厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

プロテオミクスを用いた骨髄異形成症候群関連蛋白の同定とその解析

分担研究者　泉二　登志子　東京女子医科大学血液内科　　教授

研究要旨
骨髄異形成症候群(myelodysplastic syndromes :MDS)由来CD34陽性細胞に存在するリン酸化蛋白を、プロテオミクスの手法を用いて同定した。MDSから白血病に移行した患者および正常のCD34陽性細胞由来蛋白を用いて二次元蛋白電気泳動を行い、リン酸化蛋白を特異的に認識する蛍光試薬で染色し、検出されたスポットについて質量分析を行った。その結果、MDS由来CD34陽性白血病細胞において異常発現しているリン酸化蛋白が複数同定された。これらのリン酸化蛋白は本症の病因、病態に深く関わっていると考えられ、現在解析を進めている。

A．研究目的

細胞内で多種多様な機能を担っている蛋白は、その蛋白をコードする遺伝子情報に基づく翻訳後にさまざまな修飾、たとえばリン酸化や糖鎖の付加を受けることにより、その機能を発現している。そして、さまざまな疾患の病因、病態には細胞内蛋白の翻訳後修飾の異常が深く関与していることが知られている。とくにキナーゼおよびホスファターゼによる蛋白のリン酸化、脱リン酸化は、その蛋白の機能発現や細胞内局在などを制御しており、それらに異常が起こると細胞機能やシグナル伝達に異常を来し、腫瘍の発生につながると考えられている。
本研究はプロテオミクス解析の手法を用いて骨髄異形成症候群（myelodysplastic syndrome:MDS)の幹細胞/前駆細胞レベルの細胞（CD34陽性細胞）に存在するリン酸化蛋白を同定、解析し、MDSの病因・病態を明らかにすること目的とした。

B．研究方法

MDSから白血病に移行した（MDS overt leukemia：MDS/ OL）患者の末梢血あるいは骨髄液から比重遠心法により単核球を分離し、イムノビーズ法を用いてCD34陽性細胞を純化した。その細胞から蛋白を抽出した後，二次元蛋白電気泳動を行った。泳動後、リン酸化蛋白を検出するために Pro-Q® Diamond Phosphoprotein Gel Stain (Invitrogen)および SYPRO® Ruby Protein Gel Stain (Invitrogen)を行った。Pro-Q® Diamond染色で検出されたスポットをゲル内消化後、MALDI-TOF(autoflex II TOF/TOF:BRUKER DALTONICS) を用いて質量分析を行い、当該蛋白を同定した。また、正常CD 34陽性細胞について同様な解析を行い、MDS患者由来CD34陽性細胞の解析データと比較検討した。
(倫理面への配慮)
患者に対して本研究について説明した後，文書にて同意を得た上で検体の提供を受けた。

C．研究結果

MDS/ OL患者のCD34陽性細胞由来蛋白を二次元電気泳動したゲルに対して Pro-Q® Diamond染色を行ったところ、多数のスポットが認められた（図1）。その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、細胞骨格、酵素、遺伝子発現制御、細胞周期、プロテアソームに関連する様々な蛋白が同定された。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した結果、MDS/OL のCD34陽性細胞において発現が増減している蛋白が認められた。それらの中で、正常CD34陽性細胞と比較し、とくに発現が増加していたものは tropomyosin 3 isoform 2、splicing factor,arginine/ serine-rich 2であり、発現が減少していたものは cofilin 1、heterogenous nuclear ribonucleoproteins c1/c2 variant であった。

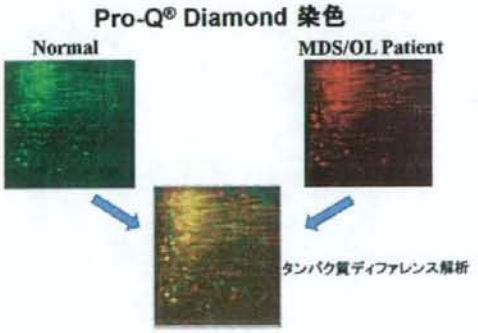


図1　MDS/OL患者および正常CD34陽性細胞に存在するリン酸化蛋白（二次元電気泳動）の解析

表 1　MDS/OL 患者 CD34 陽性細胞において同定されたリン酸化蛋白

Spot	蛋白の名称
	<細胞骨格関連>
1	Tropomyosin 3　isoform 2
2	F-actin capping protein alpha-1 subunit
3	Cofilin 1
4	Rho GDP dissociation inhibitor alpha
5	Rho GDP dissociation inhibitor beta
	<酵素>
6	Protein phosphatase 1, regulatory subunit 7
7	Chain A,crystal structure of a recombinant glutathione transferase
8	Peroxiredoxin 2 isoform a
9	TALDO1　protein
10	Enolase 1
	<遺伝子発現制御>
11	Calreticulin precursor
12	Chain A, crystal structure of Pp32
13	N-ethylmaleimide-sensitive factor attachment protein, alpha
14	Splicing factor, arginine/serine-rich 1
15	Heterogenous nuclear ribonucleoprotein K
16	Heterogenous nuclear ribonucleoproteins c1/c2 variant
17	Heterogenous nuclear ribonucleoprotein C(c1/c2),isoform CRA c
18	Nuclear autoantigenic sperm protein isoform 3
19	Eukaryotic translation elongation factor 1 beta 2
20	Eukaryotic translation initiation factor 4A isoform 1
21	Splicing factor, arginine/serine-rich 2
22	Chain A, crystal structure of the human Co-Chaperone p23
	<細胞周期関連>
23	14-3-3 protein epsilon
	<プロテアソーム>
24	Proteasome alpha 3subunit isoform 2
25	Prosome beta-subunit, HSBpros26

