Table 1.
Mutations and Single Nucleotide Polymorphisms (SNPs) for the TP53, ETV6, and TRIM28 Genes

Gene	Nucleotide Position	Amino Acid Change	Frequency, n
TP53	Missense mutation		
	T485G	I162S	1/40 (2.5%)
	A707G	Y236C	1/40 (2.5%)
	A838G	R280G	1/40 (2.5%)
	G461A	G154D	1/40 (2.5%)
	SNPs		
	G215C	R72P	20/40 (50.0%)
	G818C	R273H	6/40 (15.0%)
ETV6	SNPs		
	G171A		2/40 (5.0%)
	G258A		2/40 (5.0%)
	G632A	R211H	1/40 (2.5%)
TRIM28	SNPs		
	G1170A		5/40 (12.5%)
	C2148T		6/40 (15%)
	C2175T		1/40 (2.5%)
	C2262T		1/40 (2.5%)

(SNPs) at nucleotide positions 215 and 818 (the numbers are relative to A of the ATG start codon).

In contrast to the frequent amino acid changes reported to occur in TP53, few structural mutations in MDM2 have been reported in human hematologic malignancies. Consistently, we sequenced the coding region of *MDM2* mRNA and found neither mutations nor SNPs in our MDS samples (data not shown). Next, we investigated the nucleotide sequence for the coding region of *ETV6* mRNA. ETV6 is frequently involved in the generation of human leukemia by forming fusion proteins with various partners; however, nucleotide alterations in the *ETV6* gene that cause amino acid changes have never been reported. The results of our *ETV6* mRNA sequencing analysis identified minor alterations in the nucleotide sequence (2 silent SNPs and 1 missense SNP), each of which was found in fewer than 5% of the patients. No amino acid mutation was found, indicating that minor structural changes in the ETV6 protein are unlikely to have a role in the development of MDS. We also surveyed mutations in *TRIM28* and found only silent SNPs, indicating that the secondary structure of this molecule is highly conserved. In summary, we identified mutations in the *TP53* coding sequence at a frequency similar to the reported frequencies but found no abnormal changes in the amino acid sequences of MDM2, ETV6, and TRIM28.

The search for structural (amino acid) changes in MDM2, ETV6, and TRIM28 molecules assumes that such changes would converge on the down-regulation of the tumor suppressor activity of TP53. However, TP53 is also regulated by the expression levels of its regulatory molecules. For instance, high levels of MDM2 expression have been reported in some forms of hematologic malignancies and are associated with a poor prognosis. In biochemical analyses, overexpression of TRIM28 has been shown to promote TP53 ubiquitination and degradation, leading to a decrease in TP53-dependent transcription activity and cell cycle arrest [2]. Similarly, overexpression of ETV6 has been reported to enhance TP53-mediated apoptosis via transcription-dependent and -independent mechanisms [3]. Thus, we investigated the expression levels of MDM2, TRIM28, and ETV6 in our patient samples by means of quantitative RT-PCR analysis. RNAs were treated with deoxyribonuclease before the reverse transcription reaction, and the primers were designed to span introns so as to avoid amplification from residual genomic fragments (see Table 2 for primer sequences). The expression levels of each gene were normalized to those of the *β_2-microglobulin* gene and are presented as relative-expression values (Figure 1). There was no overexpression of the *MDM2* gene. Their expression levels in MDS samples were similar to or slightly lower than those in the samples from the healthy control individuals. This result is in line with a previous study with a smaller number of MDS samples (n = 21) in which no MDM2 mRNA overexpression was detected with Northern blot analysis. Thus, unlike some forms of leukemia, MDM2 overexpression does not seem to have a role in MDS pathogenesis. Next, we investigated the expression of the *TRIM28* gene. Recent studies have pointed to its role in regulating c-Myc and TP53, 2 major genes related to oncogenesis, and it is of interest to see if there is any dysregulated expression in patient samples. In particular, overexpression of TRIM28 could lead to the suppression of TP53 and generate oncogenicity in hematopoietic cells with wild-type TP53. However, we did not identify such overexpression in our samples.

Finally, we investigated expression of the *ETV6* gene. In a biochemical analysis, overexpression of ETV6 was reported to suppress colony transformation of the Rat1 fibroblast; thus, the tumor-suppressive nature of the molecule has been proposed [6,7]. In clinical samples, the loss of the *ETV6* allele or loss of *ETV6* expression has been implicated in leukemia progression [8,9]. We observed extremely low levels of the *ETV6* transcript in some MDS samples, with levels 1.5 SDs lower than the mean level in samples from healthy individuals (Figure 1, ETV6-C). We previously detected several isoforms of the *ETV6* transcript in MDS samples [10]. Thus, the use of a single primer pair might misleadingly show null expression simply because of the lack of target exons. Therefore, we generated another primer set in the N-terminal portion and screened for patients who showed low expression with both primer pairs (Figure 1, ETV6-C and ETV6-N). Six samples exhibited extremely low *ETV6* mRNA expression with both primer sets.

Table 2.
Quantitative PCR primers

h-mdm2-735F	5′-ccttcatcttcacatttggttt-3′
h-mdm2-856R	5′-tcagatttgtggcgttttct-3′
h-Kap1-2310F	5′-acctgaaggaggaggatgg-3′
h-Kap1-2437R	5′-gggttcgtgacagaataggg-3′
h-TEL-C-1223F	5′-cacatcatggtctctgtctcc-3′
h-TEL-C-1369R	5′-ggattctttgtcctcccatc-3′
h-TEL-N-557F	5′-ctgctgctgaccaaagagg-3′
h-TEL-N-682R	5′-agggtggaagaatggtgaaa-3′

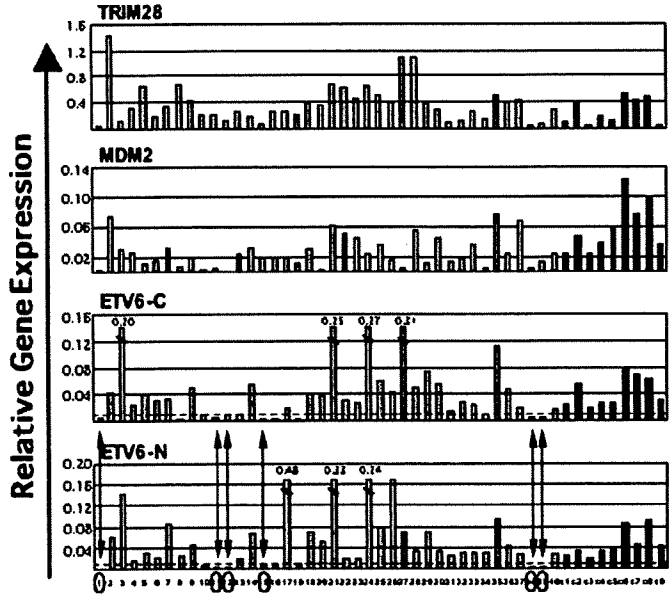

Figure 1. Relative expression of MDM2, TRIM28, and ETV6 messenger RNA. RNA was extracted from the bone marrow cells of 40 myelodysplastic syndrome patients (gray bars) and 9 healthy individuals (black bars). The difference in cycle threshold (CT) values between the gene of interest (CT_{gene}) and the control β_2–microglobulin gene ($CT_{\beta 2}$) was calculated (ΔCT), and the relative expression value for each sample was calculated as $2^{-\Delta CT}$. For *ETV6*, expression levels below the dotted lines in the 2 analyses (ETV6-C and ETV6-N) indicate expression levels lower than 1.5 SDs below the mean of samples from healthy individuals (arrows).

The clinical and cytogenetic features of the patients with *TP53* mutations or low *ETV6* expression are summarized in Table 3. In the patients with low *ETV6* expression, we detected no deletion involving 12p13 where TEL resides at the resolution level of cytogenetic analysis. To convincingly argue for the loss of the *ETV6* allele, one has to conduct fluorescence in situ hybridization analysis with probes specific for the *ETV6* allele. Even for the intact *ETV6* locus, however, shutoff of ETV6 expression has been documented [8,9]. In such a case, some epigenetic modification in the locus could account for low/null

Table 3.
Clinical and Cytogenetic Features of the Patients with *TP53* Mutation or Low *ETV6* Expression*

Patient ID No.	Sex	Age, y	FAB	Karyotype
Patients with low *ETV6* expression				
1	M	66	RAEB	46,XY,t(1;11)(q21;q13)[1]/46,idem,–2,add(3)(p13), add(4)q31),add(5)(q13),+mar1[2]/46,XY[13]
11	F	24	RA	46,XX[20]
12	M	62	RA	47,XY,+8[1]/46,XY[19]
15	M	50	RA	47,XY,del(20)(q11q13.3),+mar1[9]/47, idem,inv(19)(p13q13)[11]
38	M	80	RAEB-t	45,XY,+1,der(1;5)(q10;p10),–18[3]/44, idem,–11[2]/45,idem,–11,+r1[5]/46,XY[3]
39	M	58	RAEB	46,XY[12]/45,X,–Y[8]
Patients with *TP53* point mutation				
4	M	93	RA	44,XY,add(7)(p15),+8,–10,add(12)(p11), add(14)(p11),del(15)(q?),–16,–17,–18,–20,+mar1,+mar2[19]
8	M	67	RAEB-t	45,XY,–3,add(5)(q31),del(7)(q?),–12,+r1[12]/38, idem,–4,add(7)(q32),add(8)(q24),–10,–11,–15,–15,–17,–18, –22,–r1,+mar[1]
22	M	74	RAEB-t	46,XY,–5,del(7)(q?),add(15)(p11),–17,–18,–22,+mar1,+mar3, +mar4[9]/47,idem,add(4)(q21),add(12)(p11),add(14)(q24), –mar2,+mar5,+mar6[2]/46,XY[2]
29	M	76	CMMoL	46,XY[20]

*FAB indicates French-American-British classification; RAEB, refractory anemia with excess of blasts; RA, refractory anemia; RAEB-t, RAEB in transformation; CMMoL, chronic myelomonocytic leukemia.

ETV6 expression. For TP53, 2 of the 4 patients with TP53 mutations had the RAEB-t phenotype, an observation consistent with the previous finding that mutations in *TP53* are typically found in advanced stages of MDS [5]. In addition, we did not detect expression of the wild-type *TP53* allele in any of the 4 mutated samples, a result consistent with the initial report on MDS and other cancers showing loss of heterozygosity of the *TP53* gene.

Abnormalities in TP53 regulatory molecules could have a significant impact on its tumor suppressor activity. Therefore, we hypothesized that mutations in or aberrant expression of these molecules might affect the development of MDS, at least in a subgroup of patients with intact TP53. To our knowledge, no study has surveyed mutations in *TRIM28* or *ETV6*, or their expression levels, in MDS patients. In this analysis, we found extremely low *ETV6* expression in MDS patients.

The role of ETV6 in tumor/leukemia suppression has been documented in various aspects: (1) Loss of heterozygosity of the short arm of chromosome 12 is frequently seen in a wide range of hematologic malignancies and solid tumors [7]. (2) Loss of the wild-type *ETV6* allele is observed in childhood leukemia with the ETV6-RUNX1 fusion gene [9]. (3) Null/low ETV6 protein expression has been reported in acute myeloid leukemia patients [11]. (4) Overexpression of ETV6 suppresses the colony formation of Rat1 cells [6]. By analogy, therefore, loss of normal ETV6 function is postulated to predispose an individual to MDS, at least in a portion of disease cases.

