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Identification of a SRC-Like Tyrosine Kinase Gene, *FRK*, Fused with *ETV6* in a Patient with Acute Myelogenous Leukemia Carrying a t(6;12)(q21;p13) Translocation

Noriko Hosoya,[1] Ying Qiao,[1] Akira Hangaishi,[1] Lili Wang,[1] Yasuhito Nannya,[1] Masashi Sanada,[1] Mineo Kurokawa,[1] Shigeru Chiba,[1,2] Hisamaru Hirai,[1,2] and Seishi Ogawa[1,3]*

[1]Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[2]Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, University of Tokyo, Tokyo, Japan
[3]Department of Regeneration Medicine for Hematopoiesis, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

The SRC family of kinases is rarely mutated in primary human tumors. We report the identification of a SRC-like tyrosine kinase gene, *FRK* (Fyn-related kinase), fused with *ETV6* in a patient with acute myelogenous leukemia carrying t(6;12)(q21;p13). Both reciprocal fusion transcripts, *ETV6/FRK* and *FRK/ETV6*, were expressed. In *ETV6/FRK*, exon 4 of *ETV6* was fused in-frame to exon 3 of *FRK*, producing a chimeric protein consisting of the entire oligomerization domain of ETV6 and the kinase domain of FRK. The ETV6/FRK protein was shown to be constitutively autophosphorylated on its tyrosine residues. ETV6/FRK phosphorylated histones H2B and H4 in vitro to a greater extent than did FRK, suggesting it had elevated kinase activity. ETV6/FRK could transform both Ba/F3 cells and NIH3T3 cells, which depended on its kinase activity. Moreover, ETV6/FRK inhibited ETV6-mediated transcriptional repression in a dominant-negative manner. This report provides the first evidence that a SRC-like kinase gene, *FRK* fused with *ETV6*, could directly contribute to leukemogenesis by producing an oncoprotein, ETV6/FRK, with dual functions: constitutive activation of the ETV6/FRK tyrosine kinase and dominant-negative modulation of ETV6-mediated transcriptional repression.

INTRODUCTION

The *SRC* gene was the first protooncogene isolated as the cellular homologue of v-*SRC*, the retroviral transforming oncogene of avian Rous sarcoma virus (Brown and Cooper, 1996). Since then, it has become clear that *SRC* is the prototype for a family of genes that encode nonreceptor tyrosine kinases implicated in a variety of cellular processes, including cell growth, differentiation, and carcinogenesis. The SRC family of kinases shares common structures consisting of an N-terminal unique domain, SRC homology 3 (SH3) and SRC homology 2 (SH2) domains, a kinase domain, and a short C-terminal regulatory tail (Brown and Cooper, 1996). They are normally maintained in an inactive state through phosphorylation of a critical C-terminal tyrosine residue (Tyr 530 in human SRC, Tyr 527 in chicken SRC) by the C-terminal SRC kinase (Csk) (Brown and Cooper, 1996). The SH3 and SH2 domains also participate in this negative regulation through intramolecular interactions (Brown and Cooper, 1996; Schindler et al., 1999; Xu et al., 1999; Young et al., 2001).

The SRC and its family member kinases have long been postulated to participate in oncogenic processes. Activated variants of SRC family kinases, including the viral oncoprotein v-SRC, are capable of inducing malignant transformation in a variety of cell types (Parker et al., 1984; Cartwright et al., 1987). Activation of SRC-like kinases recently was described in *BCR-ABL1*-expressing acute lymphoblastic leukemia in mice (Hu et al., 2004). Elevated expression and/or activity of SRC have been documented in several types of primary human tumors (Bolen et al., 1987; Ottenhoff-Kalff et al., 1992; Talamonti et al., 1993). However, for many years, structural abnormalities of the SRC family of kinases have been detected rarely in primary human tumors. Although Irby et al. (1999)

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*Correspondence to: Seishi Ogawa, Department of Hematology and Oncology, Department of Regeneration Medicine for Hematopoiesis, Graduate School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
E-mail: sogawa-tky@umin.ac.jp

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reported that 12% of advanced human colon cancers had a truncating mutation at codon 531 of the *SRC* gene, determining the importance of this mutation in the generation of colorectal cancers remained elusive according to the negative results in subsequent reports (Daigo et al., 1999; Wang et al., 2000; Laghi et al., 2001). In primary hematopoietic malignancies, no studies have demonstrated structural abnormalities of the SRC family of kinases.

In this study, we performed molecular analysis of a t(6;12)(q21;p13) observed as the sole chromosomal abnormality in a case of acute myelogenous leukemia (AML) and identified a SRC-like tyrosine kinase gene, *FRK* (Fyn-related kinase or *Rak*), on 6q21 (Cance et al., 1994; Lee et al., 1994) that is fused with *ETV6* (also called *TEL*), a gene frequently involved in chromosomal translocations in a variety of human leukemias (Golub et al., 1997). We found that the resultant chimeric protein, ETV6/FRK, is a transforming oncoprotein with elevated kinase activity. We also demonstrated that ETV6/FRK inhibits ETV6-mediated transcriptional repression in a dominant-negative manner, indicating that ETV6/FRK is a unique oncoprotein with dual functions. This is the first report showing the involvement of a SRC-like kinase gene (*FRK*) in primary human cancers.

