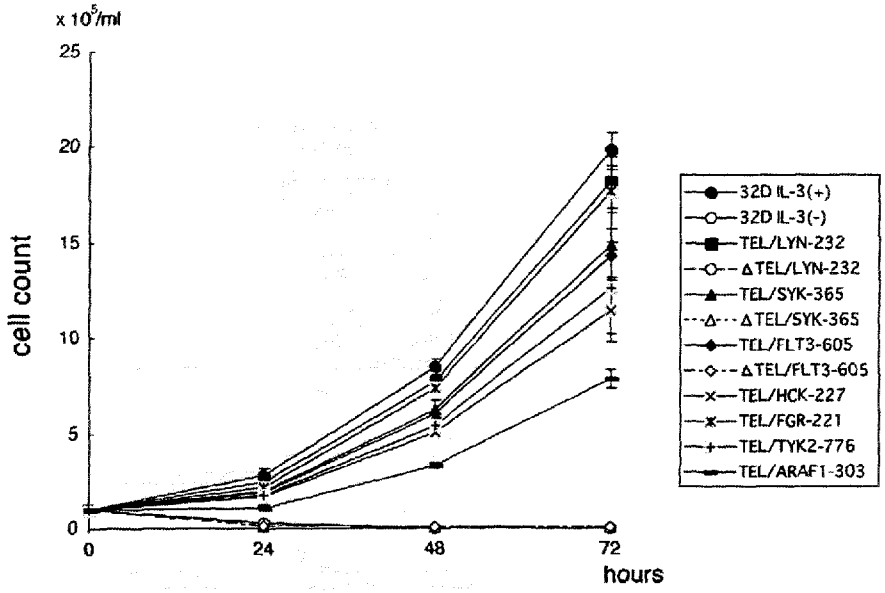

Fig. 3. Proliferation properties of 32D cells expressing fusion genes. 32D cells were infected with pHNGAP cloned with the indicated fusion cDNA. The fusion gene was introduced into 32D cells, which were then selected in the presence of G418, and cultured in IMDM with 10% FCS without IL-3. Viable cells were counted on each day for 3 days.

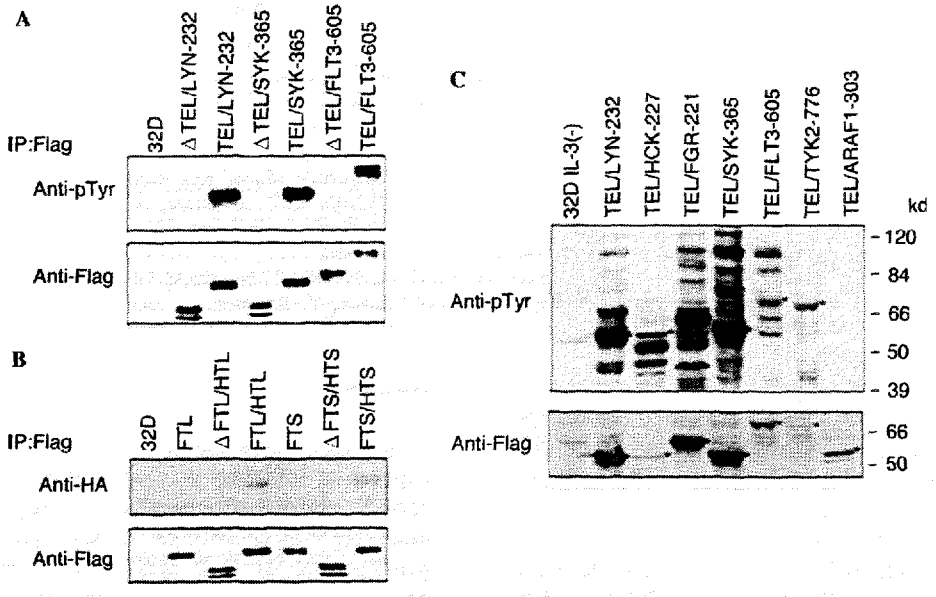

Fig. 4. Characterization of fusion genes. (A) TEL PNT-dependent phosphorylation of TEL/LYN-232, TEL/SYK-365, and TEL/FLT3-605. 32D cells transformed with each fusion gene were lysed and immunoprecipitated with an anti-Flag monoclonal antibody. Immunoprecipitated samples were detected using an HRP-conjugated anti-phosphotyrosine antibody (PY20). Stripping and reprobing were performed using an anti-Flag antibody (M2). (B) TEL PNT-dependent oligomerization of TEL/LYN-232 and TEL/SYK-365. 32D cells expressing Flag-tagged TEL/LYN-232 (FTL) or PNT-deleted TEL/LYN-232 (ΔFTL) were transformed with HA epitope-tagged TEL/LYN-232 (HTL) and selected in the presence of blasticidin resulting in ΔFTL/HTL and FTL/HTL. 32D cells with ΔFTS/HTS and FTS/HTS were similarly established. Samples immunoprecipitated with the anti-Flag antibody were detected with an HRP-conjugated anti-HA monoclonal antibody. Same samples were also detected with the anti-Flag antibody. (C) Immunodetection of whole cell lysates with anti-phosphotyrosine and anti-Flag antibodies. Whole cell lysates were separated by SDS–PAGE, transferred to PVDF membrane, and detected with the indicated antibodies. *Indicates fusion proteins.

conditions were required. First, the reading frames of the cDNA and TEL PNT have to be matched to produce a fusion protein. Second, the cDNA must be long enough to be functionally active and stimulate the cell proliferation. As the provability of positive fusion genes in the cDNA library is relatively low and cDNAs have a

polyadenylation signal which reduces the retrovirus titer [35], we infected the 32D cells with a MOI of about 5–10. Although false positive clones resulting from ectopic oncogene expression around the LTR of retrovirus integration sites or PCR amplification of false genes resulting from multiple infections may have been recovered, the proper cDNAs with the ability to transform 32D were confirmed by transduction of the recovered cDNA to 32D with a pHNGAP/FPNT vector.

We obtained 6 kinds of tyrosine kinase and 1 serine/threonine kinase fusion genes which transform the hematopoietic cell line 32D into growth factor-independent cells. In tyrosine kinase fusions, constitutively tyrosine kinase phosphorylations were observed and oligomerizations were confirmed in some of the fusion genes. Fusion gene activation was dependent on the oligomerization domain of TEL. From these results, OLAG-REX screening is applicable for cloning constitutively active protein kinase genes that stimulate cell growth by protein oligomerization. These active kinases may be useful for analyzing the downstream signalling pathway and the screening of kinase-specific inhibitors.

Exploring the unknown abnormalities of oncogenes in human cancer is an attractive subject. FLT3 is known to have an internal tandem duplication of the juxtamembrane domain in 20% of AML [36] and activating mutation in the activation loop in human hematologic malignancies [37]. The TEL/SYK fusion gene has reportedly been found in CMMoL-like MDS [26]. Since both FLT3 and SYK were shown to be cloned by OLAG-REX as fusion partners of TEL and to transform the hematopoietic cell line, we can expect that fusion genes identified by OLAG-REX are potential oncogenes and fusion partner genes are candidates with which to look for structural abnormalities in human hematological malignancy.

Acknowledgments

We thank Satoshi Suzuki and Chika Wakamatsu for excellent technical assistance. We also thank Dr. Yoshinori Yamashita for helpful discussions.

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Leukemia (2004) 18, 548–555

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TEL-Syk fusion constitutively activates PI3-K/Akt, MAPK and JAK2-independent STAT5 signal pathways

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We previously reported the fusion of the *TEL* gene to the *Syk* gene in myelodysplastic syndrome with t(9;12)(q22;p12). TEL-Syk fusion transformed interleukin-3 (IL-3)-dependent murine hematopoietic cell line BaF3 to growth factor independence. Here, we investigate the intracellular signal transduction of the stable transfectants. TEL-Syk fusion protein was associated with the p85 subunit of phosphatidyl inositol 3 kinase (PI3-K) followed by the activation of Akt in the absence of IL-3. Vav, phospholipase C-γ2 and mitogen-activated protein kinase (MAPK) were also constitutively activated. TEL-Syk also activated the signal transducer and activator of transcription 5 (STAT5) in the absence of Janus kinase 2 activation. None of these kinases were phosphorylated in the BaF3 cells transfected with TELΔPNT-Syk in which the oligomerization domain of TEL was deleted. Inhibitor analysis showed that the MAPK pathway was important in TEL-Syk-mediated cell proliferation. The immunofluorescence technique revealed that the TEL-Syk fusion protein was located in the cytoplasm. These data suggest that TEL-Syk fusion protein in the cytoplasm leads to the constitutive activation of PI3-K/Akt, MAPK and STAT5 signal pathways, which are closely involved in IL-3-independent cell proliferation of BaF3 cells.
Leukemia (2004) **18**, 548–555. doi:10.1038/sj.leu.2403266
Published online 29 January 2004
Keywords: TEL-Syk; signal transduction; PI3-K; Akt; MAPK; STAT5

Introduction

Protein tyrosine kinase plays an important role in cell growth and oncogenesis. Receptor tyrosine kinases and tyrosine kinase-associated receptors are activated by ligand binding with their extracellular domain and transduce an extracellular signal to the inside. Some tyrosine kinase-associated receptors have no inherent kinase activity but can regulate the hematopoietic system by activating nonreceptor protein tyrosine kinases. The deregulation of their signaling plays a significant role in the malignant transformation of hematological disorders.[1,2]

Syk is a nonreceptor tyrosine kinase that is widely expressed in a variety of hematopoietic cells. In the B-lineage lymphoid cell, Syk is a key regulator of signal transduction and differentiation. It has two amino-terminal Src-homology 2 domains and a carboxy-terminal kinase domain, binds to the immunoreceptor tyrosine-based activation motif (ITAM) and initiates signaling through the Ras, phosphatidyl inositol 3 kinase (PI3-K) and phospholipase C-γ (PLC-γ) signal pathways.[3–6] Syk-deficient mice show a maturational block at the pro-B to pre-B cell transition.[7,8] Aberrant splicing and protein deficiency of Syk was found in childhood pro-B cell acute lymphoblastic leukemia (ALL).[9] The TEL-Syk fusion observed in myeloid cells resulted in myeloid dysplasia and transformation in the presented case.[10] Syk is also reported to be an important suppressor of breast cancer cell growth and metastasis.[11]

Chromosomal translocation is one of the mechanisms that leads to the generation of activated tyrosine kinase fusions. A significant number of chromosomal rearrangements fuse the ETS transcriptional repressor TEL with receptor or nonreceptor tyrosine kinase in many hematological malignancies.[12,13] The TEL pointed self-association motif (PNT)-mediated oligomerization leads to the constitutive activation of tyrosine kinase fusion that is essential for cell transformation. The reported fusion genes of *TEL* with tyrosine kinases in hematological disorders are *TEL-PDGFβR* in chronic myelomonocytic leukemia,[14] *TEL-ABL* in ALL,[15] *TEL*-Janus kinase 2 (*JAK2*) in ALL and chronic myeloid leukemia (CML),[16,17] *TEL-TRKC* in acute myeloid leukemia (AML),[18] *TEL-ARG* in AML[19] and *TEL-Syk* in myelodysplastic syndrome (MDS).[20]

The JAK-STAT pathway is widely used by members of the cytokine receptor superfamily.[21,22] Dysregulation of the JAK-STAT pathway has been observed in a large number of hematopoietic malignancies.[23] TEL-JAK2 fusions in lymphoid and myeloid leukemia patients led to the constitutive activation of the tyrosine kinase and conferred cytokine-independent proliferation to the interleukin-3 (IL-3)-dependent BaF3 hematopoietic cell line.[16,17] The activation of STAT5 plays a critical role in myelo- and lymphoproliferative TEL-JAK2 diseases;[24] however, serine/threonine phosphorylation is also important in intracellular signal transduction.[25] PI3-K/Akt signal pathway is also constitutively activated in the BaF3 cell transformed by TEL-JAK2.[26] Mitogen-activated protein kinase (MAPK) is constitutively activated in a majority of acute leukemia.[27] Aberrant expression of Raf oncoproteins can abrogate cytokine dependency of the hematopoietic cell lines TF-1 and FDC-P1.[28,29]

We previously reported the fusion of the *TEL* gene to *Syk* gene in a patient with MDS with t(9;12)(q22;p12). *TEL-Syk* fusion transformed IL-3-dependent murine hematopoietic cell line BaF3 to growth factor independence. TEL-Syk was constitutively tyrosine autophosphorylated.[10,20] We here show intracellular signal components specifically activated in the *TEL-Syk*-transformed BaF3 cell.