D．考察

本研究の結果、CD34 陽性細胞由来蛋白を二次元電気泳動後、ゲルをリン酸化蛋白特異的に認識する蛍光色素で染色し、蛍光発色したスポットを質量分析器で解析することにより、CD34 陽性細胞に存在するリン酸化蛋白を網羅的に同定することが可能であることが明らかになった。この方法により、MDS 由来 CD34 陽性白血病細胞において異常発現しているリン酸化蛋白が複数同定された。これらのリン酸化蛋白の中には本症の病因、病態に深く関与する蛋白が存在している可能性が考えられる。今後、これらのリン酸化蛋白について、多数の症例における発現を検討するとともに、その遺伝子解析および機能解析を行う必要がある。

E．結論

MDS 由来 CD34 陽性白血病細胞より抽出した蛋白のプロテオーム解析の結果、本症特異的に発現異常を有する数種のリン酸化蛋白が同定された。

F．健康危険情報

なし

G．研究発表

学会発表

プロテオミクスを用いた MDS 由来白血病細胞におけるリン酸化蛋白の同定　臨床血液 49:944,2008

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

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

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

分担研究者　小川　誠司　東京大学医学部附属病院がんゲノミクスプロジェクト　特任准教授

研究要旨

高密度 SNP アレイ(Affymetrix 社)を用いた 171 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、従来の古典的な染色体分析による解析結果との比較検討を行った。ゲノム異常の検出感度は、染色体分析のみで検出された 3 例の相互転座を除き、どの染色体の異常についても、SNP アレイによる検出力が染色体分析の検出力より優れていた。とくに、染色体分析で正常核型と判定された 74 例のうち 43 例で、SNP アレイ解析により異常が検出された。また、SNP アレイによる解析では、染色体分析では原理的に検出不可能な片親性二倍体(aUPD)が全症例の 30%に検出された。MDS の予後が染色体異常と強く相関していることを考え合わせると、今後 SNP アレイで検出されるゲノムの異常に基づいた新たな予後予測の指標の導入が MDS の治療成績の向上に寄与する可能性が示唆された。

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、MDS の原因分子に直接作用する分子標的薬剤の開発が望まれるとともに、決定打となる有効な薬剤のない現状においては、そのヘテロな病態を客観的にとらえてより適切な治療を選択する指標を構築する上で有用な診断技術の構築が望まれる。このような指標としては、従来 IPSS 分類が汎用されてきた。同分類は、血球減少の程度と、骨髄ないし末梢血の芽球の割合、および染色体異常を主体として、年齢を加味した分類であるが、一方、我々は昨年度までの分担研究において、高密度 SNP アレイを用いたアレル不均衡の異常が MDS ゲノムの異常を高い精度で解析できることを示した。そこで、本年度については、従来の染色体解析法と SNP アレイ解析の性能を比較検討することにより、アレイ解析によるゲノム解析の予後分類への応用の可能性について検討を行った。

B．研究方法

解析に用いた SNP アレイデータは、昨年度までに行った 171 例の MDS におけるゲノム解析で得られたものである。具体的には、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 アレイによる解析をしたのち、我々が独自に開発したゲノムコピー数解析ツール CNAG/AsCNAR を用いてアレル特異的なゲノムコピー数を算出し、アレル不均衡の網羅的な解析を行った。これら 171 例の各症例について、上記の SNP アレイによる解析結果と、従来の核型分析により同定された染色体・ゲノムの異常の比較検討を行った。

(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。

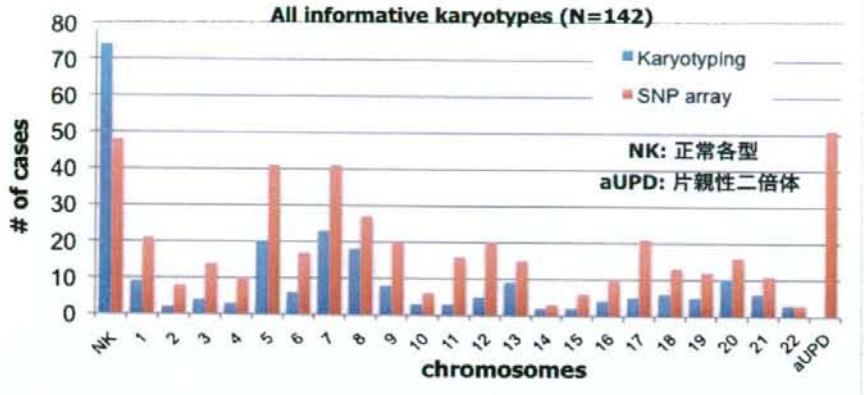
C．研究結果

SNP アレイと従来の染色体分析によるゲノム解析結果の比較を図 1 に示した。染色体分析で 43.3%を占める正常核型(異常なし)と判定される症例は、SNP アレイでは 28.1%と明らかに少なく、染色体分析で正常核型と判定された 75 例のうち 43 例で SNP アレイ解析による異常が同定された。また各染色体別にみた異常の検出率は、SNP アレイによる解析が平均で 2.59 倍となっており、またすべての染色体で SNP アレイの検出率が従来の核型分析を上回っていた。もっとも顕著な相違は aUPD の検出で、従来の各型分析では原理的に同定不可能な異常であるが、SNP アレイによる解析では、aUPD が 30%の症例で検出された。一方、SNP アレイでは原理的に同定不能の相互転座型の異常は、染色体分析によって 1.8%の症例に認められたのみであった。