MATERIALS AND METHODS

Case History

The patient was a 69-year-old Japanese woman with AML-M4, carrying the translocation t(6;12)(q21;p13) as the sole chromosomal abnormality in 8 of 20 examined bone marrow metaphase cells. After obtaining informed consent, a sample of her bone marrow was taken for use in this study. The patient did not respond to chemotherapy and died 5 months later.

Fluorescence In Situ Hybridization Analysis

Fluorescence in situ hybridization (FISH) analysis was performed as previously described (Pinkel et al., 1986) with a panel of biotin- and digoxigenin-labeled cosmid probes that contained different exons of *ETV6*, kindly provided by Dr. Peter Marynen (University of Leuven, Leuven, Belgium). The order and the relative locations of cosmids are depicted in Figure 1A.

3′-Rapid Amplification of cDNA End

To do the 3′-rapid amplification of cDNA end (RACE), total RNA was isolated from the leukemic sample as described previously (Ogawa et al., 1996). First-strand cDNA was synthesized from 2.5 μg of total RNA using the primer R2N6 as described previously by Peeters et al. (1997). The first polymerase chain reaction (PCR) was performed with primers T4F1 and R2N6R1 (Peeters et al., 1997). Then, a diluted product of the first PCR, along with primers T4F2 and R2N6R2, was used for the second, nested PCR (Peeters et al., 1997). The nucleotide sequences of the primers used in this study and the conditions for PCR are listed in Table 1. The PCR products were subcloned into the pCR® 2.1-TOPO® vector using a TOPO TA Cloning® kit (Invitrogen, Tokyo, Japan) and subjected to DNA sequencing by use of a 3100 Applied Biosystems automated sequencer (Applied Biosystems, Chiba, Japan).

Reverse Transcriptase–PCR

For the reverse transcriptase-PCR (RT-PCR), 5 μg of the total RNA was transcribed to cDNA with 2 units of Moloney murine leukemia virus reverse-transcriptase (MMLV-RT, Stratagene, La Jolla, CA) using a random hexamer. One-tenth of the synthesized cDNA was directed to PCR analysis. Primers T4F2 and FRK1198R were used to confirm the *ETV6/FRK* transcripts. The primers for detecting the reciprocal *FRK/ETV6* transcripts were FRK451F and TEL723R. For amplification of the wild-type *ETV6* and *FRK* transcripts, primers T4F2 and TEL723R and primers FRK808F and FRK1198R, respectively, were used. All the sequences of the RT-PCR products were verified by direct sequencing.

Plasmid Construction

Full-length *ETV6* cDNA tagged with a FLAG sequence at the 5′ end, a gift from Dr. Kinuko Mitani (Dokkyo University School of Medicine, Tochigi, Japan), was subcloned into the expression plasmid pME18S-neo (Invitrogen, San Diego, CA). A FLAG-tagged full-length *FRK* cDNA was isolated by RT-PCR from total RNA obtained from human placenta using primers *Eco*RI-FLAG-FRK and FRK-*Not*I-2058R and was cloned into pME18S-neo. The pME18S-neo-FLAG-ETV6/FRK vector was generated by replacement of the *Cla*I-*Not*I fragment of the pME18S-neo-FLAG-ETV6 vector with the *Cla*I-*Not*I fragment of *ETV6/FRK*, which was obtained by RT-PCR from the patient's bone marrow using primers TEL-*Cla*I-F and FRK-*Not*I-2058R, with subsequent digestion with *Cla*I and *Not*I. To construct a kinase-inactive mutant of ETV6/FRK, designated ETV6/FRK(K262R), a point mutation corresponding

