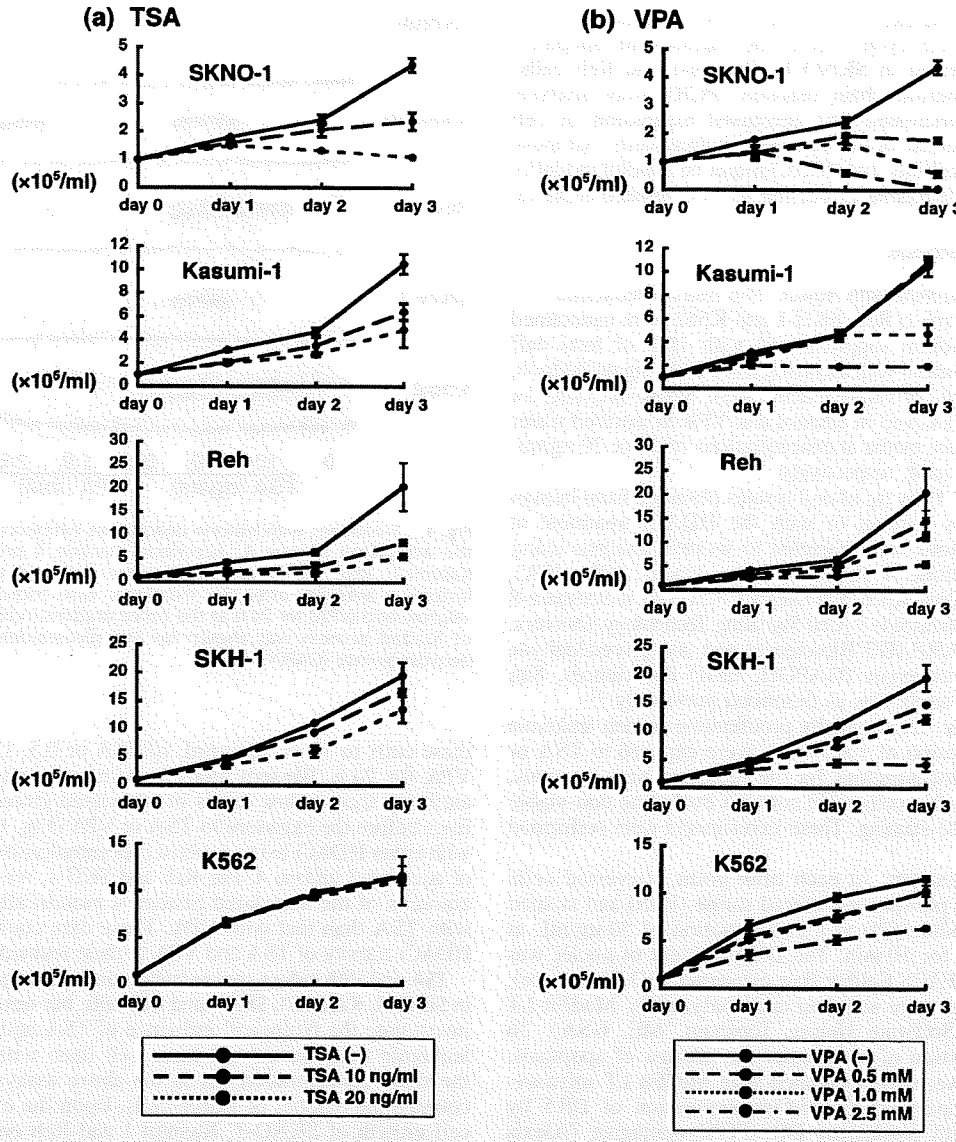

Fig. 2. Antiproliferative effects of HDACi on human leukemia cell. Human leukemia cell lines (SKNO-1, Kasumi-1, Reh, SKH-1 and K562) were cultured in the presence of HDACi (a, TSA; b, VPA) at the concentrations indicated. The numbers of viable cells were counted at each time point by Trypan blue staining. Their averages in three independent experiments are shown with standard deviations.

SKNO-1, Kasumi-1, Reh and SKH1 cells. In contrast, the S-phase population was unchanged in K562 cells with the TSA treatment. These findings suggested that the HDACi have some inhibitory effects on cell cycle progression from G0/G1 to S phase in the RUNX1 chimera-expressing leukemic cells but not in K562 cells. On the other hand, a dose-dependent increase in the sub-G1 fraction indicated induction of apoptosis after cell cycle arrest in SKNO-1, Kasumi-1 and Reh cells. However, neither TSA nor VPA caused programmed cell death in SKH-1 and K562 cells. We concluded that these HDACi are able to induce G0/G1 arrest and subsequent apoptosis in the RUNX1 chimera-expressing leukemic cells but not in the BCR/ABL-expressing cells.

TSA and VPA alter the expression of cell cycle regulators. HDAC is involved in epigenetic modulation of the gene expression. By restoring transcription of the silenced tumor suppressor genes in leukemic cells, HDACi could cause their lethality. To clarify the transcriptional events associated with delayed cell cycle progression in the presence of HDACi, we analyzed expressional changes for cell cycle regulator genes in SKNO-1, Kasumi-1, Reh and K562 cells. We employed PCR array analysis that is able to comprehensively analyze the expression levels of major cell cycle regulator genes using a quantitative PCR method. We selected genes whose expression level showed more than 1.5-fold changes in the presence of TSA or VPA relative to their absence. Of the 84 genes on the array, *CDK2*, *CCNG2* and *HERC5* showed consistent increases while *CDK4*, *CDK6*, *CUL1* and *TFDP1* showed consistent decreases in all four cell lines in the presence of the HDACi (Fig. 4). CDK2, CDK4 and CDK6 are cyclin-dependent kinases (CDK) whose activation is linked to cell cycle progression.[23] The transcription of *CUL1*, a critical component of the ubiquitin ligase involved in cell cycle transition,[24] was downregulated while its other family member, *CUL2*,[25] was activated. In addition, although not always exceeding the 1.5-fold threshold, the *CDKN1A*[26] and *CHEK2*[27] genes, which cause cell cycle arrest at the G1 phase, were upregulated in response to both the treatments. The *DIRAS3* gene, a putative tumor

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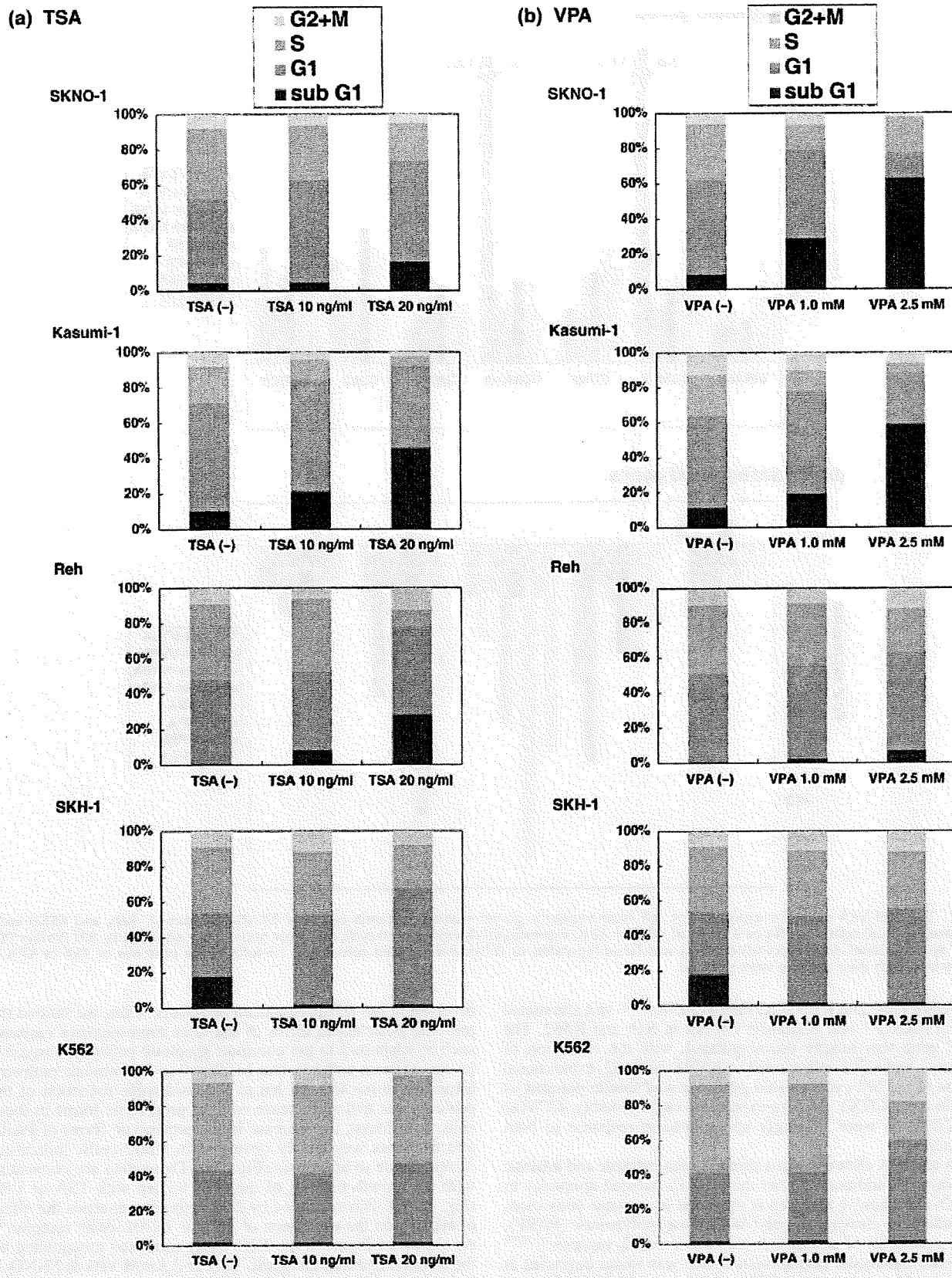

Fig. 3. HDACi inhibits cell cycle progression and induces apoptosis. Human leukemia cell lines (SKNO-1, Kasumi-1, Reh, SKH-1 and K562) were cultured in the presence of HDACi (a, TSA; b, VPA) at the concentrations indicated for 48 h. After this, the cell cycle status and the percentage of apoptotic cells were determined by flow cytometer.

