Pro-Q® Diamond で検出されたスポットを切り出し、質量分析したところ、表1に示すようなリン酸化蛋白が同定された。MDS/OL 患者と正常の CD3 4 陽性細胞のリン酸化蛋白のスポットを比較検討すると、その発現にいくつかの差異が認められ、現在その蛋白を同定・解析中である。

D. 考察

従来、リン酸化蛋白の同定およびその解析には、^{32}P の取り込みやリン酸化アミノ酸（チロシンやセリン/スレオニン）に対する抗体などを用いて行われていた。近年、リン酸化した蛋白に対して特異的に結合する蛍光色素が開発され、蛋白電気泳動したゲル上でリン酸化蛋白のみを検出することができるようになった。そのスポットを切り出し、質量分析器で解析することで、リン酸化蛋白の同定、解析が可能となっている。今回、MDS/OL および正常の CD34 陽性細胞由来蛋白について、上述の方法で検討したところ、リン酸化蛋白の検出が可能であることが明らかになった。この方法を用いれば、MDS 血球におけるリン酸化蛋白の同定、解析を網羅的に行うことができると考えられる。本研究を進めることにより、今後、様々な MDS のサブグループにおける CD34 陽性細胞のリン酸化蛋白の発現異常が明らかになり、ヘテロな疾患群と考えられている MDS の病因、病態が明らかになる可能性がある。さらに病因に関わる蛋白異常を特定できる可能性もあり、本症に対する新規治療薬の開発にもつながるものと期待される。

E. 結論

MDS 患者および正常の CD34 陽性細胞由来蛋白を用いて、二次元電気泳動を行い、ゲル上でリン酸化蛋白を認識する蛍光試薬で染色することにより、MDS 特異的なリン酸化蛋白の同定が可能である。

F. 健康危険情報

なし

G. 研究発表

なし

H. 知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他

いずれも予定なし

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分担研究報告

SNPアレイを用いたMDSの新規原因遺伝子の同定に関する研究

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研究要旨
高密度SNPアレイ(Affymetrix社)を用いた170例のMDSのゲノムコピー数異常・アレル不均衡の解析結果に基づいて、MDSの発症に関わるゲノムの変異の探索を行った。その結果、MDSでは約30%の症例で、ゲノムコピー数変化を伴わないヘテロ接合性の消失(LOH)、すなわちUPD(Uniparental disomy)が認められること、MDSの病型とUPDの生ずる染色体領域にはしばしば密接な関連があること、を見いだし、11番長腕にUPDを有する一群のMDS症例の解析により、これらのMDSの発症に関わると考えられる新規遺伝子変異を同定した。また、同遺伝子(mds3)変異の機能的解析により、機能的にドミナントであること、変異mds3遺伝子はNIH3T3細胞を強力にトランスフォームすること、造血幹細胞の活性維持をサポートすること、さらに、これらの機能的変化は、同分子のリン酸化状態の変化と密接に関わっていることを明らかにした。

A. 研究目的

骨髄異形成症候群(MDS)は、現在、造血幹細胞移植以外には根治的治療法がなく、今後、副作用の少ない有効な治療法を確立するためには、本疾患の原因分子の同定とこれを標的とした分子標的薬の開発が重要である。MDSは造血前駆細胞におけるゲノム異常の蓄積によって発症すると考えられるが、AML1などに代表される一部の例外を除いて、こうしたゲノム異常の標的となっている遺伝子に関する知見は限られている。本分担研究では、近年開発された高密度SNPアレイを用いてMDSゲノムに生ずるゲノムコピー数の異常およびヘテロ接合性の消失(LOH)を網羅的に探索することにより、MDSの発症に関わる新規遺伝子の同定を試みた。

B. 研究方法

(1)高密度SNPアレイを用いたMDSゲノムの解析

種々の病型を含む171例のMDS検体について、ゲノムDNAを抽出し、GeneChip100Kないし500Kアレイによる解析したのち、我々が独自に開発したゲノムコピー数解析ツールCNAG/AsCNARを用いて10万～50万個の各プローブのシグナルを正常対照DNAのアレイ解析で得られたシグナルと比較することにより、ゲノムコピー数の定量を行った。また、アレル特異的なゲノムコピー数の解析により、高感度なアレル不均衡の網羅的な解析を行った。

(2) 11qUPDの標的遺伝子の同定

(1)の解析により見いだされた11番染色体長腕のおけるゲノムコピー数変化を伴わないLOHの集積領域について、約1Mbの共通領域を見いだし、同領域に含まれる構造遺伝子について全エクソンのリシーケンスによる変異解析を行うことにより、11qUPDの標的遺伝子の探索を行った。

(3)変異MDSの機能解析

(2)の解析により同定された11qUPDの標的遺伝子mds3について、変異mds3遺伝子をマウス繊維芽細胞および造血幹細胞分画に遺伝子導入することにより、その機能解析を行った。

(倫理面への配慮)