Materials and methods

DNA constructs and expression plasmids

Full-length TEL-Syk and TELΔPNT-Syk (deletion of nt 222–461 in the *TEL* gene) cDNA were constructed by PCR as described

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Received 29 July 2003; accepted 1 December 2003; Published online 29 January 2004

previously.[20] PCR products were confirmed by sequencing to be devoid of mutations. We used retroviral expression vector pBabeNeo and Flag epitope-tagged expression vector pFlag (Invitrogen, San Diego, CA, USA). Retroviral expression vector pBabeNeo and pFlag have been described, respectively.[30,31] We cloned the TEL-Syk and TELΔPNT-Syk constructs into pBabeNeo (pB-TS and pB-TΔPS) and cloned the TEL-Syk into pFlag (pF-TS) (Figure 1).

Cell transfection assays

The pCMV-G-containing vesicular stomatitis virus envelope glycoprotein was cotransfected with pB-TS and pB-TΔPS or vector alone as a control into 293GP cells, a retrovirus packaging cell line expressing MMLV *gag* and *pol* genes.[32,33] The IL-3-dependent murine leukemia BaF3 cells were infected with these supernatants in the presence of 5 μg/ml polybrene. G418-resistant BaF3 expressing the indicated proteins and control cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 5 ng/ml recombinant murine IL-3.

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS. pF-TS was transfected into subconfluent COS-7 cells by Lipofectamin (Invitrogen).

Antibody

The antibodies used are as follows: anti-Syk 4D–10 monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-PI3-K p85 antisera (Upstate Biotechnology, Lake Placid, NY, USA), rabbit anti-phospho-Akt antisera (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-Akt antisera (Cell Signaling Technology), rabbit anti-Vav antisera (Santa Cruz Biotechnology), rabbit anti-PLC-γ1 antisera (Santa Cruz Biotechnology), rabbit anti-PLC-γ2 antisera (Santa Cruz Biotechnology), rabbit anti-phospho-p44/42 MAPK antisera (Cell Signaling Technology), rabbit anti-p44/42 MAPK antisera (Cell Signaling Technology), rabbit anti-phospho-MEK 1/2 antisera (Cell Signaling Technology), rabbit anti-MEK 1/2 antisera (Cell Signaling Technology), rabbit anti-STAT1 antisera (Santa Cruz Biotechnology), rabbit anti-STAT3 antisera (Santa Cruz Biotechnology), rabbit anti-STAT5 antisera (kind gift from Dr JN Ihle, St Jude Children's Research Hospital, Memphis, TN, USA), rabbit anti-phospho-STAT5 antisera (Cell Signaling Technology), rabbit anti-STAT5A antisera (R&D Systems, Minneapolis, MN, USA), rabbit anti-phospho-JAK2 antisera

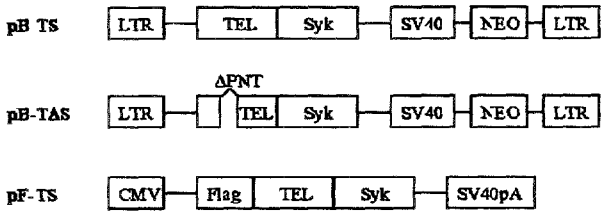

Figure 1 Schematic representation of constructed TEL-Syk and deletion mutant TELΔPNT-Syk (deletion of nt 222–461). For high expression in BaF3 cells, TEL-Syk and TELΔPNT-Syk were cloned into the pBabeNeo construct, a retroviral vector based on an MMLV-expressing neomycin-resistant gene. For immunofluorescence microscopy, TEL-Syk cDNA cloned into pFlag (the Flag epitope tagged) was used. LTR, long-terminal repeat; SV40, SV40 promoter; SV40pA, SV40 polyadenylation signal; CMV, CMV promoter.

(Upstate Biotechnology), rabbit anti-JAK2 antisera (Upstate Biotechnology), anti-phosphotyrosine mAb PY-20 (BD Biosciences, San Jose, CA, USA), HRP-linked whole anti-mouse IgG antibody (Amersham Pharmacia Biotech, Tokyo, Japan), HRP-linked whole anti-rabbit IgG antibody (Amersham Pharmacia), anti-Flag mAb (Eastman-Kodak Company, New Haven, CT, USA) and goat anti-mouse IgG (FITC) (Santa Cruz Biotechnology).

Immunoprecipitation

The BaF3 cells were grown to a density of approximately 1×10^6/ml. The cells were washed in phosphate-buffered saline (PBS) at 4°C and lysed in a lysis buffer: 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 0.5% NP-40, 1 mM Na_3VO_4, 50 mM NaF and protease inhibitor cocktail (Roche, Mannheim, Germany). The cell lysates were centrifuged and the supernatants were incubated with the indicated antibodies for 1 h followed by protein G-agarose (Invitrogen) for 2 h at 4°C. The immunoprecipitates were washed four times in the lysis buffer.

Immunoblotting

Whole-cell lysates or immunoprecipitates were boiled with the electrophoresis SDS sample buffer for 3 min. The samples were separated by SDS-PAGE (polyacrylamide gel electrophoresis) and transferred onto polyvinylidine difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane blots were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T), or 1% blocking reagent (pH 7.5) (Roche) for 1 h at 37°C followed by incubation with primary antibodies in TBS-T for 2 h at room temperature or overnight at 4°C. After being washed, the membranes were incubated with HRP-linked whole anti-mouse or anti-rabbit IgG antibody in TBS-T for 2 h at room temperature. After washing, an enhanced chemiluminescence assay was performed and positive bands were identified on the X-ray films.

Cell proliferation assay

Tetracolor One cell proliferation assay (Seikagaku Co., Tokyo, Japan) was performed as described.[34] The cells (1×10^4/well) were added to 96-well plates in 100 μl RPMI containing 10% FCS in the presence or absence of IL-3 with varying concentrations of LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benopyran-4-one] (Promega, Madison, WI, USA), the inhibitor of PI3-K or MEK inhibitor U0126 (Promega). The plates were incubated at 37°C for 72 h before the addition of a mixture of tetrazolium [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] and electron carrier (1-methoxy-5-methylphenazium methylsulfate) (final volume 110 μl/well). The cells were incubated for an additional hour at 37°C, and the absorption of 450 nm was measured using an ELISA plate reader.

Immunofluorescence microscopy

COS-7 cells were transfected with pF-TS using Lipofectamin (Invitrogen). The cells were grown on chamber slides. The transfected COS-7 cells were subjected to indirect immunofluorescence antibody staining 48 h after transfection. The cells were fixed with ice-cold methanol:acetone (1:1) and blocked in

1% BSA in PBS, and then anti-Flag mAb was added for 45 min. After washing the cells, goat anti-mouse IgG (FITC) was added for 30 min. The cells were then washed and incubated with 0.5 μg/ml propidium iodide (Sigma Aldrich, St Louis, MO, USA), which was used for fluorescent staining of the nucleic acid, for 5 min, washed again and coverslips were then mounted on chamber slides. The cells were visualized with a fluorescence microscope and photographed.

Results

TEL-Syk fusion protein activated the PI3-K/Akt signal pathway

We used the stable transfectants to unveil the intracellular signal transduction of TEL-Syk. Akt is a protein serine/threonine kinase and the major effector of the PI3-K pathway, which is activated in downstream signaling of Syk.[6,35,36] First, BaF3 cell lysates were immunoblotted with phospho-Akt antibody to assess the activation of the PI-3K/Akt signal pathway. Only in the TEL-Syk-transformed BaF3 cell was Akt activated in the absence of IL-3 (Figure 2a). Next, the BaF3 cell lysates were immunoprecipitated with antibody against p85 PI3-K and immunoblotted with the human-specific anti-Syk mAb to assess the interaction between TEL-Syk and PI3-K. Interestingly, TEL-Syk fusion products were co-immunoprecipitated with the p85 subunit of PI3-K and this association was not observed in the BaF3 cell with TELΔPNT-Syk in which the oligomerization domain was deleted (Figure 2b).

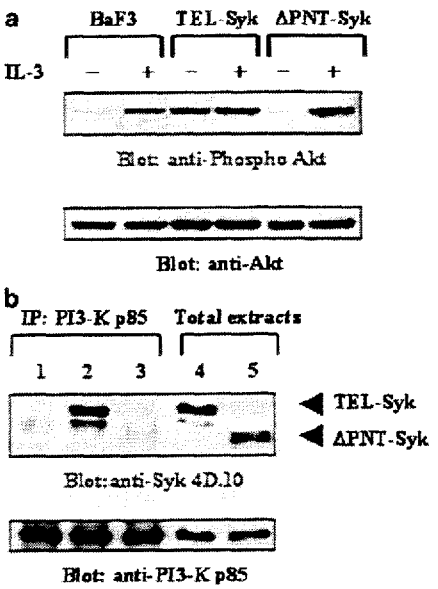

Figure 2 TEL-Syk fusion proteins were associated with PI3-K and constitutively activated Akt. (a) BaF3 cells were deprived of IL-3 for 12 h and then restimulated with 5 ng/ml of IL-3 for 30 min. Cell lysates were immunoblotted with antibody against phospho-Akt and reprobed with antibody against Akt. Akt was activated in TEL-Syk-transformed BaF3 cells in the absence of IL-3. (b) Cell lysates were immunoprecipitated with antibody against the 85K subunit of PI3-K and immunoblotted with human-specific Syk mAb. The same membrane was stripped and reprobed with antibody against the 85K subunit of PI3-K. TEL-Syk fusion products (lane 2) were co-precipitated with 85K subunit of PI3-K, and BaF3 cell lysates (lane 1) and TELΔPNT-Syk lysates (lane 3) were not. Lane 4 consisted of whole-cell lysates of TEL-Syk and lane 5 consisted of TELΔPNT-Syk.

Vav, PLC-γ2 and MAPK signal pathways were constitutively activated in TEL-Syk-transformed BaF3 cells

Vav, the proto-oncogene product, is known to bind to nonreceptor protein tyrosine kinase. Nuclear phosphorylated Vav is associated to the p85 subunit of PI3-K and to PLC-γ1 in HL-60 cells differentiated by all-*trans* retinoic acid.[37] BaF3 cell lysates were immunoprecipitated with antibody against Vav, PLC- γ1 and PLC- γ2, and immunoblotted with the mAb against phosphotyrosine. Vav and PLC-γ2 were tyrosine phosphorylated in TEL-Syk-transformed BaF3 cells in the absence of IL-3; however, PLC-γ1 was not activated (Figure 3a).

