

血栓症以外の，生存と白血病化の ET の予後予測モデルは少ない．Mayo Clinic の Gangat ら[10]は，605 例の ET の観察期間中央値 84 ヵ月，死亡 155 例，白血病化 20 例（3.3%）を解析し，予後スコアを作成している（表5）．多変量解析では，貧血（Hb：男性＜13.5g/dl，女性＜12.0g/dl）が生存と白血病化の独立したリスク因子であった．生存

図2 本態性血小板血症（ET）におけるJAK2遺伝子のV617F変異と血栓症のリスクに関するメタ解析
（文献[7]より改変引用）



n：イベント数，N：症例数

のリスク因子は貧血に加えて，60歳以上，白血球数15,000/μl以上，喫煙，糖尿病，血栓症であった．白血病化のリスク因子は貧血と血小板数100万/μl以上であり，抗がん剤治療はリスク因子ではなかった．実際に，白血病化した20例のうち4例（20%）では，抗がん剤の治療の既往がなかった．以上から予後予測モデルを作成し，生存期間中央値は低リスク278ヵ月，中間リスク200ヵ月，高リスク111ヵ月，白血病化率は低リスク0.4%，中間リスク4.8%，高リスク6.5%であった．JAK2遺伝子のV617F変異の有無は生存と白血病化に影響しなかった．

PVについては，血栓症以外の骨髄線維症への進展と白血病化のリスクを，ECLAP研究のPV 1,638例で解析した結果が報告されている[11]．全死亡は3.7%/人/年で，そのうち心血管死亡は約半分（1.7%/人/年）であり，非心血管死亡の中では骨髄線維症への進展と白血病化（全死亡の13%）が多かった．70歳以上では白血病化のリスクが高く，また罹病期間10年以上では骨髄線維症への進展のリスクが高かった．

以前から，白血病化のリスク因子として

図3 本態性血小板血症（ET）におけるJAK2遺伝子のV617F変異の状況による心血管イベントに関する無イベント生存率（文献[9]より改変引用）

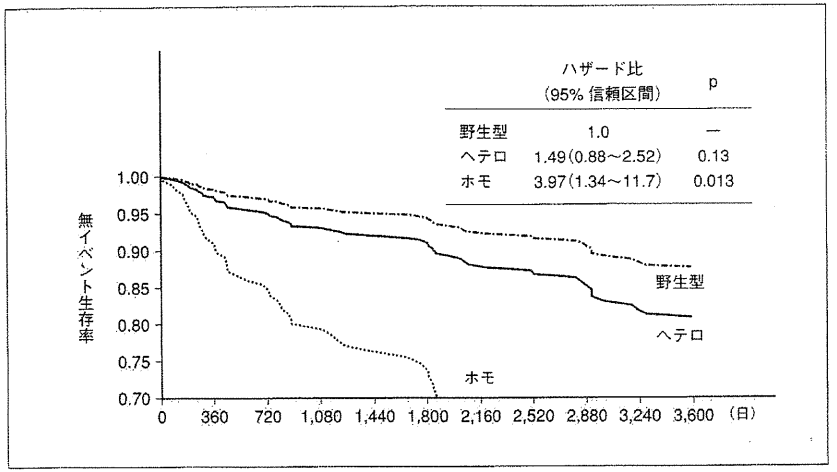


表4 真性赤血球増加症（PV）320例のJAK2 allele burdenを含む多変量解析（文献[9]より改変引用）

	イベント（95% 信頼区間，ハザード比）			
	血栓症	骨髄線維症への進展	白血病化	生存
年齢	1.05（1～1.1） p=0.01	1（0.9～1.1） p=0.8	1.08（0.9～1.2） p=0.06	1.1（1.03～1.2） p=0.002
JAK2 mutant allele burden	1（0.9～1） p=0.8	1.05（1～1.1） p=0.03	1（0.9～1） p=0.3	0.99（0.9～1） p=0.9
白血球数＞1.1万/μl	1.1（0.4～3.4） p=0.8	1.1（0.4～3.4） p=0.8	1.1（0.4～3.4） p=0.8	1.1（0.4～3.4） p=0.8
血小板数	1（0.9～1.0） p=0.9	1（0.9～1） p=0.6	1（0.9～1） p=0.4	1（0.9～1） p=0.3
ヘモグロビン濃度	0.9（0.7～1.2） p=0.5	1.3（0.8～2） p=0.2	0.9（0.6～1.3） p=0.6	0.9（0.7～1.2） p=0.4
脾サイズ	NI	1.1（0.8～1.3） p=0.4	0.5（0.1～1.1） p=0.3	0.8（0.5～1.3） p=0.3
血栓症の既往	1.9（0.7～4.9） p=0.1	NI	NI	0.3（0.1～1.3） p=0.1