D．考察

MDS は、臨床病理学的に極めて heterogeneity の大きな疾患群である。治療法選択の観点からは、現在、血球減少と芽球の割合および染色体の異常を加味した IPSS 分類が広く日常診療で用いられているところであるが、今回の解析結果からは、従来予後不良とされる 7 番染色体の異常等を含めて、染色体分析においては検出されないゲノムの異常が多数存在することが明らかとなった。このことは、染色体分析が有糸分裂の頻度に依存していること、微細な異常は検出不可能なこと、また aUPD については原理的に検出不可能なことなどによると思われるが、SNP アレイと染色体分析で、検出感度が大きく異なることは、SNP アレイ解析によって検出されるゲノム異常を考慮することにより、より精度の高い予後予測分類が構築できる可能性が示唆された。

図1. SNPアレイおよび従来の各型分析によるゲノム異常の検出率の比較



E．結論

MDS におけるゲノム異常の検出に関しては、従来の染色体分析による解析と比較して、SNP アレイによる解析が検出感度、検出する異常の質の点で優れていた。今後より詳細なゲノム解析結果に基づく予後分類システムの構築が望まれる。

F．健康危険情報

なし

G．研究発表

論文発表

1. Akagi T, Ogawa S, Dugas M, Kawamata N, Yamamoto G, Nannya Y, Sanada M, Miller CW, Yung A, Schnittger S, Haferlach T, Haferlach C, Koeffler HP, Frequent genomic abnormalities in acute myeloid leukemia/ myelodysplastic syndrome with normal karyotype. Haematologica 94: 213-223, 2009

2. Kawamata N, Ogawa S, Yamamoto G, Lehmann S, Levine RL, Pikman Y, Nannya Y, Sanada M, Miller CW, Gilliland DG, Koeffler HP. Genetic profiling of myeloproliferative disorders by single-nucleotide polymorphism oligonucleotide microarray. Exp Hematol, 36: 1471-1479,2008

3. Akagi T, Ogawa S, Dugas M, Kawamata N, Yamamoto G, Nannya Y, Sanada M, Miller CW, Yung A, Schnittger S, Haferlach T, Haferlach C, Koeffler HP. Hidden abnormalities and novel classification of t(15;17) APL based on genomic alterations. Blood 113: 1741-1748,2009

学会発表

1. Sanada M, Lee Yung S, Suzuki T,et al. Genome-wide molecular allelokaryo- typing using SNP array disclosed association between a uniparental disomy and homozygous mutations in myelodysplastic syndromes. Annual meeting of European Hematology Association, 2008

2. Sanada M, Yung SL, Suzuki T, et al. Genome-Wide Analysis of MDS/MPD Disclosed Frequent Homozygous C-Cbl mutations Tightly Associated with 11q-UPD. 50th Annual Meeting of American Society of Hematology, 2008

3. Thoennissen NH, Kawamata N, Lasho TL, et al. Genomic Changes Associated with Leukemic Transformation of Myeloproliferative Disorders. 50th Annual Meeting of American Society of Hematology, 2008

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
Maki K, Yamagata T, Mitani K.	Role of the RUNX1-EVI1 fusion gene in leukemogenesis.	Cancer Sci	99	1878- 1883	2008
Tasaka T, Tohyama K, Kishimoto M, Ohyashiki K, Mitani K, Hotta T, Kanamaru A, Okamoto S, Karasawa M, Kimura A, Tomonaga M, Uchiyama T, Ozawa K.	Myelodysplastic syndrome with chromosome 5 abnormalities: a nationwide survey in Japan.	Leukemia	22	1874-1881	2008
Eguchi-Ishimae M, Eguchi M, Maki K, Porcher C, Shimizu R, Yamamoto M, Mitani K.	Leukemia-related trans- cription factor TEL/ETV6 expands erythroid precursors and stimulates hemoglobin synthesis.	Cancer Sci		(in press)	
Kawahara M, Hori T, Chonabayashi K, Oka T, Sudol M, Uchiyama T.	Kpm/Lats2 in liked to chemosensitivity of leukemic cells through the stabilization of p73.	Blood	112	3856-3866	2008
Yamashita K, Miyoshi T, Arai T, Endo N, Itoh H, Makino K, Mizugishi K, Uchiyama T, Sasada M.	Ozone production by amino acids contributes to killing of bacteria.	Proc Natl Acad Sci USA	105	16912-16917	2008
Ishikawa Y, Xu J, Sakashita G, Urano T, Suzuki T, Tomita A, Kiyoi H,Nakamura S, Naoe T.	Abnormal cytoplasmic dyslocalisation and/or reduction of nucleophosmin protein level rarely occurs in myelodysplastic syndromes.	Leuk Lymphoma	49	2359-2364	2008
Hayakawa F, Abe A, Kitabayashi I, Pandolfi PP, Naoe T.	Acetylation of PML is involved in histone deacetylase inhibitor-mediated apoptosis.	J Biol Chem	283	24420-24425	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
Ohyashiki JH, Ohyashiki K, et al.	The oral iron chelator deferasirox represses signaling through the mTOR in myeloid leukemia cells by enhancing expression of REDD1.	Cancer Sci		(in press)	
Park J, Ohyashiki K, Akata S, Takara K, Uno R, Kakizaki D, Miyazawa K, Kimura Y, Tokuuye K.	Evaluation of cardiac iron over- load in transfusion-dependent adult marrow failure patients by magnetic resonance imaging.	Leuk Res		(in press)	