In searching for the proposed tumor suppressor property of ETV6, we identified that ETV6 induces apoptosis in myeloid cells through the activation of TP53. Thus, one of the mechanisms by which loss of ETV6 expression causes MDS could be down-regulation of the TP53 pathway. However, this effect may not be as critical as the loss of TP53 itself, a supposition that may explain the higher incidence of low-grade MDS (3 RA patients) in our patient samples (Table 3).

In conclusion, our study demonstrated that low/null expression of the *ETV6* gene is occasionally detected in patients with MDS, and even for individuals with intact *TP53* loci, low/null expression of ETV6 may disable the tumor suppressor shield that uses the ETV6-TP53 channel. Further studies are warranted to clarify the molecular mechanism responsible for MDS development, especially in cases where no genetic aberration in the *TP53* gene is detected.

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RUNX1/EVI1, which blocks myeloid differentiation, inhibits CCAAT–enhancer binding protein α function

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The RUNX1/EVI1 chimeric transcription factor produced by t(3;21) causes leukemic transformation in hematopoietic stem cell tumors, possibly through a differentiation block of malignant myeloid progenitors. A dominant negative effect over wild-type RUNX1 has been shown to constitute one of the underlying molecular mechanisms. We introduced RUNX1/EVI1 cDNA into LG-3 cells that differentiate along the myeloid lineage upon exposure to granulocyte colony stimulating factor, and confirmed that RUNX1/EVI1 suppressed the differentiation. To further investigate the molecular mechanisms of RUNX1/EVI1-mediated differentiation block, we analyzed RUNX1/EVI1's effect on the functions of CCAAT–enhancer binding protein α (C/EBPα), a key transcriptional regulator that induces granulocytic differentiation. RUNX1/EVI1 was found to associate with C/EBPα. By using a reporter assay with the *CEBPA* promoter, we observed a dominant negative effect of RUNX1/EVI1 over C/EBPα-mediated transcriptional activation via the carboxyl terminal-binding protein (CtBP)-binding site in the EVI1 portion. In a gel-shift assay, RUNX1/EVI1 downregulated the DNA-binding activity of C/EBPα. Therefore, recruitment of histone deacetylase via CtBP and disruption of DNA binding could be likely scenarios for the RUNX1/EVI1-induced dominant repression on C/EBPα. Importantly, coexpression of C/EBPα restored the differentiation ability of the RUNX1/EVI1-expressing LG-3 cells. All of these data argue that inhibition of C/EBPα function may be causatively related to the leukemogenic potential of RUNX1/EVI1. (*Cancer Sci* 2007; 98: 1752–1757)

The t(3;21)(q26;q22) translocation is a cytogenetic hallmark of chronic myelogenous leukemia in blastic crisis, myelodysplastic syndrome in leukemic transformation and *de novo* acute myelogenous leukemia (AML).[1–4] This translocation-associated leukemia is of either myeloid or megakaryocytic origin. In the joining region of t(3;21), the *RUNX1* gene on 21q22 is fused with the *EVI1* gene on 3q26.[5] The resultant *RUNX1/EVI1* fusion gene is translated in-frame to an aberrant transcription factor in which the N-terminus of RUNX1, including its DNA-binding domain Runt, is connected to almost the entire sequence of EVI1. This chimeric transcription factor could be behind the leukemogenesis caused by t(3;21).

RUNX1 is a member of the Runt family of transcription factors that regulates a number of hematopoietic cell-specific genes. Depending on the Runt domain, RUNX1 binds to a specific DNA consensus sequence named PEBP2 (ACCRCA) and forms a heterodimeric active transcription factor complex with the non-DNA binding β subunit (CBFβ–PEBP2β). RUNX1 plays an essential role in establishing definitive hematopoiesis in the fetal liver,[6,7] and maturating megakaryocytes in the adult bone marrow.[8] However, EVI1 is a zinc-finger protein that displays versatile functions such as inhibition of transforming growth factor (TGF)-β signaling,[9] repression of c-Jun *N*-terminal kinase (JNK) activity[10] and stimulation of activating protein (AP)-1 activity.[11] The molecular characterization of RUNX1/EVI1 points to two major functions: one is a dominant suppressive function over wild-type RUNX1 and the other is EVI1's own function. Recent gene-engineered studies have provided us with significant information on the *in vivo* functions of RUNX1/EVI1. *RUNX1/EVI1* knock-in heterozygous mice show defective hematopoiesis in the fetal liver similar to *Runx1* knockout mice, but possess dysplastic hematopoietic progenitors with high self-renewal capacity.[12] Notably, *RUNX1/EVI1* knock-in chimeric mice have been reported to develop acute megakaryoblastic leukemia.[13]

CCAAT/enhancer binding protein α (C/EBPα) is a leucine zipper transcription factor that regulates the expression of specific target genes containing C/EBP sites in their promoters, and thus plays distinct roles in the differentiation process of various cell types.[14,15] In the hematopoietic system, such genes include *CEBPA* itself,[16] *CEBPE*[16,17] and granulocyte colony-stimulating factor (*G-CSF*) receptor.[16,18,19] Conditional expression of C/EBPα is sufficient to trigger terminal granulocytic differentiation[20–23] and block the monocytic differentiation program.[20,22] Further, *Cebpa* knockout mice show profound defects in their granulocytic differentiation, whereas all other hematopoietic cells are present in normal numbers,[18] indicating its critical role in granulopoiesis. Several lines of evidence suggest that disturbance of C/EBPα signaling is one of the major molecular events in myeloid malignancies. Ten percent of patients with AML that belong to M1 or M2 (according to the French–American–British [FAB] classification) and do not have a frequent cytogenetic abnormality, such as t(8;21)(q22;q22), carry heterozygous *CEBPA* gene mutations resulting in production of the truncated protein with a dominant negative function.[17,24,25] RUNX1/ETO, generated by the t(8;21) translocation in AML (FAB-M2), represses the transcription of *CEBPA* mRNA by suppressing C/EBPα's autoregulatory loop,[16] whereas PML/RARα, caused by t(15;17)(q21;q22) in acute promyelocytic leukemia (FAB-M3), inhibits the function of C/EBPα.[26] Because RUNX1/EVI1 and RUNX1/ETO show high structural similarity, it could be possible that RUNX1/EVI1 mediates its differentiation block effect on myeloid progenitors through inhibiting transcription of the *CEBPA* gene.

In the present study, we investigated whether RUNX1/EVI1 affects the expression and function of C/EBPα. We first confirmed that RUNX1/EVI1 blocked granulocytic differentiation in LG-3 cells upon granulocyte colony-stimulating factor (G-CSF) exposure. Physical interaction between RUNX1/EVI1 and C/EBPα was detected in the immunoprecipitation assay. RUNX1/EVI1 significantly inhibited transcriptional activation of the *CEBPA* promoter induced by C/EBPα itself, depending on one of the two CtBP-binding sites in EVI1. Further, RUNX1/EVI1 repressed the DNA-binding activity of C/EBPα. These data indicate that RUNX1/EVI1 inhibits molecular functions of C/EBPα, possibly through recruiting the co-repressor CtBP/histone deacetylase complex to the C/EBPα-targeting promoter and suppressing DNA binding of C/EBPα. Importantly, the observation that coexpression of C/EBPα restored the RUNX1/EVI1-induced differentiation block in LG-3 cells suggests the influence of RUNX1/EVI1 on C/EBPα's biological function.

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Materials and Methods

Plasmid construction. The pcDNA3-C/EBPα and ptk81-luc-C/EBPα promoters were described previously.[16] The FLAG-tag (DYKDDDDK) was created upstream of the translation initiation site of wild-type C/EBPα cDNA by the method of polymerase chain reaction (PCR) amplification. The resultant cDNA was inserted into the *Eco*RI site of pME18S in the sense orientation to give pME18S-FLAG-C/EBPα. pME18S-RUNX1/EVI1 was described previously.[27] pME18S-FLAG-RUNX1/EVI1 was also created in the same way. FLAG-RUNX1/EVI1 and FLAG-C/EBPα cDNA were cloned into the *Eco*RI sites of the pCXN2 and pCAGIPuro expression vectors, respectively. For construction of RUNX1/EVI1 deletion mutants, new restriction enzyme sites, *Nhe*I (140), *Nhe*I (536), *Eco*RV (1821), *Pvu*II (3511), *Nhe*I (3664) and *Nhe*I (3844) (numbers in parentheses indicate nucleotide numbers from the start site of translation to the cutting site of the enzyme), were created in the pME18S-RUNX1/EVI1 expression vector by site-directed mutagenesis. Deletion mutants ΔRunt, ΔZF1, ΔZF2 and ΔAD were constructed by deleting internal fragments from mutagenic *Nhe*I (140) to mutagenic *Nhe*I (536), *Eco*RV (1102) to mutagenic *Eco*RV (1821), *Eco*473 (3227) to mutagenic *Pvu*II (3511) and mutagenic *Nhe*I (3664) to mutagenic *Nhe*I (3844), respectively. For construction of the mCtBP mutant, adenine (2816), cytosine (2818) and thymine (2819) were substituted with cytosine, thymine and cytosine, respectively, by site-directed mutagenesis. pME18S-FLAG-ΔRunt and pME18S-FLAG-mCtBP were constructed as described above.

Cell culture. LG-3 cells[28] were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 ng/mL mouse interleukin (IL)-3 and 50 μM 2-mercaptoethanol. COS-7 and CV-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS.

Establishment of stable transfectants and granulocytic differentiation assay. To establish stable transfectants of FLAG-RUNX1/EVI1, 1×10^7 LG-3 cells were electroporated with 20 μg of pCXN2-FLAG-RUNX1/EVI1 plasmid at 380 V and 975 μF using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA). Electroporated cells were selected with 0.8 μg/mL G418 (Sigma-Aldrich, St Louis, MO, USA) and cloned by limiting dilution. Surviving clones were screened for expression of RUNX1/EVI1 by western blot analysis using anti-FLAG M2 antibody (Sigma-Aldrich). To further obtain double transfectants of RUNX1/EVI1 and C/EBPα, 1×10^7 LG-3 cells stably expressing FLAG-RUNX1/EVI1 were electroporated with 20 μg of pCAGIPuro-FLAG-C/EBPα plasmid at 380 V and 975 μF using a Gene Pulser. Electroporated cells were selected with 0.75 μg/mL puromycin (Sigma-Aldrich) and cloned by limiting dilution. Surviving clones were screened for concomitant expression of RUNX1/EVI1 and C/EBPα by western blot analysis using anti-FLAG M2 antibody.

For the induction of granulocytic differentiation, LG-3 cells were washed once with phosphate-buffered saline and placed in RPMI-1640 medium supplemented with 10% FCS, 50 μM 2-mercaptoethanol and 2 ng/mL G-CSF instead of IL-3. After 7 days, morphological studies were carried out on cytospin preparations with Wright–Giemsa and myeloperoxidase stainings.

Western blotting and immunoprecipitation. COS-7 cells were transfected with full-length RUNX1/EVI1 or its mutant expression plasmids alone or in combination with the FLAG-tagged C/EBPα expression plasmid using the DEAE-dextran method as described previously.[29] Western blot analyses were carried out as described previously[29] using anti-RUNX1 antiserum (Cell Signaling Technology, Beverly, MA, USA) and anti-FLAG M2 antibody. Immunoprecipitation was carried out using anti-FLAG M2 antibody conjugated with protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and immunoprecipitates were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis.