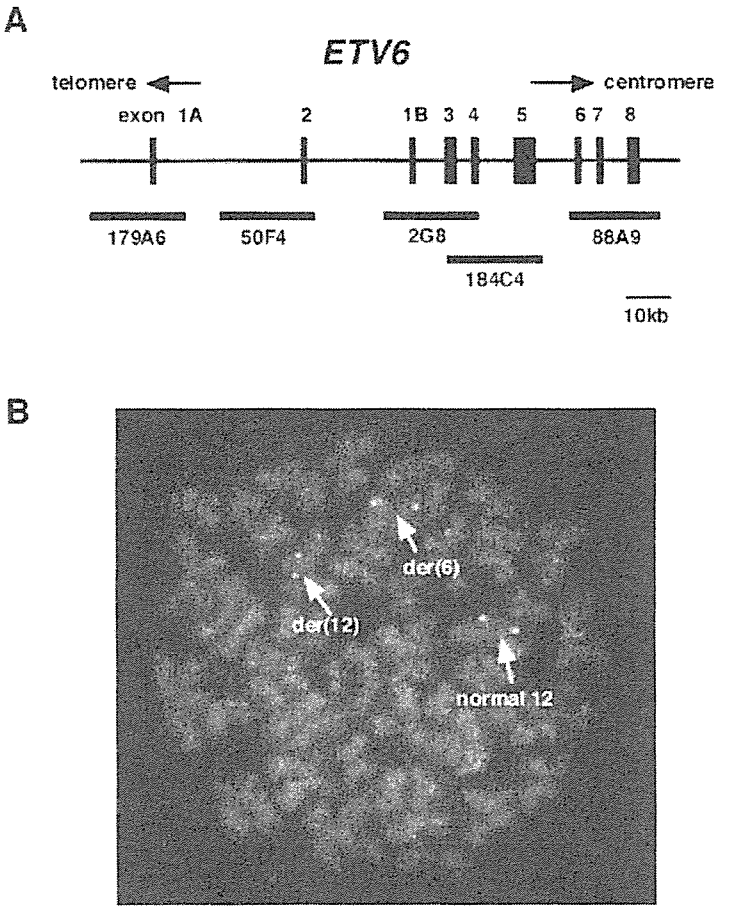

Figure 1. Analysis of breakpoint on chromosome 12. (A) A genomic map of *ETV6* and location of the cosmid probes used for FISH analysis. (B) FISH analysis of the patient's leukemic cells. The signals of the 2G8 probe (red) containing *ETV6* exons 1B, 3, and 4 are hybridized on the der(6) and on the normal 12p, whereas those of the 184C4 probe (green) containing *ETV6* exons 3–5 are found on the der(6), the der(12), and the normal 12p.

to a kinase-inactivating mutation in the ATP-binding site lysine residue (Lys262) of FRK was introduced into *ETV6/FRK* cDNA. A mutated fragment generated by PCR using the mutagenic primer FRK-K262R-*Bam*HI and the primer TEL-*Eco*RI-FLAG was spliced together with a C-terminal partial fragment of *FRK* into pME18S-neo. A FLAG-tagged full-length *FRK/ETV6* cDNA was constructed into the pME18S-neo vector by assembling partial fragments from *ETV6* and *FRK* and a fragment spanning the *FRK/ETV6* junction generated by RT-PCR using primers FRK451F and TEL723R. All the constructs were sequenced to confirm the fidelity of the sequence and conservation of the reading frame at the site of fusion.

Cell Lines, Transfection, and Cell Transformation Studies

For transient expression studies, 4×10^4 HeLa cells were seeded in each 60-mm dish and transfected with expression plasmid or plasmids 24 hr later by a lipofection method using Effectine™ Transfection Reagent (Qiagen, Hilden, Germany). Cells were incubated for 48 hr and harvested for analysis. NIH3T3 cells were transfected with expression plasmids, also using Effectine™, and selected in 400 μg/ml of G418 for 2 weeks. Ba/F3 clones stably expressing ETV6/FRK or other proteins were obtained by electroporation of each expression plasmid into Ba/F3 cells as previously described (Carroll et al., 1996) and subsequent isolation of individual G418-resistant subclones by limiting dilution. Expression of the transfected genes was evaluated by immunoblotting as previously described (Maki et al., 1999) using anti-FLAG-M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO). The soft-agar colony assay was performed as previously described (Kurokawa et al., 1996). After 21 days, all macroscopic colonies larger than 0.25 mm in diameter were counted. For growth curves, 2×10^4 G418-resistant Ba/F3 cells were washed 3 times with PBS and plated in IL-3-free medium on day 0, and viable cells were counted each day by trypan blue exclusion.

TABLE 1. Primers Used For 3′-RACE and (RT)-PCR Amplifications

Name	Sequence
R2N6	5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC(N)6-3′
T4F1	5′-CATATTCTGAAGCAGAGGAAA-3′
R2N6R1	5′-CCAGTGAGCAGAGTGACG-3′
T4F2	5′-ACACAGCCGGAGGTCATACT-3′
R2N6R2	5′-GAGGACTCGAGCTCAAGC-3′
FRK1198R	5′-CTTCCCATACTTCGCAAAC-3′
FRK451F	5′-AGCAACATCTGTCAGAGGCT-3′
TEL723R	5′-GTAGGACTCCTGGTGGTTGTT-3′
FRK808F	5′-ATCGGAAGATCAGATGCAGAG-3′
*Eco*RI-FLAG-FRK	5′-GCGAATTCGTTGTGATGGGGGACTACAAGGACGAC GATGACAAGTCCGGGAGCAACATCTGTCAGAGGCT-3′
FRK-*Not*I-2058R	5′-ATTGCGGCCGCACTGATTGTGCAGTTGGTTGA-3′
TEL-*Cla*I-F	5′-CTTTCGCTATCGATCTCCTCA-3′
TEL-*Eco*RI-FLAG	5′-GCGAATTCGTTGTGATGGGGGACTACAAGGACGAC GATGACAAGTCCGGGTCTGAGACTCCTGCTCAGTG-3′
FRK-K262R-*Bam*HI	5′-TTGGATCCATTGAACCTGGTTTTAATGTTCTCACTG-3′

Thermal cycling profile was: 94 C for 2 min, followed by 35 cycles of 94 C for 1 min, 60 C for 1 min and 72 C for 2 min, with a final extension at 72 C for 10 min.