MAPK, also known as extracellular signal-regulated kinase (ERK), is serine/threonine kinase that appears to be one of the key regulators of cell proliferation and differentiation.[25,38,39] BaF3 cell lysates were immunoblotted with the antibody against

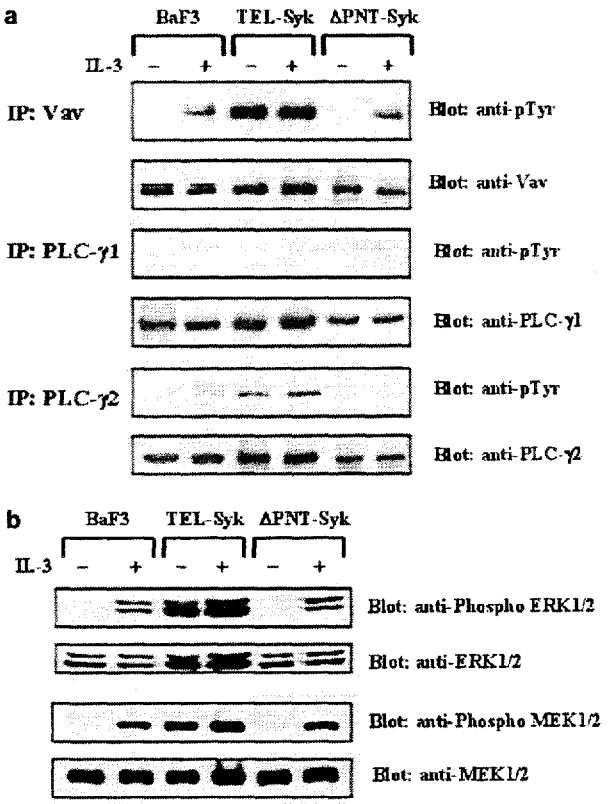

Figure 3 Constitutive kinase activation in TEL-Syk-transformed BaF3 cell. (a) BaF3 cell lysates were immunoprecipitated with antibody against Vav, PLC-γ1 and PLC-γ2, and immunoblotted with the mAb against phosphotyrosine. Vav and PLC-γ2 were tyrosine phosphorylated in TEL-Syk-transformed BaF3 cells in the absence of IL-3. These tyrosine kinase activations were not observed in the BaF3 cells with TELΔPNT-Syk. (b) BaF3 cells were deprived of IL-3 for 12 h and then restimulated with 5 ng/ml of IL-3 for 30 min. Cell lysates were immunoblotted with the antibody against phospho-ERK1/2 (p42/44 MAPK) and phospho-MEK1/2 (ERK kinases), and reprobed with antibody against ERK1/2, MEK1/2, respectively. ERK and MEK were activated in TEL-Syk-transformed BaF3 cells in the absence of IL-3.

phospho-ERK1/2 (p42/44 MAPK) and phospho-MEK1/2 (ERK kinase). Both ERK1/2 and MEK1/2 were constitutively activated in TEL-Syk-transformed BaF3 cells (Figure 3b).

Constitutive tyrosine phosphorylation of STAT5 in the absence of JAK2 activation

STAT plays an important role in growth factor-mediated intracellular signal transduction.[40,41] Dysregulation of the JAK-STAT pathway has been observed in many hematopoietic malignancies.[23] STATs were constitutively activated in some acute leukemia blasts.[42–45] To assess the potential role of STATs phosphorylation in signaling by the TEL-Syk, BaF3 cell lysates were immunoprecipitated with antibody against STAT1, STAT3 and STAT5, and immunoblotted with the mAb against phosphotyrosine. STAT5 was tyrosine phosphorylated in TEL-Syk-transformed BaF3 cells in the absence of IL-3. STAT1 and STAT3 activation were not detected because of the low expression in the BaF3 cells in the presence and absence of IL-3 (Figure 4a). JAK2 phosphorylation was not detected in the absence of IL-3 and was inhibited in the presence of IL-3 in TEL-Syk-transformed BaF3 cells (Figure 4b). The fact that the TEL oligomerization domain played a critical role in the activation of the kinases examined also strengthens our proposal that these signals are closely involved in tumor progression in TEL-Syk-induced leukemia.

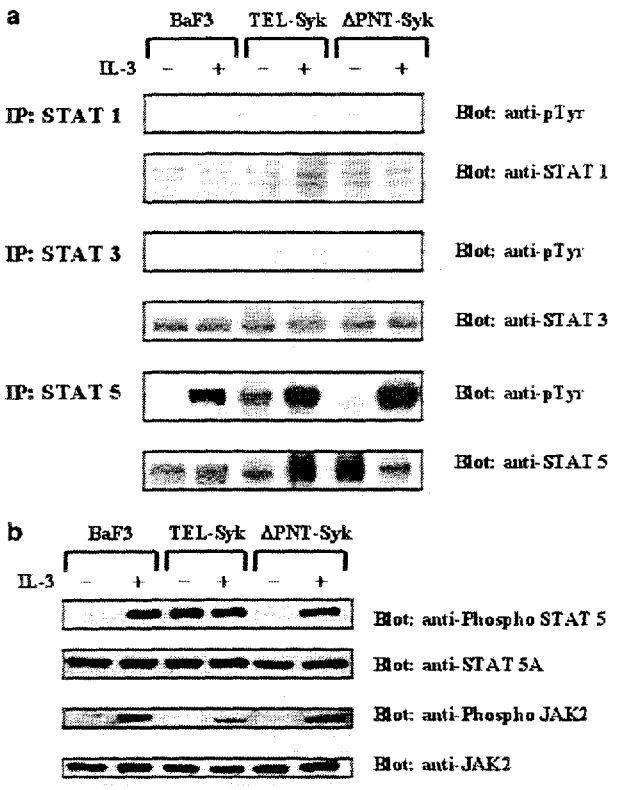

Figure 4 TEL-Syk fusion protein constitutively activates STAT5 in the absence of JAK2 activation. (a) BaF3 cell lysates were immunoprecipitated with antibody against STAT1, STAT3 and STAT5, and immunoblotted with the mAb against phosphotyrosine. STAT5 was tyrosine phosphorylated in TEL-Syk-transformed BaF3 cells in the absence of IL-3. (b) BaF3 cells were deprived of IL-3 for 12 h and then restimulated with 5 ng/ml of IL-3 for 30 min. The cell lysates were immunoblotted with the antibody against phospho-STAT5 and phospho-JAK2, and reprobed with antibody against STAT5A, JAK2, respectively. The BaF3 cell transformed by TEL-Syk was constitutively tyrosine phosphorylated of STAT5 in the absence of JAK2 activation.

MAPK signal pathway was required for TEL-Syk-mediated cell proliferation

To determine which signal pathway is important in factor-independent cell growth, we performed cell proliferation assays with several concentrations of the PI3-K inhibitor, LY294002 and MEK inhibitor U0126.

A reduction in the number of BaF3 cells and TEL-Syk-transformed BaF3 cells was observed with increasing LY294002 concentrations, even in the presence of IL-3. LY294002 suppressed both TEL-Syk-mediated and IL-3-induced BaF3 cell proliferation (Figure 5a). To determine whether the activation of Akt is dependent on PI3-K, BaF3 cells were pretreated with LY294002 and immunoblotted with the antibody against phospho-Akt. The activation of Akt by TEL-Syk and IL-3 was blocked by LY294002, although phosphorylation of ERK1/2 and STAT5 was not blocked by LY294002 (Figure 5b). These data suggest that PI3-K/Akt activation is important for both TEL-Syk-mediated and IL-3-induced cell proliferation.

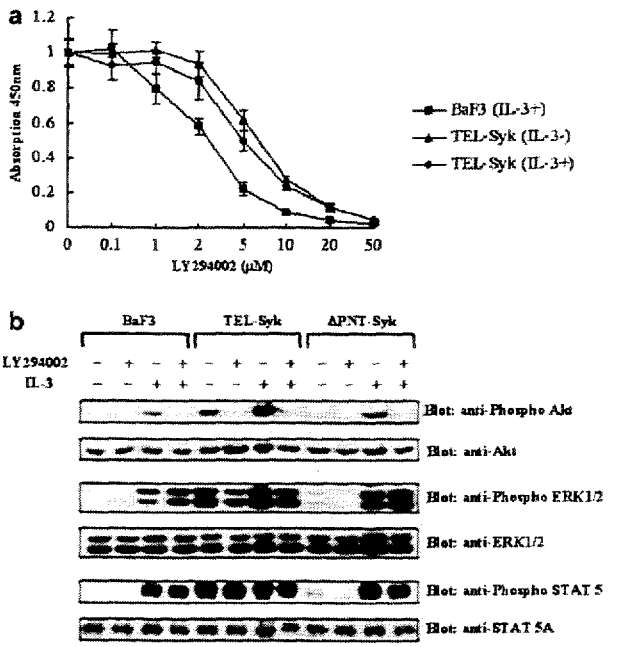

Figure 5 LY294002 inhibited the growth of TEL-Syk-transformed BaF3 cells and phosphorylation of Akt. (a) BaF3 cells and TEL-Syk-transformed BaF3 cells were added to 96-well plates with varying concentrations of LY294002 or carrier (DMSO) alone in the presence or absence of IL-3. The plates were incubated 37°C for 72 h before the addition of a mixture of tetrazolium and electron carrier. Cell proliferation assay was performed in triplicate, and mean absorption values with standard deviation were graphed. (b) BaF3 cells were deprived of IL-3 for 12 h, pretreated with 20 μM of LY294002 (+) or without LY294002 (−) (DMSO alone) for 30 min and then stimulated in the presence (+) or absence (−) of IL-3. The cell lysates were immunoblotted with the antibody against phospho-Akt, phospho-ERK1/2 and phospho-STAT5, and reprobed with antibody against Akt, ERK1/2, STAT5A, respectively. Phosphorylation of Akt by TEL-Syk and IL-3 are PI3-K dependent.

TEL-Syk-mediated BaF3 cell growth was selectively inhibited by MEK inhibitor U0126. TEL-Syk-mediated cell proliferation was suppressed more strongly than IL-3-mediated cell proliferation with 10–20 μM of U0126 (Figure 6a). To determine whether growth inhibition is dependent on the activation of MAPK, BaF3 cells were pretreated with U0126 and immunoblotted with the antibody against phospho-ERK1/2. The activation of ERK1/2 by TEL-Syk was inhibited by U0126, although phosphorylation of Akt and STAT5 were not inhibited by U0126 (Figure 6b). These data suggest that MAPK signal pathway is required for cytokine-independent cell proliferation.

TEL-Syk fusion protein was located in cytoplasm

TEL is a nuclear phosphoprotein that is widely expressed in normal tissues.[46,47] Syk is distributed in cytosol and initiates signal transduction cascades in lymphoid cells.[4,48] To determine the localization of TEL-Syk fusion protein, COS-7 cells transfected with pF-TS were stained with anti-Flag mAb and visualized with goat anti-mouse IgG (FITC). The immunofluorescence technique revealed the TEL-Syk fusion protein to be located in the cytoplasm (Figure 7a and b). TEL-Syk fusion products, not in the nucleus but in the cytoplasm, led to the constitutive activation of PI3-kinase, MAPK and STAT5 signal pathways, which are important in the IL-3-independent cell proliferation of BaF3 cells.

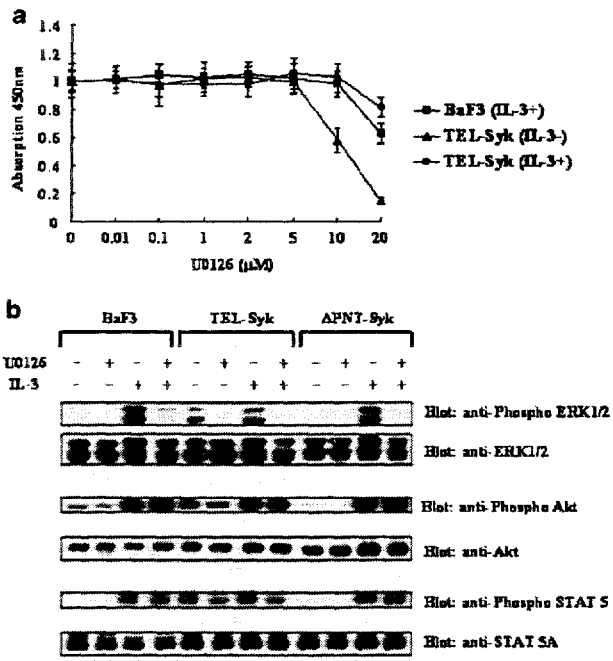

Figure 6 The MAPK signal pathway is required for TEL-Syk-mediated cell proliferation. (a) BaF3 cells and TEL-Syk-transformed BaF3 cells were added to 96-well plates with varying concentrations of U0126 or carrier (DMSO) alone in the presence or absence of IL-3. Cell proliferation assay was performed in triplicate, and mean absorption values with standard deviation were graphed. TEL-Syk-mediated cell proliferation was inhibited by U0126. (b) BaF3 cells were deprived of IL-3 for 12 h, pretreated with 20 μM of U0126 (+) or without U0126 (–) (DMSO alone) for 4 h and then stimulated in the presence (+) or absence (–) of IL-3. Cell lysates were immunoblotted with the antibody against phospho-ERK1/2, phospho-Akt and phospho-STAT5, and reprobed with antibody against ERK1/2, Akt, STAT5A, respectively. Phosphorylation of ERK1/2 by TEL-Syk was inhibited by U0126.