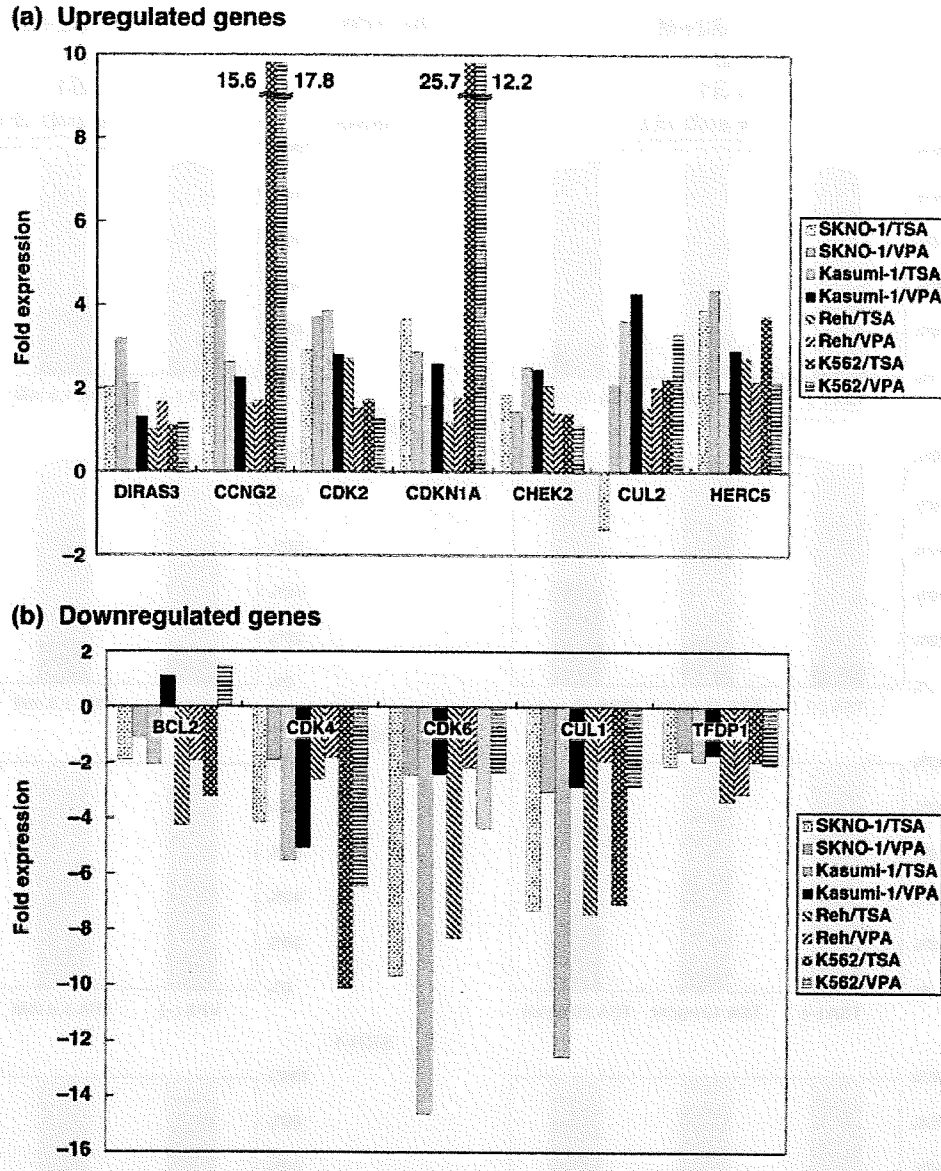

Fig. 4. TSA and VPA alter the expression of cell cycle regulator genes. Human leukemia cell lines (SKNO-1, Kasumi-1, Reh, and K562) were incubated with 20 ng/mL of TSA or 2.5 mM of VPA for 12 h. Expressional changes in cell cycle regulator genes were analyzed by RT² Profiler PCR Array system. Genes whose expression level was (a) upregulated or (b) downregulated more than 1.5-fold in the presence of TSA or VPA in comparison with their absence were selected.

suppressor gene and RAS superfamily member,[28] was stimulated only in SKNO-1 and Kasumi-1 but not in Reh and K562. The *BCL2* gene was mostly downregulated, with the exception of Kasumi-1 and K562 cells treated with VPA. In K562, expressional change of the cell cycle-related genes showed similar patterns as seen in the RUNX1 chimera-expressing cells. Notably, *CCNG2* and *CDKN1A* were strikingly upregulated in response to both treatments.

TSA and VPA elicit apoptosis through both extrinsic and intrinsic pathways. To address whether the HDACi-induced apoptosis in SKNO-1, Kasumi-1 and Reh is mediated by caspase activation, we studied the cleavage of poly(ADP-ribose) polymerase (PARP), a major substrate of executioner caspases including caspase-3,[29,30] by western analysis. Endogenous PARP was found degraded at least after 12-h culture with TSA or VPA in all RUNX1 chimera-expressing cells (SKNO-1, Kasumi-1 and Reh) (Fig. 5a–c). These data suggest that the activation of effector caspase is involved in the HDACi-induced apoptosis. Thus, we studied the pre- and postcleaved forms of the three representative caspases such as caspase-3 in the common apoptotic pathway, caspase-8 in the extrinsic pathway and caspase-9 in the intrinsic pathway. Exposure to the HDACi led to a considerable reduction of the pro-caspases and an increase of their proteolytic forms in these cells. In contrast, the increase in the proteolytic forms of PARP and caspases was hardly observed in K562 cells, indicating its resistance to apoptosis (Fig. 5d). These data are consistent with the growth kinetics of the cells treated with TSA or VPA (Fig. 2). We also examined by flow cytometer analysis the effect of HDACi on the expression of DR5, a TRAIL-death receptor.[31] As shown in Figure 6, the DR5 expression was upregulated by the addition of either 20 ng/mL TSA or 2.5 mM VPA in SKNO-1, Kasumi-1 and Reh cells. These data indicated that both the extrinsic and intrinsic apoptotic signals may contribute to the apoptotic induction in these cells.

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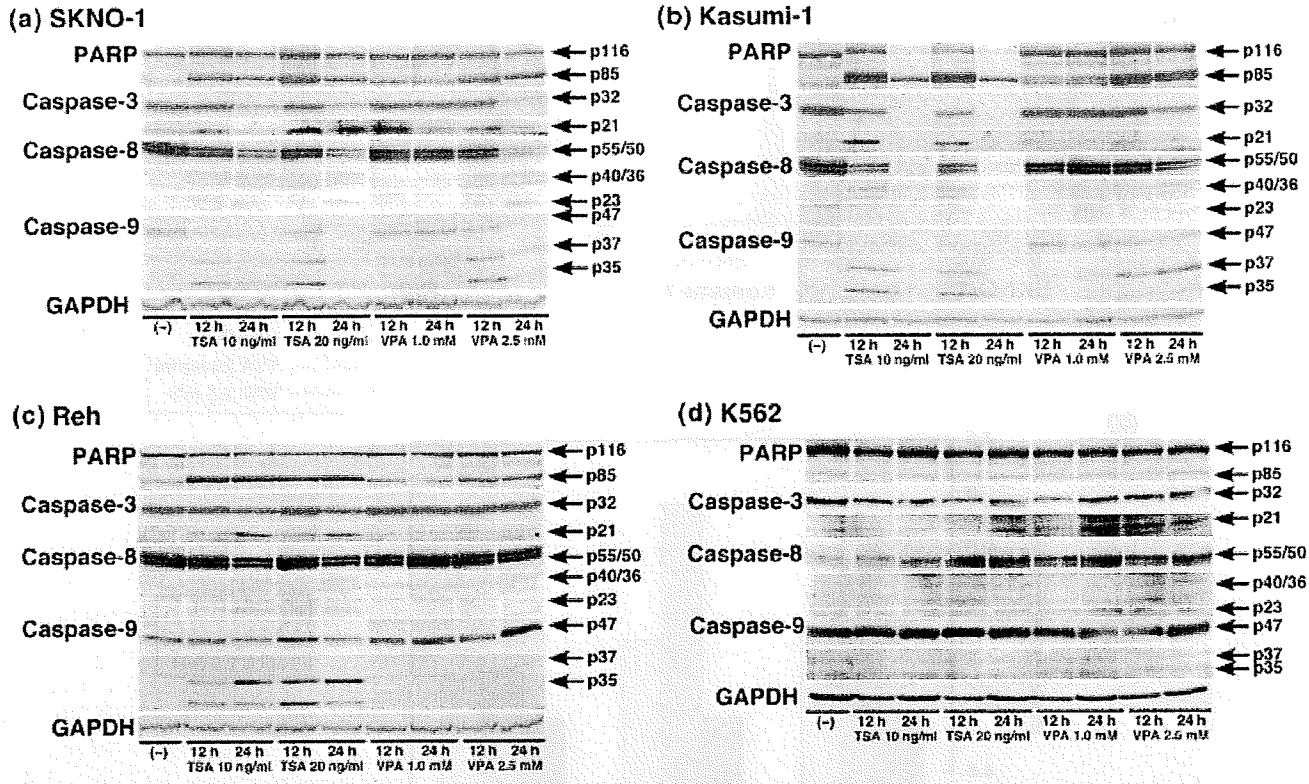

Fig. 5. Apoptotic effect of HDACi is mediated by activation of poly(ADP-ribose) polymerase (PARP), caspase-3, caspase-8 and caspase-9. Human leukemia cell lines (a, SKNO-1; b, Kasumi-1; c, Reh; and d, K562) were incubated with TSA or VPA at the concentrations indicated for 12 or 24 h. The expressions of apoptosis-related molecules (PARP, caspase-3, -8, and -9) were analyzed by western blotting. The arrows of p116 PARP, p32 caspase-3, p55/50 caspase-8, and p47 caspase-9 indicate their inactive precleaved forms, and the arrows of p85 PARP, p21 caspase-3, p40/36/23 caspase-8, and p37/35 caspase-9 indicate their cleaved (active) forms. The amount of loaded protein was shown by anti-GAPDH bolt.

Discussion

We demonstrated that treatments with TSA or VPA at concentrations sufficient to induce histone acetylation trigger apoptosis in SKNO-1, Kasumi-1 and Reh cells, but not in K562 cells. The death kinetics of SKH-1 cells after these treatments fell between that of SKNO-1, Kasumi-1 and Reh and that of K562. In the cell cycle analysis, cells in the sub-G1 fraction were accumulated in SKNO-1, Kasumi-1 and Reh cells, while cell count in the S phase was declined. Consistent with this finding, cleavage/activation of a series of caspases and PARP degradation were observed. Thus, we considered that exposure to the HDACi was associated with cell cycle arrest at the G0/G1 phase followed by apoptotic induction. SKNO-1, Kasumi-1 and Reh cells express the RUNX1-related chimera, K562 cells express the constitutive active tyrosine kinase BCR/ABL, and SKH-1 cells express both the RUNX1-related chimera and BCR/ABL. All of the RUNX1-related chimeras carried by the leukemic cells used in this study, namely RUNX1/ETO, RUNX1/EVI1 and TEL/RUNX1, elicit the dominant-negative effect over wild-type RUNX1 through recruiting a specific combination of co-repressor/HDAC complex via the portion from the fusion partners and thereby cause leukemia. It is well known that HDACi regulates gene expression by modulating histone acetylation and remodeling chromatin architecture.[15] In addition to this function, HDACi is expected to inhibit the enzymatic activity of HDAC recruited by the fusion proteins and thus restore the expression of target genes that is suppressed in the leukemic cells.[32] This notion may provide a rationale for the use of HDACi in the treatment of RUNX1 chimera-positive leukemia. On the other hand, HDACi may be less effective in the leukemic cells with higher proliferative ability mediated by active kinase such as BCR/ABL expressed in K562 and SKH-1 cells. The presence of BCR/ABL could interfere with the apoptotic induction, although some studies reported that BCR/ABL-positive cells responded to HDACi such as hydroxamic acid-derived NVP-LAQ824,[33,34] and suberoylanilide hydroxamic acid (SAHA),[35–37] and cyclic peptide apicidin.[38,39]

The gene expression analysis on the panel of cell cycle regulator genes provided an insight into possible mechanisms for the arrest in cell cycle progression in the presence of the two HDACi. Although the changes were observed in both directions, acceleration (upregulation of *CDK2* and *CCNG2*) and suppression (downregulation of *CDK4*, *CDK6*, *CUL1* and *TFDP1*, upregulation of *CDKN1A*, *CHEK2* and *HERC5*) of the cell cycle, the net effect appears to hinder the whole process, probably through downregulation of *CDK4* and *CDK6* and upregulation of *CDKN1A*. Previous reports show that several other HDACi induce the upregulation of *CDKN1A* and cell cycle arrest in cells carrying BCR/ABL.[35,40,41] In our study, although marked induction of *CDKN1A* was observed in K562 cells, they were resistant to cell cycle arrest. One mechanism could be that TSA and VPA exert specific effects on the RUNX1-related chimeras. The effect on *CDKN1B*, another key player in regulation of cyclin/CDK complex,[42] was inconsistent among our cell lines and between the two drugs (data not shown), and did not seem to be involved in the HDACi-mediated cell cycle arrest. However, an increase in protein level of CDKN1B was reported in CML-BC LAMA-84 cells by the treatment with LAQ824.[41] Considering that consistent downregulation of the *CUL1* gene, which progresses cell cycle via degradation of CDKN1B,[43] was observed, the protein