検討に用いた検体は、当該患者からインフォームドコンセントを得たのちに連結可能匿名化を施して検討に用いた。当院の倫理委員会の承認済みである。

C. 研究結果

(1)高密度SNPアレイを用いた171例のMDSの解析により、MDSにおけるゲノムコピー数の異常およびアレル不均衡の網羅的な解析を行った。MDSでは従来報告されている-5/5q-、-7/7q-、+8、12q-、13q-、17q-、20q-などの典型的な染色体変化に加えて、染色体分析では同定不可能な微小なゲノム領域の異常が多数同定された。さらに、アレル不均衡の解析により、正常核型を含むMDSの約30%内外の症例でUPDの異常が認められることが明らかとなった。これらのUPDは、1p, 1q, 4q, 7q, 11q, 14q, 17q, 21qを含む幾つかの染色体領域に集積して認められ、上述のゲノムコピー数の異常とあわせて、MDSがゲノム異常の観点から幾つかの亜型に分類されることが示された。

(2)MDSで繰り返し認められるUPDのうち、11qを含むUPDについて、その最小の共通領域として1Mbの領域を同定した。当該領域内にコードされる標的遺伝子の候補について、蛋白をコードするエクソンについて変異解析を行った結果、mds3(研究室

名)において、リンカードメインをコードする領域に集積する新規遺伝子変異を同定した。変異は、11qUPD を認める MDS のほぼ全例でみとめられ、予測されたとおり、変異は全てホモ接合を呈していた。(3)(2)の変異解析から同定された MDS の新規標的遺伝子 mds3 についてその機能解析を行った。NIH3T3 を用いたコロニーアッセイおよびマウス造血幹細胞分画を用いた replating アッセイの結果、変異 mds3 遺伝子は、正常 mds3 に対してドミナントに作用し、NIH3T3 を強くトランスフォームすること、またマウス造血幹細胞分画における replating 活性を上昇されることが示された。

D. 考察

MDS は、形態学的に分類しうる以上にヘテロな疾患群であることは日常経験するところであるが、高密度 SNP アレイによる MDS のゲノムプロファイリングの結果から、MDS をゲノム異常のパターンに基づいて再分類できる可能性が示唆された。本研究の結果は、今後新たな MDS のゲノム分類法を確立する上で極めて有用である。一方、これらの異常の多くが、しばしば特異的な遺伝子変異と関連しているという事実を勘案すると、こうしたゲノム異常に基づく MDS の分類によって、治療法の選択、予後判定を含む MDS の診療を行う上で、より有用な情報が得られる可能性がある。実際、11q の UPD を有する MDS は臨床的にも特徴的な一群を形成するが、本研究でその標的遺伝子が同定されたことにより、その分子診断が可能となった。さらに今後、同変異分子を標的とした有効な分子標的薬が開発されれば、現在有用な治療手段に乏しい MDS の治癒率向上に貢献しうると期待される。

E. 結論

高密度 SNP アレイを用いた MDS における網羅的なゲノムコピー数異常およびアレル不均衡の解析から、MDS がゲノム異常の観点から複数の亜型に分類されることが示された。
これらの亜型のうち、11 番染色体長腕の UPD を有する一群について、11qUPD の標的遺伝子として mds3 遺伝子を同定した。
変異 mds3 遺伝子は、正常アレルに対してドミナントに作用する活性化型分子をコードしており、強いトランスフォーミング活性を有すること、また、マウス造血幹細胞活性の増強を示すことが示され、その MDS 発症における関与が強く示唆された。

F. 健康危険情報

なし

G. 研究発表

論文発表

1. Yamamoto G, Nannya Y, Kato M, Sanada M, Levine RL, Kawamata N, Hangaishi A, Kurokawa M, Chiba S, Gilliland DG, Koeffler HP, Ogawa S. Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays. Am J Hum Genet 81: 114-126, 2007
2. Sanada M, Uike N, Ohyashiki K, Ozawa K, Lili W, Hangaishi A, Kanda Y, Chiba S, Kurokawa M, Omine M, Mitani K, Ogawa S. Unbalanced translocation der(1;7)(q10;p10) defines a unique clinicopathological subgroup of myeloid neoplasms. Leukemia 21: 992-997, 2007
3. Ogawa S, Nannya Y, Yamamoto G, Genome-wide Copy Number Analysis on GeneChip_ Platform Using Copy Number Analyzer for Affymetrix Gene Chip 2.0 Software. Method in Molecular Biology 369 Comparative Genomics Volume2. Humana Press.185-206, 2007

学会発表

第 67 回米国血液学会（平成 19 年 12 月、米国アトランタ）
Sanada M, Shih Ly, Suzuki T, Yamamoto G, Nannya Y, Yanagimoto-Sakata M, Kato M, Kumano K, Mori H, Kurokawa M, Chiba S, Omine M, Koeffler HP, Ogawa S. SNP Chip Analysis of Myelodysplastic Syndromes Disclosed High Frequency of Uniparental Disomy and a Novel Dominant Mutation as the Target of 11q UPD