NI：not included as covariate

抗がん剤が言われていた．スウェーデンの人口ベースのデータを用いた解析の結果をBjörkholmら[12]が報告している．スウェーデンの全国規模のMPNコホート11,039例のうち，白血病化した162例（急性白血病153例と骨髄異形成症候群9例）と，マッチした対照242例を用いた症例対照研究である．MPNで白血病化した162例のうち41例（25%）は，アルキル化薬，^{32}P，ヒドロキシ尿素（HU）のいずれの投与歴もなかった．HU

表5 Mayo Clinic の 605 例の多変量解析による本態性血小板血症（ET）の生存と白血病化のリスク分類
（文献[18]より改変引用）

全生存のリスク層別化

リスク因子	因子の数	リスクカテゴリー	症例数（%）	イベント数（%）	生存期間中央値	
年齢≧60 歳 ヘモグロビン＜正常値* 白血球数≧15,000/μl	0	低リスク	254 例（42%）	31 例（12%）	278 ヵ月	p<0.0001
	1	中間リスク	249 例（41%）	77 例（31%）	200 ヵ月	
	2～3	高リスク	102 例（17%）	47 例（46%）	111 ヵ月	

白血病化のリスク層別化

リスク因子	因子の数	リスクカテゴリー	症例数（%）	イベント数（%）	
ヘモグロビン＜正常値* 血小板数≧100 万/μl	0	低リスク	239 例（40%）	1 例（0.4%）	p<0.0009
	1	中間リスク	289 例（48%）	14 例（4.8%）	
	2	高リスク	77 例（13%）	5 例（6.5%）	

*女性＜12.0g/dl，男性＜13.5g/dl

の投与歴のなかった例に比べて，白血病化リスクのオッズ比は，HU 累積投与量 1～499g では 1.5，500～999g では 1.4，1,000g 以上では 1.3 であり，有意差はなかった．^{32}P の 1,000MBq 以上の例では 4.6，アルキル化薬 1 g 以上では 3.4 であり，白血病化リスクが高かった．2 つ以上の抗がん剤の投与歴のある例では 2.9 で，白血病化リスクは高かった．MPN における白血病化リスクはアルキル化薬あるいは ^{32}P で高く，HU では変わらなかった．しかし，MPN から白血病化した 1/4 の例では抗がん剤の投与歴がなく，治療とは関連しない因子の大きな関与が考えられた．

PMF の予後（表6）

これまでに PMF の予後スコアは幾つか報告されていたが，2009 年に International Working Group for Myelofibrosis Research and Treatment（IWG-MRT）によって，International Prognostic Scoring System（IPSS）が発表された[13]．7 施設の 1,054 例を解析し，全生存期間中央値は 69 ヵ月で，診断時の項目の多変量解析から予後不良因子は年齢＞65 歳，症状（constitutional symptoms）（＋），Hb＜10g/dl，白血球数＞2.5万/μl あるいは末梢血芽球≧1％であり，そのスコアに基づく 4 グループの生存期間中央値は 135 ヵ月，95 ヵ月，48 ヵ月，27 ヵ月であり，以前の予後モデルに比べて各群の予後の違いがより明瞭となった．

IPSS は診断時の項目を用いたスコアであるが，経過中の随時の項目を用いたスコアに改変した Dynamic International Prognostic Scoring System（DIPSS）がその後に報告された[14]．DIPSS では，65 歳未満のための DIPSS（age-adjusted DIPSS）も用意された．その後 Passamonti ら[15]は，DIPSS の各グループの急性期（blastic phase）への移行率は low risk 0.3%/人/年，intermediate-1 risk 0.7%/人/年，intermediate-2 risk 2.6%/人/年，high risk 8.6%/人/年であることを追加報告している．