Luciferase assay. CV-1 cells were transfected with 200 ng of ptk81-luc-C/EBPα reporter plasmid alone or along with 100 ng of expression plasmids using Lipofectamine2000 (Invitrogen, Rockville, MD, USA). Luciferase assays were carried out using a Dual-luciferase Reporter Assay System (Promega, Madison, WI, USA). The phRL/CMV plasmid (10 ng; Promega) was cotransfected as an internal control of transfection efficacy and the data were normalized to *Rennilla* luciferase activity. All transfections were carried out at least three times and similar results were obtained.

Electrophoretic mobility shift assay. Preparation of FLAG-C/EBPα or FLAG-RUNX1/EVI1-expressing COS-7 lysates and binding reactions were carried out as described previously.[16] The G-CSF receptor promoter oligonucleotide had a sequence of 5′-GGAAGGTGTTGCAATCCCCAGC-3′, in which the C/EBP binding site is underlined. In competition studies, a 300-fold molar excess of unlabeled specific or non-specific oligonucleotide was added with the probe. The non-specific oligonucleotide had a sequence of 5′-GGAAGGTGTTGGATACCCCAGC-3′, in which the C/EBP binding site was substituted with the GATA binding site. For supershift experiments, 5 μL of anti-C/EBPα polyclonal antibody 14AA (Santa Cruz Biotechnology, Santa Cruz, CA) was added. Reactions were electrophoresed at 165 V on 10% Tris-borate-EDTA (TBE) gels in 0.25 × TBE at 25°C.

Results

RUNX1/EVI1 suppresses granulocytic differentiation in LG-3 cells upon G-CSF treatment. RUNX1/EVI1 has been reported to inhibit granulocytic differentiation in 32D cells upon G-CSF stimulation.[27] We confirmed the same effect of RUNX1/EVI1 using another murine IL-3-dependent myeloid progenitor cell line, LG-3, which differentiates into mature granulocyte in response to G-CSF. By transfecting the FLAG-tagged RUNX1/EVI1 expression plasmid (pCXN2-FLAG-RUNX1/EVI1) into LG-3 cells, we established several stable cell lines overexpressing RUNX1/EVI1. Western blot analysis with anti-FLAG antibody verified that clones R/E11 and R/E14 expressed high levels of the 210-kDa RUNX1/EVI1 protein (Fig. 1a). Two clones transfected with the empty plasmid were used as mock-transfected controls (M5 and M20). R/E11 and R/E14 showed more rapid proliferation than M5 and M20 in the presence of IL-3, although only to a slight degree (data not shown). However, the RUNX1/EVI1-overexpressing cells did not become growth factor independent as IL-3 was required for their continued growth. To test the effect of overexpressed RUNX1/EVI1 on granulocytic differentiation, LG-3 cells were induced into terminal granulocytic differentiation by treatment with G-CSF. As expected, Wright–Giemsa staining of the mock cells before and after 7 days of treatment with G-CSF demonstrated dramatic morphological changes with myeloid blasts seen at day 0 and polymorphonuclear cells appearing at day 7 (data not shown). In contrast, the RUNX1/EVI1-overexpressing cells hardly differentiated into mature granulocytes even after 7 days of the treatment. Differential counts of these cells at day 7 of culture are shown in Fig. 1b. The RUNX1/EVI1-expressing clones reproducibly displayed lower percentages of mature granulocyte (percentages of the stab and segmented forms; 37% in M5 and 50% in M20 versus 10% in R/E11 and 12% in R/E14) and higher percentages of blast (6% in M5 and 10% in M20 versus 43% in R/E11 and 48% in R/E14) than the controls. Myeloperoxidase positivity, which indicates mature granulocytes, was also significantly lower in the RUNX1/EVI1-expressing cells compared to the mock cells. These data demonstrate that RUNX1/EVI1-positive LG-3 cells arrest at the myeloblast stage even after induction with G-CSF.

RUNX1/EVI1 associates with C/EBPα *in vivo*. To clarify the molecular effect of RUNX1/EVI1 on C/EBPα, we first tested whether

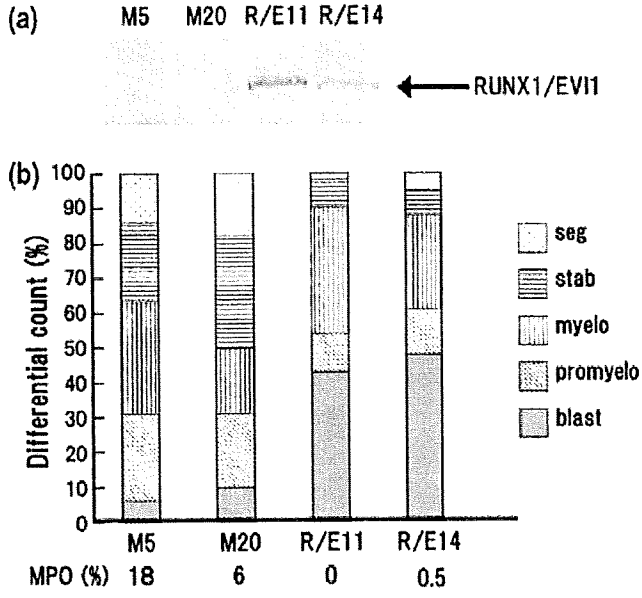

Fig. 1. RUNX1/EVI1 represses granulocyte colony-stimulating factor (G-CSF)-induced granulocytic differentiation in LG-3 cells. (a) Western blot analysis using anti-FLAG M2 antibody for RUNX/EVI1 proteins from whole cell lysates of RUNX1/EVI1-expressing LG-3 clones, R/E11 and R/E14, as well as mock clones, M5 and M20. (b) Granulocytic differential counts of the mock and RUNX1/EVI1-expressing clones after 7 days of treatment with 2 ng/mL of G-CSF are shown. Percentages of myeloperoxidase-positive cells in each clone are shown below the graph. This experiment was carried out four times independently and similar results were obtained. Representative data are shown.

RUNX1/EVI1 and C/EBPα physically interact *in vivo*. Mock or FLAG-tagged C/EBPα expression plasmid (pME18S-FLAG-C/EBPα) was cotransfected with RUNX1/EVI1 expression plasmid (pME18S-RUNX1/EVI1) in COS-7 cells, and immunoprecipitation was carried out using an antibody against FLAG. Expression of RUNX1/EVI1 (Fig. 2, upper panel) and C/EBPα (middle panel) was confirmed by western blot analysis with anti-RUNX1 and anti-FLAG antibodies, respectively. RUNX1/EVI1 was immunoprecipitated with anti-FLAG antibody only when FLAG-tagged C/EBPα was co-expressed (lower panel). This indicates that RUNX1/EVI1 binds to C/EBPα *in vivo*. To determine the C/EBPα-binding region in RUNX1/EVI1, we then transfected a set of RUNX1/EVI1 plasmids expressing its deletion mutants ΔRunt, ΔZF1, ΔZF2 and ΔAD. ΔRunt is a mutant lacking the Runt domain of RUNX1, whereas ΔZF1, ΔZF2 and ΔAD are mutants lacking the first and second zinc finger, and the acidic domains of EVI1, respectively. The plasmid designated pME18S-mCtBP, which produces the RUNX1/EVI1 point mutant harboring normal N-terminal (PF*DLT*) but substituted C-terminal (PL*DLS* to PL*ASS*) CtBP-binding motifs in the EVI1 portion, was also included in this study. This amino acid mutation has been reported to eliminate EVI1's binding to CtBP.[30] All of these mutants were shown to be expressed at a comparable level by western blot analysis (Fig. 2, upper panel). Surprisingly, all of these mutants were again immunoprecipitated with anti-FLAG antibody in the presence of FLAG-tagged C/EBPα (lower panel). These data suggest that RUNX1/EVI1 associates with C/EBPα via regions other than the DNA-binding domain in RUNX1 and the functional domains in EVI1, and that destruction of the critical CtBP-binding motif does not modify C/EBPα-binding activity of RUNX1/EVI1.

RUNX1/EVI1 inhibits C/EBPα-mediated transcriptional activity. Because a physical association between RUNX1/EVI1 and C/EBPα was demonstrated, we sought the effect of RUNX1/EVI1 on C/EBPα-dependent transcription. C/EBPα has been shown to stimulate transcription of the reporter gene containing the human *CEBPA* promoter and so far is the only factor known to activate the promoter in synergy with the ubiquitous upstream stimulatory factor. We thus investigated whether RUNX1/EVI1 alters C/EBPα-mediated transcriptional activity by transient transfection assay with a luciferase construct driven by a 562-bp fragment of the human *CEBPA* promoter, ptk81-luc-CEBPA.[16] To confirm that C/EBPα autoregulates its own promoter, we transfected the *CEBPA* reporter along with mock or C/EBPα expression plasmid into African green monkey kidney cell line CV-1, in which C/EBPα is shown to activate its own promoter,[16] and evaluated luciferase activities. Consistent with a previous report, cotransfection of the C/EBPα expression plasmid resulted in a 1.5-fold increase in luciferase activity compared with that obtained with the mock plasmid (Fig. 3). RUNX1/EVI1 alone had no effect on the *CEBPA* promoter. Importantly, coexpression of RUNX1/EVI1 almost completely abolished the C/EBPα-dependent activation of the promoter. These data suggest that RUNX1/EVI1 interferes with the autoregulatory loop of C/EBPα.

To identify the critical portion of the RUNX1/EVI1 protein that contributes to the repression of C/EBPα transcriptional activity, we analyzed the functions of the RUNX1/EVI1 mutants

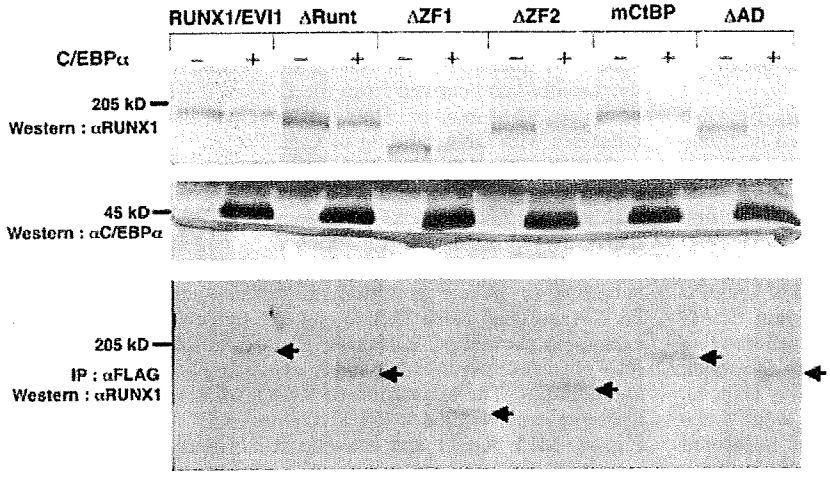

Fig. 2. RUNX1/EVI1 binds to CCAAT/enhancer binding protein α (C/EBPα) *in vivo*. COS-7 cells were transfected with 5 μg pME18S-RUNX1/EVI1, pME18S-ΔRunt, pME18S-ΔZF1, pME18S-ΔZF2, pME18S-mCtBP or pME18S-ΔAD with or without 5 μg pME18S-FLAG-C/EBPα and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 48 h before harvesting. Western blot analyses were carried out with anti-RUNX1 antiserum to detect RUNX1/EVI1 or its mutant proteins (upper panel) or with anti-FLAG M2 antibody to detect C/EBPα (middle panel) expressed in COS-7 cells. RUNX1/EVI1 or its mutant proteins immunoprecipitated with anti-FLAG M2 antibody were detected using anti-RUNX1 antiserum (lower panel). Arrows indicate immunoprecipitated RUNX1/EVI1 and its mutant proteins.