Immunoprecipitation, Immunoblotting, and Immune Complex Kinase Assay

Lysates were prepared by washing cells (1×10^6–1×10^7) with phosphate-buffered saline and then adding lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% NP-40, 1 mM EDTA, and 1 mM Na3VO4] containing 5 mM phenylmethyl-sulfonylfluoride and 1 μg/ml of aprotinin. After 10 min on ice, the samples were centrifuged at 12,000 g to remove insoluble particles. For immunoprecipitation, 1 mg of total cell lysate was incubated with anti-FLAG-M2 antibody for 1 hr at 4°C, after which 50 μl of Protein G-Sepharose beads (Amersham Biosciences, Uppsala, Sweden) was added. After rotating for 1 hr at 4°C, immunoprecipitates were washed 3 times and boiled in loading buffer for 5 min. Protein samples were separated on 6.5%–15% gradient SDS-polyacrylamide gels and transferred onto PVDF membranes (Millipore, Bedford, MA). Immunoblotting was performed as previously described (Maki et al., 1999) using either anti-FLAG-M2 antibody or antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology Incorporated, Lake Placid, NY) as a primary antibody.

For the immune complex kinase assay, immunoprecipitates were washed 3 times and suspended in kinase buffer [40 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 5 mM $MnCl_2$]. For determination of kinase activity, 2.5 μg of either histone H2B or histone H4 (Roche Diagnostics K. K., Tokyo, Japan) was added to each reaction. Kinase reactions were initiated by the addition of 10 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol; Amersham Biosciences Corp., Piscataway, NJ) and incubated at 30°C for 15 min. Reactions were stopped by the addition of loading buffer and analyzed by SDS-PAGE and exposure to a film.

Luciferase Assay

For the luciferase assay, 4×10^4 HeLa cells were transfected with 1 μg of the reporter plasmid (EBS)3tkLuc (Waga et al., 2003), a kind gift of Dr. Kinuko Mitani, along with the indicated amounts of the expression vectors. The total amount of DNA in weight was adjusted to be equal by adding pME18S-neo plasmid. Luciferase activities were determined as described previously (Maki et al., 1999). All transfection experiments were performed in duplicate at least 3 times.

RESULTS

Identification of the Breakpoint on Chromosome 12

We performed FISH experiments using several probes from the *ETV6* locus, on 12p13 (Fig. 1A). The signals from the cosmids containing exons 1–4 (179A6, 50F4, and 2G8) were found on the der(6) (Fig. 1B), whereas the signals from the cosmid containing exons 3–5 (184C4) were split to the der(6) and the der(12) (Fig. 1B), suggesting that the breakpoint on 12p13 was localized to *ETV6* exons 4–5. The signals on the normal 12p were always observed with all the indicated cosmid probes of the *ETV6* locus, suggesting that the non-

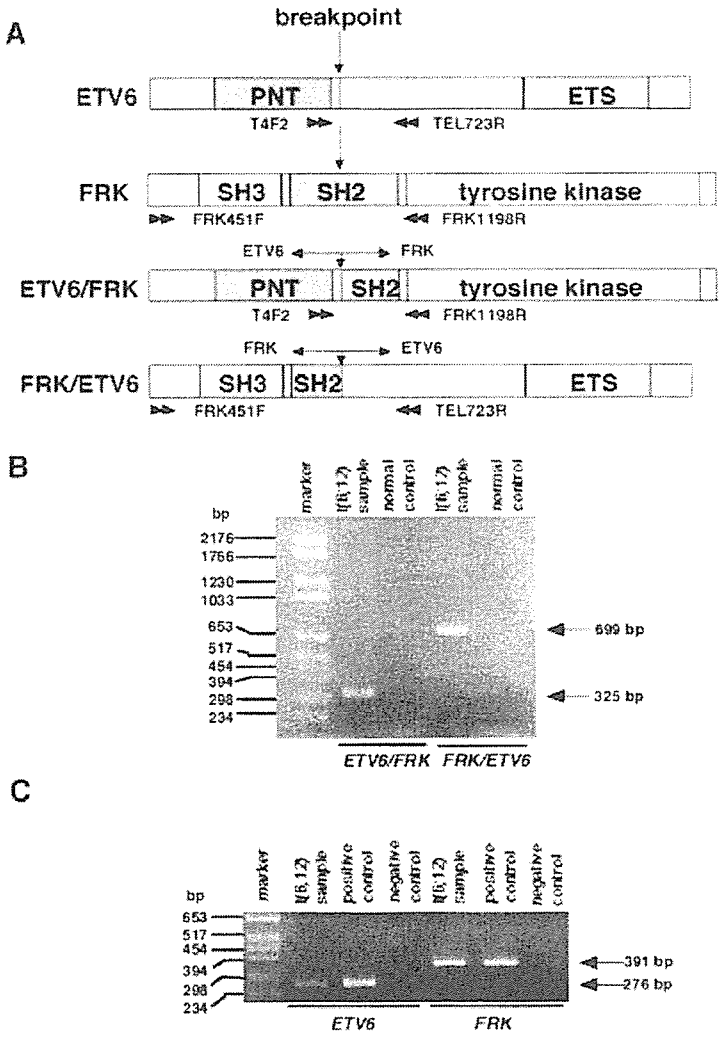

Figure 2. Identification of *ETV6/FRK* and *FRK/ETV6* fusion transcripts. (A) Schematic representation of wild-type *ETV6*, *FRK*, and the fusion transcripts. The breakpoints are indicated by vertical arrows. Horizontal arrows indicate the positions of RT-PCR primers (described in the Materials and Methods section). (B) Detection of *ETV6/FRK* as well as *FRK/ETV6* fusion transcripts by RT-PCR in the patient's leukemic sample. (C) Expression of *ETV6* and *FRK* in the patient's leukemic sample by RT-PCR.

translocated allele of *ETV6* was grossly intact with no large deletions.