Tam ら[16]は，M. D. Anderson Cancer Center の 256 例の PMF の核型と，臨床像

表6 原発性骨髄線維症（PMF）の予後予測スコア

予後スコア	リスク因子	スコアリングシステム			
		ポイント	ポイント合計	リスクカテゴリー	生存期間中央値
Lille スコア	ヘモグロビン＜10g/dl	1	0	low risk	93 ヵ月
	白血球＜4,000 または＞3 万/μl	1	1	intermediate risk	26 ヵ月
			2	high risk	13 ヵ月
Cervantes スコア	ヘモグロビン＜10g/dl	1	0～1	low risk	176 ヵ月
	発熱，1 ヵ月以上持続する盗汗，1 年に 1 割以上の体重減少	1	2～3	high risk	33 ヵ月
	末梢血骨髄芽球≧1％	1			
Mayo スコア	ヘモグロビン＜10g/dl	1	0	low risk	173 ヵ月
	白血球＜4,000 または＞3 万/μl	1	1	intermediate risk	61 ヵ月
			2～4	poor risk	26 ヵ月
	血小板＜10 万/μl	1			
	単球＞1,000/μl	1			
特発性造血障害研究班（谷本ほか）	男性	1	0～1	低リスク	111 ヵ月
	ヘモグロビン＜10g/dl	1	2～4	高リスク	34 ヵ月
	末梢血骨髄芽球≧1％	1			
	発熱，夜間盗汗，体重減少の持続	1			
IPSS[13]	年齢＞65 歳	1	0	low risk	135 ヵ月
	発熱，1 ヵ月以上持続する盗汗，1 年に 1 割以上の体重減少	1	1	intermediate risk-1	95 ヵ月
			2	intermediate risk-2	48 ヵ月
			3～5	high risk	27 ヵ月
	末梢血骨髄芽球≧1％	1			
	ヘモグロビン＜10g/dl	1			
	白血球＞25,000/μl	1			
DIPSS[14]	年齢＞65 歳	1	0	low risk	not reached
	発熱，1 ヵ月以上持続する盗汗，1 年に 1 割以上の体重減少	1	1～2	intermediate risk-1	14.2 年
			3～4	intermediate risk-2	4 年
			5～6	high risk	1.5 年
	末梢血骨髄芽球≧1％	1			
	ヘモグロビン＜10g/dl	2			
	白血球＞25,000/μl	1			
age-adjusted DIPSS[14]	発熱，1 ヵ月以上持続する盗汗，1 年に 1 割以上の体重減少	2	0	low risk	not reached
			1～2	intermediate risk-1	9.8 年
			3～4	intermediate risk-2	4.8 年
	末梢血骨髄芽球≧1％	2	5～7	high risk	2.3 年
	ヘモグロビン＜10g/dl	2			
	白血球＞25,000/μl	1			
DIPSS Plus[16]	血小板＜10 万/μl	1	0	low risk	185 ヵ月
	予後不良核型*	1	1	intermediate risk-1	78 ヵ月
	赤血球輸血依存性	1	2～3	intermediate risk-2	35 ヵ月
	DIPSS intermediate risk-1	1	4～6	high risk	16 ヵ月
	DIPSS intermediate risk-2	2			
	DIPSS high risk	3			

*予後不良核型：複雑核型，あるいは＋8，－7/7q－，i(17q)，－5/5q－，12p－，inv(3) または 11q23 再構成
他の略語：巻末の「今月の略語」参照

の関連に関する後方視的解析を報告している．診断時の核型は生存に有意に影響し，予後良好核型（13q⁻単独，20q⁻単独，trisomy 9±その他の1つの異常）の生存期間（中央値63ヵ月）は正常核型（46ヵ月）と差はなく，予後不良核型（第5，7，3番染色体の異常）は不良で生存期間中央値15ヵ月であり，第17番染色体の異常では5ヵ月であった．経過中に新たな染色体異常の出現（clonal evolution）が見られた73例の解析では，予後不良核型あるいは第17番染色体の異常の出現した症例の生存期間中央値は，それぞれ18ヵ月と9ヵ月であった．

Mayo Clinic の Gangat ら[17]は，DIPSS に核型と血小板数，輸血依存性を含めることを試みた．PMF 793例の解析で，DIPSS，予後不良核型，血小板数10万/μl未満，輸血依存性が独立した予後不良因子であり，これを用いた予後スコア DIPSS Plus を作成した．

Mayo Clinic の Vaidya ら[18]は，PMF におけるモノソミーについて報告している．PMF 793例のうち62例が予後不良核型を呈し，41例は複雑核型，+8単独21例であった．予後不良核型の41例中17例41%がモノソミーであった．生存期間中央値は，モノソミーでは6ヵ月，モノソミーなしの複雑核型では24ヵ月，+8単独では20ヵ月であり，2年の白血病化の頻度は，モノソミーでは29.4%，モノソミーなしの複雑核型では8.3%，+8単独では0%であった．モノソミーは DIPSS には含まれていないが，極端に生存期間は短く，白血病化の頻度が高い予後不良因子である．

進行期（移行期と急性期）の予後因子

M. D. Anderson Cancer Center の Tam ら[19]は，74例の bcr-abl 陰性 MPN の急性期（blastic phase）の解析の結果を報告している．全体の生存期間中央値は5ヵ月，支持療法（16%）のみでは生存期間は6週間で，performance status などを含めた多変量解析でも，治療法が生存の独立した予後因子であった．

さらに Tam ら[20]は，PMF と ET/PV から移行したものを含む骨髄線維症における移行期（accelerated phase）の定義を決める解析を報告している．骨髄線維症370例を解析して生存期間12ヵ月以下である症例の臨床的特徴を調べ，移行期の定義とした．移行期の特徴として3項目，すなわち①骨髄あるいは末梢血芽球≧10%，②血小板数＜5万/μl，③第17番染色体異常が同定された．それぞれの生存期間中央値は，①10～12ヵ月，②12～15ヵ月，③5～6ヵ月であった．移行期は急性期への移行で必ず通過する病期であり，慢性期から移行期を経ずに直接急性期へ移行することは10年で3%と頻度が低い．

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Risk Stratification of Myeloproliferative Neoplasms: Prognostic Factors

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