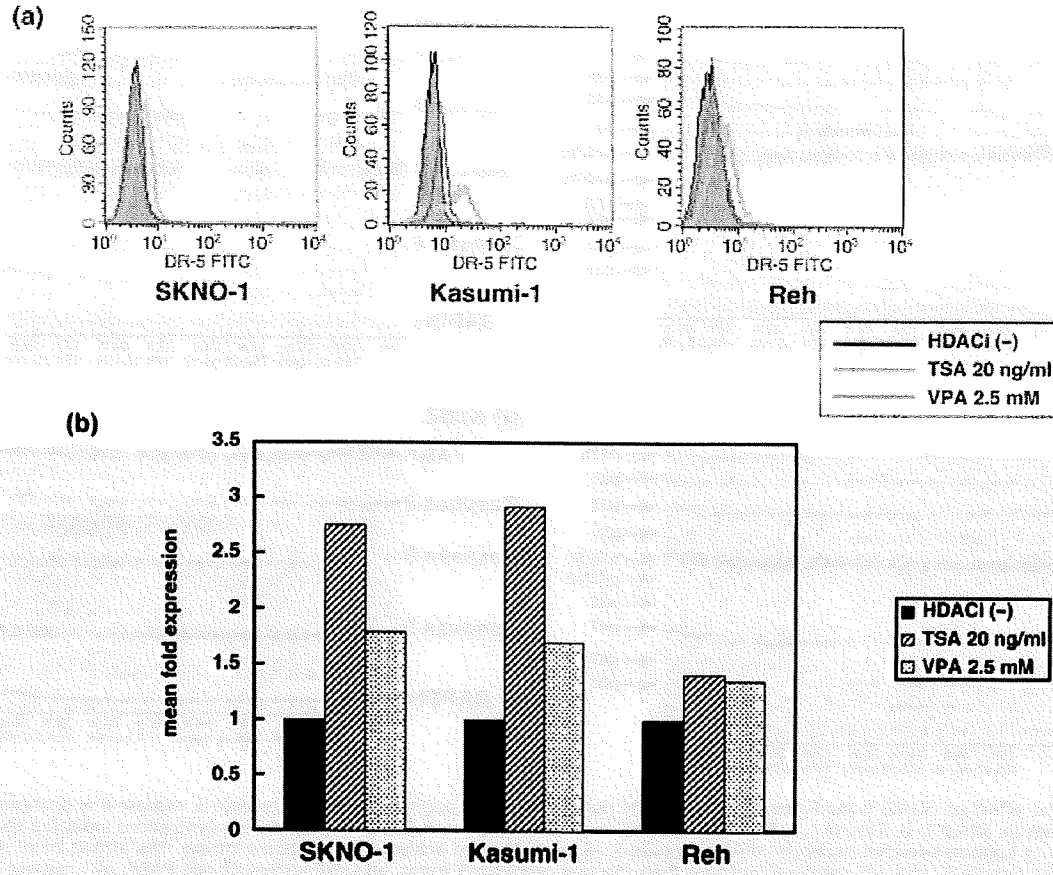

Fig. 6. The cell surface expression of DR5 is upregulated with HDACi. Human leukemia cell lines (SKNO-1, Kasumi-1 and Reh) were incubated with 20 ng/mL of TSA or 2.5 mM of VPA for 24 h. After this, the cell surface expression of DR5 was analyzed by flow cytometer. (a) The horizontal and vertical axes indicate the fluorescence intensity and event numbers, respectively. Black (filled), red and blue histograms represent untreated, TSA- and VPA-treated cells, respectively. (b) Mean fold expressions of DR5 were calculated with the mean expressions of untreated cells as standards and indicated in bars.

expression may have been upregulated in our leukemic cells. CHEK2 is a cell cycle checkpoint regulator and a putative tumor suppressor whose activation is associated with inhibition of CDC25c phosphatase activity that prevents entry into mitosis, and stabilization of p53.[44] The levels of *CHEK2* transcript were increased by the treatment with the HDACi, which may have contributed to the cell cycle blockade by these reagents. In contrast to *CUL1*, its homolog *CUL2* was mostly upregulated in the leukemic cells. CUL2 forms a protein complex consisting of Elongin C, Elongin B and von Hippel-Lindau tumor suppressor (VHL), and supports VHL to suppress tumor growth.[45] Because VHL is known to stabilize p53,[46] the increased expression of the *CUL2* gene caused by the HDACi could have interrupted a transition from G1 to S phase in the leukemic cells. TFDP1 belongs to the E2F transcription factor family that regulates the expression of genes involved in the cell cycle.[47] The downregulation of the *TFDP1* gene may have caused general paucity in cell cycle machineries. Lastly, the upregulation of *HERC5*, a HERC family of ubiquitin ligases that is implicated in cyclin degradation,[48] may also have caused the cell cycle delay. Because the directions of change in most of the cell cycle regulator genes are similar between the RUNX1 chimera-positive and -negative cells, we speculate that the changes in expression of the cell cycle regulator genes are not sufficient to explain the preferential growth retardation of RUNX1 chimera-positive cells treated with HDACi. Genes other than the cell cycle regulators are under investigation to discover novel HDACi targets.

The majority of the leukemic cells arrested in the G0/G1 phase most likely underwent apoptosis within 24 h, as judged from the accumulation of the sub-G1 fraction in the cell cycle analysis. Cleavage of PARP that is a hallmark of apoptosis was also detected. The proteolysis is mediated by caspase-3, a critical downstream apoptotic caspase that becomes activated by both the extrinsic and intrinsic pathways largely through caspase-8 and -9, respectively. We outlined the major steps of programmed cell death in these leukemic cells by analyzing the functional status of upstream caspases. Importantly, we observed activation of both caspase-8 and -9 in addition to caspase-3. The concomitant activation of caspase-8 and -9 was also reported in K562 after the treatment with apicidin,[38] and U937 cells after that with NVP-LAQ824.[49] Interestingly, flow cytometry analysis demonstrated the increase of the cell surface expression of DR5, a TRAIL-death receptor, in cells treated with TSA or VPA, suggesting their sensitization to the extrinsic apoptotic pathway. All these data collectively indicated that both the extrinsic and intrinsic apoptotic pathways are involved in the HDACi-induced cytotoxicity in the RUNX1 chimera-positive leukemic cells. However, we did not observe the production of reactive oxygen species that could represent a primary event in the HDACi-mediated apoptosis (data not shown).

In summary, we showed that TSA and VPA have significant antileukemic activity *in vitro* in the cells showing transcriptional dysregulation caused by the aberrant transcription factors. This activity correlates well with its ability to arrest cell cycle and induce

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apoptosis. Collectively, these findings generate the rationale to investigate the clinical efficacy of the HDACi in the treatment of core binding factor (CBF) leukemia such as t(8;21)- and inv(16)-type leukemia resulting from functional dysregulation of wild-type RUNX1. Although most HDACi, including TSA, are of limited therapeutic value due to their poor bioavailability *in vivo* as well as their potential toxic side-effects, effectiveness of VPA alone or in combination with *all-trans* retinoic acid has been demonstrated in myelodysplastic syndrome,[50] and relapsed or refractory myelogenous leukemia.[51] Thus, it is worth investigating whether CBF leukemia responds to administration of VPA. Although transplant therapy is not a primary option in the first remission of CBF leukemia, this type of leukemia sometimes relapses especially in the central nervous system and extramedullary region. HDACi therapy could be a powerful modality for the maintenance therapy for CBF leukemia.

Acknowledgments

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Review Article

Role of the *RUNX1-EVI1* fusion gene in leukemogenesis

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RUNX1-EVI1 **is a chimeric gene generated by t(3;21)(q26;q22) observed in patients with aggressive transformation of myelodysplastic syndrome or chronic myelogenous leukemia. RUNX1-EVI1 has oncogenic potentials through dominant-negative effect over wild-type RUNX1, inhibition of Jun kinase (JNK) pathway, stimulation of cell growth via AP-1, suppression of TGF-β-mediated growth inhibition and repression of C/EBPα. *Runx1-EVI1* heterozygous knock-in mice die in uteri due to central nervous system (CNS) hemorrhage and severe defects in definitive hematopoiesis as *Runx1-/-* mice do, indicating that RUNX1-EVI1 dominantly suppresses functions of wild-type RUNX1 *in vivo*. Acute myelogenous leukemia is induced in mice transplanted with bone marrow cells expressing RUNX1-EVI1, and a *Runx1-EVI1* knock-in chimera mouse developed acute megakaryoblastic leukemia. These results suggest that RUNX1-EVI1 plays indispensable roles in leukemogenesis of t(3;21)-positive leukemia. Major leukemogenic effect of RUNX1-EVI1 is mainly through histone deacetyltransferase recruitment via C-terminal binding protein. Histone deacetyltransferase could be a target in molecular therapy of RUNX1-EVI1-expressing leukemia. (*Cancer Sci* 2008; 99: 1878–1883)**

The t(3;21)(q26;q22) chromosomal translocation occurs in patients with aggressive transformation of myelodysplastic syndrome (MDS) or chronic myelogenous leukemia (CML). The presence of this chromosomal translocation indicates poor prognosis.[1–3] In the chromosomal joining region of t(3;21)(q26;q22), the *RUNX1* gene on 21q22 is fused with the *EVI1* (ecotropic viral integration site-1) gene on 3q26.5. Previously, we cloned the *RUNX1-EVI1* fusion gene from a case with blastic crisis of CML developing additional to the t(3;21) translocation.[4] This chimeric transcription factor is believed to be a molecular culprit for the leukemic progression of stem cell malignancies caused by t(3;21)(q26;q22).

Molecular and biological function of wild-type RUNX1

The RUNX1 protein mainly consists of two functional domains; the Runt homology domain (which is known as a DNA-binding domain), and the proline-, serine- and threonine-rich (PST) region (which is known as a putative transcriptional activation domain) (Fig. 1). RUNX1 forms a heterodimeric active transcriptional complex with the non-DNA-binding β subunit (CBFβ/PEBP2β) and binds to a specific DNA consensus sequence (ACCRCA) named PEBP2.[5–9] Runx1- or Cbfβ-deficient mice are embryonic lethal at day 12.5 of gestation (E12.5), showing massive hemorrhage in the central nervous system (CNS) and lack of hematopoiesis in the fetal liver.[10–14] A recent study demonstrated that inactivation of *Runx1* in adult mice results in megakaryocyte maturation arrest, block in T- and B-lymphocyte development and increase in hematopoietic precursor cells.[15] A

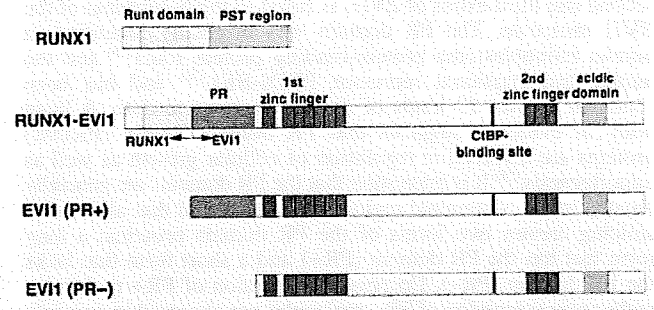

Fig. 1. Schematic structure of wild-type RUNX1, EVI1 and RUNX1-EVI1 molecules. Wild-type RUNX1 has Runt homology domain at the N-terminus and proline-, serine- and threonine-rich (PST) region at the C-terminus. In RUNX1-EVI1, RUNX1 protein is truncated at the end of the Runt homology domain and followed by almost the entire coding region of EVI1 molecule including the PRDI-BF1-RIZ1 (PR) domain.

subsequent study by the same group showed that the number of quiescent hematopoietic stem cells (HSC) is also negatively regulated by RUNX1.[16] Clinically, mutations in *RUNX1* have been identified in 15% to 40% of MDS-refractory anemia with excess of blasts (RAEB) and MDS/acute myelogenous leukemia (AML).[17,18] Patients with MDS/AML with the *RUNX1* mutations have a significantly worse prognosis than those without them.