Discussion

We investigated the intracellular signal transduction in TEL-Syk-transformed BaF3 cells. Syk binds to ITAM and initiates signaling through the Ras, PI3-K and PLC-γ signal pathways.[5,6] We showed the association between the p85 subunit of PI3-K and TEL-Syk fusion protein followed by the constitutive activation of Akt. Akt is the serine/threonine kinase and an important component of cell survival signaling. Akt phosphorylates the BCL family member BAD and suppresses apoptosis.[49] Subsequently, apoptosis is inhibited by phosphorylation and inactivation of caspase-9.[50] TEL-Syk may play a critical role in antiapoptosis and cell survival of IL-3-depleted BaF3 through the suppression of the caspase pathway. Beitz *et al*[6] demonstrated that Syk plays a major role in linking the B-cell receptor to PI3-K activation; however, the Syk-dependent pathways for

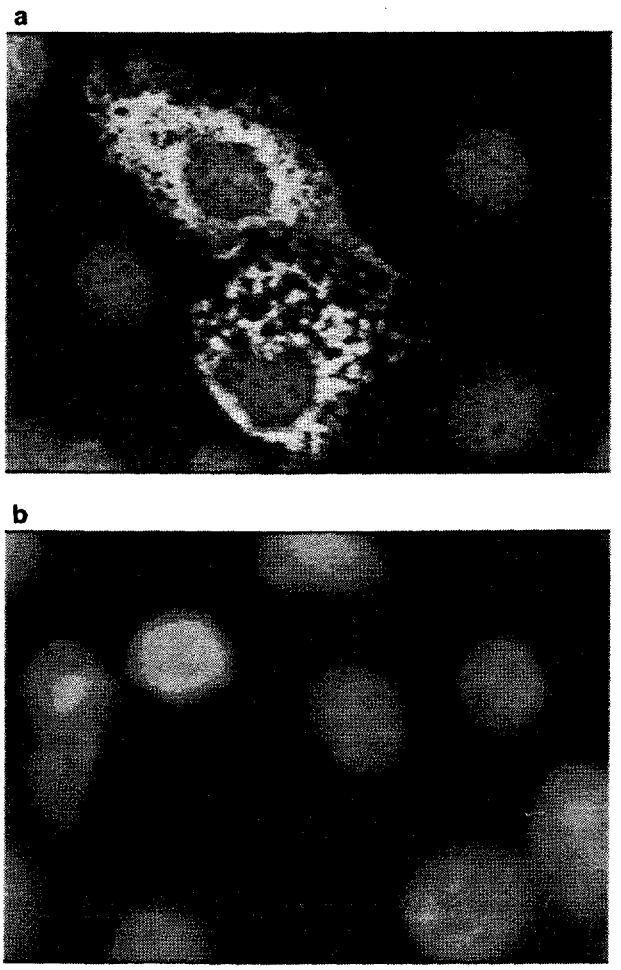

Figure 7 Immunofluorescence localization of TEL-Syk fusion protein. (a) COS-7 cells transfected with pF-TS was stained with anti-Flag mAb and visualized with goat anti-mouse IgG (FITC). (b) COS-7 cells transfected with pF-TS was stained with goat anti-mouse IgG (FITC) (secondary antibody alone). Propidium iodide (Sigma Aldrich) was used for the fluorescent staining of the nucleic acid.

PI3-K activation is not shown. Our data suggest that Syk and the p85 subunit of PI3-K may form a protein complex.

Deregulation of the JAK-STAT pathway is observed in many hematopoietic malignancies.[23] STAT5 is phosphorylated and activated in a variety of leukemia cells containing tyrosine kinase oncogenes, including BCR-ABL, TEL-JAK2, TEL-PDGFβR and FLT3 with internal tandem duplication.[24,51–53] However, the constitutive JAK activation is not necessarily required for transformation.[54] A constitutively active mutant of STAT5 is sufficient to induce factor independence of BaF3 cells.[55] We have previously reported the constitutive kinase activation of Syk and cytokine-independent proliferation in TEL-Syk-transformed BaF3 cells.[20] We here show the constitutive tyrosine phosphorylation of STAT5 in the absence of JAK2 activation and the suppression of IL-3-induced JAK2 phosphorylation in TEL-Syk-transformed BaF3 cells. The expression of JAB (also called SOCS-1) was unable to inhibit the growth of TEL-Syk-transformed BaF3 cells and tyrosine phosphorylation of STAT5 (data not shown), although JAB can inhibit JAK signaling.[56,57] The constitutive tyrosine kinase activation of TEL-Syk led to the activation of STAT5, not through the JAK-family members, while the exact transduction mechanism from TEL-Syk to STAT5 is not known. Klejman *et al*[58] recently reported hematopoietic cell kinase-coupled BCR-ABL to STAT5 activation. *In vitro* phosphorylation assay and *in vitro* interaction assay between STAT5 and TEL-Syk may reveal whether the interaction is direct. We do not know the reasons behind the downregulation of IL-3-induced JAK2 phosphorylation in TEL-Syk-transformed BaF3 cells, but hope to examine the functional activity of IL-3 receptor in TEL-Syk-transformed BaF3 cells in the future.

MAPK, also known as ERK, is serine/threonine kinase that appears to be one of a key regulator of cell proliferation and differentiation, and is constitutively activated in a majority of acute leukemia.[25,27,38] The upstream components of the MAPK pathway contain the Ras signaling cascade. ERK1/2 and MEK1/2, which were constitutively activated in the TEL-Syk-transformed BaF3 cells, might be activated through the Ras/Raf/MEK/ERK cascade downstream of TEL-Syk signaling. MEK inhibitor U0126 selectively inhibited the TEL-Syk-mediated BaF3 cell growth and activation of MAPK. Constitutively activated form of MEK1 can transform NIH 3T3 and Chinese hamster fibroblast,[59,60] and conditionally active form of MEK1 can also abrogate cytokine dependency of hematopoietic cell lines TF-1, FDC-P1 and FL5.12.[61,62] These data suggest that the MAPK signal pathway is important in TEL-Syk-mediated BaF3 cell proliferation and might be one of the targets for chemotherapy in TEL-Syk disease.

Tyrosine kinase inhibitors are being explored in leukemia therapy.[2] The BCR-ABL tyrosine kinase inhibitor STI571 (Imatinib mesylate) has significant antileukemic activity in patients with CML.[63] Piceatannol, which is known as a Syk-selective tyrosine kinase inhibitor,[64,65] suppressed the survival and proliferation of TEL-Syk-transformed BaF3 cells and inhibited the phosphorylation of STAT5, but piceatannol also suppressed IL-3-induced BaF3 cell growth (data not shown). Piceatannol not only inhibits protein tyrosine kinase Syk but also interferes with other kinases and induces apoptosis in leukemic lymphoblasts.[66,67] TEL-Syk fusion is a useful tool in the search for another Syk-specific inhibitor and for future investigation of Syk.

Recent reports suggest that Syk is a tumor suppressor. Goodman *et al*[9] reported that childhood pro-B-cell ALL is caused by Syk deficiency, and Coopman *et al*[11] showed that Syk suppresses breast cancer cell growth and metastasis. However, the *TEL-Syk* fusion gene was discovered in the presented case of myeloid dysplasia with unique clinical features, including skin involvement, dry cough and eosinophilia, and Syk was constitutively activated in hematopoietic cells.[10,20] The presented evidence reveals another function of Syk constitutively activated by oligomerization through TEL, that of a stimulator of tumor progression.

Acknowledgements

We thank Dr JN Ihle for providing anti-STAT5 antisera, Dr Kazuhito Yamamoto and Hitoshi Kiyoi for helpful discussions, and Satoshi Suzuki and Chika Wakamatsu for their excellent technical assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Osaka Foundation for Promotion of Clinical Immunology, the Akiyama Foundation, the Suhara Memorial Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research and the Uehara Memorial Foundation.

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High incidence of somatic mutations in the *AML1/RUNX1* gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia

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A high incidence of somatically acquired point mutations in the *AML1/RUNX1* gene has been reported in poorly differentiated acute myeloid leukemia (AML, M0) and in radiation-associated and therapy-related myelodysplastic syndrome (MDS) or AML. The vast majority of AML1 mutations identified in these diseases were localized in the amino (N)–terminal region, especially in the DNA-binding Runt homology domain. In this report, we show that AML1 point mutations were found in 26 (23.6%) of 110 patients with refractory anemia with excess blasts (RAEB), RAEB in transformation (RAEBt), and AML following MDS (defined these 3 disease categories as MDS/AML). Among them, 9 (8.2%) mutations occurred in the carboxy (C)–terminal region, which were exclusively found in MDS/AML and were strongly correlated with sporadic MDS/AML. All patients with MDS/AML with an AML1 mutation expressed wild-type AML1 protein and had a significantly worse prognosis than those without AML1 mutations. Most AML1 mutants lost *trans*-activation potential, regardless of their DNA binding potential. These data suggested that AML1 point mutation is one of the major driving forces of MDS/AML, and these mutations may represent a distinct clinicopathologic-genetic entity. (Blood. 2004;103: 2316-2324)

Introduction

Somatically acquired point mutations of critical genes have been demonstrated to contribute to the development of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). Genes encoding key regulatory factors for cell division, differentiation, or cell survival of hematopoietic progenitors, such as Ras, receptors for stem cell factor (c-Kit) and Flt-3 ligand, and transcription factors are frequent mutation targets. However, there is no strong correlation between point mutations of these genes and morphology, drug sensitivity, or prognosis. This is in sharp contrast with certain chimeric oncoproteins that result from recurrent chromosomal translocations and that form distinct clinicopathologic-genetic entities.[1] For instance, a majority of leukemias associated with AML1-ETO (MTG8) chimera are de novo AML with maturation, and it is relatively easy to obtain complete remission in these cases by standard chemotherapy, and these patients have favorable prognoses.[2] Thus, the AML1-ETO chimera is considered to be the decision factor of the biologic features of leukemia, whereas the point mutations mentioned earlier might merely play a complementary role that contributes to the progression of disease.

The *AML1* gene was also found to be altered by point mutations in AML and MDS. Beginning with the first report by Osato et al,[3] the unique features of this mutation have been revealed by several studies. First, although the frequency of AML1 mutations in de novo AML is low (< 5%), they have been detected at a substantially higher frequency in a specific subtype of AML, poorly differentiated AML M0 (12%-33%).[3-6] We also reported a high frequency (42%) of AML1 mutations among radiation-associated and therapy-related MDS and AML.[7] These results suggest that AML1 mutations might play, in certain situations, critical roles in developing hematopoietic malignancy. Second, germ line mutations of *AML1* have been shown to occur in a rare autosomal dominant disorder, familial platelet disorder with predisposition to AML (FPD/AML).[8-10] All mutations with one exception identified in patients with AML and FPD/AML are in the amino (N)–terminal region of this transcription factor, especially in the Runt homology domain (RHD), which mediates its ability to bind to DNA and core binding factor β (CBFβ). Nearly half of them are missense mutations that replace amino acid residues directly contacting DNA, as shown by analysis of the crystal structure of the RHD–CBFβ–DNA ternary complex.[11-14] Most of the other mutations are frame shift or nonsense mutations that abolish the function of the RHD.[3,7] The majority of AML1 point mutations abrogate its DNA-binding potential, suggesting that loss of function is the main mechanism through which mutated AML1 contributes to the malignant transformation of hematopoietic progenitors.