Identification of the Fusion Partner of *ETV6*

To identify the unknown fusion partner of *ETV6*, 3′-RACE-PCR was performed. After two rounds of PCR, 3′-RACE-PCR products were successfully obtained. Sequencing analysis of the PCR products showed that exon 4 of *ETV6* was fused to exon 3 of *FRK* on 6q21, creating an *ETV6/FRK* fusion gene. The *FRK* gene encodes a SRC-like nonreceptor tyrosine kinase, consisting of the N-terminal SH3 and SH2 domains, the C-terminal kinase domain, and a short regulatory tail (Fig. 2A). The *ETV6/FRK* fusion gene produced a chimeric protein in which the entire pointed (PNT) oligomerization domain (also called helix–loop–helix domain) of ETV6 and the kinase domain of FRK were fused in-frame (Fig. 2A).

Detection of the *ETV6/FRK* and *FRK/ETV6* Fusion Transcripts

RT-PCR analysis was performed to confirm the fusion transcripts of the *ETV6* and *FRK* genes. Both reciprocal fusion transcripts, *ETV6/FRK* and *FRK/ETV6*, were specifically amplified from the leukemic sample but not from control bone marrow (Fig. 2B). Expression of wild-type *ETV6* and *FRK* also was detected in the leukemic sample (Fig. 2C). There were no mutations in the entire coding sequences of *ETV6*, *FRK*, *ETV6/FRK*, and *FRK/ETV6* (data not shown).

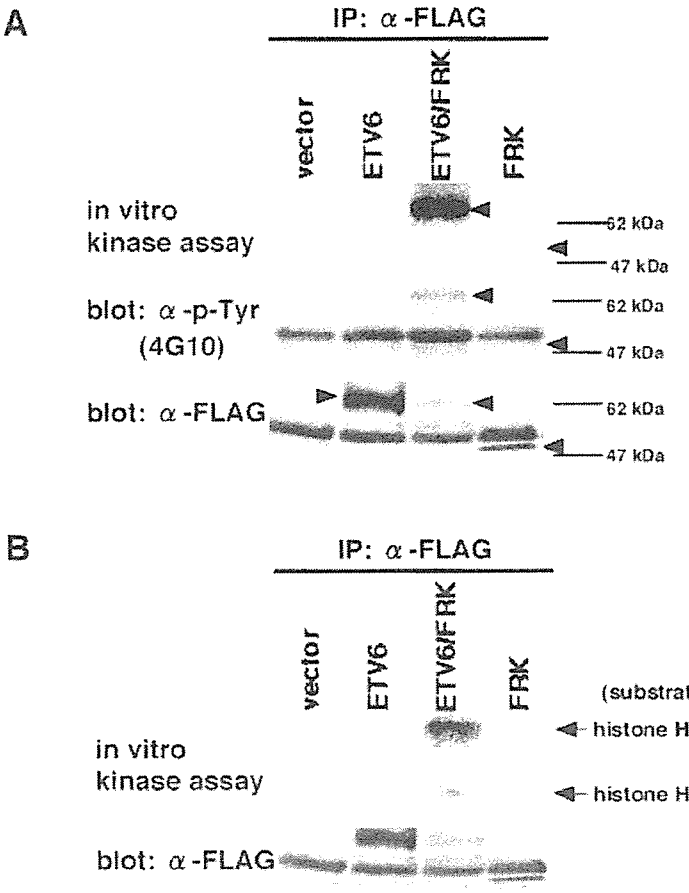

Figure 3. The ETV6/FRK tyrosine kinase is constitutively activated in HeLa cells. (A) Lysates of HeLa cells transfected with the indicated expression vectors were immunoprecipitated with an anti-FLAG-M2 monoclonal antibody and then analyzed by immune complex kinase assay (top) or immunoblotting with an antiphosphotyrosine antibody 4G10 (middle). The total amount of each protein was also assessed by immunoblotting with anti-FLAG-M2 antibody (bottom). Arrowheads show the proteins expressed or phosphorylated at an expected size. (B) Results of kinase assay performed with histones H2B (top) and H4 (middle).

Constitutive Activation of the ETV6/FRK Tyrosine Kinase

Because the ETV6/FRK fusion protein retained the kinase domain but lacked the SH3 domain and most of the SH2 domain, we examined its kinase activity. First, we compared the autophosphorylation status of ETV6/FRK and wild-type FRK. Either the ETV6/FRK fusion protein, wild-type FRK, or wild-type ETV6 FLAG-tagged at the N-terminus was introduced into HeLa cells, immunoprecipitated with an anti-FLAG-M2 monoclonal antibody, and then analyzed by the kinase assay or immunoblotting with an antiphosphotyrosine antibody 4G10 (Fig. 3A, top and middle). To compare expression levels, the same amounts of immunoprecipitate were also subjected to anti-FLAG blot (Fig. 3A, bottom). A high level of tyrosine phosphorylation occurred only in the ETV6/FRK protein (Fig. 3A, top and middle). A basal level of autophosphorylation also was detectable in the wild-type FRK (Fig. 3A, top), a finding in agreement with the previous data (Cance et al., 1994). However, the level of autophosphorylation was significantly lower than that of ETV6/FRK (Fig. 3A, top and middle). Next, we compared the ability of ETV6/FRK and wild-type FRK to phosphorylate exogenous substrates. When histone H2B or H4 was added to the kinase reaction, they were found to be phosphorylated to a greater extent in ETV6/FRK-expressing cells than in FRK-expressing cells (Fig. 3B), suggesting that the ETV6/FRK protein had elevated tyrosine kinase activity.