Molecular and biological function of wild-type EVI1

The *Evi1* gene was initially identified as a frequent retrovirus integration site in myeloid tumors in AKXD mice.[19] EVI1 expression is low in normal hematopoietic cells, but it is highly expressed in some patients of MDS or AML.[20,21] EVI1 has four major functional domains; the two zinc finger (ZF) domains, the C-terminal binding protein (CtBP)-binding site, and the acidic domain (AD) (Fig. 1). The CtBP-binding site is located between the two ZF domains, and the AD is located in the most C-terminus. EVI1 is reported to interfere with transforming growth factor-β (TGF-β) signaling, and antagonize its growth inhibitory effect through targeting an intracellular signal transducer Smad3.[22] EVI1 is also known to enhance AP-1 activity[23] and to block c-Jun N-terminal kinase (JNK) activity,[24] as described below. *Evi1-/-* mice die at E10.5, and HSC in *Evi1-/-* embryos are markedly decreased in numbers, with defective self-renewing proliferation and repopulating capacity.[25] The study also shows that Evi1 directly regulates the transcription of Gata-2, which controls both the maintenance and proliferation of HSC. It was also recently reported that the decreased colony forming

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capacity of *Evi1–/–* para-aortic splanchnopleural (P-Sp) cells could be rescued by retroviral Gata-2 expression, and by blocking of TGFβ signaling using an *in vitro* cultivation system of murine P-Sp regions.[26]

The *MDS1* gene was originally identified as a distinct gene from the *EVI1* gene, and the putative intergenic splicing form between *MDS1* and *EVI1* found in normal and leukemic cells was designated as *MDS1/EVI1*.[27] Because the breakpoint in the chromosome 3 of t(3;21)(q26;q22) is located in the 5′ region of the *MDS1* gene, and the resultant *RUNX1-EVI1* fusion gene includes the first and second exons of the *MDS1* gene between *RUNX1* and *EVI1* sequences, *RUNX1-EVI1* is also called as *RUNX1-MDS1/EVI1*. However, at present, *MDS1* is recognized as one of the alternative spliced form of the *EVI1* gene.[28] In MDS1/EVI1, a 188 amino-acid region called the PR domain, which consists of the first and second exons of *MDS1* and the second and third exons of *EVI1*, is fused to the N-terminal of the EVI1 molecule. The PR domain was originally identified in human retinoblastoma protein-binding protein RIZ1[29] and the human transcriptional repressor PRDI-BF1,[30] and has been found in at least 17 kinds of diverse proteins to date. A large body of evidence suggests that PR domain family (PRDM) proteins are involved in regulation of cellular growth as well as tumorigenesis.[28] It is interesting that the PR domains are generally located in the N-terminal region of proteins, and that alternative splicing creates two forms of the PR domain proteins; a long form that has the PR domain (PR+) and a short form that lacks the PR domain (PR–). Decreased expression of PR+ molecules and/or overexpression of PR– molecules are observed in a variety of cancer cells, suggesting a functional antagonism, in which the PR+ forms contribute to tumor suppression while the PR– forms are oncogenic.

For the *EVI1* gene, EVI1 (PR-) is highly expressed in cases with human AML or MDS as a consequence of chromosomal rearrangements involving 3q26. Increased expression of EVI1 (PR-) is also observed without 3q26 abnormalities. Recent studies have shown that increased expression of EVI1 (PR-) in AML, which occurs in approximately 10% of the cases, is associated with unfavorable outcomes.[20,21]

Molecular structure and function of RUNX1-EVI1

Molecular structure of RUNX1-EVI1. The *RUNX1-EVI1* fusion gene is translated in frame to generate a chimeric transcription factor in which *RUNX1-EVI1* chimeric complementary DNA (cDNA), an open reading frame of 4185 nucleotides, encodes a 1395 amino-acid protein. The N-terminal of RUNX1 molecule including its Runt DNA-binding domain is fused to almost the entire portion of EVI1 molecule (Fig. 1). Therefore, the RUNX1-EVI1 fusion protein is a chimeric transcription molecule that consists of the Runt domain of RUNX1 and two ZF domains, CtBP-binding site and AD of EVI1.

Dominant-negative function over wild-type RUNX1. RUNX1-EVI1 dominantly suppresses the transactivation capacity of RUNX1 through the PEBP2 sites (Fig. 2).[5–9] Competition for the PEBP2 site-binding is proposed to be a mechanism of such dominant negative effects, since RUNX1-EVI1 binds to the PEBP2 sites with higher affinity than RUNX1 does. On binding to the PEBP2 site, RUNX1-EVI1 is believed to recruit corepressor complex via CtBP, since it is reported that RUNX1-EVI1 requires interaction with CtBP to repress RUNX1-induced transactivation.[31] In addition, the association with CtBP is also required for RUNX1-EVI1 to block myeloid differentiation of 32Dcl3 cells induced by granulocyte colony-stimulating factor (G-CSF), indicating that the association with CtBP is critical for RUNX1-EVI1 to exert its biological functions *in vivo*. Taken together, it is suggested that one of the mechanisms for RUNX1-EVI1-meidated leukemogenesis is the dominant-negative effects over wild-type

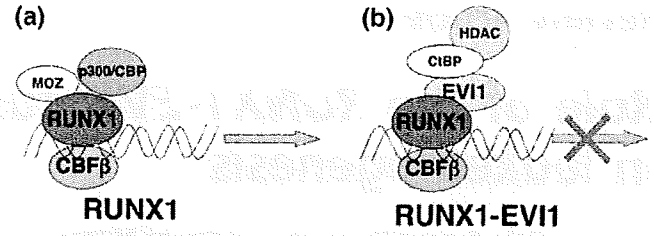

Fig. 2. (a) RUNX1-EVI1 exerts dominant-negative effects over wild-type RUNX1. RUNX1 forms an active transcription factor by heterodimerizing with CBFβ through Runt homology domain. CBFβ increases the DNA-binding ability of RUNX1 and protects RUNX1 from degradation. (b) Because RUNX1-EVI1 binds CBFβ more strongly than RUNX1, RUNX1-EVI1 competes RUNX1 out from the PEBP2 sites. RUNX1-EVI1 associates with CtBP and recruits HDAC. RUNX1-EVI1 actively represses the transcription of the RUNX1 target genes.

RUNX1 through an aberrant recruitment of a transcriptional corepressor complex.

Stimulation of cell growth through AP-1. The transcription factor AP-1 (Fos/Jun heterodimer or Jun/Jun homodimer) represents a prototype of regulatory protein that converts extracellular signals into gene expressions. AP-1 is activated by growth stimuli, including growth factors, phorbol esters such as 12-0-tetradecanoylphorbol 13-acetate (TPA), and various transforming oncogene products. In addition, AP-1 functions as a positive or negative regulator in a variety of cellular differentiation processes, such as changes in the differentiation processes of embryonal carcinoma cell lines, preadipocytes and myoblasts. Previously, we showed that Rat1 cells expressing RUNX1-EVI1 chimeric protein formed macroscopic colonies in soft agar,[32] indicating that RUNX1-EVI1 has oncogenic potential. Removal of the second ZF domain within the EVI1 sequence totally abrogated the ability of RUNX1-EVI1 to transform Rat1 cells. We showed that the transforming effect is correlated with the AP-1 activation induced by RUNX1-EVI1.[33]

Inhibition of JNK pathway. JNK is activated preferentially by extracellular stress stimuli including UV light, γ-radiation, osmotic shock, protein synthesis inhibitors, tumor necrosis factor-α and interleukin-1.[34] The JNK pathway is thought to play an important role in triggering apoptosis in response to cellular stresses. The activated JNKs translocate into the nucleus where they phosphorylate transcription factors such as c-Jun, and strongly augment their transcriptional activity. We showed that EVI1 physically interacts with and thereby inhibits the function of JNK.[24] For instance, EVI1 inhibits stress-induced cell death by inhibiting JNK. This inhibition of cell death by Evi1 could contribute to oncogenic transformation of cells. It has not been determined whether RUNX1-EVI1 possesses the similar antiapoptotic effect as EVI1 does. However, given that this antiapoptotic function is dependent on the first ZF domain in EVI1, it is reasonable to speculate that RUNX1-EVI1 also inhibits cell apoptosis by inhibiting JNK.

Suppression of TGF-β -mediated growth inhibition. TGF-β is one of the best characterized members of growth inhibitory factors. TGF-β inhibits proliferation of a wide range of cell types including epithelial, endothelial and hematopoietic cells.[35,36] Intracellular mechanisms that transmit TGF-β signaling have been clarified in detail. When TGF-β binds to its receptors, the down-stream signaling molecules Smad2 and Smad3 are phosphorylated by the activated TGF-β receptors, followed by oligomerization with Smad4. The Smad2/4 or Smad3/4 complexes accumulate in the nucleus, interact with DNA, and activate the transcription of TGF-β-responsive genes.[37,38] This process is apparently simple, but many proteins, including inhibitory Smads, participate in regulating the process and modify cellular responses to the stimuli.

Previously we reported that EVI1 antagonizes the growth-inhibitory effects of TGF-β in the epithelial cells.[22] Consistent with this finding, RUNX1-EVI1 also represses TGF-β-mediated growth inhibition of a murine hematopoietic precursor cell line, 32Dcl3.[39] The ability of RUNX1-EVI1 to repress TGF-β signaling depends on the two separate regions of the EVI1 portion, one of which is the first ZF domain. RUNX1-EVI1 interacts with Smad3 through this domain, and represses the Smad3 activity as EVI1 does.

Recently we have demonstrated that EVI1 represses Smad-induced transcription by recruiting CtBP as a corepressor,[40] indicating that RUNX1-EVI1 represses Smad3 activity through recruitment of CtBP.[31] EVI1 and RUNX1-EVI1 associate with CtBP through one of the consensus binding motifs, and this association is required for efficient inhibition of TGF-β signaling. A specific inhibitor for histone deacetylase (HDAC) alleviates EVI1-mediated repression of TGF-β signaling, suggesting that HDAC is involved in transcriptional repression by EVI1. The association with CtBP is required for RUNX1-EVI1 to block myeloid differentiation of 32Dcl3 cells induced by G-CSF.

Several chimeric proteins, such as RUNX1-ETO,[41,42] PML-RARα[43] and BCR-ABL[44] have self-interaction domains that are critical for their oncogenic capacity. RUNX1-EVI1 was reported to homo-oligomerize through at least three oligomerization regions,[45] i.e. the Runt domain, the first and the second ZF domains. Deletion of the second ZF domain significantly reduces the differentiation block of primary murine bone marrow progenitors by RUNX1-EVI1. A point mutation that inhibits CtBP binding also completely abrogates the effects of RUNX1-EVI1 on differentiation. These results imply the importance of homo-oligomerization for RUNX1-EVI1 chimeric proteins to exert its leukemogenic potentials.