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
Miyazawa K, Ohyashiki K, Urabe A, Hata T, Nakao S, Ozawa K, Ishikawa T, Kato J, Tatsumi Y, Mori H, Kondo M, Taniguchi J, Tanii H, Rojkjaer L, Omine M.	A safety, pharmacokinetic and pharmacodynamic investigation of deferasirox (Exjade, ICL670) in patients with transfusion-dependent anemias and iron-overload: a Phase I study in Japan.	Int J Hematol	4	73-81	2008
Yokoyama T, Miyazawa K, Naito M, Toyotake J, Tauchi T, Itoh M, Yuo A, Hayashi Y, Georgescu MM, Kondo Y, Kondo S, Ohyashiki K.	Vitamin K2 induces autophagy and apoptosis simultaneously in leukemia cells	Autophagy	88	629-640	2008
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	13	199-208	2008
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	111	4297-4301	2008
Yoshinaga K, Mori N, Wang YH, Tomita K, Shiseki M, Motoji T.	JAK2 V617F mutation is rare in idiopathic erythrocytosis: a difference from polycythemia vera.	Int J Hematol	88	82-87	2008
Mori N, Yoshinaga K, Tada M, Wang YH, Shiseki M, Motoji T.	Infrequent V617F mutation of the JAK2 gene in myeloid leukemia and its absence in lymphoid malignancies in Japan.	Genet Mol Biol	31	427-430	2008
Yamada O, Kawauchi K, Akiyama M, Ozaki K, Motoji T, Adachi T, Aikawa E.	Leukemic cells with increased telomerase activity exhibit resistance to imatinib.	Leuk Lymphoma	49	1168-1177	2008
Kawamata N, Ogawa S, Yamamoto G, Lehmann S, Levine RL, Pikman Y, Nannya Y, Sanada M, Miller CW, Gilliland DG, Koeffler HP.	Genetic profiling of myeloproliferative disorders by single-nucleotide polymorphism oligonucleotide microarray.	Exp Hematol	36	1471-1479	2008
Akagi T, Ogawa S, Dugas M, Kawamata N, Yamamoto G, Nannya Y, Sanada M, Miller CW, Yung A, Schnittger S, Haferlach T, Haferlach C, Koeffler HP.	Hidden abnormalities and novel classification of t(15;17) APL based on genomic alterations.	Blood	113	1741-1748	2009
Akagi T, Ogawa S, Dugas M, Kawamata N, Yamamoto G, Nannya Y, Sanada M, Miller CW, Yung A, Schnittger S, Haferlach T, Haferlach C, Koeffler HP.	Frequent genomic abnormalities in acute myeloid leukemia/myelodysplastic syndrome with normal karyotype.	Haematologica	94	213-223	2009

Ⅳ．　研究成果の刊行物・別刷

Review Article

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

Kazuhiro Maki, Tetsuya Yamagata and Kinuko Mitani[1]

Department of Hematology, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu-machi, Shimotsuga-gun, Tochigi 321-0293, Japan

(Received June 2, 2008/Revised June 19, 2008 /Accepted June 24, 2008/Online publication October 3, 2008)

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

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

Molecular and biological function of wild-type RUNX1

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

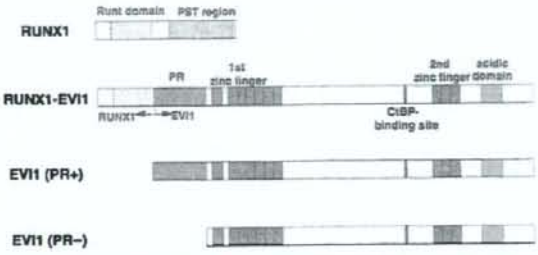


Fig. 1. Schematic structure of wild-type RUNX1, EVI1 and RUNX1-EVI1 molecules. Wild-type RUNX1 has Runt homology domain at the N-terminus and proline-, serine- and threonine-rich (PST) region at the C-terminus. In RUNX1-EVI1, RUNX1 protein is truncated at the end of the Runt homology domain and followed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

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

Molecular and biological function of wild-type EVI1

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

[1]To whom correspondence should be addressed. E-mail: kinukom-tky@umin.ac.jp

doi: 10.1111/j.1349-7006.2008.00956.x

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

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

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

Molecular structure and function of RUNX1-EVI1

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

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

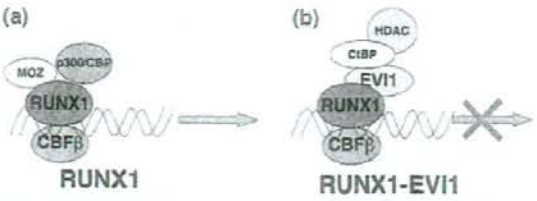


Fig. 2. (a) RUNX1-EVI1 exerts dominant-negative effects over wild-type RUNX1. RUNX1 forms an active transcription factor by heterodimerizing with CBFβ through Runt homology domain. CBFβ increases the DNA-binding ability of RUNX1 and protects RUNX1 from degradation. (b) Because RUNX1-EVI1 binds CBFβ more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

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

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

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

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

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

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

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

Biological function of RUNX1-EVI1

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

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

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

doi: 10.1111/j.1349-7006.2008.00956.x

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

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

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

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

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

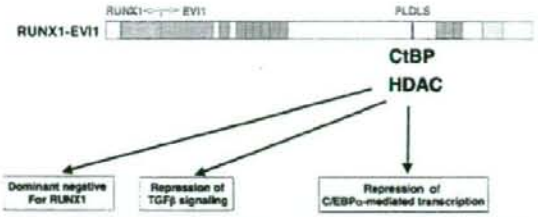


Fig. 3. RUNX1-EVI1 is a multifunctional oncoprotein. RUNX1-EVI1 exerts oncogenic function through three pathways: dominant-negative effect over RUNX1, repression of TGFβ signaling and inhibition of C/EBPα activity.