H. 知的財産権の出願・登録状況

1. 特許取得
2. 実用新案登録
3. その他

いずれも予定なし

III.　研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表（論文）

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Yamagata T, Nakamura Y, Mitani K.	Low expression of ETV6/TEL found in patients with myelodysplastic syndrome.	Int J Hematol	86	282-285	2007
Tokita K, Maki K, Mitani K.	RUNX1/EVI1 that blocks myeloid differentiation inhibits CCAAT-enhancer binding protein a function.	Cancer Sci	98	1752-1757	2007
Sasaki K, Yamagata T, Mitani K.	Histone deacetylase inhibitors trichostatin A and valproic acid circumvent apoptosis in human leukemic cells expressing the RUNX1 chimera.	Cancer Sci	99	414-422	2008
Ishikawa T, Tohyama K, Nakao S, Yoshida Y, Teramura M, Motoji T, Takatoku M, Kurokawa M, Mitani K, Uchiyama T, Omine M.	A prospective study of cyclosporine a treatment of patients with low-risk myelodysplastic syndrome: presence of CD55-CD59-Blood cells predicts platelet response.	Int J Hematol	86	150-157	2007
Shindo T, Ishikawa T, Fukunaga A, Hori T, Uchiyama T.	Growth and Differentiation Advantages of CD4+OX40+ T Cells from Allogeneic Hematopoietic Stem Cell Transplantation Recipients.	Biol. Blood Marrow Transplant	14	268-281	2008
Xu J, Suzuki M, Niwa Y, Hiraga J, Nagasaka T, Ito M, Nakamura S, Tomita A, Abe A, Kiyoi H, Kinoshita T, Naoe T.	Clinical significance of nuclear non-phosphorylated beta-catenin in acute myeloid leukaemia and myelodysplastic syndrome.	Br J Haematol	140	394-401	2008
Iwasaki T, Sugisaki C, Nagata K, Takagi K, Takagi A, Kojima T, Ito M, Nakamura S, Naoe T, Murate T.	Wilms' tumor 1 message and protein expression in bone marrow failure syndrome and acute leukemia.	Pathol Int	57	645-651	2007
Kajiguchi T, Chung EJ, Lee S, Stine A, Kiyoi H, Naoe T, Levis MJ, Neckers L, Trepel JB.	FLT3 regulates beta-catenin tyrosine phosphorylation, nuclear localization, and transcriptional activity in acute myeloid leukemia cells.	Leukemia	21	2476-2484	2007
Ito Y, Ohyashiki K, Yoshida I, Takeuchi M, Aoyama Y, Mugitani A, Matsuura Y, Wakita H, Matsuda M, Sakamoto E, Kiguchi T, Urabe A, Tamura K, Kanamaru A, Masaoka T.	The prophylactic effect of itraconazole capsules and fluconazole capsules for systemic fungal infections in patients with acute myeloid leukemia and myelodysplastic syndromes: a Japanese multicenter randomized, controlled study.	Int J Hematol	85	121-127	2007
Ohyashiki K, Hori K, Makino T, Ohyashiki JH.	Automated JAK2V617F quantification using a magnetic filtration system and sequence-specific primer-single molecule fluorescence detection (SSP-SMFD) .	Cancer Genet Cytogenet	179	19-24	2007

研究成果の刊行に関する一覧表（論文）

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Ohyashiki K, Tauchi T, Kuroda M, Kodama A, Ohyashiki JH.	Recurrent chromosomal aberration at 12q15 in chronic idiopathic myelofibrosis with or without JAK2(V617F) mutation.	Leukemia	21	1578-1580	2007
Watanabe-Okochi N, Kitaura J, Ono R, Harada H, Harada Y, Komeno Y, Nakajima H, Nosaka T, Inaba T, Kitamura T.	AML1 mutations induced MDS and MDS/AML in a mouse BMT model.	Blood		(in press)	
Aki D, Minoda Y, Yoshida H, Watanabe S, Yoshida R, Takaesu G, Chinen T, Inaba T, Hikida M, Kurosaki T, Saeki K, Yoshimura A.	Peptidoglycan and lipopolysaccharide activate PLCg2, leading to enhanced cytokine production in macrophages and dendritic cells.	Genes Cells		(in press)	
Shiseki M, Kitagawa Y, Wang YH, Yoshinaga K, Kondo T, Kuroiwa H, Okada M, Mori N, Motoji T.	Lack of nucleophosmin mutation in patients with myelodysplastic syndrome and acute myeloid leukemia with chromosome 5 abnormalities.	Luek Lymphoma	48	2141-2144	2007
Yasunami T, Wang YH, Tsuji K, Takanashi M, Yamada Y, Motoji T.	Multidrug resistance protein expression of adult T-cell leukemia/lymphoma.	Leukemia Res	31	465-470	2007
Ishikawa T, Tohyama K, Nakao S, Yoshida Y, Teramura M, Motoji T, Takatoku M, Kurokawa M, Mitani K, Uchiyama T, Omine M.	A prospective study of cyclosporine A treatment of patients with low-risk myelodysplastic syndrome: presence of CD55-CD59- blood cells predicts platelet response.	Int J Hematol	86	150-157	2007
Ogawa S, Nannya, Yamamoto G.	Genome-wide Copy Number Analysis on GeneChip_ Platform Using Copy Number Analyzer for Affymetrix Gene Chip 2.0 Software Method in Molecular Biology 369.	Comparative Genomics	2	185-206	2007
Yamamoto G, Nannya Y, Kato M, Sanada M, Levine RL, Kawamata N, Hangaishi A, Kurokawa M, Chiba S, Gilliland DG, Koeffler HP, Ogawa S.	Highly sensitive method for genomewide detection of allelic composition in nonpaired, primary tumor specimens by use of affymetrix single-nucleotide-polymorphism genotyping microarrays.	Am j Hum Genet	81	114-126	2007
Sanada M, Uike N, Ohyashiki K, Ozawa K, Lili W, Hangaishi A, Kanda Y, Chiba S, Kurokawa M, Omine M, Mitani K, Ogawa S.	Unbalanced translocation der(1;7)(q10;p10) defines a unique clinicopathological subgroup of myeloid neoplasms.	Leukemia	21	992-997	2007