Cell Transformation by ETV6/FRK in a Kinase-Dependent Manner

To assay the transforming activities of ETV6/FRK, we stably expressed the cDNA-encoding ETV6/FRK or other proteins into the fibroblast cell line NIH3T3. We established 3 NIH3T3 clones expressing ETV6/FRK, 2 clones expressing FRK/ETV6, 2 clones expressing FRK, 2 clones expressing ETV6, and 2 clones expressing ETV6/FRK(K262R) (Fig. 4A), the kinase-inactive

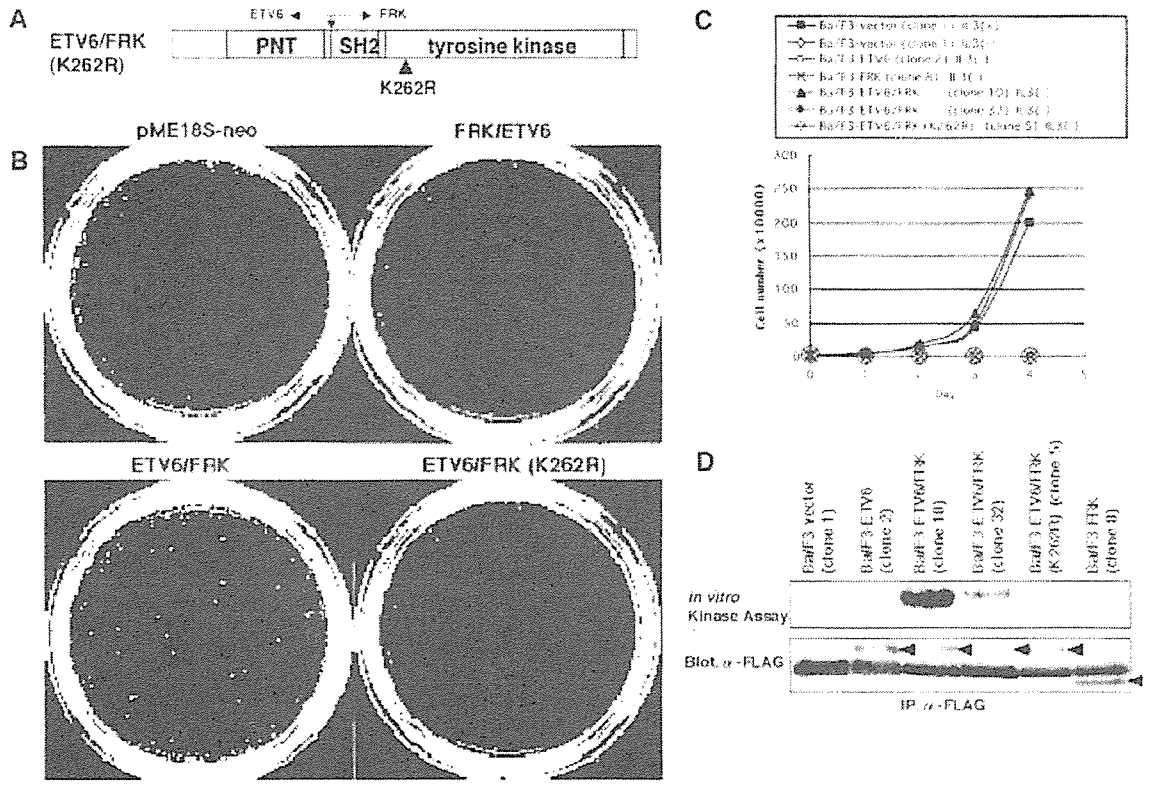

Figure 4. ETV6/FRK transforms NIH3T3 cells and Ba/F3 cells in a kinase-dependent manner. (A) Schematic representation of the kinase-inactive ETV6/FRK(K562R) mutant with a lysine-to-arginine mutation at the ATP binding site. (B) Soft-agar assay demonstrating macroscopic colony formation in ETV6/FRK-expressing NIH3T3 cells. (C) 2 × 10^4 Ba/F3 cells stably transfected with the indicated expression vectors were washed free of IL-3 and plated on day 0 in growth medium without IL-3. Viable cells were counted each day. Data of the representative clone(s) for each protein are presented. (D) Cell lysates of the indicated Ba/F3 clones were immunoprecipitated with an anti-FLAG-M2 antibody and then subjected to kinase assay (top) and immunoblotting with anti-FLAG-M2 antibody (bottom). Arrowheads show the proteins expressed at an expected size.

mutant of ETV6/FRK, confirmed by immunoblotting analysis (data not shown). The soft-agar assay was performed on each clone. Comparable results were obtained for the clones expressing the same proteins, and the representative data are presented. Only the NIH3T3 cells expressing intact ETV6/FRK were able to produce macroscopic colonies, whereas the NIH3T3 cells transfected with the empty vector or cells expressing the kinase-inactive mutant ETV6/FRK(K262R), the reciprocal FRK/ETV6 fusion protein, wild-type FRK, or wild-type ETV6 failed to grow colonies (Fig. 4B, Table 2). These results suggest that ETV6/FRK but not FRK/ETV6 contributes to neoplastic transformation in a kinase-dependent manner.