Repression of C/EBPα. C/EBPα (CCAAT/enhancer binding protein α) is a leucine zipper transcription factor that regulates the expression of target genes containing C/EBP sites in their promoter regions.[46,47] Of the genes related to hematopoiesis, these genes include *CEBPA* itself,[48] *CEBPE*[48,49] and *granulocyte colony-stimulating factor (G-CSF) receptor*.[48,50] Inducible expression of C/EBPα is sufficient to trigger terminal granulocytic differentiation[51–54] and blocks monocytic differentiation program.[51,53] Conversely, inactivation of C/EBPα in *Cebpa* knock-out mice showed profound defects in the granulocytic differentiation, while all other hematopoietic cells are present in normal number, indicating its critical role in the granulopoiesis.[55] Several lines of evidence suggest that mutations in *CEBPA* is one of the major molecular events that could cause myeloid malignancies. Ten per cent of patients with AML M1 or M2, according to the French-American-British (FAB) classification, without frequent cytogenetic abnormalities such as t(8;21)(q22;q22), carry heterozygous *CEBPA* gene mutations that result in truncated proteins with a dominant negative effects.[49,56,57] The RUNX1-ETO fusion protein generated by the t(8;21) translocation represses the transcription of *CEBPA* by suppressing its auto-regulatory loop in gene transcription,[48] while the PML-RARα fusion protein generated by t(15;17)(q21;q22) inhibits the function of C/EBPα.[58] We confirmed that RUNX1-EVI1 suppressed the differentiation of LG-3 cells that differentiate along the myeloid lineage upon exposure to G-CSF.[59] Coexpression of C/EBPα restored the differentiation ability of the RUNX1-EVI1-expressing LG-3 cells. We also found that RUNX1-EVI1 associates with C/EBPα. RUNX1-EVI1 suppresses C/EBPα-mediated transcription of the *CEBPA* promoter in a CtBP-binding site-dependent fashion. In a gel-shift assay, RUNX1-EVI1 down-regulated the DNA-binding activity of C/EBPα. Therefore, the recruitment of HDAC by RUNX1-EVI1 and interference with the DNA binding of C/EBPα could be the mechanisms for the repression of C/EBPα by RUNX1-EVI1. These results indicate that inhibition of C/EBPα is related to the leukemogenic potential of RUNX1-EVI1. Helbling *et al.*[60] have reported that RUNX1-EVI1 represses C/EBPα in a different way. They reported that RUNX1-EVI1 reduces the level of C/EBPα protein but not the level of its mRNA in U937 cells and in leukemic blasts of patients carrying the *RUNX1-EVI1* translocation, and that RUNX1-EVI1 up-regulates the expression of calreticulin, a putative inhibitor of CEBPα translation. Calreticulin has calcium storage and chaperone function, and is postulated to be involved in the development of leukemia.[61] The small interference RNA (siRNA) against calreticulin showed that RUNX1-EVI1 inhibited C/EBPα expression in a post-transcriptional mechanism through calreticulin.

Biological function of RUNX1-EVI1

RUNX1-EVI1 transforms the most immature hematopoietic cells. As described above, Senyuk *et al.* showed that RUNX1-EVI1 transforms primary murine bone marrow progenitors, depending on both of the CtBP-binding site and the second ZF domain.[45,62] Recently, Takeshita *et al.*[63] introduced *RUNX1-EVI1* and its mutants in murine bone marrow cells and evaluated their transforming activities by colony replating assays. The transforming activity of RUNX1-EVI1 was lost when any of the known functional domains of EVI1, the first and the second ZF domains, AD at the C-terminus or CtBP-binding site, was deleted from the chimeric protein. Although RUNX1-EVI1 is known to repress function of wild-type RUNX1 dominantly, forced expression of EVI1 did not transform the *Runx1–/–* bone marrow cells, indicating that the existence of RUNX1-EVI1 means more than a simple combination of the presence of EVI1 and the absence of RUNX1. Interestingly, unlike the MLL-ENL or RUNX1-ETO leukemia-related chimeric proteins, which transform hematopoietic progenitors, RUNX1-EVI1 transforms only the hematopoietic stem cell fraction (c-kit + Sca-1 + Lin–). Moreover, RUNX1-EVI1-transformed cells show a cell-marker profile distinct from that of the cells transformed by RUNX1-ETO, which also suppresses RUNX1 function. The nature of RUNX1-EVI1-leukemia as hematopoietic stem cell tumors might be a consequence of these oncogenic preference of RUNX1-EVI1.

A bone marrow transplantation model. Cuenco *et al.*[64] analyzed the effect of the human *RUNX1-EVI1* fusion gene in mouse bone marrow cells using retroviral transduction system. Mice transplanted with RUNX1-EVI1-expressed bone marrow cells developed acute leukemia 5–13 months after the transplantation. The disease can be transferred into secondary recipient mice with a much shorter latency period. Morphological analysis of peripheral blood and bone marrow smears demonstrated the presence of myeloid blast cells and immature cells differentiated into both myelocytic and monocytic lineages. Cytochemical and flow cytometric analysis confirmed that these mice had a disease recapitulating human AML. These observations indicate that the expression of RUNX1-EVI1 can induce AML in mice, with extended latency period suggesting a requirement for additional perturbations. A study by the same group has demonstrated a cooperation of BCR-ABL and RUNX1-EVI1 in blocking myeloid differentiation and rapid induction of AML in mouse model.[65] The study showed that RUNX1-EVI1 alone does not block myeloid differentiation in the mouse bone marrow during the 4 months of preleukemia stage, while coexpression of BCR-ABL and RUNX1-EVI1 can block myeloid differentiation and induce AML rapidly. They also showed that both RUNX1 and EVI1 portions are required for RUNX1-EVI1 to cooperate with BCR-ABL in the induction of AML in mice.[66]

Recently, it has been shown in a virus transduced experiment that mice transplanted with bone marrow cells expressing RUNX1 mutants developed MDS/AML within 4–13 months.[67] Interestingly, the analysis of the viral integration sites showed that EVI1 seemed to be a collaborating gene for the RUNX1(D171N) mutant for the induction of MDS/AML. The disease has common

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phenotype characterized by marked hepatosplenomegaly, myeloid dysplasia, leukocytosis and biphenotypic surface markers. The collaboration between RUNX1(D171N) and EVI1 was confirmed by a bone marrow transplant (BMT) model, where coexpression of RUNX1(D171N) and EVI1 induced acute leukemia of the same phenotype with much shorter latency. These results suggest that a combination of dominant-negative effect over RUNX1 and the oncogenic property of EVI1, both of which are components of RUNX1-EVI1, could cause MDS or MDS/AML without additional hits. It seems important that RUNX1 should not be inactivated completely in order to cooperate with EVI1, because expression of EVI1 did not transform the *Runx1*-deleted murine bone marrow cells.[63]

***Runx1-EVI1* knock-in mice.** We knocked-in the *Runx1-EVI1* chimeric gene into the mouse *Runx1* genomic locus to explore the effect of Runx1-EVI1 in developmental hematopoiesis *in vivo*.[68] Our knock-in expression of *Runx1-EVI1* fusion gene results in embryonic lethality between E12.5 and E14.5, with CNS hemorrhage and a lack of fetal liver hematopoiesis. Post-enucleated erythrocytes were absent in the peripheral blood from E12.5 *Runx1-EVI1/+* embryos, whereas nucleated erythroblasts were abundantly observed. These findings indicate that *Runx1-EVI1/+* heterozygous mice fail to establish definitive hematopoiesis in the fetal liver as *Runx1–/–* mice. Therefore, RUNX1-EVI1 was first demonstrated to have *in vivo* dominant inhibitory effects over RUNX1. Electron microscopic examination of the E13.5 fetal liver showed that a fewer number of erythroid and myeloid progenitors, and dysplastic megakaryocytes that were defective for demarcation membrane exist in the *Runx1-EVI1/+* fetal liver.

On *in vitro* hematopoietic colony forming assays, the fetal liver from E13.5 *Runx1-EVI1/+* embryos contained multilineage hematopoietic progenitors, while that from E12.5 *Runx1–/–* embryos was reported to contain no definitive hematopoietic progenitors.[10,11] No erythroid colonies were seen in the livers at both E12.5 and E13.5. The CFU-GEMM-derived colonies from E13.5 *Runx1-EVI1/+* embryos included few erythroblasts, numerous agranular granulocytes with a delayed differentiation, and dysplastic megakaryocytes. Moreover, serial *in vitro* replating assays showed higher self-renewal capacity of hematopoietic progenitors in E13.5 *Runx1-EVI1/+* fetal livers than that in wild-type fetal livers.

On semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method with fetal livers, the expression of *Pu.1* mRNA in *Runx1-EVI1/+* fetal liver cells was comparable with that in the wild-type cells, whereas its expression level was markedly decreased in *Runx1–/–* fetal liver cells, as has been reported previously.[69] The maintained expression of PU.1 in *Runx1-EVI1/+* fetal liver cells may support the survival of their multilineage hematopoietic progenitors up to E13.5, and enhance their monocyte/macrophage lineage differentiation. These differences in gene expression pattern provide logical explanation for the distinct hematopoietic capacity between *Runx1–/–* and *Runx1-EVI1/+* fetal liver cells. These data suggest that the sufficient expression of the *Pu.1* gene is the prerequisite for definitive hematopoiesis in the fetal liver.

***Runx1-EVI1* knock-in chimeric mice.** Of the six chimeric mice created, one mouse which died at 5 months of age showed marked hepatosplenomegaly. Wright-Giemsa staining of stump preparation from the enlarged spleen demonstrated massive infiltration of large dysplastic cells, some of which contained multilobulated nuclei with various size of cytoplasm reminiscent of megakaryoblastic leukemia.[70] Histology section showed disrupted gross architecture of the spleen, with white and red pulp intermingling, and the electron microscopic analysis of the infiltrating cells in the spleen showed 20% of the cells positive for platelet-peroxidase, indicating that this chimeric mouse developed megakaryoblastic leukemia. The important aspect of our observation is that RUNX1-EVI1 protein could be leukemogenic per se, in contract to RUNX1-ETO, which requires additional hits to induce leukemia.[70–72] This clear difference in the pathophysiological outcome likely arises from the EVI1 portion of RUNX1-EVI1 protein. Oncogenic functions of EVI1 described above may cause stronger oncogenic capacity of RUNX1-EVI1 than RUNX1-ETO. Another important aspect is that the affected lineage in human leukemia is recapitulated in the experimental animal. Our observation indicates strong causal relationship between the expression of RUNX1-EVI1 protein and megakaryoblastic leukemia. Indeed, the *RUNX1-EVI1* chimeric gene was isolated from a patient developing megakaryoblastic crisis in chronic myelocytic leukemia with additional chromosome t(3;21).

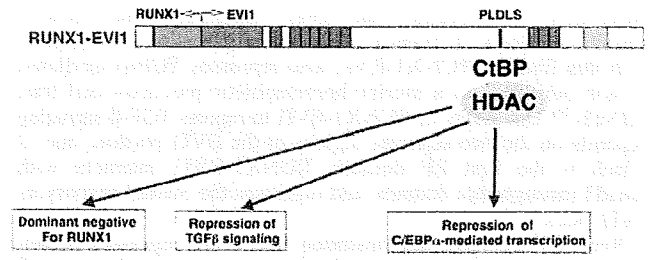

Fig. 3. RUNX1-EVI1 is a multifunctional oncoprotein. RUNX1-EVI1 exerts oncogenic function through three pathways: dominant-negative effect over RUNX1, repression of TGFβ signaling and inhibition of C/EBPα activity.