Clinical aspects

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

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

Summary

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

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

References

1 Thompson PW, Whittaker JA. Translocation 3;21 in Philadelphia chromosome positive chronic myeloid leukemia at diagnosis. *Cancer Genet Cytogenet* 1989; **39**: 143–6.
2 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.
3 Chen Z, Morgan R, Baer MR, Ligorsky R, Sandberg AA. Translocation (3;21) characterizes crises in myeloid stem cell disorders. *Cancer Genet Cytogenet* 1991; **57**: 153–9.
4 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.
5 Ito Y. Oncogenic potential of the RUNX gene family: 'overview'. *Oncogene* 2004; **23**: 4198–208.
6 van Wijnen AJ, Stein GS, Gergen JP *et al.* Nomenclature for Runt-Related (RUNX) Proteins. *Oncogene* 2004; **23**: 4209–10.
7 Levanon D, Groner Y. Structure and regulated expression of mammalian RUNX genes. *Oncogene* 2004; **23**: 4211–19.
8 Durst KL, Hiebert SW. Role of RUNX family members in transcriptional repression and gene silencing. *Oncogene* 2004; **23**: 4220–4.
9 Cameron ER, Neil JC. The Runx genes: lineage-specific oncogenes and tumor suppressors. *Oncogene* 2004; **23**: 4308–14.
10 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.
11 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.
12 Wang Q, Stacy T, Miller JD *et al.* The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo. *Cell* 1996; **87**: 697–708.
13 Sasaki K, Yagi H, Bronson RT *et al.* Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta. *Proc Natl Acad Sci USA* 1996; **93**: 12359–63.
14 Niki M, Okada H, Takano H *et al.* Hematopoiesis in the fetal liver is impaired by targeted mutagenesis of a gene encoding a non-DNA binding subunit of the transcription factor, polyomavirus enhancer binding protein 2/core binding factor. *Proc Natl Acad Sci USA* 1997; **94**: 5697–702.
15 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.
16 Ichikawa M, Goyama S, Asai T *et al.* AML1/Runx1 negatively regulates quiescent hematopoietic stem cells in adult hematopoiesis. *J Immunol* 2008; **180**: 4402–8.
17 Harada H, Harada Y, Niimi H, Kyo T, Kimura A, Inaba T. High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. *Blood* 2004; **103**: 2316–24.
18 Harada H, Harada Y, Tanaka H, Kimura A, Inaba T. Implications of somatic mutations in the AML1 gene in radiation-associated and therapy-related myelodysplastic syndrome/acute myeloid leukemia. *Blood* 2003; **101**: 673–80.
19 Morishita K, Parker DS, Mucenski ML, Jenkins NA, Copeland NG, Ihle JN. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. *Cell* 1988; **54**: 831–40.
20 Morishita K, Parganas E, William CL *et al.* Activation of EVI1 gene expression in human acute myelogenous leukemias by translocations spanning 300–400 kilobases on chromosome band 3q26. *Proc Natl Acad Sci USA* 1992; **89**: 3937–41.
21 Ogawa S, Kurokawa M, Tanaka T *et al.* Increased Evi-1 expression is frequently observed in blastic crisis of chronic myelocytic leukemia. *Leukemia* 1996; **10**: 788–94.
22 Kurokawa M, Mitani K, Irie K *et al.* The oncoprotein Evi-1 represses TGF-beta signalling by inhibiting Smad3. *Nature* 1998; **394**: 92–6.
23 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**: 24020–6.
24 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.
25 Yuasa H, Oike Y, Iwama A *et al.* Oncogenic transcription factor Evi1 regulates hematopoietic stem cell proliferation through GATA-2 expression. *Embo J* 2005; **24**: 1976–87.
26 Sato T, Goyama S, Nitta E *et al.* Evi-1 promotes para-aortic splanchnopleural hematopoiesis through up-regulation of GATA-2 and repression of TGF-b signaling. *Cancer Sci* 2008; **99**: 1407–13.
27 Fears S, Mathieu C, Zeleznik-Le N, Huang S, Rowley JD, Nucifora G. Intergenic splicing of MDS1 and EVI1 occurs in normal tissues as well as in myeloid leukemia and produces a new member of the PR domain family. *Proc Natl Acad Sci USA* 1996; **93**: 1642–7.
28 Morishita K. Leukemogenesis of the EVI1/MEL1 gene family. *Int J Hematol* 2007; **85**: 279–86.
29 Buyse IM, Shao G, Huang S. The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A protein. *Proc Natl Acad Sci USA* 1995; **92**: 4467–71.
30 Ren B, Chee KJ, Kim TH, Maniatis T. PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes Dev* 1999; **13**: 125–37.
31 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.
32 Kurokawa M, Ogawa S, Tanaka T *et al.* The AML1/Evi-1 fusion Protein t(3;21) translocation exhibits transforming activity on Rat1 fibroblasts with dependence on the Evi-1 sequence. *Oncogene* 1995; **11**: 833–40.
33 Tanaka T, Mitani K, Kurokawa M *et al.* Dual functions AML1/Evi-1 chimeric protein in the mechanism of leukemogenesis in t(3;21) leukemias. *Mol Cell Biol* 1995; **15**: 2383–92.
34 Derijard B, Hibi M, Wu IH *et al.* JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* 1994; **76**: 1025–37.
35 Kingsley DM. The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994; **8**: 133–46.
36 Kreider BL, Orkin SH, Ihle JN. Loss of erythropoietin responsiveness in erythroid progenitors due to expression of the Evi-1 myeloid-transforming gene. *Proc Natl Acad Sci USA* 1993; **90**: 6454–8.
37 Nakao A, Imamura T, Souchelnytskyi S *et al.* TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. *Embo J* 1997; **16**: 5353–62.
38 Nakao A, Roijer E, Imamura T *et al.* Identification of Smad2, a human Mad-related protein in the transforming growth factor beta signaling pathway. *J Biol Chem* 1997; **272**: 2896–900.
39 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-beta-mediated growth inhibition of myeloid cells. *Blood* 1998; **92**: 4003–12.
40 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.
41 Lenny N, Meyers S, Hiebert SW. Functional domains of the t(8;21) fusion protein, AML-1/ETO. *Oncogene* 1995; **11**: 1761–9.
42 Lutterbach B, Sun D, Schuetz J, Hiebert SW. The MYND motif is required for repression of basal transcription from the multidrug resistance 1 promoter by the t(8;21) fusion protein. *Mol Cell Biol* 1998; **18**: 3604–11.
43 Perez A, Kastner P, Sethi S, Lutz Y, Reibel C, Chambon P. PMLRAR homodimers: distinct DNA binding properties and heteromeric interactions with RXR. *Embo J* 1993; **12**: 3171–82.
44 McWhirter JR, Galasso DL, Wang JY. A coiled-coil oligomerization domain of Bcr is essential for the transforming function of Bcr-Abl oncoproteins. *Mol Cell Biol* 1993; **13**: 7587–95.
45 Senyuk V, Li D, Zakharov A, Mikhail FM, Nucifora G. The distal zinc finger domain of AML1/MDS1/EVI1 is an oligomerization domain involved in induction of hematopoietic differentiation defects in primary cells in vitro. *Cancer Res* 2005; **65**: 7603–11.
46 Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem J* 2002; **365**: 561–75.
47 Schuster MB, Porse BT. C/EBPalpha: a tumour suppressor in multiple tissues? *Biochim Biophys Acta* 2006; **1766**: 88–103.
48 Pabst T, Mueller BU, Harakawa N *et al.* Aml1-ETO downregulates the granulocytic differentiation factor C/Ebpalpha t(8;21) myeloid leukemia. *Nat Med* 2001; **7**: 444–51.
49 Pabst T, Mueller BU, Zhang P *et al.* Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet* 2001; **27**: 263–70.