Next, we also examined the ability of ETV6/FRK to transform the murine hematopoietic cell line Ba/F3, which is strictly dependent on IL-3 for survival and proliferation. Following stable transduction by electroporation, we obtained 6 Ba/F3 clones expressing ETV6/FRK, 2 clones expressing FRK, 2 clones expressing ETV6, and 3 clones expressing ETV6/FRK(K262R), confirmed by immunoblotting analysis (data not shown). To assay the ability to confer independent proliferation of IL-3, each Ba/F3 clone was switched to growth medium without IL-3. Comparable results were obtained for the clones expressing the same proteins, and the representative data are presented. The Ba/F3 clones expressing ETV6/FRK showed sustained proliferation in the absence of IL-3 (Fig. 4C). In contrast, Ba/F3 cells transfected with the empty vector or cells expressing kinase-inactive mutant ETV6/FRK(K262R), wild-type FRK, and wild-type ETV6 were all unable to proliferate in the absence of IL-3 (Fig. 4C). Although the ETV6/FRK proteins expressed in the stable clones were constitutively autophosphorylated, the ETV6/FRK(K262R) mutants were not (Fig. 4D). These observations indicate that ETV6/FRK is a dominant oncoprotein and that constitutive activa-

TABLE 2. Transformation of NIH3T3 Cells By ETV6/FRK

Transfected DNA	No. of colonies[a]
pME18S-neo (vector)	0
pME18S-neo-ETV6	0
pME18S-neo-FRK	0
pME18S-neo-ETV6/FRK	15
pME18S-neo-ETV6/FRK(K262R)	0
pME18S-neo-FRK/ETV6	0

NIH3T3 cells were transfected with the indicated constructs, and stable transfectants were selected in G418. Cells were plated in soft agar. Macroscopic colonies were counted at day 21.
[a]Average of four experiments.

tion of the ETV6/FRK tyrosine kinase is necessary for ETV6/FRK-induced transformation.

Inhibition of ETV6-Mediated Transcription Repression by ETV6/FRK

Because ETV6 is an ETS transcription factor that acts as a transcriptional repressor (Lopez et al., 1999), we also investigated the transcriptional regulatory property of ETV6/FRK and its ability to modulate the function of wild-type ETV6. We transfected a previously described (EBS)3tkLuc reporter, in which the luciferase gene is placed under the control of an ETS responsive promoter (Waga et al., 2003), along with either wild-type ETV6, ETV6/FRK, or FRK/ETV6 into HeLa cells and evaluated luciferase activity. The results showed, in agreement with the previous finding (Waga et al., 2003), that there was decreased luciferase activity after cotransfection of (EBS)3tkLuc with the wild-type ETV6 expression plasmid (Fig. 5A). In contrast, no repression was observed when ETV6/FRK or FRK/ETV6 was expressed with the (EBS)3tkLuc reporter (Fig. 5A).

Because the oncoprotein ETV6/FRK lacks the ETS DNA binding site but still retains the PNT oligomerization domain, it is possible that it might affect ETV6-mediated transcriptional repression by heterodimerizing with ETV6. Notably, coexpression of ETV6/FRK abolished the transcriptional repression by ETV6 in a dose-dependent manner (Fig. 5B), suggesting that ETV6/FRK has a dominant-negative effect on ETV6-mediated transcriptional repression. In contrast, coexpression of the reciprocal FRK/ETV6 protein did not affect ETV6-mediated transcriptional repression (Fig. 5B). In control experiments, dose-dependent expression of the ETV6, ETV6/FRK, or FRK/ETV6 protein was confirmed by immunoblotting analysis (data not shown).

DISCUSSION

The t(6;12)(q21;p13) is a rare but recurrent reciprocal chromosome translocation in human leukemia (Hayashi et al., 1990; Katz et al., 1991; Raimondi et al., 1997). In this article, we report our finding that it generated novel fusion genes *ETV6/FRK* and *FRK/ETV6* in a case of AML. FRK belongs to a family of SRC kinases, as at the amino acid level, it has the highest homology, 50%, with FYN (Cance et al., 1994; Lee et al., 1994). Although several tyrosine kinase (TK) genes have been identified as fusion partners of *ETV6* (Golub et al., 1994; Papadopoulous et al., 1995; Lacronique et al., 1997; Peeters et al., 1997; Cazzaniga et al., 1999; Eguchi et al., 1999; Iijima et al., 2000; Kuno et al., 2001), this is the first report of a SRC-family tyrosine kinase gene being fused with *ETV6* and structurally altered in human cancers. In the resultant ETV6/FRK fusion protein, the entire PNT oligomerization domain of ETV6 and the kinase domain of FRK are fused in frame. We demonstrated that this ETV6/FRK fusion protein constitutively underwent autophosphorylation on its tyrosine residues. ETV6/FRK had elevated kinase activity compared to that in wild-type FRK. ETV6/FRK showed transforming activities in two cell lines, Ba/F3 and NIH3T3, indicating that ETV6/FRK is a dominant transforming oncoprotein. The kinase-inactive mutant ETV6/FRK(K262R) transformed neither of these two cell lines, indicating that the kinase activity of ETV6/FRK was essential for transformation. The reciprocal fusion protein FRK/ETV6, whose mRNA also was transcribed in the patient sample, did not have transforming activity. These data strongly suggest that the elevated kinase activity of the ETV6/FRK fusion protein directly contributes to the pathogenesis of leukemia with a t(6;12)(q21;p13).