Germ line mutations of the *RUNX2* gene, which encodes another member of the Runx family of transcription factors that contains the RHD, have been detected in patients with Cleidocranial dysplasia (CCD), an autosomal dominant disorder characterized by skeletal anomalies (reviewed in Otto et al[15]). Although

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Submitted September 8, 2003; accepted November 5, 2003. Prepublished online as *Blood* First Edition Paper, November 13, 2003; DOI 10.1182/blood-2003-09-3074.

Supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

An Inside *Blood* analysis of this article appears in the front of this issue.

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more than 70% of mutations detected in CCD are in the RHD, around one fourth of them are in the carboxy (C)–terminal region. These data prompted us to test whether AML1 point mutations in the C-terminal region occur in hematopoietic malignancy, especially in MDS, because only 6 patients with MDS were so far tested for the C-terminal mutations of AML1.[3] Here we report a high frequency of AML1 mutations in the C-terminal, as well as in the N-terminal region of the protein in patients with MDS, especially those with refractory anemia with excess blast (RAEB), RAEB in transformation (RAEBt), and AML following MDS. Our data suggest that AML1 point mutations are strongly associated with these specific types of hematopoietic malignancy.

Patients, materials, and methods

Patients

We examined 160 cases of MDS (46 refractory anemia [RA] and RA with ringed sideroblasts [RARS], 41 RAEB, 35 RAEBt, 34 AML following MDS, 4 chronic myelomonocytic leukemia [CMMoL]) and 115 cases of AML without antecedent MDS, all of whom were diagnosed at Hiroshima University Hospital and its affiliated hospitals between 1995 and 2003. We divided these patients into "sporadic" and "secondary" disease categories according to their past history. The secondary group included (1) atomic-bomb survivors in Hiroshima, who were exposed within 3 Km of the hypocenter[7]; (2) patients who developed disease after radiotherapy and/or chemotherapy for malignancy or myeloproliferative disorder (MPD); and (3) an individual who was occupationally exposed to mustard gas. We also examined 51 cases of MPD in the chronic phase (15 myelofibrosis [MF], 21 essential thrombocythemia [ET], 13 polycythemia vera [PV], and 2 atypical MPD), 23 cases of chronic myeloid leukemia (CML; 20 in the chronic phase and 3 in blast crisis), and 28 cases of acute lymphoid leukemia (ALL).

Diagnosis was based on morphologic and immunophenotypic studies according to the French-American-British (FAB) classification.[16,17] Most of the patients in this study were treated in Hiroshima University Hospital or Hiroshima Red Cross Hospital and Atomic Bomb Survivors Hospital with a protocol containing intensive chemotherapy and bone marrow transplantation. Cytogenetic analyses using standard procedures were performed according to the International System of Human Cytogenetic Nomenclature (1995).[18] Patient samples were taken after obtaining informed consent and approval from the institutional review board at Hiroshima University. Mononuclear cells were isolated from bone marrow or peripheral blood samples by Ficoll-Conray density gradient centrifugation. Genomic DNA was extracted with a Puregene Kit (Gentra, Minneapolis, MN), and total RNA was extracted using a TRIzol Kit (Gibco Life Technologies, Rockville, MD), according to the manufacturers' instructions.

Identification of *AML1* mutations

Polymerase chain reaction–single-strand conformation polymorphism (PCR-SSCP) was performed as follows: 100 ng genomic DNA was amplified by PCR in a total volume of 20 μL containing 1 × PCR buffer (Perkin-Elmer, Foster City, CA), 0.2 mM dNTP (deoxynucleotide triphosphate; Perkin-Elmer), 0.2 μM of each primer, and 0.5 U AmpliTaq (Perkin-Elmer). PCR of exons 3 through 8 of the *AML1* gene was performed by using the following flanking intronic, forward/reverse primers: 5′-AGCTGTTTG-CAGGGTCCTAA-3′/5′-GTCCTCCCACCACCCTCT-3′ for exon 3, 5′-CATTGCTATTCCTCTGCAACC-3′/5′-CCATGAAACGTGTTTCAA-GC-3′ for exon 4, 5′-CCACCAACCTCATTCTGTTT-3′/5′-AGACATG-GTCCCTGAGTATA-3′ for exon 5, 5′-GGGGGCCCATTCTGCT-GAGAGG-3′/5′-GAGCATCAAGGGGAAACCCC-3′ for exon 6, 5′-AATCCCACCCCACTTTACAT-3′/5′-CTCAGCTGCAAAGAATGT-GT-3′ for exon 7b, and 5′-TCCGCTCCGTTCTCTTGC-3′/5′-GCTT-GTCGCGAACAGGAG-3′ for exon 8. To identify *AML1* mutations, SSCP analysis was performed on a GenePhor system (Amersham Pharmacia Biotech, Buckinghamshire, England) using 12.5% GeneGel Excel (Amersham Pharmacia Biotech). After electrophoresis, gels were silver-stained to visualize the bands. All PCR products with abnormal SSCP bands were confirmed by an independent amplification and SSCP analysis.

PCR products that showed abnormal bands were subcloned into a pCR2.1 vector (Invitrogen, Carlsbad, CA), and 8 independent clones were sequenced in both directions using a BigDye Terminator Cycle sequencing kit (Perkin-Elmer) and were analyzed on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer). To confirm the mutations, PCR products from cDNA were also sequenced. First-strand cDNA was synthesized by using total RNA and random hexamers with SuperScript II reverse transcriptase (Gibco). The cDNA products were amplified with the following primers: 5′-GCAGGGTCCTAACTCAATCG-3′/5′-GCTCGGAAAAGGACAAG-CTC-3′ for the N-terminal region (corresponding to exons 3 through 5), 5′-CTACCGCAGCCATGAAGAAC-3′/5′-TTCTGCAGAGAGGGTT-GTCA-3′ (corresponding to exons 4 to 7b) and 5′-CCAATACCTGGGATC-CATTG-3′/5′-CCTCAGTAGGGCCTCCACAC-3′ (corresponding to exons 7b to 8) for the C-terminal region. Subcloned PCR products were sequenced as described earlier.

Cell culture and transfection

The cell lines Cos-7 and HeLa were cultured in Dulbecco modified Eagle medium (DMEM; Gibco), and U937 cells in RPMI1640 (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine at 37°C in a humidified atmosphere with 5% CO_2. Cos-7 cells were transfected by using SuperFect (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For the reporter assay, HeLa cells were transfected by the calcium phosphate precipitation method, and U937 cells were transfected by Effectene (Qiagen).

A cDNA encoding CBFβ-MYH11 (kindly provided by Dr T. Watanabe, Tohoku University)[19] and PCR-generated fragments encoding AML1, AML1 mutants, or CBFβ with or without an N-terminus FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) epitope were also subcloned into the pcDNA3.1 expression vector (Invitrogen). The integrity of the amplified sequence was confirmed by DNA sequencing. A reporter plasmid containing a macrophage colony-stimulating factor receptor (M-CSFR) promoter (pM-CSF-R-luc) was kindly provided by Dr D. Zhang (Beth Israel Hospital and Harvard Medical School).[20]

Immunoprecipitations and immunoblot analysis

Cos-7 cells were transfected by using Superfect (Qiagen) with 5 μg pcDNA3.1-CBFβ and 5 μg FLAG-tagged AML1 or AML1 mutant expression plasmid. After 24 hours, the cells were lysed in the lysis buffer (20 mM Tris (tris(hydroxymethyl)aminomethane)–HCl, pH 7.5; 150 mM NaCl; 1% Nonidet P40; 1 mM phenylmethlsulfonyl fluoride [PMSF]; 1 μg/mL leupeptin). The lysates were sonicated and then incubated with protein G (Amersham Pharmacia Biotech) to block nonspecific binding of proteins. A portion of each lysate was removed for immunoblot analysis. A 20 μL volume of a 50% slurry of anti-FLAG M2 beads (Sigma, St Louis, MO) was added to the lysates, incubated for 4 hours at 4°C, and washed 3 times with lysis buffer. FLAG beads were blocked in phosphate-buffered saline (PBS) containing 1% bovine serum albumin prior to addition to the lysates. Immunoblot analysis was performed as reported previously.[7] The primary antibodies used in this study were anti-M2 antibody (Sigma) and anti-CBFβ polyclonal antibody (Oncogene Research Products, Boston, MA). Bound antibodies were detected by enhanced chemiluminescence (ECL) using a Western blotting kit (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assay (EMSA) and transcriptional assay

Nuclear extracts from Cos-7 cells that were transiently transfected with the corresponding expression plasmid were prepared as described previously.[21] Protein concentrations were determined with Bradford reagents (Bio-Rad, Hercules, CA). EMSA was performed as described previously.[22] For supershift analysis, 1 μL rabbit AML1 antiserum that was raised against a

polypeptide (Arg-Ile-Pro-Val-Asp-Ala-Ser-Thr-Ser-Arg-Arg-Phe-Thr-Pro-Pro-Ser, corresponding to the N-terminal region of AML1 of human and mouse origin) was used. Transcription assays using HeLa and U937 cells were performed by the procedure described elesewhere.[7]

Statistical methods

Kaplan-Meyer analysis was used to estimate survival of patients with RAEB, RAEBt, and AML following MDS with or without AML1 point mutations.[23] The log-rank test was used to compare the probabilities of survival for both groups.

Results

High frequency of *AML1* mutations in the N-terminal region in MDS and AML

We previously reported 11 cases of secondary MDS or AML (definition of "secondary" given in "Patients, materials, and methods") and 2 cases of sporadic MDS that harbor somatic mutations of the *AML1* gene in exons 3 through 5 (corresponding to amino acids 1-177, including the Runt homology domain, Table 1; Figure 1).[7] After publication of this report, one of the sporadic MDS patients (case 8) was found to have a history of receiving chemotherapy for AML (M3). Continuous complete remission (CCR) was achieved for 3 years and then she developed MDS (RAEBt). Because no mutation in the *AML1* gene was detected before development of MDS, we reclassified her as secondary MDS. The other sporadic MDS patient (case 7) had a history of manufacturing poison gas (mustard gas) during World War II. Thus, we categorized all of the 13 patients with AML1 mutations as secondary MDS or AML. We extended the N-terminal mutation analysis by PCR-SSCP assay by using genomic DNA extracted from mononuclear cells in the bone marrow of patients. Mutations were further confirmed by sequence analysis of reverse trasnscriptase (RT)–PCR products, and we thereby found 10 new cases (cases 14-23). One patient with sporadic AML (M0) had a frame-shift mutation (case 14), 4 patients with sporadic MDS had silent and missense mutations (cases 15, 19, 20, and 21), 3 patients with sporadic MDS had a frame-shift mutation (cases 16-18), and 2 patients with secondary MDS had a mutation corresponding to an amino acid insertion (cases 22 and 23). The clinical findings of these patients are summarized in Table 1 (cases 14-23). The overall incidence of N-terminal mutations in sporadic and secondary RAEB, RAEBt, and AML following MDS was 8% (7 of 88) and 45.4% (10 of 22), respectively (Table 2). We found only 1 patient (1.1%) with an N-terminal AML1 mutation among 95 patients with sporadic AML without antecedent MDS, and no N-terminal AML1 mutations were found in the patients with ALL, CML, or MPD. These data confirmed findings previously reported by us and others that AML1 mutations in the N-terminal region are not associated with ALL, MPD, or CML, but they are implicated in sporadic AML (except M0) at low frequency, and they are frequently found in secondary AML and MDS (Figure 1; Table 2).[3,4,7,8,24]

Frequent occurrence of C-terminal mutations of the *AML1* gene in MDS

To investigate the *AML1* mutations in the C-terminal portion of AML1, we analyzed exons 6 through 8. Point mutations were detected in patients with MDS and were found preferentially in sporadic MDS. Nine of 110 patients (8.2%) with RAEB, RAEBt, and AML following MDS had C-terminal AML1 mutations, whereas no mutation was detected in the following patients: 115 AML without antecedent MDS, 45 RA/RARS, 28 ALL, 23 CML, and 51 MPD in accordance with previous studies.[3,8] The clinical findings of these patients are summarized in Table 1 (cases 24-32). All but one patient (case 32, an atomic-bomb survivor) had sporadic MDS. Eight of 15 (53%) patients with sporadic MDS had an AML1 point mutation that occurred in the C-terminal region. By contrast, only 1 of 12 (8%) patients with secondary MDS with AML1 point mutations had mutations occurring in the C-terminal region; this difference was statistically significant ($P = .013$; chi-square test).