doi: 10.1111/j.1349-7006.2008.00956.x

50 Smith LT, Hohaus S, Gonzalez DA, Dziennis SE, Tenen DG. PU.1 (Spi-1) and C/EBP alpha regulate the granulocyte colony-stimulating factor receptor promoter in myeloid cells. *Blood* 1996; **88**: 1234–47.
51 Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* 1998; **18**: 4301–14.
52 Wang X, Scott E, Sawyers CL, Friedman AD. C/EBPalpha 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.
53 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.
54 Tavor S, Park DJ, Gery S, Vuong PT, Gombart AF, Koeffler HP. Restoration of C/EBPalpha expression in a BCR-ABL+ cell line induces terminal granulocytic differentiation. *J Biol Chem* 2003; **278**: 52651–9.
55 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 alpha-deficient mice. *Proc Natl Acad Sci USA* 1997; **94**: 569–74.
56 Gombart AF, Hofmann WK, Kawano S *et al.* Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. *Blood* 2002; **99**: 1332–40.
57 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.
58 Mueller BU, Pabst T. C/EBPalpha and the pathophysiology of acute myeloid leukemia. *Curr Opin Hematol* 2006; **13**: 7–14.
59 Tokita K, Maki K, Mitani K. RUNX1/EVI1, which blocks myeloid differentiation, inhibits CCAAT-enhancer binding protein alpha function. *Cancer Sci* 2007; **98**: 1752–7.
60 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**: 13312–7.
61 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.
62 Senyuk V, Chakraborty S, Mikhail FM, Zhao R, Chi Y, Nucifora G. The leukemia-associated transcription repressor AML1/MDS1/EVI1 requires CtBP to induce abnormal growth and differentiation of murine hematopoietic cells. *Oncogene* 2002; **21**: 3232–40.
63 Takeshita M, Ichikawa M, Nitta E *et al.* AML1-Evi-1 specifically transforms hematopoietic stem cells through fusion of the entire Evi-1 sequence to AML1. *Leukemia* 2008; **22**: 1241–9.
64 Cuenco GM, Nucifora G, Ren R. Human AML1/MDS1/EVI1 fusion protein induces an acute myelogenous leukemia (AML) in mice: a model for human AML. *Proc Natl Acad Sci USA* 2000; **97**: 1760–5.
65 Cuenco GM, Ren R. Cooperation of BCR-ABL and AML1/MDS1/EVI1 in blocking myeloid differentiation and rapid induction of an acute myelogenous leukemia. *Oncogene* 2001; **20**: 8236–48.
66 Cuenco GM, Ren R. Both AML1 and EVI1 oncogenic components are required for the cooperation of AML1/MDS1/EVI1 with BCR/ABL in the induction of acute myelogenous leukemia in mice. *Oncogene* 2004; **23**: 569–79.
67 Watanabe-Okochi N, Kitaura J, Ono R *et al.* AML1 mutations induced MDS and MDS/AML in a mouse BMT model. *Blood* 2008; **111**: 4297–308.
68 Maki K, Yamagata T, Asai T *et al.* Dysplastic definitive hematopoiesis in AML1/EVI1 knock-in embryos. *Blood* 2005; **106**: 2147–55.
69 Okada H, Watanabe T, Niki M *et al.* AML1(-/-) embryos do not express certain hematopoiesis-related gene transcripts including those of the PU.1 gene. *Oncogene* 1998; **17**: 2287–93.
70 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.
71 Rhoades KL, Hetherington CJ, Harakawa N *et al.* Analysis of the role of AML1-ETO in leukemogenesis, using an inducible transgenic mouse model. *Blood* 2000; **96**: 2108–15.
72 Higuchi M, O'Brien D, Kumaravelu P, Lenny N, Yeoh EJ, Downing JR. Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. *Cancer Cell* 2002; **1**: 63–74.
73 Duvic M, Talpur R, Ni X *et al.* Phase 2 trial of oral vorinostat (suberoylanilide hydroxamic acid, SAHA) for refractory cutaneous T-cell lymphoma (CTCL). *Blood* 2007; **109**: 31–9.
74 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* 2008; **99**: 414–22.
75 Shackelford D, Kenific C, Blusztajn A, Waxman S, Ren R. Targeted degradation of the AML1/MDS1/EVI1 oncoprotein by arsenic trioxide. *Cancer Res* 2006; **66**: 11360–9.