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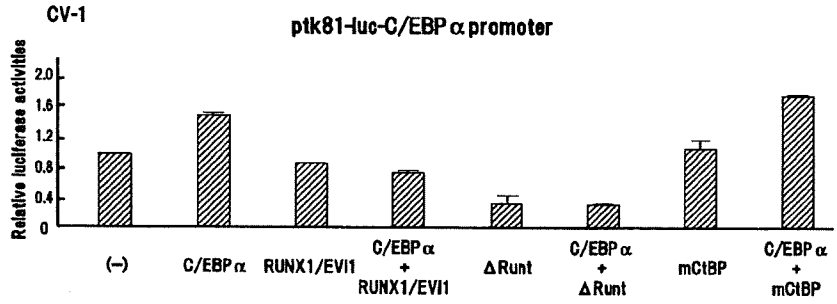

Fig. 3. RUNX1/EVI1 represses CCAAT/enhancer binding protein α (C/EBPα)-mediated transcriptional activity. CV-1 cells were transfected with 200 ng ptk81-luc-C/EBPα reporter plasmid alone or along with 50 ng indicated expression plasmid (C/EBPα, pME18S-C/EBPα; RUNX1/EVI1, pME18S-RUNX1/EVI1; ΔRunt, pME18S-ΔRunt; mCtBP, pME18S-mCtBP) and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 48 h before harvesting. Bars show relative luciferase activities to the level when a control plasmid pME18S was cotransfected and present average results of duplicate experiments.

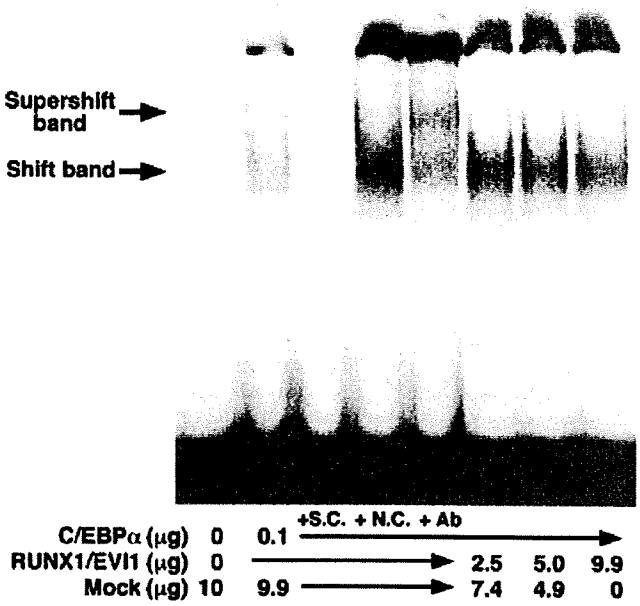

Fig. 4. RUNX1/EVI1 reduces the DNA-binding affinity of CCAAT/enhancer binding protein α (C/EBPα). Electrophoretic mobility shift assay was carried out using a [^{32}P]-labeled probe and lysates from COS-7 cells transfected with pME18S, pME18S-FLAG-C/EBPα or pME18S-FLAG-RUNX1/EVI1. A 300-fold molar excess of cold specific competitor (SC) or non-specific competitor (NC) was added to the reaction. Anti-C/EBPα antiserum (14 AA) was also added to the reaction.

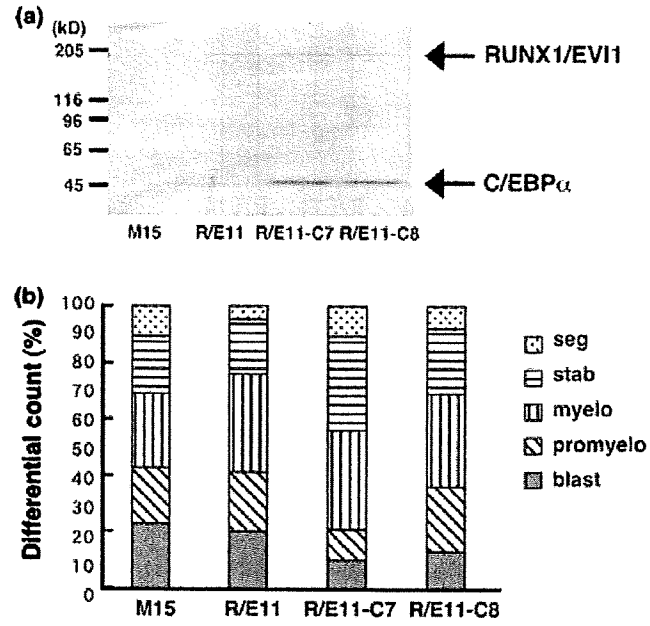

Fig. 5. Co-expression of CCAAT/enhancer binding protein α (C/EBPα) restores granulocytic differentiation in LG-3 cells expressing RUNX1/EVI1. (a) Expression of RUNX1/EVI1 and C/EBPα proteins in the mock (M15), RUNX1/EVI1-expressing (R/E11) or both RUNX1/EVI1- and C/EBPα-expressing (R/E11-C7 and R/E11-C8) clones. (b) Granulocytic differential counts of these LG-3 clones after 7 days of treatment with 2 ng/mL granulocyte colony-stimulating factor (G-CSF) are shown. This experiment was carried out four times independently and similar results were obtained. Representative data are shown.

ΔRunt and mCtBP. The ΔRunt mutant suppressed the basal *CEBPA* promoter activity, and still suppressed it when C/EBPα was co-expressed. Notably, the mCtBP mutant lost the ability to repress C/EBPα-mediated transcription, whereas it did not affect the promoter activity in the absence of C/EBPα. Therefore, we speculate that CtBP binding followed by histone deacetylase recruitment is required for RUNX1/EVI1 to suppress the molecular function of C/EBPα.

RUNX1/EVI1 decreases the DNA-binding affinity of C/EBPα. Because RUNX1/EVI1 associated with C/EBPα and disturbed its transcriptional activity, we analyzed whether RUNX1/EVI1 influenced the DNA binding of C/EBPα. For this purpose, cell lysates prepared from COS-7 cells expressing C/EBPα were at first subjected to electrophoretic mobility shift assay (EMSA) using a radioactive C/EBP-site oligonucleotide derived from the G-CSF receptor promoter (–57 to –38 bp).[16,19] The expression of C/EBPα was demonstrated by western blot analysis with anti-FLAG antibody (data not shown). In EMSA, C/EBPα generated a specific DNA–protein complex that was not seen in the mock lysate and was supershifted with anti-C/EBPα antibody (Fig. 4). This band represented the specific binding of C/EBPα to the probe as the binding was reduced by the addition of the unlabeled wild-type C/EBP site oligonucleotide but not the oligonucleotide mutated in the C/EBP site. We then carried out the EMSA assay in the same manner with the lysates expressing RUNX1/EVI1 added. The presence of RUNX1/EVI1 decreased the intensity of the specific band derived from the DNA–C/EBPα complex in a dose-dependent manner. Thus, we conclude that RUNX1/EVI1 interferes with DNA binding of C/EBPα.

Coexpression of C/EBPα restores the granulocytic differentiation suppressed by RUNX1/EVI1 in LG-3 cells. Because it is conceivable that RUNX1/EVI1 blocks granulocytic differentiation at least partly by repressing the functions of endogenous C/EBPα in LG-3 cells, we studied whether coexpression of C/EBPα was sufficient to induce granulocytic differentiation in RUNX1/EVI1-expressing cells. To this end, we transfected the C/EBPα expression plasmid pCAGIPuro-C/EBPα into R/E11 and successfully obtained several clones stably expressing both RUNX1/EVI1 and C/EBPα. The expression of RUNX1/EVI1 and C/EBPα in the representative clones R/E11-C7 and R/E11-C8 is shown in Fig. 5a. M15, R/E11 and the two R/E11-derived clones were

treated with G-CSF and the degree of granulocytic differentiation was compared among them (Fig. 5b). Notably, R/E11-C7 and R/E11-C8 morphologically restored granulocytic differentiation suppressed by RUNX1/EVI1. The percentages of mature granulocytes were 44% in R/E11-C7 and 31% in R/E11-C8 versus 24% in R/E11. We speculated that restoration of C/EBPα function partly overcomes the block in differentiation mediated by RUNX1/EVI1 and progresses the granulocytic differentiation.

Discussion

We demonstrated in this study that RUNX1/EVI1 disturbs the C/EBPα-mediated transcriptional activity of the *CEBPA* promoter containing the C/EBP site. Because we could not identify the PEBP2 site in the promoter used in the assay and expression of RUNX1/EVI1 alone had no effect on it, RUNX1/EVI1 is thought to inhibit autoregulation of C/EBPα. We observed the association between RUNX1/EVI1 and C/EBPα *in vivo*. However, analysis with a set of RUNX1/EVI1 mutants failed to identify the C/EBPα-binding region, because none of the mutants tested lost their ability to bind to C/EBPα. This indicates that regions outside of the functional domains deleted in this study, the Runt domain in RUNX1 and the zinc finger and acidic domains in EVI1, are required for interaction with C/EBPα, or that RUNX1/EVI1 associates with C/EBPα through multiple binding sites including the functional domains. It is interesting to remember that RUNX1/ETO generated by t(8;21) in AML (FAB-M2) also associates with C/EBPα and inhibits its transcriptional activity.[16] The association between RUNX1/ETO and C/EBPα occurs at the DNA-binding domains of both proteins, namely the Runt domain in RUNX1/ETO and the basic-region leucine zipper domain in C/EBPα. Despite the structural similarity between RUNX1/EVI1 and RUNX1/ETO, deletion of the Runt domain did not abolish the C/EBPα binding of RUNX1/EVI1, suggesting that the domain is the sole binding domain in RUNX1/EVI1.

Based on the observation of a physical association between these molecules, we propose two possible underpinning mechanisms in the suppressive effect of RUNX1/EVI1 on C/EBPα function. One is recruitment of histone deacetylase via CtBP bound to the EVI1 portion and the other is interference of C/EBPα's DNA-binding activity. Considering that introduction of the point mutation in the C-terminal CtBP-binding motif in the EVI1 portion of RUNX1/EVI1 significantly repressed RUNX1/EVI1's negative effect on C/EBPα-induced transcription, binding with the co-repressor CtBP and subsequent recruitment of histone deacetylase could play a critical role in the suppression of C/EBPα function. There are two putative CtBP-binding motifs, PFDLT (amino acid 553–557) and PLDLS (584–588) located between the two zinc finger domains of EVI1. Of the two motifs, the C-terminal PLDLS motif has been shown to be responsible for the interaction between EVI1 and CtBP.[30] Collectively with the previous report, the indirect association with histone deacetylase via the C-terminal CtBP-binding motif could be required for RUNX1/EVI1 to disturb the molecular function of C/EBPα. Notably, the ΔRunt mutant that retained both the C/EBPα- and CtBP-binding abilities appeared to be able to repress C/EBPα function. However, RUNX1/EVI1 inhibits the DNA-binding activity of C/EBPα in a dose-dependent manner. Thus, dissociation of C/EBPα from DNA also contributes to the suppressive function of RUNX1/EVI1 on C/EBPα. However, if C/EBPα leaves the DNA, recruitment of histone deacetylase by RUNX1/EVI1 should not be effective to suppress C/EBPα function. Therefore, the former mechanism is not compatible with the latter.