Ⅳ．　研究成果の刊行物・別刷

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

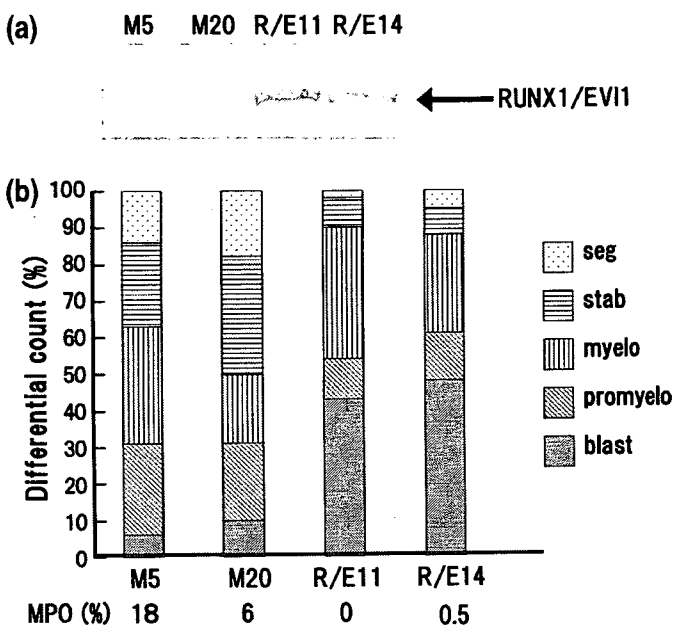

Fig. 1. RUNX1/EVI1 represses granulocyte colony-stimulating factor (G-CSF)-induced granulocytic differentiation in LG-3 cells. (a) Western blot analysis using anti-FLAG M2 antibody for RUNX/EVI1 proteins from whole cell lysates of RUNX1/EVI1-expressing LG-3 clones, R/E11 and R/E14, as well as mock clones, 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*DL*T) but substituted C-terminal (PL*DL*S to PL*AS*S) 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

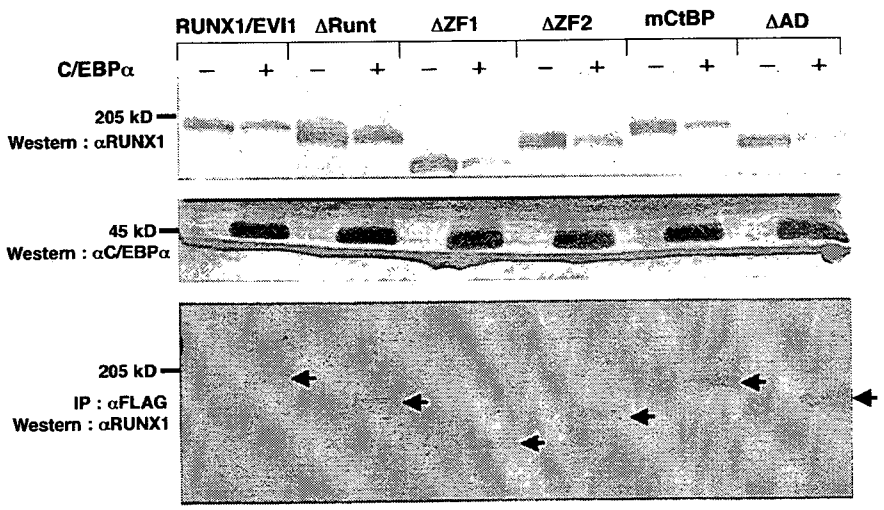

Fig. 2. RUNX1/EVI1 binds to CCAAT/enhancer binding protein α (C/EBPα) *in vivo*. COS-7 cells were transfected with 5 μg pME18S-RUNX1/EVI1, pME18S-ΔRunt, pME18S-ΔZF1, pME18S-ΔZF2, pME18S-mCtBP or pME18S-ΔAD with or without 5 μg pME18S-FLAG-C/EBPα and cultured in Dulbecco's modified Eagle's medium containing 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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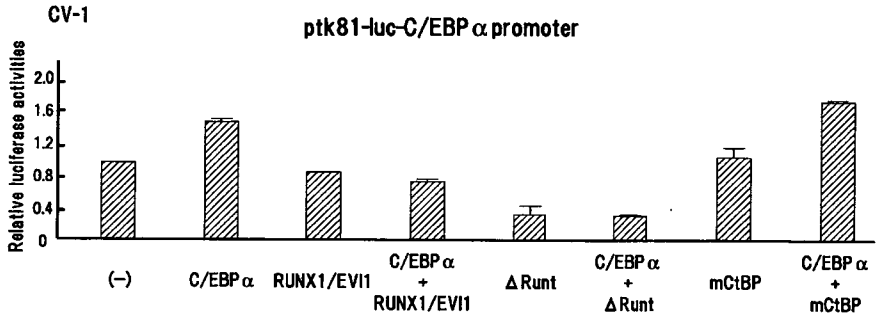