Clinical aspects

As mentioned above, the major leukemogenic effect of RUNX1-EVI1 is through HDAC-recruitment via CtBP (Fig. 3). Thus repression of HDAC activity is thought to suppress oncogenic effect of RUNX1-EVI1. HDAC inhibitors (HDACi) are the member of a new class of chemical agents that epigenetically modulate gene transcription by enhancing the acetylation of core nucleosomal histones and are expected to be promising anticancer agents against various types of tumors. Suberoylanilide hydroxamic acid (vorinostat), one of the newly synthesized HDACi, is the most clinically successful HDACi that controls cutaneous T-cell lymphoma (CTCL) effectively.[73] Conventional HDACi, such as valproic acid and butylate, are also reported to be effective in treating some types of hematological malignancies in clinical trials. Recently, we have reported that HDACi (trichostatin A and VPA) triggers apoptosis in human leukemic cell lines expressing RUNX1-related chimeric proteins such as RUNX1-ETO, TEL-RUNX1 or RUNX1-EVI1.[74] A cell line without RUNX1-related chimeras is less affected by the HDACi treatment. These data suggest that HDACi seems to be an attractive choice in the molecular targeting therapy of RUNX1-EVI1-expressing leukemia.

Another potential therapeutic agent targeted to RUNX1-EVI1 is arsenic trioxide (ATO). Shackelford *et al.*[75] found that ATO degrades RUNX1-EVI1. The ATO treatment induces the differentiation and apoptosis of RUNX1-EVI1-expressing leukemic cells *in vitro* and elongates the survival of mice transplanted with these cells *in vivo*. They also demonstrated that ATO targets RUNX1-EVI1 via two moieties, MDS1 and EVI1 moieties. The EVI1 induces a ubiquitin-proteasome pathway and MDS1 induces a proteasome-independent pathway. With abundant experiences of clinical use in treating acute promyelocytic leukemia, ATO could be used clinically as a targeted therapy for RUNX1-EVI1-positive human leukemia.

Summary

The fusion protein RUNX1-EVI1 is a multifunctional protein demonstrated by its diverse role in regulating differentiation,

proliferation, apoptosis and self-renewal capacity. It functions as a dominant-negative suppressor of RUNX1 and has oncogenic properties of EVI1. There is no doubt that RUNX1-EVI1 plays a major role in t(3;21)-related MDS and MDS/AML. It remains to be determined whether RUNX1-EVI1 induces leukemia by itself, or needs additional genetic events. A conditional knock-in experiment of the *RUNX1-EVI1* gene would help clarify this question.

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ORIGINAL ARTICLE

Myelodysplastic syndrome with chromosome 5 abnormalities: a nationwide survey in Japan

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Chromosome 5 abnormalities, deletion of the long arm of chromosome 5 (del(5q)) or monosomy 5 (−5), arise in about 10% of myelodysplastic syndromes (MDS), either as the sole cytogenetic abnormality or as part of complicated karyotype, and has distinct clinical implications for MDS. However, the prognostic factors of MDS patients with chromosome 5 abnormalities are not determined yet. In this study, 183 Japanese MDS patients with chromosome 5 abnormalities were analyzed. Estimated incidence of del(5q) and 5q- syndrome among MDS patients was 8.4 and 1.3%, respectively. Significant shorter overall survival (OS) and leukemia-free survival (LFS) were observed in −5 patients than del(5q) patients. Among del(5q) patients, addition of monosomy 7 or complex karyotype with more than three abnormalities were significantly related to shorter OS.

LFS of del(5q) patients was divided into two risk groups by international prognostic scoring system (IPSS): low/intermediate (Int)-1 and Int-2/high groups. LFS sorted by World Health Organization classification-based prognostic scoring system (WPSS) was also divided into two groups: very low/low/Int and high/very high, and WPSS was able to predict the outcome of del(5q) patients more clearly than IPSS.

Together with additional cytogenetic data, WPSS might be useful for clinical decision making in MDS patients with del(5q).
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Introduction

Loss of part of the long arm of chromosome 5 (del(5q)) is a frequent clonal chromosomal abnormality in patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML),[1] and is thought to contribute to the pathogenesis of these diseases by deleting one or more tumor-suppressor genes.[2] MDS with del(5q) is a heterogeneous disease,[3,4] apart from 5q- syndrome,[5,6] and often accompanies additional cytogenetic abnormalities leading to the poor-risk karyotypes, or an increase of bone marrow and/or peripheral blasts irrespective of chromosomal complexity. These distinct disease subgroups have dramatically different prognostic features.[3,5,7,8]

The international prognostic scoring system (IPSS), defined by bone marrow blast percentage, number of peripheral cytopenias and cytogenetic pattern, has become a benchmark for clinical decision making.[9] Recently, on the other hand, the World Health Organization (WHO) classification-based prognostic scoring system (WPSS) has been proposed based on WHO classification,[10] cytogenetic pattern and transfusion dependency as independent indicators of disease severity.[11]

To elucidate the prognostic features of Japanese del(5q) MDS patients, we adapted IPSS, WHO criteria and WPSS to 131 MDS patients with del(5q), 52 patients with monosomy 5 (−5) and 375 MDS patients who did not carry chromosome 5 abnormality, to estimate the mortality rates and life expectancy of these groups as the base for adapting treatments.

Materials and methods

Patient data

A total of 50 MDS patients with chromosome 5 abnormalities were collected in the first series of the survey within 425 MDS patients recorded by the Japanese Cooperative Study Group for Intractable Bone Marrow Diseases. Of 375 patients who did not carry chromosome 5 abnormality in this series were used as controls. In addition to these 50 patients, we conducted a retrospective survey on MDS patients with chromosome 5 abnormalities across 285 hospitals in Japan. A total of 133 cases were newly collected.

A total of 558 MDS patients were collected and 183 patients with chromosome 5 abnormalities and 375 patients with a morphologically normal chromosome 5 were analyzed for (1) additional chromosomal abnormalities, (2) French American and British (FAB) and WHO criteria, (3) IPSS and WPSS,[9–12] (4) clinical outcome and (5) degree of cytopenia. MDS patients with chromosome 5 abnormalities were classified according to FAB and WHO criteria in Table 1.

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Table 1 Classification of MDS with chromosome 5 abnormalities

Case number	*Total*		*del(5q)*[a]		*Monosomy 5*		*Isolated del(5q)*[b]	
Classification	*183*		*131*		*52*		*35*	
	FAB	*WHO*	*FAB*	*WHO*	*FAB*	*WHO*	*FAB*	*WHO*
RA	61	25	51	18	10	7	24	
RCMD		14		11		3		2
RARS	5	3	4	2	1	1	1	
RCMD-RS		1		1				
RAEB-1	81	46	56	30	25	16	8	4
RAEB-2		45		31		14		5
RAEB-t	20		10		10			
CMML	2	2	2	2				
AML	14	24	8	14	6	10	2	2
5q- syndrome		21		21				21
Others		2		1[c]		1[d]		1[c]

Abbreviations: AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; FAB, French American and British; RA, refractory anemia; RAEB-t, refractory anemia with excess blast in transformation; RARS, RA with ringed sideroblast; RCMD-RS, refractory cytopenia with multilineage dysplasia with ringed sideroblast; WHO, World Health Organization.
[a]Includes all del(5q) cases with or without other chromosomal abnormalities.
[b]Includes the cases having only del(5q) and some cases besides 5q- syndrome.
[c]MDS/MPD, unclassifiable case.
[d]MDS, unclassifiable case.

Categorization of del(5q) MDS by additional chromosomal abnormalities

Fifty-two patients with −5 were initially separated, and then del(5q) patients were assigned to one of four cytogenetic categories according to their karyotypes: del(5q) alone as 5q- that included 5q- syndrome, del(5q) with additional chromosome 7 abnormality as (7+), del(5q) with more than three abnormalities as 'complex' and del(5q) patients with cytogenetic aberrations other than 7+ and complex defined as 'other'. Of 131 del(5q) patients, 35 patients were categorized as 5q-, 47 were 7+, 35 were complex and 14 were other.

Statistical analysis

First, statistical test of homogeneity between the two patient data collections was carried out. Actuarial probability of overall survival (OS) and leukemia-free survival (LFS) were estimated using the Kaplan–Meier product limit method. OS was defined as the time between diagnosis and death of any cause or end of follow-up. LFS was calculated from diagnosis to leukemic progression or end of follow-up. Patients who died before leukemic progression were considered as censored at the time of death and those who received stem cell transplantation were censored at the time of transplantation. Comparisons between Kaplan–Meier curves were carried out by Gehan's Wilcoxon's test. To assess the relation of cytogenetic abnormalities with hematological values, Mann–Whitney's *U*-test was carried out using the hemoglobin concentration, neutrophil and platelet count.

Univariate analyses were performed by χ^2-test and multivariate analyses were performed by Cox proportional hazard regression model. The data were considered statistically significant if *P*-values were less than 0.05. These analyses were carried out using SPSS for Windows version14.0. The whole study was in accordance with the modified Declaration of Helsinki.

Results

Patient characteristics

As the data of patients with chromosome 5 abnormalities were collected in two cohorts, we performed the statistical test of homogeneity between the two data collections on such factors as patient ages, gender, WHO classification, hemoglobin concentration, neutrophil and platelet counts, degree of red cell transfusion dependency and confirmed that the two groups were not statistically different each other.

Total patient characteristics are listed in Table 1. The median age of patients with chromosome 5 abnormalities was 69 years and the male-to-female ratio was 113/70, the median age of patients with del(5q) was 69 years and the male/female ratio was 80/51, consistent with the well-known male predominance of MDS. Of the 183 patients, 131 (71.5%) were del(5q) patients with or without other chromosomal abnormalities, 21 (11%) were 5q- syndrome and 52 (28.4%) were −5. Here we defined the cases with macrocytic red cells, isolated del(5q), bone marrow blasts less than 5% as 5q- syndrome. Two refractory cytopenia with multilineage dysplasia (RCMD) cases with isolated del(5q) were excluded because of microcytic anemia without iron deficiency or detection of t(5;17) by further analysis.

Of 425 Japanese MDS patients recorded in the Japanese Cooperative Study Group for Intractable Bone Marrow Diseases, 50 (11.8%) had chromosome 5 abnormalities; therefore, the estimated rate of del(5q) patients and 5q- syndrome among MDS patients was 8.4 and 1.3%, respectively. The incidence of 5q- syndrome proved to be quite rare in Japan.

Impact of chromosome 5 abnormalities on cytopenia

As compared with MDS patients who did not have chromosome 5 abnormality, patients with chromosome 5 abnormalities had significantly severe anemia, whereas no significant difference in the degree of anemia was observed between patients with del(5q) without 5q- syndrome, −5 and 5q- syndrome (Figure 1a and inset table of Figure 1c). Except for 5q- syndrome patients, neutropenia was significantly severe in patients with chromosome 5 abnormalities (Figure 1b and inset table of Figure 1c). Significant thrombocytopenia was observed in −5 patients as compared with del(5q) patients and patients without chromosome 5 abnormality (Figure 1c and inset table). As expected, the platelet count of 5q- syndrome patients remained within the normal range, which was significantly higher than that of del(5q) excluding 5q- syndrome.

Impact of chromosome 5 abnormalities on survival

Although the median OS of MDS patients without chromosome 5 abnormality was 2358 days, that of patients with chromosome 5 abnormalities was 454 days and significantly short (Figure 2a). To analyze more precisely, patients with chromosome 5 abnormalities were divided into 5q- syndrome, del(5q) excluding 5q- syndrome and (−5) patients. In total, 52 patients were classified as −5, 110 patients were classified as del(5q) excluding 5q- syndrome and 21 were categorized as 5q- syndrome. The median OS of 5q- syndrome was over 6000 days but that of del(5q) excluding 5q- syndrome and −5 was 501 days and 210 days, respectively.