Our data postulate the possibility that RUNX1/EVI1 could reduce the transcription of C/EBPα's target genes *in vivo*. By using real-time reverse transcription–PCR assay, we compared the mRNA levels of its target genes including Cebpa, Cebpe and G-CSF receptor between the mock and RUNX1/EVI1-expressing LG-3 cells. The levels of Cebpa and Cebpe mRNA were unchanged and that of G-CSF receptor mRNA was rather increased in the presence of RUNX1/EVI1. Because the amount of Cebpa mRNA was extremely small in parental and mock-transfected LG-3 cells, it may have been difficult to detect a decrease in expression, if present. Notably, higher expression of G-CSF receptor mRNA was also observed in the LG-3 cells ectopically expressing RUNX1/ETO.[31] We used western blot analysis to evaluate the levels of the C/EBPε and G-CSF receptor proteins, but found no differences between the mock and RUNX1/EVI1-expressing cells. Helbling *et al.* have reported that RUNX1/EVI1 reduces the level of C/EBPα protein but not its mRNA in U937 cells, and that a putative inhibitor of *CEBPA* translation (calreticulin) is upregulated by RUNX1/EVI1.[32] Calreticulin is a ubiquitous protein with calcium storage and chaperone functions and is postulated to be involved in the development of leukemia.[32,33] In an experiment with small interfering RNA for the calreticulin gene, they concluded that RUNX1/EVI1 inhibits C/EBPα expression through a post-transcriptional mechanism of calreticulin. However, the level of calreticulin protein was unaltered in the RUNX1/EVI1-expressing LG-3 cells compared to the mock cells (data not shown), suggesting that the post-transcriptional mechanism of calreticulin may not be activated by RUNX1/EVI1 in LG-3 cells. RUNX1/EVI1 could modify C/EBPα expression at either the transcriptional or translational level in a context-dependent manner.

We demonstrated that exogenous expression of RUNX1/EVI1 in LG-3 cells resulted in the maturation block induced by G-CSF, as reported in 32D cells.[27] Co-expression of C/EBPα in the RUNX1/EVI1-expressing cells clearly restored their ability to differentiate along the myeloid lineage. These data support the concept of RUNX1/EVI1 as an inhibitor of C/EBPα-mediated transcription required for myeloid differentiation. However, we could not identify which target genes of C/EBPα are transcriptionally repressed by RUNX1/EVI1 in LG-3 cells, because the levels of the candidate mRNA tested were not decreased as described above. Other critical target genes may be regulated by C/EBPα in LG-3 cells and downregulation of those genes could lead to the differentiation block in the RUNX1/EVI1-expressing cells.

RUNX1/EVI1 causes various kinds of leukemia, including *de novo* or therapy-related AML, myelodysplastic syndrome-transformed leukemia and blastic crisis of chronic myelogenous leukemia, through the following mechanisms:[34] dominant negative effect over wild-type RUNX1,[27,35] blockade of TGF-β-mediated signal,[36] inhibition of JNK[10] and stimulation of AP-1 activity.[37] Our study points to another function for RUNX1/EVI1, that is, suppression of C/EBPα, as the molecular mechanism leading to the block in maturation seen in myeloid leukemia characterized by the t(3;21) translocation. From these data, we argue that transfer of exogenous C/EBPα protein into leukemia cells could represent a specific therapeutic option for the treatment of this type of leukemia by recovering their differentiation ability. Further, considering that recruitment of histone deacetylase seems to be critical for RUNX1/EVI1 to block the autoregulatory loop and suppress the molecular function of C/EBPα, administration of histone deacetylase inhibitor could be another potential modality to restore the function of C/EBPα and thereby differentiate the leukemic cells expressing RUNX1/EVI1.

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HEMATOLOGY

A Prospective Study of Cyclosporine A Treatment of Patients with Low-Risk Myelodysplastic Syndrome: Presence of CD55$^-$CD59$^-$ Blood Cells Predicts Platelet Response

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Abstract

Although immunosuppressive therapy using antithymocyte globulin or cyclosporine A (CSA) is effective in selected patients with low-risk myelodysplastic syndrome, the response rates reported so far are inconsistent, and the indication of immunosuppressive therapy for myelodysplastic syndrome has not been clearly defined. We treated 20 myelodysplastic syndrome patients (17 refractory anemia cases [RA], 2 RA with excess blasts, and one RA with ringed sideroblasts) with 4 mg/kg per day of CSA for 24 weeks. Among the 19 patients evaluated, 10 showed hematologic improvement; 8 patients showed an erythroid response, 6 showed a platelet response, and one showed a neutrophil response. Most patients with hematologic improvement continued CSA thereafter, and the progressive response was observed until the latest follow-up (median, 30 months). Most toxicities associated with CSA usage were manageable, and no patient had developed acute leukemia up to this point. Short duration of illness, refractory anemia with minimal dysplasia determined by bone marrow morphology, and the presence of paroxysmal nocturnal hemoglobinuria-type cells were significantly associated with the platelet response. A minority of RA patients who did not possess such predictive variables achieved an isolated erythroid response. In conclusion, CSA may be a therapeutic option for patients with RA who do not have adverse prognostic factors.
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Key words: Myelodysplastic syndromes; Cyclosporine A

1. Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by peripheral cytopenia, morphological dysplasia, and an elevated likelihood of progression to acute leukemia [1]. The international prognostic scoring system (IPSS) is the most reliable tool for evaluating the risk of leukemic transformation in individuals [2]. According to IPSS, MDS are divided into 4 groups. Complications of bone marrow failure are more likely to influence survival than leukemic transformation in patients with low and intermediate-1 risk categories [3-5]. Therefore, therapeutic approaches for low and intermediate risk patients are mainly aimed at restoring hematopoiesis.

Several studies documented that erythropoietin with or without granulocyte colony–stimulating factor may improve anemia and reduce the requirement of red cell transfusion in approximately 30% of MDS patients [6,7]. However, the median response duration was short (around 2 years), and the

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use of erythropoietin did not increase survival in a controlled trial. According to recent reports, the use of lenalidomide resulted in hematologic improvement (HI), which was defined by International Working Group (IWG) criteria [8], in 56% of enrolled patients, and the response rate was especially high in patients harboring a clonal interstitial deletion involving chromosome 5q31.1 [9,10]. However, the response rate of lenalidomide for MDS patients without chromosome 5q abnormality appears to be less than 50% [11].

Immunosuppressive therapy raises the blood cell count in some MDS patients. Antithymocyte globulin (ATG) leads to a sustained increase in red blood cell, platelet, and neutrophil production in about one third of patients with low-risk MDS, who are not at increased risk of leukemic transformation. A series of phase II trials demonstrated that lasting transfusion independence is obtained in about one third of patients who also achieved a long survival without added risk of leukemic progression [12-14]. Younger age, shorter duration of illness, diagnosis of French-American-British (FAB) refractory anemia (RA), expression of D-related human leukocyte antigen 15 (HLA-DR15), and the presence of a minor clone with the paroxysmal nocturnal hemoglobinuria (PNH) phenotype have been postulated as pretreatment characteristics correlated with ATG responsiveness [12-16]. Since ATG therapy causes severe adverse events, such as serum sickness, patients must be carefully selected for this modality. Cyclosporine A (CSA) also improves cytopenias in selected MDS patients [17-23]. Previously, we collected the results from individual pilot studies investigating CSA treatment for MDS in Japan, and reported that 30 of 50 patients responded to CSA [24]. These promising results prompted us to perform a prospective trial to evaluate the efficacy and safety of CSA for patients with low-risk MDS.

2. Patients and Methods

2.1. Study Design

In May 2001, we initiated an open-labeled, prospective, multicenter, phase II study to evaluate the efficacy and safety of 24-week oral cyclosporine in patients with low and intermediate-1 risk MDS according to IPSS. The primary endpoint was the rate of HI according to the criteria of IWG. Secondary endpoints were the duration of responses beyond 24 weeks and the rate of adverse events. The study was conducted in complete concordance with the declaration of Helsinki and approved by the ethics committees of the participating institutions. Patients fulfilled all of the inclusion criteria: morphologically proven MDS according to FAB classification; IPSS score of less than 1.5; presence of cytopenia (either hemoglobin value less than 10 g/dL, platelet count less than 100,000/μL, or neutrophil count less than 1500/μL); age range from 18 to 70 years; Zubrod performance status less than 2; and written informed consent. The exclusion criteria were: presence of clinically significant coexisting medical illness; prior history of malignancy or cytotoxic therapy; prior usage of CSA or ATG; and pregnant or lactating women. In all patients registered, the diagnosis of MDS was re-examined by central morphological evaluation in a blinded fashion by independent reviewers (K.T. and Y.Y.) who were not involved in the treatment of these patients. In addition, peripheral blood samples were subjected to the following analysis: the detection of PNH-type cells, the genetic typing of HLA-DR molecules, and the analysis of abnormally expanded T-cell clones. These tests were not compulsory, and written informed consent was taken independently.

Registered patients initially received 2 mg/kg of body weight twice per day of CSA (Neoral), which was supplied by Novartis Pharma K.K. (Tokyo, Japan). Thereafter, the dose was adjusted to keep the blood trough value at 150 to 200 ng/mL. The response to treatment, adverse events, and blood trough level of CSA were assessed every 2 weeks. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC) version 2.0. In cases with more than grade II nonhematologic toxicity or serum creatinine elevation of 1.5 or higher from the baseline, the treatment of CSA was withheld until the patients recovered. Except for treatment during infectious episodes, the use of or corticosteroid was not permitted. If progressive cytopenia or sign of leukemic transformation developed during the course of CSA treatment, patients were considered nonresponders and were allowed to choose alternative therapy. The daily dose, targeted trough level of CSA, and the appropriate treatment period to evaluate the response were based on our previous retrospective survey.

Bone marrow aspiration was performed before starting CSA and just after 24 weeks of therapy. Patients who met the criteria of HI in any of the 3 hematologic lineages at the 24th week received CSA for an additional 8 weeks to confirm the stability of the response. Patients with a sustained response until the 32nd week were regarded as hematologic responders. The treatment of enrolled patients after the response judgment was not identified. The status of the enrolled patients was monitored every 6 months for 36 months.

2.2. Detection of Minor Populations of PNH-Type Cells

Heparinized peripheral blood was drawn from patients, and minor populations of PNH-type cells were detected by high-resolution 2-color flow cytometry, as described previously [25,26]. Identification of the presence or absence of PNH-type cells was performed by S.N., who did not know the clinical response to CSA at the time of each experiment.