Fig. 3. RUNX1/EVI1 represses CCAAT/enhancer binding protein α (C/EBPα)-mediated transcriptional activity. CV-1 cells were transfected with 200 ng ptk81-luc-C/EBPα reporter plasmid alone or along with 50 ng indicated expression plasmid (C/EBPα, pME18S-C/EBPα; RUNX1/EVI1, pME18S-RUNX1/EVI1; ΔRunt, pME18S-ΔRunt; 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.

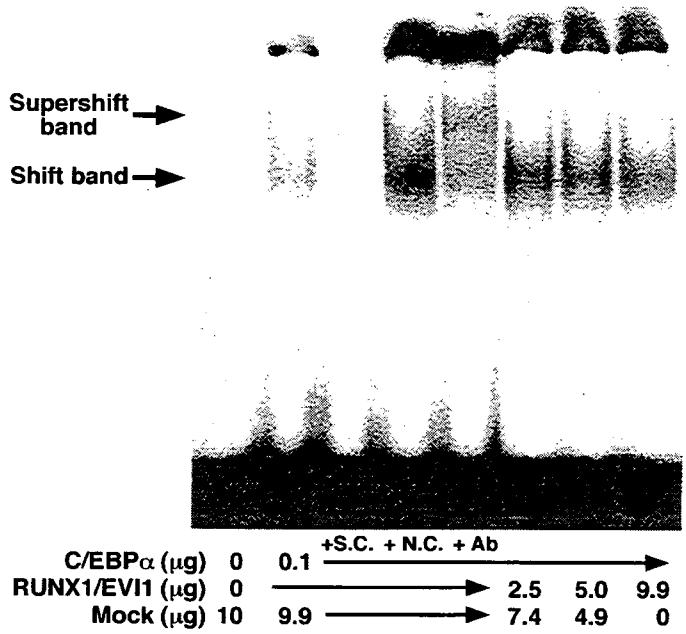

Fig. 4. RUNX1/EVI1 reduces the DNA-binding affinity of CCAAT/enhancer binding protein α (C/EBPα). Electrophoretic mobility shift assay was carried out using a [^{32}P]-labeled probe and lysates from COS-7 cells transfected with pME18S, pME18S-FLAG-C/EBPα or pME18S-FLAG-RUNX1/EVI1. A 300-fold molar excess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

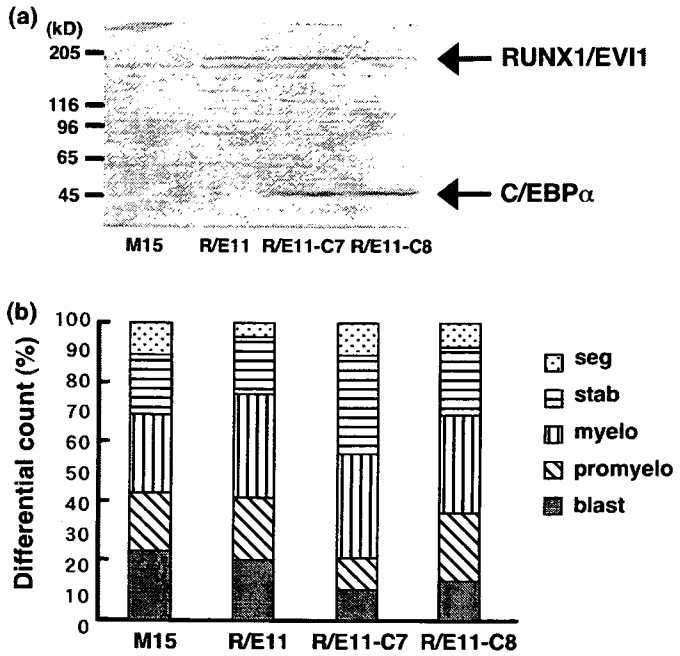

Fig. 5. Co-expression of CCAAT/enhancer binding protein α (C/EBPα) restores granulocytic differentiation in LG-3 cells expressing RUNX1/EVI1. (a) Expression of RUNX1/EVI1 and C/EBPα proteins in the mock (M15), RUNX1/EVI1-expressing (R/E11) or both RUNX1/EVI1- and C/EBPα-expressing (R/E11-C7 and R/E11-C8) 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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University Graduate School of Medicine, Osaka, Japan). Recombinant human G-CSF and murine IL-3 were provided by KIRIN Brewery. This work was financially supported in part by the Japan Health Sciences Foundation, Grants-in-Aid from the Ministries in Japan of Education, Culture, Sports, Science and Technology, and Health, Labour and Welfare, and from the Japanese Society for the Promotion of Science.