Leukemia (2008) 22, 1874–1881

www.nature.com/leu

ORIGINAL ARTICLE

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

T Tasaka[1], K Tohyama[1], M Kishimoto[2], K Ohyashiki[3], K Mitani[4], T Hotta[5], A Kanamaru[6], S Okamoto[7], M Karasawa[8], A Kimura[9], M Tomonaga[10], T Uchiyama[11] and K Ozawa[12] on behalf of the Japanese Cooperative Study Group for Intractable Bone Marrow Diseases

[1]Department of Laboratory Medicine, Kawasaki Medical School, Okayama, Japan; [2]Department of Medical Technology, Kawasaki College of Allied Health Professions, Okayama, Japan; [3]First Department of Internal Medicine, Tokyo Medical University, Tokyo, Japan; [4]Department of Hematology, Dokkyo Medical University School of Medicine, Tochigi, Japan; [5]Department of Hematology and Oncology, Tokai University School of Medicine, Kanagawa, Japan; [6]Department of Internal Medicine, Division of Hematology, Kinki University School of Medicine, Osaka, Japan; [7]Department of Medicine, Keio University, Tokyo, Japan; [8]Blood Transfusion Service, University Hospital, Gunma University, Gunma, Japan; [9]Department of Hematology and Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan; [10]Department of Hematology, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan; [11]Department of Hematology and Oncology, Kyoto University, Kyoto, Japan and [12]Department of Medicine, Division of Hematology, Jichi Medical University, Tochigi, Japan

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

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

Together with additional cytogenetic data, WPSS might be useful for clinical decision making in MDS patients with del(5q).
Leukemia (2008) **22,** 1874–1881; doi:10.1038/leu.2008.199; published online 31 July 2008
Keywords: myelodysplastic syndrome; chromosome 5 abnormality; deletion of 5q; IPSS; WPSS

Introduction

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

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

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

Materials and methods

Patient data

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

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

Correspondence: Professor K Tohyama, Department of Laboratory Medicine, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192, Japan.
E-mail: ktohyama@med.kawasaki-m.ac.jp
Received 29 March 2008; revised 16 June 2008; accepted 27 June 2008; published online 31 July 2008