2.3. Analysis of Abnormally Expanded T-Cell Clones

RNA isolated from peripheral blood mononuclear cells was converted to double-stranded complementary DNA, and T-cell receptor (TCR) β chain variable region (Vβ) repertoires were analyzed with an adaptor ligation polymerase chain reaction (PCR)–based microplate hybridization assay [27]. Then, complementarity-determining region 3 size spectratyping was performed [28]. Using peripheral blood mononuclear cells from 4 healthy donors, we confirmed that a normal spectratype was distributed in a Gaussian fashion with 6 to 10 different size classes at 3 nucleotide intervals, as reported previously [29,30]. We defined the spectratype as skewed if more than one oligoclonal or monoclonal pattern was detected.

2.4. Statistical Analysis

Fisher's exact probability test was used to define predictive parameters for responses at the 24th week. All statistical analyses were performed using Stat View software (version 5.0; SAS Institute, Cary, NC, USA).

3. Results

3.1. Patient Characteristics at Registration

From May 2001 to April 2004, 22 patients were registered for this protocol. Two patients could not be evaluated because careful follow-up revealed coexisting illness negatively affecting hematopoiesis: smoldering multiple myeloma in one patient and chronic alcoholism in the other. The diagnosis of MDS and the eligibility based on criteria were confirmed in the remaining 20 patients. Primary data for the 20 patients are presented in Table 1. The median age was 52 years old, and 11 patients were male. The median duration of illness before CSA treatment was 5 months (range, 1-168 months). At the time of registration, 19 of 20 patients had anemia (hemoglobin less than 10 g/dL), and 10 patients were transfusion-dependent. Thrombocytopenia with platelet counts of less than 100,000/μL was seen in 18 patients, and one patient required regular platelet transfusion. Neutropenia of less than 1500/μL was observed in 15 patients. Central morphological review identified that 17 patients (85%) had RA according to FAB classification (FAB-RA). Two other patients had RA with excess of blasts (RAEB) and one had RA with ring sideroblasts (RARS). According to a World Health Organization (WHO) classification system, 17 patients with FAB-RA were categorized either with RA (WHO-RA, eight patients) or refractory cytopenia with multilineage dysplasia (RCMD, 9 patients). Patients with RAEB by FAB classification were diagnosed as RAEB-1 and RARS as RCMD-RS by WHO classification. Bone marrow cellularity was normo- or hyper-cellular in 18 patients. A total of 17 patients had a diploid karyotype. Among 8 patients with WHO-RA, 6 patients had persistent unexplained cytopenia with mild morphological abnormalities. For these patients, central reviewers carefully examined not only smear preparations, but also complete blood counts and biochemical data at diagnosis as well as follow-up periods, as recommended by Yoshida et al [31], and finally diagnosed as RA by WHO classification.

3.2. Hematologic Response

One patient was excluded from HI evaluation; this patient (No. 20) suffered from acute cholecystitis and pneumonia at the 11th week of therapy, and, after full recovery from an infectious episode, he refused further CSA treatment. Two more patients did not complete the 24 weeks of CSA treatment because of grade 4 cytopenia (No. 18), and progressively elevated values of peripheral blood Wilm's tumor gene products (No. 19), which is reportedly predictive of evolution into acute leukemia [32]. Both patients received allogeneic bone marrow transplantation from HLA-matched sibling donors. These 2 patients were regarded as nonresponders. The therapeutic responses are shown in Table 2. Ten patients (53%) showed HI at the 24th week of therapy, according to IWG criteria, and all responses were continuously observed for 8 successive weeks. Improvement in anemia (HI-E) was observed in 8 of 18 anemic patients. Four of 10 patients became transfusion-independent within 32 weeks. The improvement in thrombocytopenia (HI-P) and neutropenia (HI-N) was observed in 6 of 17 thrombocytopenic (35%) and in one out of 14 (7%) neutropenic patients, respectively.

3.3. Adverse Events within 6 Months of CSA Treatment

Adverse events were assessed in the 20 patients available for evaluation (Table 3). The most common adverse events observed were impaired renal function tests, elevated liver enzymes, and hypomagnesemia, the majority of which were categorized as grade 1 toxicities. One patient required temporal cessation of CSA because of elevated serum creatinine values. Over grade 2 toxicities were documented in 4 patients. A patient with acute cholecystitis and pneumonia was described. One patient (No. 17), who showed therapy-unresponsive severe neutropenia (neutrophil count of less than 200/μL), developed fatal pneumonia. Progressive anemia and thrombocytopenia were documented in one patient, respectively. No patient demonstrated increased blast counts in the bone marrow examination performed at the 24th week of therapy.

3.4. Variables Associated with Response

We determined the effect of pretreatment parameters on the probability of response to CSA at the 24th week by univariate analysis. Variables compared with response included age, sex, bone marrow cellularity, pretreatment blood cell counts, transfusion dependence, FAB and WHO classifications, karyotypes, IPSS score values, and genetically typed HLA-DR. As the distribution of patients with platelet and erythroid responses was not similar, patients were also individually analyzed. As shown in Table 4, we could not detect any variables predictive of the overall as well as erythroid response. In contrast, 3 variables were significantly associated with the platelet response: disease duration of less than 4 months, the presence of PNH-type cells, and the bone marrow morphology (judged as RA with minimal dysplasia).

3.5. Follow-up

Among the 20 patients, the follow-up of one patient was lost. In addition, 2 patients who received allogeneic stem cell transplantation were not included in the analysis of the long-term outcome. As shown in Table 2, 16 patients are currently alive without disease progression with a median follow-up of 30 months. In 9 responders, 8 patients maintain hematologic responses with the continuous use of CSA. One patient (No. 16) with an isolated erythroid response refused to continue CSA after 32 weeks and lost the response. Retreatment with CSA was not successful. Another patient who stopped CSA therapy (No. 5) also lost the platelet response, which recovered with the resumption of CSA. She was categorized

Table 1.
Characteristics of Patients*

UPN	Age/Gender	Interval, m†	Red Cell Transfusion	Pretreatment Value‡ PLT	Pretreatment Value‡ Neut	BM Central Review Cellularity	BM Central Review FAB/WHO	Cytogenetics	IPSS Score	PNH-Type Cell	HLA-DR15 (DRB1)
1	18/M	1	No	2.4	612	Hypo	RA§/RA	46, XY	0.5	No	Yes (1501)
2	55/F	1	No	1.6	1308	Normo	RA§/RA	46, XX	0.5	Yes	Yes (1502)
3	61/F	1	Dependent	1.2	906	Normo	RA§/RA	46, XX	0.5	Yes	Yes (1502)
4	31/M	1	No	4.6	957	Hyper	RA§/RA	46, XY	0.5	Yes	Yes (1501)
5	52/F	1	No	1.5	1240	Hyper	RA§/RA	46, XX	0.5	Yes	Yes (1501)
6	31/M	14	No	2.2	1492	Hyper	RA§/RA	46, XY	0.5	No	No
7	40/F	12	Dependent	4.1	420	Hyper	RA/RA	46, XX	0.5	Yes	No
8	52/M	8	No	1.8	1717	Hyper	RA/RA	46, XY	0.5	No	No
9	47/F	5	No	6.4	1820	Hyper	RA/RCMD	46, XX	0.5	No	No
10	27/F	3	No	2.3	1930	Hyper	RA/RCMD	46, XX	0	Yes	No
11	44/M	168	Dependent	22.4	2155	Hyper	RA/RCMD	46, XY	0	NT	NT
12	55/F	1	Dependent	3.6	749	Hyper	RA/RCMD	46, XX	0.5	Yes	Yes (1501)
13	67/F	5	Dependent	1.2	687	Normo	RA/RCMD	46, XX	0.5	No	No
14	55/F	12	Dependent	5.1	1237	Hyper	RA/RCMD	46, XX	0.5	No	Yes (1501)
15	64/M	5	No	6.7	765	Hyper	RA/RCMD	47, XY, +Y, add(9)(q13)	1	No	Yes (1501)
16	50/M	12	Dependent	8.1	340	Hypo	RA/RCMD	47,XY +8	1	No	Yes (1501)
17	50/M	14	Dependent	4.5	70	Hyper	RAEB/RAEB-1	46, XY	1	No	No
18	35/M	2	Dependent	13.2	2153	Hyper	RAEB/RAEB-1	46, XY	0.5	No	No
19	52/M	1	Dependent	2.2	1358	Normo	RARS/RCMDRS	46, XY	0.5	No	Res (1502)
20	63/M	2	No	7.4	901	Hyper	RA/RCMD	46,XY, del20q	0.5	Yes	No

*UPN indicates unique patient number; PLT, platelet count ($\times 10^4/\mu L$); Neut, neutrophil count ($/\mu L$); BM, bone marrow; FAB, French-American-British classification; WHO, World Health Organization classification; IPSS, International prognostic scoring system for myelodysplastic syndromes; PNH, paroxysmal nocturnal hemoglobinuria; HLA-DR; D-related human leukocyte antigen; RA, refractory anemia; RCMD, refractory cytopenia with multilineage dysplasia; NT, not tested; RAEB, refractory anemia with excess of blasts; RARS, refractory anemia with ringed sideroblasts; RCMD-RS, RCMD with ringed sideroblasts.

†Interval between diagnosis and enrollment.

‡Average of 2 measurements at least 2 weeks apart.

§Patients showing bone marrow morphology as RA with minimal dysplasia.

Table 2.
Outcome of Treatment with Cyclosporine A (CSA)*

UPN	HI at 24th Week (Duration of CSA before Achieving HI) HI-E	HI-P	HI-N	Therapy after Study Period	Present Status
1	Major (8 wks)	Minor (6 wks)	No	Unknown	Lost follow-up at 6 mo
2	Minor† (6 wks)	Minor† (4 wks)	No	CSA	Alive at 30 mo, continuous response
3	Minor† (24 wks)	Minor† (24 wks)	Major (6 wks)	CSA	Alive at 36 mo, continuous response
4	Major (2 wks)	Major (4 wks)	No‡ (18 mo)	CSA	Alive at 36 mo, continuous response
5	No‡ (24 mo)	Minor† (20 wks)	No	CSA	Alive at 36 mo, continuous response
6	No	No	No	Androgen	Alive at 24 mo
7	No‡ (24 mo)	No‡ (10 mo)	No	CSA	Alive at 24 mo, continuous response
8	No	No	—	Observation	Alive at 36 mo
9	No	No	—	Observation	Alive at 36 mo
10	—	Minor (4 wks)	—	CSA	Alive at 36 mo
11	Minor† (22 wks)	—	—	CSA	Alive at 24 mo, continuous response
12	No	No	No	Observation	Alive at 30 mo
13	Major (16 wks)	No‡ (14 mo)	No‡ (14 mo)	CSA	Alive at 24 mo, continuous response
14	Major (18 wks)	No	No	CSA	Alive at 30 mo, continuous response
15	No	No	No	Observation	Alive at 36 mo
16	Major (8 wks)	No	No	mPSL, androgen	Alive at 30 mo, lost response
17	No	No	No	Observation	Died at 7 mo
18	No	—	—	Allo-SCT	WT-1 value elevated
19	No	No	No	Allo-SCT	Grade 4 cytopenia developed
20	NE	NE	NE	Androgen	Cyclosporine withheld due to infection, alive at 18 mo

*UPN indicates unique patient number; HI, hematologic improvement according to the response criteria from the International Working Group; HI-E, erythroid response; HI-P, platelet response, HI-N, neutrophil response; mPSL, methyl-predonisolone; allo-SCT, allogeneic stem cell transplantation.
†Maximal hematologic response turned out to be the most major until the latest follow-up.
‡Hematologic response was obtained until the latest follow-up.

as HI-E as well. Hematologic responses were durable and progressive. Figure 1 shows the kinetics of hematologic increments in patients who showed responses before the latest follow-up. Except for one patient (No. 10), a minor response at the 24th week turned out to be the most major response at the time of the latest follow-up. The patient (No. 10) has had a continued minor response for 36 months. In addition, many responders gained further responses that were not attained by patients treated with CSA for 24 weeks. Until now, 2 erythroid, one platelet, and 2 neutrophil responses have been further documented. In addition, one patient (No. 7), who was judged as a nonresponder at the 24th week, continued to take CSA thereafter, and obtained platelet and erythroid responses at the 10th and 24th months, respectively.