References

1 Rubin CM, Larson RA, Bitter MA *et al.* Association of a chromosomal 3;21 translocation with the blast phase of chronic myelogenous leukemia. *Blood* 1987; **70**: 1338–42.
2 Coyle T, Najfeld V. Translocation (3;21) in Philadelphia chromosome-positive chronic myelogenous leukemia prior to the onset of blast crisis. *Am J Hematol* 1988; **27**: 56–9.
3 Rubin CM, Larson RA, Anastasi J *et al.* t(3;21)(q26;q22): a recurring chromosomal abnormality in therapy-related myelodysplastic syndrome and acute myeloid leukemia. *Blood* 1990; **76**: 2594–8.
4 Schneider NR, Bowman WP, Frenkel EP. Translocation (3;21)(q26;q22) in secondary leukemia. Report of two cases and literature review. *Ann Genet* 1991; **34**: 256–63.
5 Mitani K, Ogawa S, Tanaka T *et al.* Generation of the AML1-EVI-1 fusion gene in the t(3;21)(q26;q22) causes blastic crisis in chronic myelocytic leukemia. *EMBO J* 1994; **13**: 504–10.
6 Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 1996; **84**: 321–30.
7 Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the *Cbfa2* gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci USA* 1996; **93**: 3444–9.
8 Ichikawa M, Asai T, Saito T *et al.* AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nat Med* 2004; **10**: 299–304.
9 Kurokawa M, Mitani K, Irie K *et al.* The oncoprotein Evi-1 represses TGF-β signalling by inhibiting Smad3. *Nature* 1998; **394**: 92–6.
10 Kurokawa M, Mitani K, Yamagata T *et al.* The evi-1 oncoprotein inhibits c-Jun N-terminal kinase and prevents stress-induced cell death. *EMBO J* 2000; **19**: 2958–68.
11 Tanaka T, Nishida J, Mitani K, Ogawa S, Yazaki Y, Hirai H. Evi-1 raises AP-1 activity and stimulates c-fos promoter transactivation with dependence on the second zinc finger domain. *J Biol Chem* 1994; **269**: 24 020–6.
12 Maki K, Yamagata T, Asai T *et al.* Dysplastic definitive hematopoiesis in AML1/EVI1 knock-in embryos. *Blood* 2005; **106**: 2147–55.
13 Maki K, Yamagata T, Yamazaki I, Oda H, Mitani K. Development of megakaryoblastic leukaemia in Runx1-Evi1 knock-in chimaeric mouse. *Leukemia* 2006; **20**: 1458–60.
14 Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* 2002; **365**: 561–75.
15 Schuster MB, Porse BT. C/EBPα: a tumour suppressor in multiple tissues? *Biochim Biophys Acta* 2006; **1766**: 88–103.
16 Pabst T, Mueller BU, Harakawa N *et al.* AML1-ETO downregulates the granulocytic differentiation factor C/EBα in t(8;21) myeloid leukemia. *Nat Med* 2001; **7**: 444–51.
17 Pabst T, Mueller BU, Zhang P *et al.* Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-α (C/EBPα), in acute myeloid leukemia. *Nat Genet* 2001; **27**: 263–70.
18 Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein α-deficient mice. *Proc Natl Acad Sci USA* 1997; **94**: 569–74.
19 Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG. PU.1 (Spi-1) and C/EBPα regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood* 1996; **88**: 1234–47.
20 Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein α is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* 1998; **18**: 4301–14.
21 Wang X, Scott E, Sawyers CL, Friedman AD. C/EBPα bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. *Blood* 1999; **94**: 560–71.
22 Miyamoto T, Iwasaki H, Reizis B *et al.* Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. *Dev Cell* 2002; **3**: 137–47.
23 Tavor S, Park DJ, Gery S, Vuong PT, Gombart AF, Koeffler HP. Restoration of C/EBPα expression in a BCR-ABL+ cell line induces terminal granulocytic differentiation. *J Biol Chem* 2003; **278**: 52 651–9.
24 Gombart AF, Hofmann WK, Kawano S *et al.* Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein α in myelodysplastic syndromes and acute myeloid leukemias. *Blood* 2002; **99**: 1332–40.
25 Snaddon J, Smith ML, Neat M *et al.* Mutations of CEBPA in acute myeloid leukemia FAB types M1 and M2. *Genes Chromosomes Cancer* 2003; **37**: 72–8.
26 Mueller BU, Pabst T. C/EBPα and the pathophysiology of acute myeloid leukemia. *Curr Opin Hematol* 2006; **13**: 7–14.
27 Tanaka T, Mitani K, Kurokawa M *et al.* Dual functions of the AML1/Evi-1 chimeric protein in the mechanism of leukemogenesis in t(3;21) leukemias. *Mol Cell Biol* 1995; **15**: 2383–92.
28 Kinashi T, Lee KH, Ogawa M *et al.* Premature expression of the macrophage colony-stimulating factor receptor on a multipotential stem cell line does not alter differentiation lineages controlled by stromal cells used for coculture. *J Exp Med* 1991; **173**: 1267–79.
29 Waga K, Nakamura Y, Maki K *et al.* Leukemia-related transcription factor TEL accelerates differentiation of Friend erythroleukemia cells. *Oncogene* 2003; **22**: 59–68.
30 Izutsu K, Kurokawa M, Imai Y, Maki K, Mitani K, Hirai H. The corepressor CtBP interacts with Evi-1 to repress transforming growth factor beta signaling. *Blood* 2001; **97**: 2815–22.
31 Shimizu K, Kitabayashi I, Kamada N *et al.* AML1-MTG8 leukemic protein induces the expression of granulocyte colony-stimulating factor (G-CSF) receptor through the up-regulation of CCAAT/enhancer binding protein epsilon. *Blood* 2000; **96**: 288–96.
32 Helbling D, Mueller BU, Timchenko NA *et al.* The leukemic fusion gene AML1-MDS1-EVI1 suppresses CEBPA in acute myeloid leukemia by activation of Calreticulin. *Proc Natl Acad Sci USA* 2004; **101**: 13 312–17.
33 Helbling D, Mueller BU, Timchenko NA *et al.* CBFB-SMMHC is correlated with increased calreticulin expression and suppresses the granulocytic differentiation factor CEBPA in AML with inv(16). *Blood* 2005; **106**: 1369–75.
34 Mitani K. Molecular mechanisms of leukemogenesis by AML1/EVI-1. *Oncogene* 2004; **23**: 4263–9.
35 Izutsu K, Kurokawa M, Imai Y *et al.* The t(3;21) fusion product, AML1/Evi-1, blocks AML1-induced transactivation by recruiting CtBP. *Oncogene* 2002; **21**: 2695–703.
36 Kurokawa M, Mitani K, Imai Y, Ogawa S, Yazaki Y, Hirai H. The t(3;21) fusion product, AML1/Evi-1, interacts with Smad3 and blocks transforming growth factor-β-mediated growth inhibition of myeloid cells. *Blood* 1998; **92**: 4003–12.
37 Kurokawa M, Ogawa S, Tanaka T *et al.* The AML1/Evi-1 fusion protein in the t(3;21) translocation exhibits transforming activity on Rat1 fibroblasts with dependence on the Evi-1 sequence. *Oncogene* 1995; **11**: 833–40.