All of the C-terminal mutations (cases 24-32) resulted in frame shifts similar to the Runx2 mutations identified in CCD.[15] The consequences of the C-terminal mutations were unusual (Figure 2A). Usually, frame-shift mutations are created by insertions or deletions of nucleotides in exons or by loss of splice donor or acceptor sites, and they result in truncation of the authentic protein followed by a relatively short additional stretch of amino acid residues originating from the wrong reading frame or from intronic sequences. Among the 9 C-terminal AML1 mutations found, only 3 cases (cases 24, 27, and 29) obeyed this general rule. In 4 cases (cases 25, 28, 31, and 32), small nucleotide insertions or deletions (4-14 base pair [bp]) occurred in exons, but the stretches of additional amino acids resulting from the wrong reading frame were 195 to 335 residues in length (Table 1), because the wrong frame contained an in-frame termination codon 353 bp downstream (3′) of the authentic termination codon (Figure 2B). Thus, the mutated AML1 proteins in these 4 cases were even longer than wild-type AML1 and appeared to be fusion proteins rather than truncations of AML1 (Figure 2A).

The other 2 cases (cases 26 and 30) had mutations within introns. Case 26 had a single nucleotide replacement (T > A) in the intron between exons 6 and 7b that formed a new splice acceptor, resulting in the insertion of 11 nucleotides in the mRNA, thus creating a frame-shift mutation (Figure 3A). Case 30 had a deletion of the splice donor signal adjacent to the end of exon 7b (Figure 3B). As a result, 2 types of mRNA were created from the single mutation. One mRNA, designated 30a, was made by a cryptic splice donor site (212 bp downstream from the authentic splice donor signal), resulting in the insertion of an intronic sequence (208 bp) that contained an in-frame termination codon. The predicted mutated protein was a truncation of the wild-type AML1 protein at Thr296 with 42 additional amino acid residues that originated from the intronic sequence. The other message, designated 30b, was created by another cryptic splice donor site (112 bp downstream from the authentic site), resulting in the insertion of an intronic sequence (108 bp), but no frame shift in exon 8. Consequently, the mutated AML1 protein had an insertion of 36 amino acid residues that originated from intronic sequences in the middle of the *trans*-activation domain of AML1.

We observed virtually equal intensities of normal and shifted bands in the PCR-SSCP analysis of all the samples with AML1 mutations, including case 30, and obtained comparable frequencies of normal and mutated sequences by the sequence analysis. In addition, germ line genomic DNA sequences were examined in specimens obtained from nonleukemic organs in 6 cases (cases 4, 5, 6, 9, 10, and 12), and these were found to be normal (data not shown), suggesting that mutations of the *AML1* gene were monoallelic at the somatic level.

To test whether AML1 point mutations affect the prognosis of MDS, we tracked the overall survival of patients with RAEB, RAEBt, and AML following MDS, comparing survival of those

Table 1. Clinical features and mutation characteristics of the patients with AML1 mutations

Case no.	Age, y/sex	Category and diagnosis	History*	Chromosome	AML1 mutation		
					Genome	cDNA	Protein
1	66/F	Secondary MDS, RA	A-bomb (2.5 km)	46, XX	471G>T	471G>T	Pro157syn
2	81/F	Secondary MDS, RAEBt	A-bomb (2.0 km)	45, XX, −5	303T>C	303T>C	Thr101syn
3	69/M	Secondary MDS, RAEBt	A-bomb (2.7 km)	46, XY, del(12)(p12), der(14)t(1;14)(p10;p10)	124G>C	124G>C	Gly42Arg
4	59/F	Secondary MDS, RAEBt	A-bomb (1.7 km)	46, XX, del(3)(p14)	211delC†	211delC†	Leu71fsTer94†
5	68/M	Secondary MDS, RAEBt	A-bomb (0.8 km)	47, XY, t(3;5)(q27;p13), +4, +5, −7	511G>A	511G>A	Asp171Asn
6	80/F	Secondary MDS, AML following MDS	A-bomb (2.5 km)	47, XX, +8, t(9;11)(q22;q23)	124G>C	124G>C	Gly42Arg
7	80/M	Secondary MDS, RAEBt	Manufacturing mustard gas	46, XY	316_338dup	316_338dup	Tyr113Ter
8	43/F	Secondary MDS, RAEBt	AML(M3)>CCR> RAEBt	45, XX, −7	511G>A	511G>A	Asp171Asn
9	39/F	Secondary MDS, RAEBt	Astrocytoma; Rad/Chem Tx	46, XX	530G>A	530G>A	Arg177Gln
10	62/M	Secondary AML, M0	Lymphoma; Rad/Chem Tx	46, XY	413_414ins CGGGGG	413_414ins CGGGGG	Gly138_Arg139 insGlyGly
11	61/F	Secondary AML, M1	Myelofibrosis; Rad/Chem Tx	46, XX	343_364dup	343_364dup	Ala122fsTer123
12	57/F	Secondary AML, M4	ET; Chem Tx	45, XX, −18	517C>T	517C>T	Pro173Ser
13	64/M	Secondary AML, M1	Polycythemia vera; Chem Tx	46, XY	511G>A	511G>A	Asp171Asn
14	29/M	Sporadic AML, M0	—	46, XY	314_342insAGGC	341_342 insAGGC	Ser114fsTer117
15	80/M	Sporadic MDS, RAEB	—	47, XY, +8	261C>A	261C>A	Ile87syn
16	74/M	Sporadic MDS, AML following MDS	—	46, XY	342G>CCTTCC	342G>CCTTCC	Ser114fsTer119
17	65/M	Sporadic MDS, RAEB	—	47, XY, +8	388delA	388delA	Arg130fsTer148
18	47/F	Sporadic MDS, RAEBt	—	46, XX	416_423dup	416_423dup	Arg142fsTer151
19	79/M	Sporadic MDS, RAEBt	—	46, XY	422_427 + 3dup	427_428ins GTAGAAGAG	Gly143_Lys144 insArgArgGly
20	64/F	Sporadic MDS, RAEB	—	46, XX	497T>C	497T>C	Ile166Thr
21	75/M	Sporadic MDS, RAEBt	—	45, XY, add(3)(q?13.2), −7	512A>G	512A>G	Asp171Gly
22	41/M	Secondary MDS, RAEB	AML(M5b)>CCR> RAEB	46, XY, add(6)(p21.3), del(20)(q11.2q13.3)	253_254insCCC	253_254 insCCC	Thr84_Leu85insPro
23	62/M	Secondary MDS, RAEBt	Esophageal carcinoma; Rad Tx.	46, XY, add(17)(p11.2)	411C>AGGA	411C>AGGA	Val137_Gly138 insGly
24	62/M	Sporadic MDS, AML following MDS	—	46, XY	669_670ins ACCGT	669_670ins ACCGT	Ala224fsTer228
25	75/M	Sporadic MDS, RAEBt	—	46, XY	695_708del	695_708del	Phe232fsTer567
26	73/M	Sporadic MDS, RAEBt	—	46, XY	Intron 7a 725-13T>A	724_725ins ATTCTCTTCAG	Asp242fsTer287
27	55/F	Sporadic MDS, AML following MDS	—	46, XX	871delT	871delT	Ser291fsTer300
28	75/M	Sporadic MDS, AML following MDS	—	46, XX	878G> AGGGCCCA	878G>AGGGCCCA	Arg292fsTer574
29	70/F	Sporadic MDS, RAEBt	—	46, XY	876_886 + 3dup	886_887ins GTATCGACTCTCAA	Thr296fsTer305
30	54/M	Sporadic MDS, RAEB	—	46, XY	Intron 7b 886 + 1_4del	886_887ins (208 bp) 886_887ins (108 bp)	Thr296fsTer338 Thr296Ser ins(36aa)
31	64/M	Sporadic MDS, RAEB	—	47, XY, +1, der(1;7)(q10;p10), +8	1091_1095del	1091_1095del	Ala364fsTer570
32	87/F	Secondary MDS, RAEBt	A-bomb (2.0 km)	46, XX	1130_1133dup	1130_1133dup	Leu378fsTer573

Cases 1 through 13 were reported previously.[7] CCR indicates continuous complete remission; Rad Tx, radiation therapy; Chem Tx, chemotherapy; ET, essential thrombocythemia; and —, no history that belongs to the "secondary" categories described in "Patients, materials, and methods."

*Distances in parentheses indicate how far from the center of the explosion the patient was.

†The previous description (208delC; Ser70fsTer93)[7] was incorrect.

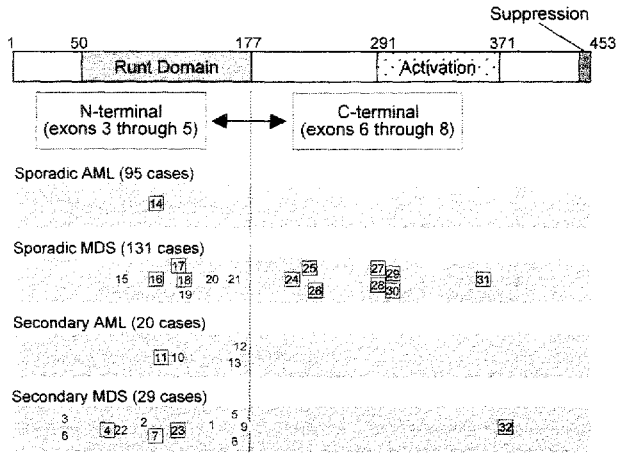

Figure 1. AML1 point mutations identified in AML and MDS. Horizontal bars show the AML1 protein diagrammatically (453 aa), indicating the Runt domain (50-177), the *trans*-activation domain (291-371), and the suppression domain (446-453). AML1 is divided into N-terminal and C-terminal regions. Case numbers (Table 1) with AML1 mutations are indicated according to disease categories (sporadic AML, sporadic MDS, secondary AML, and secondary MDS). Numbers in white circles indicate missense/silent mutations, and numbers in squares indicate frame-shift/nonsense mutations.

associated with AML1 mutations (n = 26) with that of patients without the mutations (n = 81) (Figure 4). There was no significant difference in the distribution of age (mean of 66.2 years, range 39-87, versus mean of 66.7 years, range 17-82) and sex (16 male to 10 female, versus 52 male to 29 female) between the 2 groups. AML1 point mutations were proven to be a risk factor by a log-rank test ($P < .01$).

Abilities of AML1 mutants with C-terminal region mutations to bind to DNA and to heterodimerize with CBFβ

To obtain insight into molecular mechanisms through which the newly discovered C-terminal mutations of AML1 contribute to malignant transformation of hematopoietic progenitors, we analyzed the DNA binding potential of the mutant proteins. A radiolabeled oligonucleotide probe containing the consensus binding sequence for AML1 and nuclear extracts from Cos-7 cells transfected with AML1 mutants were used in EMSA. A DNA/protein complex was detected when using nuclear extract from a transfectant expressing FLAG-tagged wild-type AML1 (Figure 5A, lane 10) that was not observed when using an extract from the mock transfectant (lane 1). This complex was competed for by the nonradiolabeled oligonucleotide containing the AML1 binding site (lane 11) but was not competed for by those containing a mutated AML1 binding site (lane 12). Moreover, this complex was supershifted by a specific serum against AML1 (lanes 13 and 15), indicating that the complex contains AML1. Among C-terminal AML1 mutants we tested, mutants 24 and 27 had a markedly enhanced DNA-binding potential compared with that of wild-type AML1 (lanes 2 and 5). Mutants 26 and 30b also showed a substantially higher DNA binding potential (lanes 4 and 7). By contrast, mutants 25, 31, and 32, which had long stretches of additional amino acid residues originating from the wrong reading frame, bound to the probe, but only weakly (lanes 3, 8, 9, 17, 19, and 21). These findings were not due to uneven expression levels of AML1 mutants, because an approximately equal amount of protein was detected by immunoblot analysis (lower panel).