Table 1 Classification of MDS with chromosome 5 abnormalities

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

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

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

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

Statistical analysis

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

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

Results

Patient characteristics

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

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

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

Impact of chromosome 5 abnormalities on cytopenia

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

Impact of chromosome 5 abnormalities on survival

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

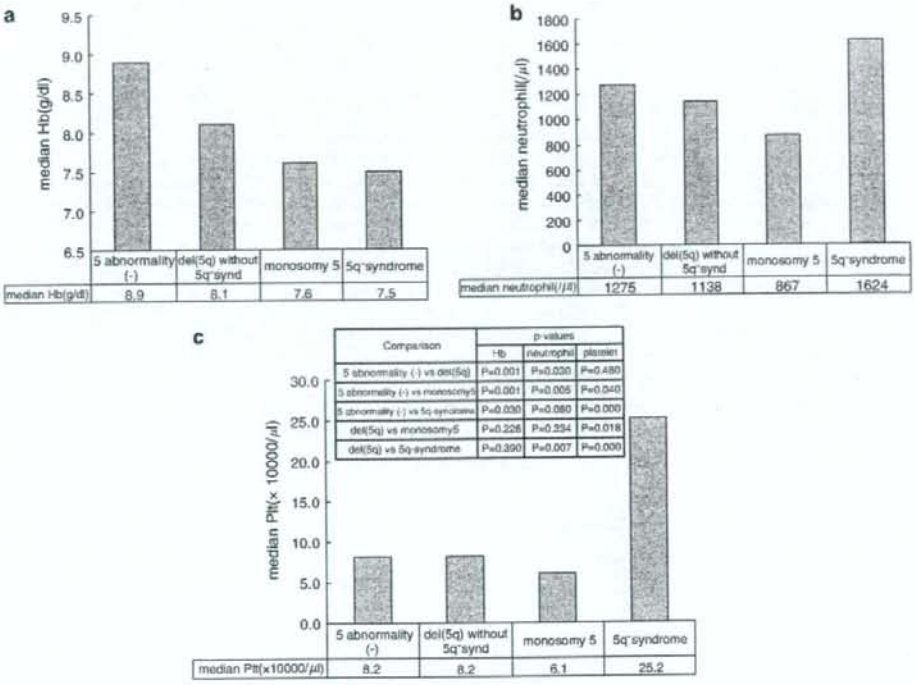


Figure 1 Cytopenias in four categories of myelodysplastic syndromes (MDS) with chromosome 5 abnormalities. (**a**) Hemoglobin concentration, (**b**) neutrophil count and (**c**) platelet count. Median values are indicated by a column and numerals. MDS patients were categorized into chromosome 5 abnormality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

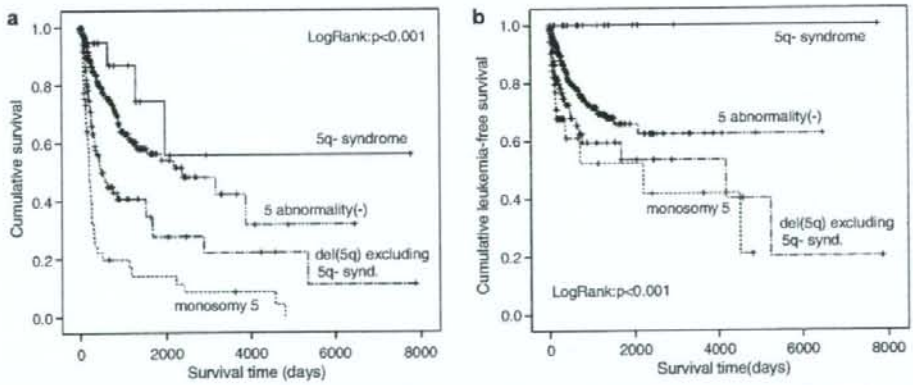


Figure 2 Impact of chromosome 5 abnormalities by three categories on the survival of myelodysplastic syndromes (MDS) patients. MDS patients were categorized into no chromosome 5 abnormality (375 cases), del(5q) (110 cases), monosomy 5 (52 cases) and 5q- syndrome (21 cases). (**a**) Overall survival curves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

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

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

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

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

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

Survival analysis according to IPSS

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

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

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

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

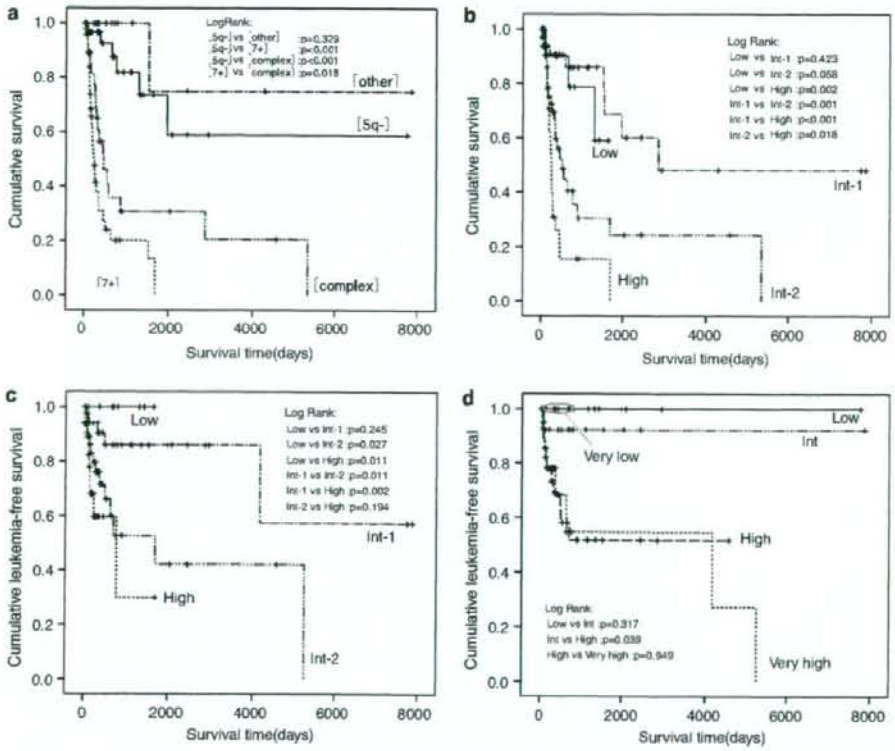


Figure 3 Impact of several factors on the survival of myelodysplastic syndromes (MDS) patients with del(5q). (**a**) Overall survival of MDS patients with del(5q) categorized by additional chromosomal abnormalities. A total of 131 MDS patients with del(5q) were categorized into 5q- (35 cases), 7+ (47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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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