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Letter to the Editor

Low-Level Expression of ETV6/TEL in Patients with Myelodysplastic Syndrome

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Myelodysplastic syndrome (MDS) is a group of myeloid disorders characterized by varying degrees of cytopenia with morphologic abnormalities in multiple blood cell lineages. The disease exhibits a broad clinical spectrum, from moderate anemia requiring occasional transfusion to progressive leukemia that needs immediate cytoreductive chemotherapy. In accordance with this variability, the pathogenesis of the disease is believed to be heterogeneous, including genetic abnormalities, immune dysregulation, and toxic environmental factors.

Of the genetic abnormalities, point mutations in key signaling molecules have been detected in MDS patients, implying that the dysregulation of signaling pathways plays a crucial role in the disease [1]. The mutations that cause constitutive activation of the receptor tyrosine kinase and RAS/MAPK pathways are key to the pathophysiology of this disease. These mutations generate constitutive proliferation signals in the cells, causing dysregulated expansion of the affected clones and replacement of the normal hematopoietic tissues, leading to progression into leukemia.

Point mutations in the *TP53* gene constitute one of the key genetic alterations in MDS. Mutations in *TP53* cause defects in apoptosis and cell cycle arrest, which allow the survival and expansion of the clones harboring other oncogenic alterations, thereby leading to the development of cancer or leukemia. Resistance to apoptosis and defects in cell cycle arrest have been documented in bone marrow cells from MDS patients. Thus, the mutation or deletion of *TP53* is thought to promote the development of MDS.

The activity of TP53 is controlled by various upstream molecules. MDM2 is a TP53-specific E3 ligase that binds to TP53 and promotes its ubiquitination and subsequent degradation. Recently, a KRAB-associated transcriptional corepressor, TRIM28 (also known as KAP1), has been reported to regulate TP53 via protein-protein interaction with MDM2 [2]. More recently, our group showed that the proapoptotic function of TP53 is enhanced by the overexpression of ETV6/TEL, an ETS family transcriptional repressor involved in various forms of leukemia [3]. Furthermore, we have used mass spectrometry analysis in an independent search for molecules that interact with ETV6 and have reported TRIM28 to be a possible candidate [4]. Abnormalities in TP53 regulatory molecules have been implicated in the pathogenesis of hematopoietic malignancies. Thus, in the present study we searched for structural alterations in or aberrant expression of MDM2, TRIM28, and ETV6 that might influence the tumor suppressor activity of TP53 and lead to the generation of MDS.