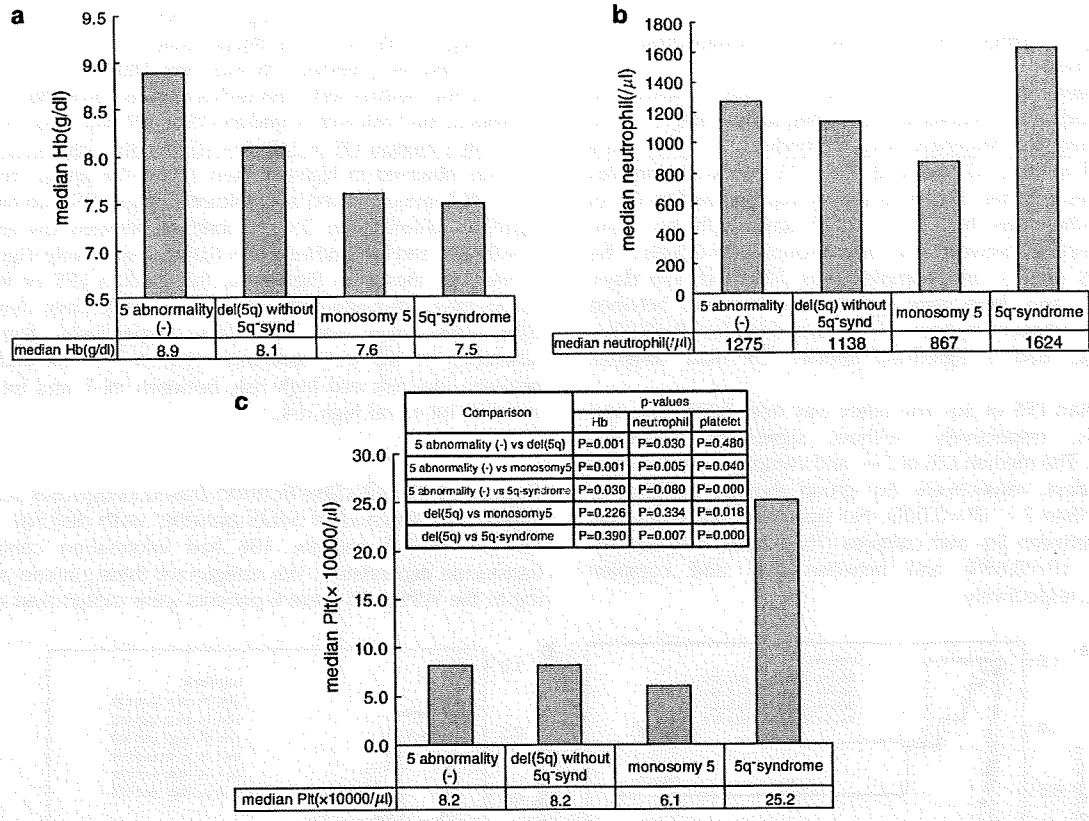

Figure 1 Cytopenias in four categories of myelodysplastic syndromes (MDS) with chromosome 5 abnormalities. (**a**) Hemoglobin concentration, (**b**) neutrophil count and (**c**) platelet count. Median values are indicated by a column and numerals. MDS patients were categorized into chromosome 5 abnormality (−; 375 cases), del(5q) without 5q- syndrome (110 cases), monosomy 5 (52 cases) and 5q- syndrome (21 cases). Mann–Whitney's *U*-test was performed between the groups and the *P*-values are indicated collectively in the inset table of Figure 1c.

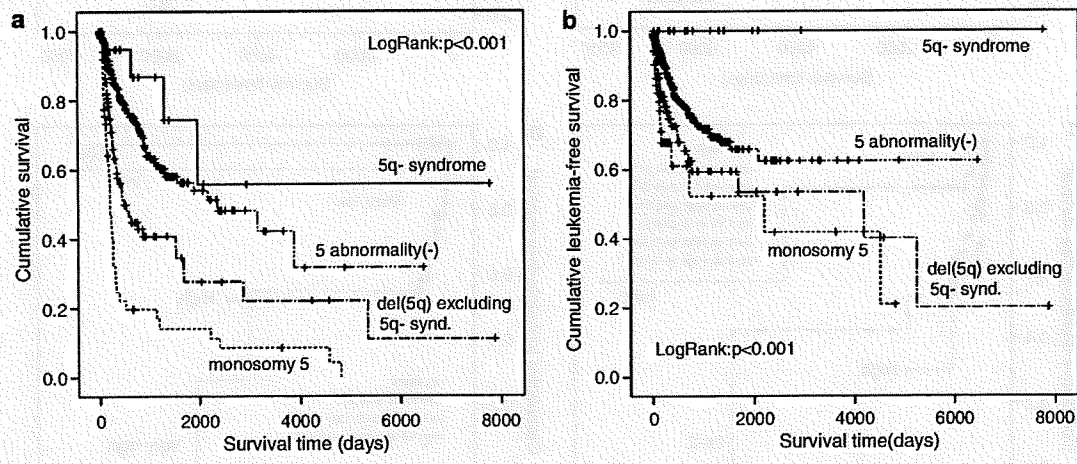

Figure 2 Impact of chromosome 5 abnormalities by three categories on the survival of myelodysplastic syndromes (MDS) patients. MDS patients were categorized into no chromosome 5 abnormality (375 cases), del(5q) (110 cases), monosomy 5 (52 cases) and 5q- syndrome (21 cases). (**a**) Overall survival curves and (**b**) leukemia-free survival curves were drawn by Kaplan–Meier method, and Wilcoxon's Log-rank test was performed in a lump.

It is noteworthy that none of the 5q- syndrome patients died of leukemic progression. The median LFS of patients without chromosome 5 abnormality was over 6000 days but that of patients with del(5q) excluding 5q- syndrome was 4176 days and significantly short (Figure 2b). The median LFS of −5 was 2199 days.

Of 52 patients with −5, 37 carried monosomy 7 together, whereas 47 of 131 del(5q) patients did, which indicated that MDS patients with −5 carry monosomy 7 together in a significantly higher incidence than del(5q) patients (χ^2-test, $P<0.001$).

Survival analysis of MDS patients with del(5q) according to categories of additional chromosomal abnormalities

We paid attention to the outcome of 131 MDS patients with del(5q) including 5q- syndrome. According to the categorization as mentioned in 'Materials and methods', 35 cases were categorized as 5q-, 47 cases as 7+, 35 cases as complex and 14 cases as other. Figure 3a shows that the median OS of 5q- and other was both over 6000 days with no significant difference between the two groups ($P=0.329$). The median OS of 7+ and complex was 240 and 458 days, respectively, and there were significant differences between 5q- and 7+ ($P<0.001$), between 5q- and complex ($P<0.001$). 7+ patients had a significant shorter OS than complex ($P=0.018$).

The median LFS of 5q- and other was over 6000 days and 4176 days, respectively, without significant difference ($P=0.699$). The median LFS of 7+ and complex was 770 days and 5247 days, respectively. 5q- group showed significantly longer LFS than 7+ ($P=0.006$). No significant difference was observed between 5q- and complex ($P=0.069$), between 5q- and other ($P=0.699$) and between 7+ and complex ($P=0.236$), respectively.

Survival analysis according to IPSS

According to IPSS, of 131 del(5q) patients, 11 were categorized as low-risk and their median OS was over 1800 days, 37 patients were as Intermediate (Int)-1 risk with a median OS of 2863 days, 50 patients as Int-2 risk with a median OS of 501 days and 33 as high risk with a median OS of 248 days (Figure 3b). Significant shorter OS was observed in high-risk than in low-risk group, Int-1 and Int-2. Int-1 group showed a significantly longer OS than Int-2. No significant difference in OS was observed between low and Int-1 ($P=0.423$), and low and Int-2 ($P=0.058$), respectively (Figure 3b).

Next, as shown in Figure 3c, the median LFS of low-risk group, Int-1, Int-2 and high risk was over 1800 days, over 6000 days, 1682 days and 770 days, respectively. Significant difference in LFS was observed between low-risk and Int-2, between low risk and high risk, between Int-1 and Int-2 and between Int-1 and high risk.

Impact of WHO classification-based prognosis scoring system on survival of MDS patients with del(5q)

Of 131 del(5q) patients, 106 had information concerning transfusion dependency. We categorized these patients according to the WPSS. Of these 6 patients were categorized as very

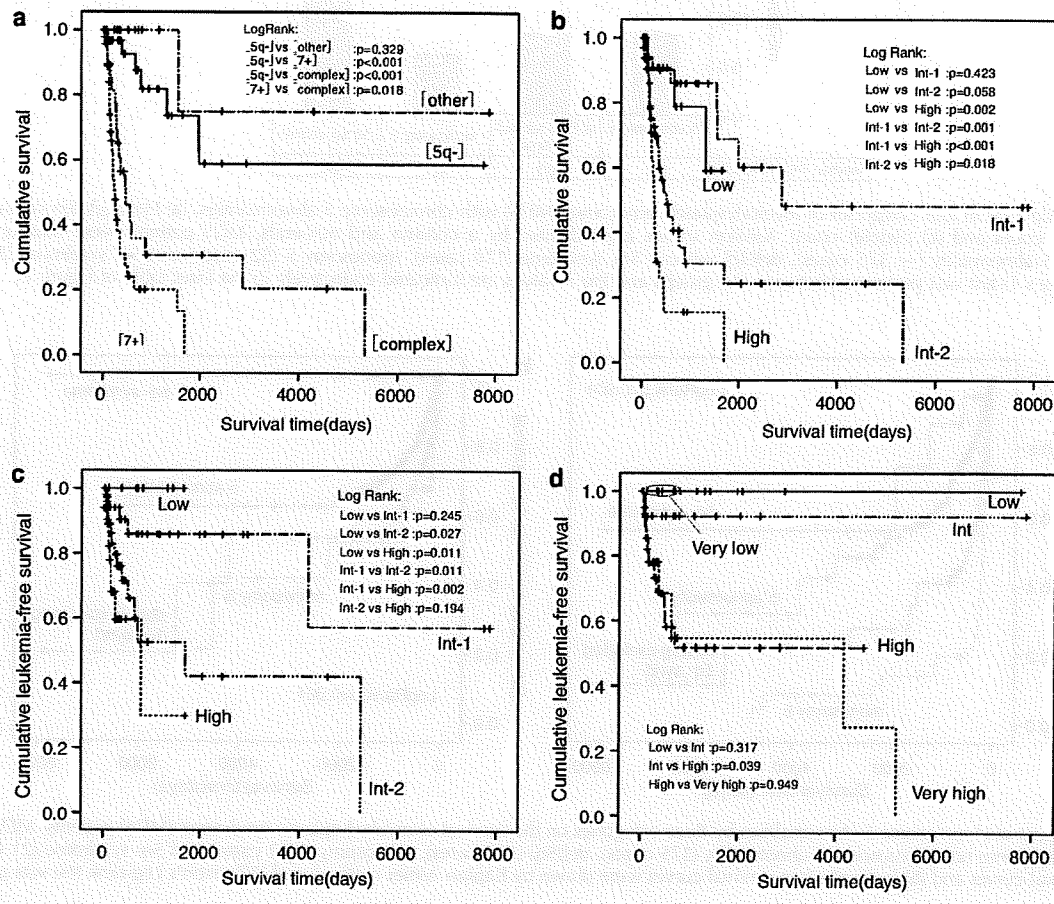

Figure 3 Impact of several factors on the survival of myelodysplastic syndromes (MDS) patients with del(5q). **(a)** Overall survival of MDS patients with del(5q) categorized by additional chromosomal abnormalities. A total of 131 MDS patients with del(5q) were categorized into 5q- (35 cases), 7+ (47 cases), complex (35 cases) and other (14 cases). **(b)** Overall survival of MDS patients with del(5q) categorized by IPSS. A total of 131 MDS patients with del(5q) were categorized into low cases, intermediate (Int-1, 37 cases), Int-2 (50 cases) and high (33 cases). **(c)** Leukemia-free survival of MDS patients with del(5q) categorized by IPSS. A total of 131 MDS patients with del(5q) were categorized into low (11 cases), Int-1 (37 cases), Int-2 (50 cases) and high (33 cases). **(d)** Leukemia-free survival of MDS patients with del(5q) categorized by WPSS. A total of 106 MDS patients with del(5q) were categorized into very low (6 cases), low (13 cases), Int (16 cases), high (29 cases) and very high (42 cases). The survival curves were drawn by Kaplan–Meier method, and Wilcoxon's Log-rank test was performed in the indicated pairs.

low risk, 13 as low risk, 16 as Int, 29 as high and 42 as very high. As for patients categorized by WPSS, the patterns of LFS were divided into two groups such as very low/low/Int and high/very high Figure 3d). Although the duration of observation was short, none of the very low-risk group and only one case of the low-risk group progressed to leukemia.