3.6. Analysis for the Presence of Minor T-Cell Clones

To gain insights into the mechanism of CSA-induced hematological responses, the presence or absence of minor T-cell clones was examined. We could examine the pretreatment T-cell repertoires in 13 patients, and samples drawn at the 24th week of CSA therapy were also available in 6 of them. Before CSA treatment, 11 patients showed skewed complementarity-determining region 3 spectratypes, and 2 patients displayed normal patterns. The presence or absence as well as the number of minor T-cell clones did not correlate with therapeutic outcomes (data not shown). As shown in Figure 2, the comparison of TCR-Vβ spectratypes between those obtained at pretreatment and after 24 weeks of CSA demonstrated that abnormally expanded minor T-cell clones present before CSA therapy persisted with a similar frequency even in CSA responders.

4. Discussion

In this prospective trial, 10 of 19 patients available for evaluation (53%) showed HI within 24 weeks of CSA therapy, and their responses lasted for at least 2 years if CSA was continuously administered. Reports documenting the efficacy of CSA against low-risk MDS are limited. In addition, most reports were single center experiences or a retrospective survey. Only 3 multicenter prospective trials exist, which reported

Table 3.
Adverse Events Observed within 6 Months of Cyclosporine A Treatment

	Grade According to CTC Version 2.0				
	0	1	2	3	4
Cardiovascular	19	1	0	0	0
Infectious	16	0	2	1*	1†
Renal	9	10	1	0	0
Hepatic	8	10	1	1*	0
Gastrointestinal	17	2	1	0	0
Metabolic	8	11	1	0	0
Cutaneous	17	3	0	0	0
Neutropenia	18	0	1	1†	0
Thrombocytopenia	18	1	0	1	0
Anemia	19	0	0	0	1

*†Adverse events developed in the same patients.

Table 4.
Pretreatment Variables Associated with Hematologic Response at 24th Week*

	Overall Response			Erythroid Response			Platelet Response		
	Responder	Nonresponder	*P*	Responder	Nonresponder	*P*	Responder	Nonresponder	*P*
Age, y			.81			.96			1
More than 50	5	4		4			3	5	
50 or younger	5	5		4	6		3	6	
Disease duration			.83			1			.009
4 mo or longer	4	5		4	5		0	8	
Less than 4 mo	6	4		4	5		6	3	
Karyotype			1			1			.51
Normal	9	8		7	9		6	9	
Abnormal	1	1		1	1		0	2	
PNH-type cells			.33			.64			.03
Yes	5	2		3	3		5	2	
No	4	7		4	7		1	9	
HLA DRB1 1501			.33			.35			.64
Yes	5	2		4	3		3	4	
No	4	7		3	7		3	7	
RA with minimal dysplasia			.44			.71			.03
Yes	5	2		4	3		5	2	
No	5	7		4	7		1	9	

*See Table 1 for abbreviations.

exceedingly high CSA response rates. Janasova demonstrated a response rate of 82% (14/17) [17], Dixit showed that 14 of 19 patients (74%) gained HI according to IWG criteria [19], and Chen reported the response rate at 62.5% (20/32) [23]. Although Atoyebi reported unsuccessful outcomes in 6 patients with FAB-RA and RARS [22], inconsistent results are inevitable in small-sized studies, as documented in studies using ATG [12,33]. The response rate of 53% in our study is consistent with previous reports as well as our retrospective survey [24]. Low-risk MDS, especially FAB-RA, is highly diverse in both clinical presentations and pathophysiology. In addition, a recent report from Matsuda demonstrated that the frequency of WHO-RA among FAB-RA is much higher in Japanese than in German patients [34,35]. Thus, comparison of response rates in patients of different ethnic background seems to have limited value. Rather, the predictive variables of the CSA response, which can be universally applicable, should be elucidated and compared between trials. We found that 3 variables: the presence of PNH-type cells, short duration of illness (less than 4 months), and the diagnosis of RA with minimal dysplasia, were significantly associated with the platelet response at the 24th week. In contrast, we could not find any predictive variables for overall and erythroid responses. The predictive value of having HLA-DRB1 1501, which was associated with the CSA response in our retrospective survey, was not confirmed in this study.

To our surprise, hematologic responses were durable and progressive after the 24th week of evaluation. Six additional HI were documented in four patients after the 24th week. RA patients with minimal dysplasia or PNH-type cells gathered into one cohort (RAminiD/PNH cohort) (UPN 1-7, 10, 12, 20) had an elevated probability of multi-lineage responses. HI was obtained in 7 of 9 patients, and 6 of 7 responses were multilineage. In particular, the platelet response was almost restricted in this cohort. Some patients in the other cohort, who showed neither the feature of RA with minimal dysplasia nor PNH-type cells (No. 8, 9, 11, 13-19), also gained a hematologic response to CSA (hematologic response rate of 4/10). However, most responses were restricted to the erythroid lineage. The significance of the presence of PNH-type cells in the prediction of the response

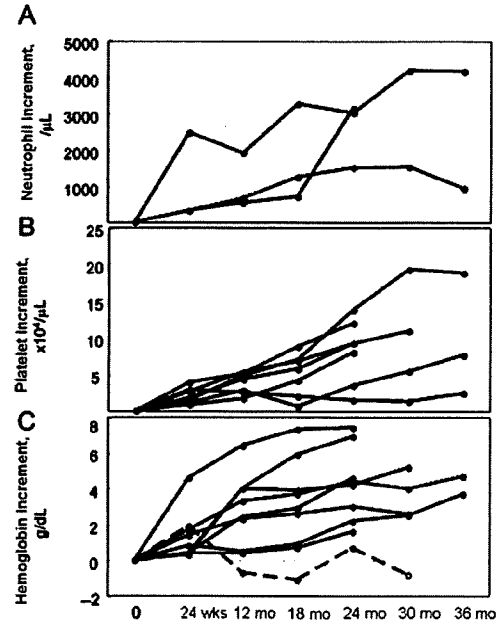

Figure 1. Hematologic increments in patients who showed a response up to the latest follow-up. A, Increments in neutrophil counts from baseline values in three patients who showed neutrophil responses. B, Increments in platelet counts in 7 responders. C, Increments in hemoglobin values in 9 responders. One patient who lost the response after the 12th month is indicated as a broken line.

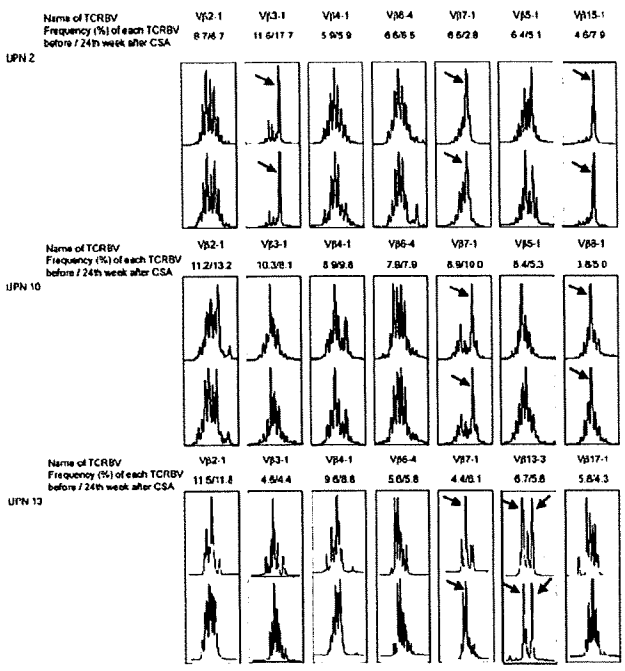

Figure 2. Representative T-cell receptor β chain variable region (TCR-Vβ) spectratype before and after cyclosporine A (CSA) therapy. The spectratypes listed were the eight frequent Vβ families; the frequency was defined by microplate hybridization assay. The name of TCR Vβ, the relative frequency of each Vβ, and spectratypes are shown. All patients shown here were CSA responders.

to immunosuppressive therapy against aplastic anemia as well as MDS has been reported [15,25,26]. A retrospective survey demonstrated that RA patients harboring PNH-type cells showed less pronounced morphologic abnormality, rare progression into acute leukemia, a higher incidence of HLA-DR15, and higher CSA response rate [26]. The results of this study are in agreement with previous observations.

Molldrem and Kochenderfer showed that MDS patients exhibited a skewed TCR-Vβ repertoire, indicating the presence of a clonal T-cell population. The clonal population was diminished only in responders to immunosuppressive therapy and ATG [36,37], which is consistent with clinical findings that ATG produces long-lasting HI without additional therapy. In this study, we also detected the presence of abnormally expanded T-cell clones in most MDS patients. However, CSA administration for 24 weeks did not affect expanded T-cell clone frequencies, even in responders. Thus, CSA does not appear to eliminate pathologic T-cells, but inhibits their marrow-suppressive function, thus bringing about a CSA-dependent hematological response, as reported in aplastic anemia [38].

In addition to its promising effects, CSA has a limited toxicity profile and can be safely administered in outpatient clinics. CSA produced less severe adverse events than ATG or lenalidomide, which caused effects defined as over grade 2 toxicities. Stadler reported that the administration of horse- or rabbit-derived ATG produced higher than grade 2 toxicities in 23 of 35 MDS patients [13]. Higher than grade 2 neutropenia (65%) or thrombocytopenia (53%) was documented in a phase I trial of lenalidomide [9]. Most of the adverse events seen in CSA-treated patients were slightly elevated liver enzymes or marginally impaired renal function tests (elevated creatinine or potassium values); dose reduction or interruption of CSA was rarely needed.

In conclusion, the use of CSA was associated with HI in selected patients with FAB-RA without severe adverse events. Patients harboring minor populations of PNH-type cells or showing minimal dysplastic features have an elevated likelihood of recovering from thrombocytopenia and anemia. Erythropoiesis is generally restored in patients who do not have PNH-type cells. As the hematologic response is CSA-dependent, long-term outcomes, including the possibility of accelerating leukemic transformation, must be carefully observed.

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Original Article

Wilms' tumor 1 message and protein expression in bone marrow failure syndrome and acute leukemia

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Wilms' tumor 1 (WT1) is a useful marker for the diagnosis of acute leukemia and myelodysplastic syndromes (MDS). In the current study quantitative reverse transcription–polymerase chain reaction and immunostaining were used simultaneously to examine the relationship between *WT1* RNA and protein level and also to evaluate WT1 as a tool to differentiate aplastic anemia (AA) and MDS refractory anemia (RA). Three types of *WT1* messages (total, exon 5(+) and KTS(+)) and WT1 immunostaining of these diseases were analyzed. An increase of all three *WT1* messages in high-grade MDS and acute leukemia was observed as compared with the normal control, whereas there was no significant difference in *WT1* message between AA and RA, suggesting that *WT1* message is not a good tool to discriminate AA and RA. No significant difference was observed between normal and RA, except for exon 5 message. Three *WT1* message levels had a significant correlation, suggesting that the total WT1 message is sufficient for clinical practice. Positive immunostaining of WT1 was observed only in the portion of acute leukemia and overt leukemia (OL) transformed from MDS with a high *WT1* message level, suggesting the relatively high detection threshold of WT1 protein with the immunostaining method.