To test whether AML1 mutants are able to interact with CBFβ, we performed immunoblot analysis after immunoprecipitation.

Table 2. Frequency of AML1 mutations in the specific types of hematopoietic disease

Diagnosis	History	No. cases	AML1 mutation		
			Total, no. (%)	N-terminal, no. (%)	C-terminal, no. (%)
RAEB, RAEBt, and AML following MDS (MDS/AML)					
RAEB					
	Sporadic	36	5 (13.9)	3 (8.3)	2 (5.6)
	Secondary	5	1 (20.0)	1 (20.0)	0 (0.0)
	Total	41	6 (14.6)	4 (9.8)	2 (4.9)
RAEBt					
	Sporadic	23	6 (26.1)	3 (13.0)	3 (13.0)
	Secondary	12	9 (75.0)	8 (66.7)	1 (8.3)
	Total	35	15 (42.9)	11 (31.4)	4 (11.4)
AML following MDS					
	Sporadic	29	4 (13.8)	1 (3.4)	3 (10.3)
	Secondary	5	1 (20.0)	1 (20.0)	0 (0.0)
	Total	34	5 (14.7)	2 (5.8)	3 (8.8)
Total MDS/AML					
	Sporadic	88	15 (17.0)	7 (8.0)	8 (9.1)
	Secondary	22	11 (50.0)	10 (45.5)	1 (4.5)
	Total	110	26 (23.6)	17 (15.4)	9 (8.2)
AML without antecedent MDS (de novo AML)					
AML without antecedent MDS					
	Sporadic	95	1 (1.1)	1 (1.1)	0 (0.0)
	Secondary	20	4 (20.0)	4 (20.0)	0 (0.0)
	Total	115	5 (4.3)	5 (4.3)	0 (0.0)
Others					
RA/RARS	Total	45	1 (2.2)	1 (2.2)	0 (0.0)
CMMoL	Total	4	0 (0.0)	0 (0.0)	0 (0.0)
MPD	Total	51	0 (0.0)	0 (0.0)	0 (0.0)
ALL	Total	28	0 (0.0)	0 (0.0)	0 (0.0)
CML/CML-BC	Total	23	0 (0.0)	0 (0.0)	0 (0.0)

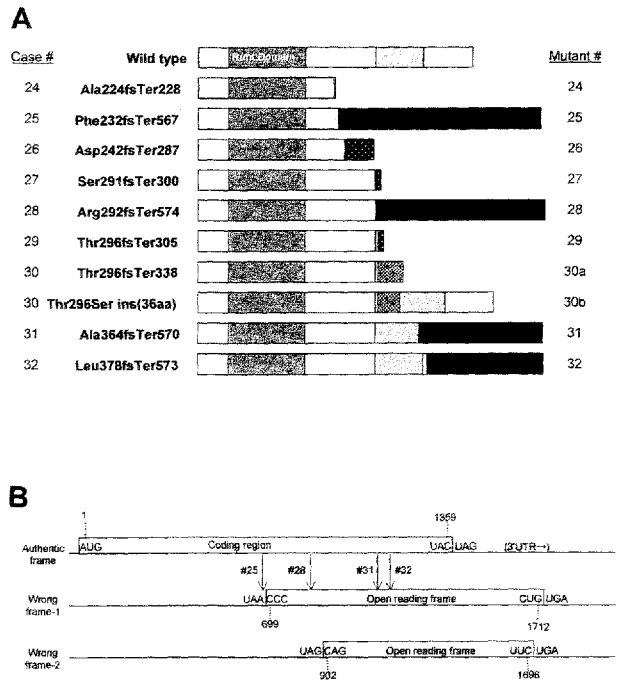

Figure 2. AML1 mutations in the C-terminal region. (A) Horizontal bars show the wild-type (453 aa) and mutant AML1 sequences, indicating the Runt domain (50-177), the *trans*-activation domain (291-371), and the C-terminal mutants. The numbers in the left-hand column indicate the case numbers (24 through 32) described in Table 1. Patient 30 expressed 2 types of mutated AML1 mRNA and protein that originated from one genomic mutation (described in "Results"). Cases 25, 28, 31, and 32 expressed extraordinary long stretches originating from a wrong reading frame (indicated by black bars). (B) Open reading frames (ORFs) of the human *AML1* gene. Boxes showed ORFs including the authentic frame that encodes the wild-type AML1 protein. Vertical arrows show locations of frame shifts in cases 25, 28, 31, and 32. Only relevant ORFs are shown.

Cos-7 cells were cotransfected with the FLAG-tagged wild-type or mutated AML1 (Figure 5B, upper panel), together with CBFβ. CBFβ was coimmunoprecipitated with FLAG-tagged wild-type AML1 by beads coated with anti-FLAG antibody (lower panel, lane 9). CBFβ was also coimmunoprecipitated with AML1 proteins having mutations in the C-terminal domain (lanes 1-8), indicating that these mutants interacted with CBFβ. Taking account of expression levels of mutant proteins, the binding potential of mutants 24 and 27 seemed to be increased.

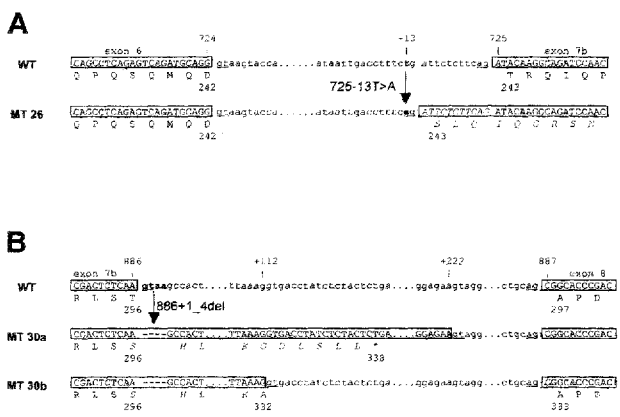

Figure 3. Introduction of alternative splice sites in cases 26 and 30. (A) Case 26, the T>A replacement in the intron results in a frame shift that makes a new splice acceptor 11 bp upstream (5') of the authentic splice acceptor. (B) Case 30, a 4-bp deletion, including splice donor sites, resulted in use of 2 cryptic splice donor sites 208 bp and 108 bp downstream (3') of the authentic splice donor site. WT indicates wild-type; MT, mutants.

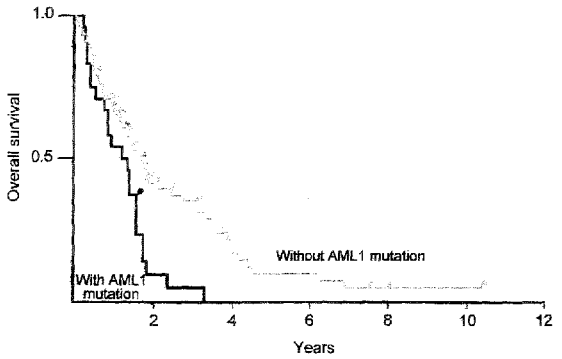

Figure 4. Kaplan-Meier analysis comparing overall survival of patients with MDS/AML (RAEB, RAEBt, and AML following MDS) and AML1 point mutations with the survival of patients without an AML1 mutation. The black line indicates patients with an AML1 point mutation; the gray line indicates patients without an AML1 mutation.

Transcriptional potential of C-terminal AML1 mutants

To investigate the transcriptional activities of the C-terminal AML1 mutants, reporter experiments were performed by using the promoter of M-CSFR, which is known to be transcriptionally regulated by AML1.[20] When wild-type AML1 and CBFβ were cotransfected in

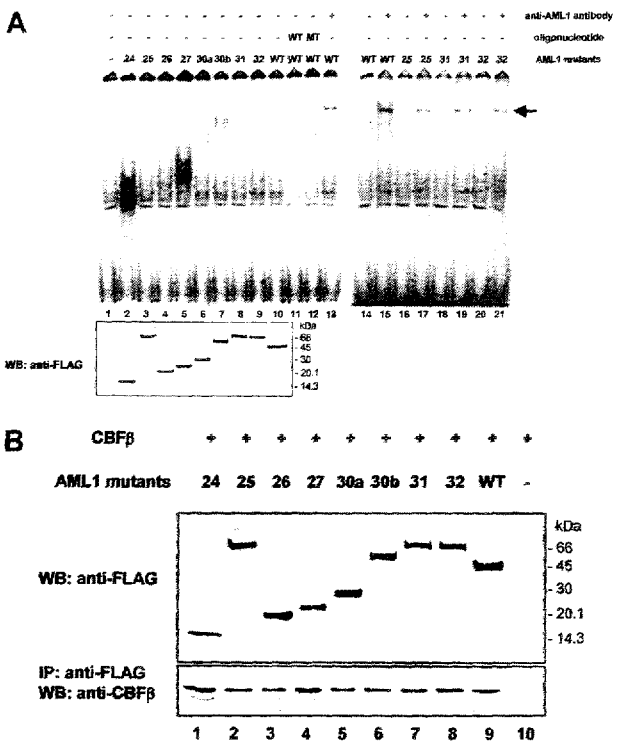

Figure 5. Abilities of C-terminal AML1 mutant proteins to bind DNA and to heterodimerize with CBFβ. (A) DNA binding potential of AML1 mutants was analyzed by EMSA using nuclear extract from Cos-7 cells transfected with wild-type AML1 or mutated AML1 expression plasmid vectors. The oligonucleotides used as competitors were as follows: W, containing one wild-type AML1 binding site (CGAGTATTGTGGTTAATACG); and M, containing one mutated AML1 binding site (CGAGTATTGTTAGTAATACG). Equal expression of each mutant was demonstrated by immunoblot analysis (WB) using a FLAG antibody, as shown in the lower panel. An arrow indicates supershifted bands. (B) Heterodimerization ability of AML1 mutants with CBFβ. Cos-7 cells were cotransfected with an expression vector containing CBFβ cDNA and with vectors containing either wild-type AML1 or mutated AML1 cDNA. The expression levels of AML1 in total cell lysates were detected by immunoblot analysis with an anti-FLAG antibody (upper panel). Cell lysates were immunoprecipitated (IP) with an anti-FLAG antibody, and then proteins were detected by immunoblot analysis (WB) using an anti-CBFβ antibody (lower panel).

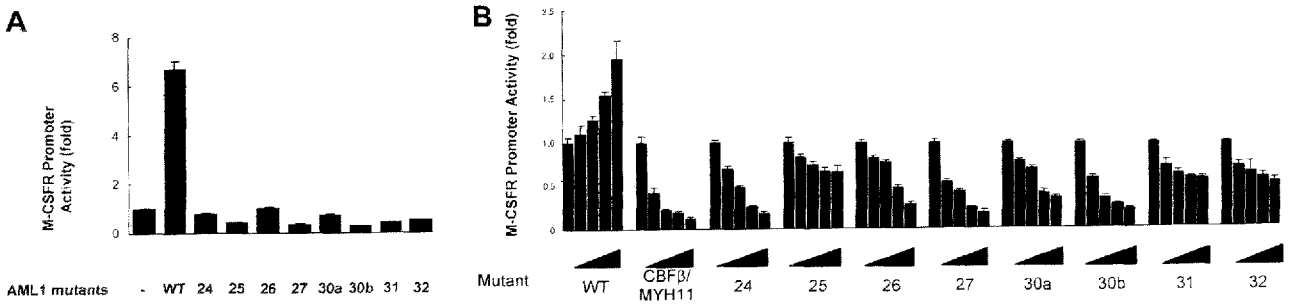

Figure 6. Transcriptional potential of AML1 mutants. (A) Transcriptional activities of the AML1 mutants in HeLa cells. Cells were transfected with 5 μg pM-CSF-R-luc, 3 μg FLAG-tagged AML1 or AML1 mutant expression vector, 1 μg CBFβ expression vector, and 0.25 μg pRL-tk as an internal control to normalize luciferase activities for transfection efficiency. (B) Transcriptional activities of the AML1 mutants in U937 cells. Cells were transfected with 2 μg pM-CSF-R-luc reporter plasmid, the indicated amounts of AML1 expression constructs, and 0.2 μg pRL-tk as an internal control to normalize luciferase activities for evaluation of transfection efficiency. The expression vector containing wild-type AML1 (0.2 μg) was cotransfected with increasing doses (0, 0.1, 0.2, 0.4, and 0.6 μg) of expression vectors containing CBFβ-MYH11 or various AML1 mutations. Each value represents the mean of 3 independent experiments. The error bars indicate the mean ± standard deviation.