We collected bone marrow cells according to protocols approved by the institutional review board at Dokkyo Medical University after obtaining written informed consent from the patients. RNA was extracted from cells and analyzed in our laboratory in the same institution. We conducted reverse transcriptase–polymerase chain reaction (RT-PCR) direct sequence analysis with primers that cover most of the regions encoding TP53, MDM2, TRIM28, and ETV6 proteins. The results are summarized in Table 1. In the 40 patient samples examined (refractory anemia [RA], 13 patients; RA with ringed sideroblasts, 1 patient; RA with excess of blasts (RAEB), 18 patients; RAEB in transformation [RAEB-t], 6 patients; chronic myelomonocytic leukemia, 2 patients), we identified 4 mutations in *TP53* messenger RNA (mRNA) that cause amino acid substitutions (Gly154Asp, Ile162Ser, Arg280Gly, and Tyr236Cys). The first 3 mutations have been reported in the literature, and the fourth mutation, Tyr236Cys, was discovered in our analysis. All 4 amino acid changes were located within the DNA-binding region of the TP53 protein. The frequency of mutations found in our analysis (4/40) is consistent with the frequencies described in earlier reports [5] and with updated statistics in a TP53 database (82/646; http://www.iarc.fr/p53/). Besides these 4 mutations, we have identified 2 missense single nucleotide polymorphisms

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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′

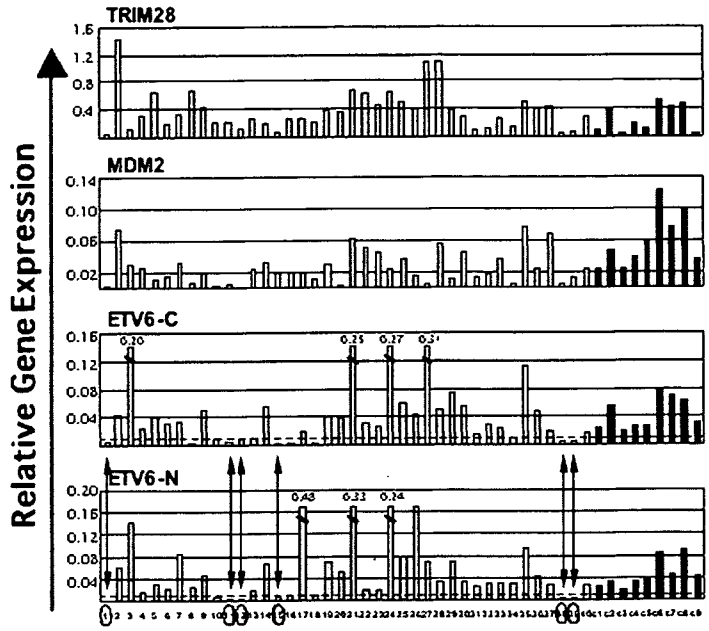

Figure 1. Relative expression of MDM2, TRIM28, and ETV6 messenger RNA. RNA was extracted from the bone marrow cells of 40 myelodysplastic syndrome patients (gray bars) and 9 healthy individuals (black bars). The difference in cycle threshold (CT) values between the gene of interest (CT_{gene}) and the 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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References

1. Fenaux P. Chromosome and molecular abnormalities in myelodysplastic syndromes. *Int J Hematol.* 2001;73:429-437.
2. Wang C, Ivanov A, Chen L, et al. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. *EMBO J.* 2005;24:3279-3290.
3. Yamagata T, Maki K, Waga K, Mitani K. TEL/ETV6 induces apoptosis in 32D cells through p53-dependent pathways. *Biochem Biophys Res Commun.* 2006;347:517-526.
4. Nakamura Y, Yamagata T, Maki K, Sasaki K, Kitabayashi I, Mitani K. TEL/ETV6 binds to corepressor KAP1 via the HLH domain. *Int J Hematol.* 2006;84:377-380.
5. Mitani K, Hangaishi A, Imamura N, et al. No concomitant occurrence of the N-ras and p53 gene mutations in myelodysplastic syndromes. *Leukemia.* 1997;11:863-865.
6. Rompaey LV, Potter M, Adams C, Grosveld G. Tel induces a G1 arrest and suppresses Ras-induced transformation. *Oncogene.* 2000;19:5244-5250.
7. Montpetit A, Boily G, Sinnett D. A detailed transcriptional map of the chromosome 12p12 tumour suppressor locus. *Eur J Hum Genet.* 2002;10:62-71.
8. Patel N, Goff LK, Clark T, et al. Expression profile of wild-type ETV6 in childhood acute leukaemia. *Br J Haematol.* 2003;122:94-98.
9. Cave H, Cacheux V, Raynaud S, et al. ETV6 is the target of chromosome 12p deletions in t(12;21) childhood acute lymphocytic leukemia. *Leukemia.* 1997;11:1459-1464.
10. Sasaki K, Nakamura Y, Maki K, et al. Functional analysis of a dominant-negative ΔETS TEL/ETV6 isoform. *Biochem Biophys Res Commun.* 2004;317:1128-1137.
11. Barjesteh van Waalwijk van Doorn-Khosrovani S, Spensberger D, de Knegt Y, Tang M, Lowenberg B, Delwel R. Somatic heterozygous mutations in ETV6 (TEL) and frequent absence of ETV6 protein in acute myeloid leukemia. *Oncogene.* 2005;24:4129-4137.

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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