Key words: acute leukemia, aplastic anemia, exon 5, immunostaining, KTS, MDS, quantitative RT-PCR, WT1 message

Wilms' tumor gene (*WT1*) is a tumor suppressor gene coding for a zinc finger transcription factor located on chromosome 11p13, which was originally identified for its involvement in the pathogenesis of Wilm's tumor.[1] WT1 is expressed in a variety of normal tissues, including ovary, testis, and spleen, while WT1 expression is low in normal bone marrow.[1] On the contrary, the *WT1* message was increased in acute leukemia and myelodysplastic syndromes (MDS)[2–5] and was regarded as an oncogene in some situations. The usefulness of *WT1* message level quantification has been reported in the detection of minimal residual disease and the relapse of acute leukemia.[2,6,7]

MDS are acquired clonal hematopoietic stem cell disorders characterized by ineffective dysplastic hematopoiesis involving one of more cell lineages and characterized by peripheral-blood cytopenias and a high risk of progression to acute leukemia.[8] Recently, the term bone marrow failure syndromes (BFS) was used from the standpoint of stem cell disorder, and both MDS and aplastic anemia were included in this category. However, because of the difference in treatment strategy, the differential diagnosis of MDS, especially refractory anemia (RA) and aplastic anemia (AA), is crucial but sometimes difficult.

Cilloni *et al.* reported the statistical significance of *WT1* message between normal bone marrow and RA bone marrow.[9] Even in their study, however, considerable overlap was observed between the *WT1* message of RA and those of normal control. Patmasiriwat *et al.* reported no statistically significant difference in the *WT1* message level between RA and normal control,[10] and the *WT1* message was not compared between RA and AA. Because the differential diagnosis between RA and non-severe AA is especially difficult when chromosomal abnormality and morphological dysplasia are not present, a new clinical parameter is needed. The aim of the present study was to analyze the *WT1* message level in BFS and also to evaluate the significance of *WT1* message level for the differential diagnosis of RA and AA.

WT1 is known to have four major isoforms due to the alternative splicing,[11] and it is also of interest to know the significance of these isoforms in MDS pathogenesis as well as the relative importance of each isoform as a clinical marker.

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The gene expression level of each *WT1* splice variant in MDS has not been reported to date. Thus, we sought to analyze the message level of total *WT1*, exon 5(+) *WT1* and KTS(+) *WT1* in BFS and de novo acute leukemia.

The comparison of WT1 protein and *WT1* message level in MDS and acute leukemia are clinically important to determine the usefulness of immunostaining for routine laboratory examination of these diseases. Hence, we performed WT1 immunostaining of the clot section in patients whose *WT1* message level was measured. Previous histochemical analysis showed that many solid tumors and cancer cell lines express increased WT1 protein, but it is located sometimes in the cytoplasm, not the nucleus.[12–14] This might suggest that the role of WT1 in such cases is not that of a transcription factor. It was also shown that the *WT1* mRNA expression level does not correlate with the WT1 protein expression level.[13] Therefore, the significance of enhanced WT1 expression in solid tumor as well as in hematological malignancies remains to be determined.

MATERIALS AND METHODS

Clinical samples

After obtaining informed consent, bone marrow cells were collected from seven patients with AA, 28 with de novo acute myelocytic leukemia (AML) and 38 with MDS (17 RA, 21 refractory anemia with excessive blast (RAEB) and RAEB in transformation (RAEB-T) according to French–American–British classification) along with seven patients having overt leukemia developed from the previous MDS stage (OL). We used RAEB and RAEB-T as a single category because of the limited number of RAEB cases. Samples were obtained before any treatment. For the normal control, bone marrow samples for the disease staging were used from 11 patients with non-Hodgkin's lymphoma without bone marrow invasion. The clinical characteristics of AML and MDS patients are given in Table 1.

Quantitative reverse transcription–polymerase chain reaction

Mononuclear cells were separated from the bone marrow samples. RNA was extracted, and first-strand cDNA was prepared as described previously.[15] The primer set and reaction condition were according to Siehl *et al.*[16] After preliminary confirmation of the validity of the measurement of total *WT1*, exon 5(+) and KTS(+) *WT1* messages but not of exon 5(–) and KTS(–) messages by their method, we examined total *WT1*, exon 5(+) *WT1* and KTS(+) *WT1* messages of the present patient cohort. Primers and Taqman probe are listed in Table 2. PCR amplification was 40 cycles with the denaturing temperature at 95°C for 10 s followed by 60°C for 15 s.

Table 1 Patient characteristics

FAB	No. patients	Sex M	Sex F	Age (mean) (years)	Chromosome abnormality *n* (%)
AA	7	2	5	42.9	0 (0)
RA	17	11	6	60.9	4 (23.5)
RAEB/RAEB-T	21	14	7	66.3	9 (42.9)
OL	7	3	4	68.7	6 (85.7)
AML	28	19	9	51.7	12 (42.9)

AA, aplastic anemia; AML, acute myelocytic leukemia; FAB, French–American–British; MDS-OL, overt leukemia transformed from myelodysplastic syndromes; RA, refractory anemia; RAEB/RAEB-T, refractory anemia with excessive blast/refractory anemia with excessive blast in transformation.

Table 2 Primer and TaqMan probe sequences for quantitative RT-PCR

Total WT-1	
Forward	5′-CGCTATTCGCAATCAGGGTTAC-3′
Reverse	5′-ATGGGATCCTCATGCTTGAATG-3′
TaqMan Probe	5′-FAM-CGGTCACCTTCGACGGGACGC-TAMRA-3′
EXON 5(+)	
Forward	5′-TGGACAGAAGGGCAGAGCA-3′
Reverse	5′-GGATGGGCGTTGTGTGGT-3′
TaqMan Probe	5′-FAM-CCACAGCACAGGGTACGAGAGCGA-TAMRA-3′
KTS(+)	
Forward	5′-GCTCAAAAGACACCAAAGGAGAC-3′
Reverse	5′-AGCTGAAGGGCTTTTCACTTGTTT-3′
TaqMan Probe	5′-FAM-TTCTCCCGGTCCGACCACCTGAA-TAMRA-3′

Sequences are shown according to Siehl *et al.*[16] for of each primer set and Taqman probe used in quantitative RT-PCR.
RT-PCR, reverse transcription–polymerase chain reaction.

Quantification of total-*WT1* and splice variants (exon 5(+) *WT1* and KTS(+) *WT1*) was performed with the Light Cycler system (Roche Diagnostics, Mannheim, Germany). The housekeeping gene *ABL* mRNA was also measured as the internal control according to the recommendation of Beillard *et al.*[17] The relative ratio (*WT1/ABL*) was calculated and presented. Briefly, 1 μL of each cDNA was diluted to a volume of 19 μL PCR mix (LightCycler TaqMan Master, Roche Diagnostics), containing 0.5 pmol of each primer and 0.2 pmol of the TaqMan probe. The standard curve of *WT1* was created using the cDNA fragment of each *WT1*, which was produced by PCR and inserted into the cloning vector, pBluescript (Stratagene, La Jolla, CA, USA). The standard curve of *ABL* plasmid was also prepared. The specificity of each PCR product was confirmed in the preliminary experiments using leukemia cell lines.

Immunostaining

Formalin-fixed, paraffin-embedded bone marrow tissues were used for the immunostaining. Antigen retrieval was performed by microwave in 10 mmol/L citrate buffer (pH 7.4). Sections were blocked with PBS containing 5% skim milk, and then were reacted with anti-WT1 monoclonal antibody (clone 6F-H2, Dakocytomation, Carpinteria, CA, USA) at 4°C overnight. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol solution. The detection was performed using the ChemMate EnVision Detection Kit/HRP (DAB) (Dakocytomation). The grading of WT1-positive cells was as follows: positive (+), >5% of total nuclei; weakly positive (+/–), 1–5% of total nuclei.

Statistical analysis

Results of clinical samples were analyzed using Microsoft Excel and Statview version 5 (SAS Institute, Cary, NC, USA). The statistical significance of differences between each group was assessed by one-way factorial analysis of variance and multiple comparison tests (Fisher's method). $P < 0.05$ was regarded as statistically significant.

RESULTS

Total *WT1* message level in AA, MDS, MDS-OL and AML

According to Table 1 the mean age of AA and AML patients is slightly lower than those of other groups, chromosome abnormality was absent in AA, and was increased with the progression of MDS stage (from RA to RAEB/RAEB-T). Figure 1 illustrates the distribution of total *WT1* message in normal, AA, MDS, OL and AML. In MDS bone marrow a statistically significant difference was observed between RA and RAEB/RAEB-T. OL and AML had a much higher expression level than RA but no statistically significant differences were observed between normal and AA, between normal and RA or between AA and RA, respectively.

WT1 immunostaining of bone marrow clot section

Nuclear staining was evident using our staining method (Fig. 1). Positive WT1 staining in each disease category was also shown in red (+) or blue (+/–) on the left side of Fig. 1. Strong positive staining was observed only in AML (8/28 cases, 28.6%) and OL (1/7 cases, 14.3%). In MDS, weakly positive (+/–) staining but not strong positive staining was observed in some cases (4/21 RAEB/RAEB-T, 19%; 4/17 RA, 23.5%). No positive staining was observed in AA or normal groups. The staining intensity of each positive cell was apparently higher in AML and OL than in MDS. When combined with *WT1* message and immunostaining data, the positive staining clearly required the high *WT1* message expression in whole bone marrow (Fig. 1). The threshold is approximately 1/10 of the *ABL* message level. Normal bone marrow contained approximately 10^5 copies of *ABL*/μg RNA according to previous reports[17,18] and our preliminary experiments. Therefore, the threshold for positive WT1 staining was 10^4 copies/μg RNA of total bone marrow. In other words, a value exceeding this threshold can indeed confirm the presence of leukemic bone marrow. It is interesting to examine the CD34 population of MDS bone marrow because the present data suggest that the *WT1* message might be proportional to the blast cell percentage in MDS. But we could not examine this point in the present study because of the paucity of samples. It is of note that the *WT1* message level in AML and OL is heterogeneous, suggesting that the WT1 staining pattern of bone marrow is not always proportional to the percentage of blast cells. It can be said that the sensitivity of WT1 staining is relatively low as compared with reverse transcription–polymerase chain reaction (RT-PCR).

Exon 5 and KTS+ *WT1* message level in BFS and AML

The message level of exon 5(+) and KTS(+) *WT1* is shown in Fig. 2. Statistically significant differences were also observed in exon 5 *WT1* message between RA and RAEB/RAEB-t (Fig. 2). Only in this isoform was a statistically significant difference observed between normal and MDS RA; no similar tendency was observed between normal and AA, or between AA and RA. Analysis of KTS(+) *WT1* message found a similar tendency (Fig. 2), that is, an increase of message during the progression of MDS and the lack of significant differences between normal and AA, normal and RA, or AA and RA, respectively.