Hence, we divided IPSS and WPSS classifications into lower-risk and higher-risk groups; that is, low/Int-1 and Int-2/high in IPSS and very low/low/Int and high/very high in WPSS described above, respectively. We then applied our patient data to each scoring system and calculated the statistic sensitivity to divide the outcome of each patient into: (1) 'alive or dead' and (2) 'leukemia-free or leukemic transformation'. The sensitivity for dividing the events 'alive or dead' was 75.6% in IPSS and 85.4% in WPSS. Likewise, the sensitivity to divide into 'leukemia-free or leukemic transformation' was 78.3% in IPSS and 95.7% in WPSS. These data demonstrated that WPSS was able to predict the outcome of MDS patients with del(5q) more clearly than IPSS.

Determination of prognostic factors in MDS patients with del(5q)

As the univariate analysis, age, gender, hemoglobin concentration, platelet count, neutrophil count, percentage of bone marrow blasts, cytogenetic pattern, red cell transfusion dependency and platelet transfusion dependency were examined. Table 2 indicates the relevant factors for death and leukemic progression rates in the del(5q) patients. This analysis showed

Table 2 Prognostic risk factors of MDS patients with del(5q) by univariate analysis

Variables	*Number*	*Death rate*				*Leukemic progression rate*			
		Odds ratio	*95% CI*	*χ^2-value*	*P-value*	*Odds ratio*	*95% CI*	*χ^2-value*	*P-value*
Gender									
Male	65								
vs		1.630	0.717–3.704	1.370	0.242	0.976	0.379–2.516	0.003	0.96
Female	41								
Age									
>60	83								
vs		1.155	0.436–3.058	0.084	0.772	1.336	0.403–4.428	0.225	0.635
≤60	22								
Hb									
>9 g per 100 ml	77								
vs		2.485	0.949–6.504	3.559	0.059[a]	0.635	0.236–1.710	0.815	0.367
⩾9 g per 100 ml	29								
Plt									
<100 000 per μl	53								
vs		1.491	0.679–3.273	0.994	0.319	0.895	0.355–2.255	0.056	0.814
⩾100 000 per μl	53								
Neutrophil									
<1800 per μl	69								
vs		1.261	0.551–2.887	0.301	0.583	3.135	0.978–10.044	3.966	0.046[b]
⩾1800 per μl	37								
BM blasts									
⩾5%	50								
vs		2.500	1.121–5.576	5.114	0.024[b]	2.031	0.791–5.216	2.212	0.137
<5%	56								
Additional chromosome abnormalities									
7 Abnormality/complex	61								
vs		6.836	2.640–17.700	17.629	<0.001[b]	2.511	0.900–7.004	3.22	0.073
Isolated (5q) or others	45								
Red cell transfusion dependency									
Dependent	80								
vs		2.006	0.758–5.310	2.007	0.157	0.679	0.243–1.892	0.554	0.457
Independent	26								
Platelet transfusion dependency									
Dependent	31								
vs		3.425	1.406–8.344	7.662	0.006[b]	2.045	0.745–5.617	1.971	0.16
Independent	66								

Abbreviations: BM, bone marrow; CI, confidence interval; Plt, platelet
[a]Shows nearly significant difference.
[b]Shows statistically significant differences.

that the major risk factors for death rate were the percentage of bone marrow blasts, cytogenetic pattern and platelet transfusion dependency. It was not significant whether the degree of anemia influences the outcome. The only major factor for leukemic progression was neutrophil count.

We further investigated the prognostic factors by multivariate analyses using Cox proportional hazard regression model with fixed covariates and found that the most significant independent risk factors for determining outcome were the percentage of bone marrow blasts, cytogenetic pattern and platelet transfusion dependency (Table 3). The major factors predictive of leukemia progression were the cytogenetic pattern, the presence of neutropenia and thrombocytopenia. Multivariate analyses excluding the influence of red cell and platelet transfusion dependency revealed that the risk factors for OS were bone marrow blasts ($P=0.044$) and cytogenetic pattern ($P<0.001$), and the risk factors for LFS were neutrophil count ($P=0.026$), platelet count ($P=0.023$) and cytogenetic pattern ($P=0.008$). In contrast, Multivariate analyses excluding the influence of hemoglobin concentration and platelet count revealed that cytogenetic pattern was a risk factors for OS ($P=0.003$) but neither red cell nor platelet transfusion dependency was a statistically significant risk factor for OS.

Discussion

The biological and clinical significances of −5 and deletion of the long arm of chromosome 5 (del(5q)) are accepted as equivalent, or at least quite similar, in patients with AML and

Table 3 Prognostic risk factors of MDS patients with del(5q) by multivariate Cox hazards regression analysis

Variables	*Number*	*Overall survival*			*Leukemia-free survival*		
		P-value	*Hazard ratio*	*95% CI*	*P-value*	*Hazard ratio*	*95% CI*
Gender							
Male	65						
vs		0.162	1.796	0.790–4.085	0.688	1.248	0.424–3.678
Female	41						
Age							
>60	83						
vs		0.351	0.626	0.235–1.672	0.682	1.344	0.327–5.533
⩽60	22						
Hb							
<9 g per 100 ml	77						
vs		0.106	2.212	0.844–5.795	0.642	0.760	0.239–2.418
⩾9 g per 100 ml	29						
Platelets							
<100 000 per μl	53						
vs		0.145	0.501	0.197–1.271	0.038[a]	0.277	0.082–0.934
⩾100 000 per μl	53						
Neutrophil							
<1800 per μl	69						
vs		0.787	1.116	0.504–2.469	0.045[a]	3.377	1.030–11.072
⩾1800 per μl	37						
BM blasts							
⩾5%	50						
vs		0.047[a]	2.288	1.011–5.175	0.416	1.589	0.521–4.852
<5%	56						
Additional chromosome abnormalities							
7 Abnormality/ complex	61						
vs		0.002[a]	4.421	1.692–11.552	0.028[a]	4.333	1.169–16.056
Isolated 5q- or others	45						
Red cell transfusion dependency							
Dependent	80						
vs		0.398	0.637	0.224–1.812	0.422	0.578	0.152–2.202
Independent	26						
Platelet transfusion dependency							
Dependent	31						
vs		0.047[a]	2.403	1.013–5.703	0.316	1.841	0.558–6.068
Independent	66						

Abbreviations: BM, bone marrow; CI, confidence interval.
[a]Shows statistically significant differences.

MDS.[13–15] In fact, the prognostic value of these two chromosome aberration groups was not significantly different among AML patients,[15] but sufficient data for MDS were lacking. The present study demonstrates that the prognosis of patients who carry −5 and del(5q) are significantly different, as OS and LFS of −5 group were shorter than del(5q) patients even if 5q- syndrome patients were excluded from del(5q) patients (Figure 2 and data not shown). AML patients with monosomy 7 rather than with deleted 7q chromosome were reported to lead to poor prognosis.[16] Significantly poor prognosis of −5 group in our series might be explained by the observation that −5 was significantly correlated with presence of monosomy 7 as compared to del(5q) group ($P<0.001$). The co-presence of chromosomes 5 and 7 abnormalities has been associated with poor outcomes in MDS and AML.[17–22]

Although the cause of leukemic progression is unknown, susceptibility to leukemia clearly leads to higher mortality of −5 patients compared to del(5q) patients (Figure 2b). The −5 group also had significantly more severe neutropenia and thrombocytopenia and might exacerbate the survival of this group (Figures 1b and c). Neutropenia and thrombocytopenia, but not severe anemia, are reported to be common findings of patients with monosomy 7.[23] Taken together, laboratory findings shown in −5 group and high incidence of the co-presence of −5 and monosomy 7 might result in poor prognosis of the corresponding patients.

In this survey, the incidence of 5q- syndrome was quite rare in Japan. Recent studies suggest different genetic or environmental backgrounds between Asian and Western MDS populations.[24,25] According to the recent report by Haase *et al.*,[28] isolated del(5q) was seen in 14%, del(5q) with one additional abnormality in 5%, and complex abnormalities including del(5q) were seen in 11% of patients with clonal abnormalities. In the Korean study, isolated del(5q) was seen in 1.7% of the patients.[24] The incidence of del(5q), isolated del(5q) and 5q- syndrome patients was 8.4, 2.2 and 1.3%, respectively, in Japanese MDS study. These data are lower than those of Western patients but more similar to those of Asian patients.[24–26]

In the present study, we paid particular attention to MDS patients with del(5q) and classified them into four groups according to the cytogenetic complexity: 5q-, 7+, complex and other. 5q- patients have previously been well defined as having relatively good prognosis, whereas poor prognosis was indicated when it was combined with other anomalies.[19,20,26,27] OS and LFS of 7+ group were significantly shorter than 5q- group and OS of complex was significantly shorter than 5q-(Figure 3a and data not shown). It is suggested that the clinical outcome of MDS patients with del(5q) depends on the prognostic value of combined chromosomal abnormalities.

The most significant independent prognostic variables in MDS are the percentage of bone marrow blasts, the number of cytopenias and cytogenetic pattern. By weighting these variables according to their statistic power, IPSS separates MDS patients into four distinct risk groups regarding survival and the potential for leukemic progression: low risk, Int-1, Int-2 and high risk.[10] Even in the del(5q) patient group, which is considered to have a better prognosis, univariate analysis and multivariate analysis of del(5q) patients in our series showed that the cytogenetic pattern, percentage of bone marrow blasts and platelet transfusion dependency were the most relevant risk factors (Tables 2 and 3). Figures 3b and c show that IPSS critically determines OS and LFS of del(5q) patients. All 21 patients classified as 5q- syndrome were low risk of IPSS, and the patients classified as high risk were AML and refractory anemia with excess blasts (RAEB)-2 cases with adverse chromosomal abnormalities.

As for the del(5q) patients who are far from the risk of leukemic progression, red cell transfusion dependency often has an adverse impact on survival.[28] Although red cell transfusion dependency was not a significant prognostic factor by the present analyses, the degree of anemia had a tendency to affect survival of these patients from the result of univariate analysis ($P=0.059$, Table 2).

Although IPSS is based on FAB classification and does not take into account other prognostic factors such as dysplasia and transfusion requirement, WPSS has a relevant prognostic value.[29] Therefore, we applied WPSS to our patient data (Figure 3d) and confirmed that WPSS can predict the prognosis of del(5q) MDS patients more clearly than IPSS.

Our study demonstrated that −5 and del(5q) belong to different clinical entities and their biological behaviors are different from each other, and that del(5q) patients can be stratified according to their additional chromosomal abnormalities and IPSS or WPSS status. Severe anemia requires frequent transfusions, reduces quality of life and becomes often the major clinical problem for MDS patients with del(5q). The prognosis of del(5q) patients is related to their status of chromosomal abnormalities and transfusion dependency, but new agents such as lenalidomide improve the disorder and might provide new insights into more precise understanding of the disease.[30]

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