HeLa cells, the promoter activity was induced 7-fold compared with transfection with CBFβ alone (Figure 6A). In contrast, none of the C-terminal mutants induced significant *trans*-activation, regardless of their DNA-binding potentials (Figure 5A). To examine whether AML1 mutants act as dominant negative inhibitors of wild-type AML1, we performed the same reporter assay by using U937 monocytic cells, in which the activity of the M-CSFR promoter was *trans*-activated by transfecting AML1 alone in a dose-dependent manner (Figure 6B). As a positive control, we used CBFβ-MYH11, which is formed as a result of inv(16) and is a well-established negative regulator of AML1.[25] All mutants suppressed the *trans*-activation activity of wild-type AML1 in a dose-dependent fashion. Inhibition of the *trans*-activation seemed to be related to DNA- and CBFβ-binding potential (Figure 5A-B). Thus, the C-terminal AML1 mutants identified in this study lacked *trans*-activation potential and could act as dominant negative inhibitors of wild-type AML1.

Discussion

In this study, we established strong correlations between point mutations of the *AML1* gene and subgroups of MDS and AML (ie, RAEB, RAEBt, and AML) following MDS. Here we define these 3 disease categories as MDS/AML. Of the 110 patients with MDS/AML who were tested, 26 (23.6%) had an AML1 mutation (Table 2). Conversely, of 32 patients with AML1 point mutation, 26 (81%) belonged to this category (Table 1). Moreover, the prognosis for the patients with AML1 mutations was significantly worse than for patients without AML1 mutations (Figure 4).

The MDS/AML category we defined here is characterized by (1) relatively a low blast percentage in the bone marrow, (2) multilineage dysplasia, (3) resistance to chemotherapy, and (4) poor prognosis. In the FAB classification, this disease entity was designated as MDS, and a substantial number of patients are classified into the RAEBt subgroup.[17] In the World Health Organization (WHO) classification proposed in 2001,[26] the RAEBt category was eliminated, and it was designated as AML. It is generally accepted that leukemogenesis of MDS/AML is different from that of de novo AML without significant myelodysplastic features. De novo AML is often associated with oncogenic chimeras, which are considered as the major cause of malignant transformation of hematopoietic progenitors. In this subgroup, point mutations are likely a complement for chimeras, as in recent reports that c-Kit mutations are often associated with AML1-ETO or Flt-3 mutations with MLL chimeras.[27-29] By contrast, MDS/AML, which is rarely associated with chimeras, develops as a result of accumulation of gene deletions and point mutations.[30,31] *AML1* is the first gene that was demonstrated to have a high frequency of mutations in patients with MDS/AML. Moreover, AML1 point mutations are rarely detected in other types of hematologic malignancies or MPD, except secondary AML and AML (M0). Furthermore, patients with MDS/AML with AML1 point mutations had a significantly worse prognosis than patients without such mutations, suggesting that this is one of the major decision factors of the biologic features of myeloid malignancy. Thus, "low blast percentage myeloid leukemia with AML1 point mutation" may represent a new subgroup of AML, which would be considered for inclusion in the recurrent genetic abnormalities in the WHO classification.

This report also provides the first demonstration that AML1 point mutations in the C-terminal region correlate with MDS/AML (overall 9 of 110, 8.2%; Table 2). By contrast, of 115 cases of de novo AML (including 13 M0 cases), we found no AML1 mutations in the C-terminal region. These data were supported by Osato et al,[3] who tested 109 cases of de novo AML including 9 patients with AML (M0) and did not find mutations in the C-terminal region. Thus, we believe that C-terminal AML1 mutations are rare, if they occur at all, in overall de novo AML. However, there remains a possibility that they are involved in AML (M0) at a relatively low frequency, because, although we and others tested a total of 121 patients with AML (M0) for AML1 point mutations and found 22 (18%) patients with N-terminal mutations,[3-6] only 22 patients have been tested so far for C-terminal mutations. Only 1 of 12 mutations identified in secondary MDS/AML was in the C-terminal region. By contrast, *AML1* mutations in sporadic MDS/AML were distributed throughout the gene (Figure 1). A simple hypothesis to interpret these data are that ionizing radiation (IR) or alkylating agents preferentially damage the N-terminal region of AML1, whereas there is a source of DNA damage other than IR or alkylating agents that can injure the C-terminal region and ultimately lead to development of sporadic MDS/AML. Alternatively, gene abnormalities frequently induced by radiation or alkylating agents somehow contribute to development of MDS/AML in cooperation with N-terminal AML1 mutants but not with C-terminal ones.

Although the contribution of AML1 point mutations to the development of AML (M0) and MDS/AML is convincing, the following question may be raised: Why do mutations in one gene

Mutation	MDS/AML	AML (M0)	FPD/AML
N-terminal, missense or insertion	Gly42Arg (cases 3 and 6) *	Leu29Ser[5] His58Asn[3] *	—
	Thr84_Leu85 ins Pro (case 22)	Trp79Cys[5, 6]	Lys83Glu[10] †
	Val137_Gly138 ins Gly (case 23) Arg139Gly[24] † Gly143_Lys144insArgArgGly(case 19)	Arg135Gly[4] Gly138Asp[4] Gly138_Arg139 ins GlyGly (case 10) † Ser140_Gly141 ins SerArg[4]	Arg139Gln[8] †
	—	Ile150Thr[5]	—
	Ile166Thr (case 20) **Asp171Gly**(case 21) Asp171Asn (cases 5 and 8) † Arg177Gln (case 9) †	**Asp171Gly**[4] **Arg174Gln**[4] Arg174Gly[4]	Asp171Tyr[9] **Arg174Gln**[8] †
N-terminal, nonsense or frame-shift	Phe13fsTer26[32]	—	—
	Leu71fsTer94 (case 4) †	Ser67fsTer109[5] Phe70fsTer93[5] Cys72fsTer111[3] †	—
	—	Val91fsTer101[4]	Lys90fsTer101[8] †
	Val105Ter[24] †	—	—
	Tyr113Ter (case 7) † Ser114fsTer119 (case 16)	Ser114Ter[3] † Ser114fsTer117 (case 14)	—
	Ala122fsTer123(case 11) †	**Ala122fsTer123**[4]	—
	Arg130fsTer148(case 17)	Phe131fsTer180[4]	—
	—	Arg135fsTer[4]	Arg135fsTer177[10] †
	Arg142fsTer151(case 18)	Arg139Ter[4] (2 cases) Gly143fsTer153[5]	—
	—	**Arg177Ter**[3] †	Arg174Ter[8] † **Arg177Ter**[6] †
C-terminal, frame-shift	Ala224fsTer228 (case 24) § Phe232fsTer567 (case 25) ‡ Asp242fsTer287 (case 26) § Ser291fsTer300 (case 27) § Arg292fsTer574 (case 28) Thr296fsTer305 (case 29) Thr296fsTer338(case 30a) ¶ Thr296Ser ins(36aa) (case 30b) § Ala364fsTer570 (case 31) ‡ Leu378fsTer573 (case 32) ‡	—	Tyr260Ter[10]

Figure 7. AML1 mutations in MDS/AML, AML (M0), and FPD/AML. Items in bold show the identical mutation. Boxes outlined with solid lines indicate N-terminal missense/insertion mutations that replace amino acid residues in three loops directly contacting DNA. Boxes outlined with dashed lines indicate N-terminal nonsense/frame-shift mutants resulting in similar truncated forms of the protein. — indicates previously not reported. * indicates mutant belonging to Type b (described in "Discussion"); †, mutant belonging to Type a-1 (described in "Discussion"); ‡, mutant belonging to Type a-2 (described in "Discussion"); §, mutant belonging to Type a-3 (described in "Discussion"); and ¶, mutant belonging to Type a-4 (described in "Discussion").

result in myeloid malignancies with such totally different phenotypes? One possibility is that residual wild-type AML1 contributes to the differentiation of leukemic blasts. Of 22 patients with AML (M0) harboring *AML1* gene mutations so far reported, including 2 patients (cases 10 and 14) in this study, 13 (60%) lost the wild-type *AML1* gene. In addition, 2 patients with M0 were reported to have a total homozygous deletion of *AML1*.[6] By contrast, all 26 patients with MDS/AML with AML1 mutations in this study and an additional 3 patients reported by others expressed wild-type AML1,[3,24] suggesting that this transcription factor differentiates not only normal hematopoietic progenitors in the process of development, but also pathologic blasts in leukemogenesis. This hypothesis is supported by the fact that wild-type AML1 is usually expressed in leukemic blasts harboring AML1-ETO chimeras, which cause AML with maturation (M2). Another possibility is that different AML1 mutations result in different phenotypes. Indeed, C-terminal mutations we found so far only in MDS/AML, suggesting that this type of mutation may induce MDS/AML but not de novo AML. However, there seemed to be little difference in N-terminal mutations between MDS/AML, AML (M0), and FPD/AML (Figure 7). A limited number of amino acid residues were replaced by missense/insertion mutations, and these types of

mutations were found in each of the 3 disease categories. Nonsense/frame-shift mutations in each disease category also resulted in similar, or even identical, truncated forms. This finding suggested that, in the case of N-terminal AML1 mutations, the phenotype of the resulting leukemia would be determined by the presence of wild-type AML1 or other genetic abnormalities that contributed to leukemogenesis in cooperation with the AML1 mutation.

Most amino acid residues replaced by missense or insertion type mutations in the N-terminal region of AML1 were located in 3 protein loops, called loop(βA′-B), loop(βE-F), and βG′tail, which mediate the DNA-binding potential, as demonstrated by analysis of the crystal structure of the RHD-CBFβ-DNA ternary complex (Figure 7).[11-14] Nonsense and frame-shift mutations in the N-terminal region result in partial deletion of the Runt homology domain. Thus, these mutants are predicted to lose DNA-binding ability and *trans*-activating potential (we designate this type of mutation as Type a-1), and indeed, we and others have confirmed this type for 18 mutants (Figure 7).[3,7,10,24] However, there are 4 missense mutations (Leu29Ser, Gly42Arg, His58Asn, and Ile150Thr) that replace amino acids out of the 3 loops. The Gly42Arg and His58Asn mutant proteins were shown to bind to DNA more avidly than wild-type AML1 and to have enhanced *trans*-activation potential (Type b).[3,7] Analysis of C-terminal mutants provided 3 additional types of mutation: attenuated DNA-binding potential without *trans*-activation potential (Type a-2, mutants 25, 31, and 32), enhanced DNA-binding ability but no *trans*-activation potential (Type a-3, mutants 24, 26, 27, and 30b), and DNA-binding ability equivalent to wild-type AML1 but no *trans*-activation potential (Type a-4, mutant 30a). Mutants belonging to Type a-3 have a similar structure to that of an alternative splice variant, AML1a, which also binds to DNA more avidly than wild-type AML1b but lacks *trans*-activation potential.[33] Currently, we can offer no adequate interpretation for the question as to why mutations resulting in such divergent biochemical features contribute equally to the same type of myeloid malignancy. However, we can say that, with a few exceptions such as Gly42Arg or His58Asn, AML1 mutants lose their *trans*-activation potential, suggesting a loss of function mechanism. We believe that further careful analysis of these AML1 mutants will reveal the key molecular system that induces myeloid malignancy.

Acknowledgment

We thank Ryoko Yamaguchi for excellent technical support in carrying out these experiments.

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