Although activated variants of the SRC family kinases show transforming activities (Parker et al., 1984; Cartwright et al., 1987), the *SRC* and its family of genes rarely have been reported as being mutated or structurally altered in primary human tumors. Irby et al. (1999) reported that 12% of advanced human colon cancers in the United States had a truncating mutation at codon 531 of the *SRC* gene and that the mutation elevated kinase activity and promoted the potential for malignancy. However, three subsequent large-scale studies on advanced colorectal cancers in Japanese, northern European, Chinese, and Italian patients failed to detect the mutation (Daigo et al., 1999; Wang et al., 2000; Laghi et al., 2001), making the

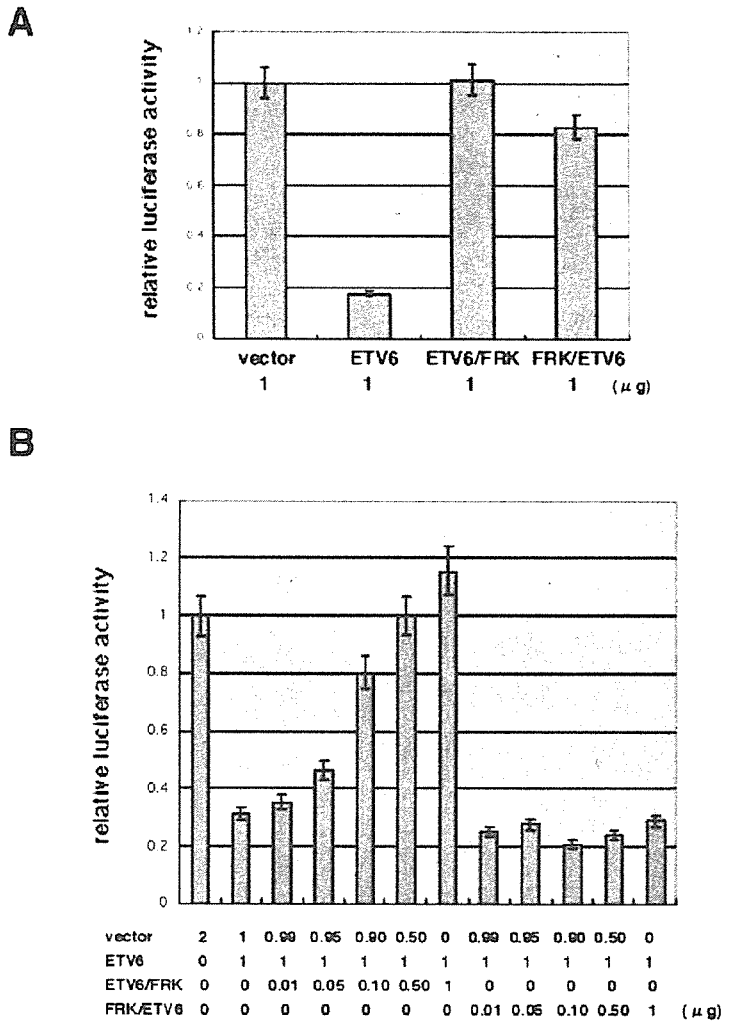

Figure 5. ETV6/FRK is a dominant-negative regulator of ETV6-mediated transcriptional repression in HeLa cells. (A) HeLa cells were transfected with 1 μg of (EBS)3tkLuc reporter plasmid along with 1 μg of the indicated expression vector. Bars show relative luciferase activities to the level when a control plasmid pME18S-neo was cotransfected with the corresponding reporter plasmid, and they present average results of duplicate experiments. (B) HeLa cells were transfected with 1 μg of (EBS)3tkLuc reporter plasmid along with 1 μg of pME-18S-neo-FLAG-ETV6 expression vector together with indicated amounts of pME18S-neo-FLAG-ETV6/FRK or pME18S-neo-FLAG-FRK/ETV6 expression vector. The results are presented as relative luciferase activities.

importance of this mutation controversial. In hematopoietic malignancies, two human T-cell acute lymphoblastic leukemia cell lines have been shown to have rearrangement of *LCK*, a SRC-family kinase gene (Tycko et al., 1991; Wright et al., 1994). In these two cell lines, HSB-2 and SUP-T12, the upstream promoter of the *LCK* gene was juxtaposed to the *TCRB* locus without any accompanying large structural abnormality of the LCK protein. *LCK* mRNA was elevated in the two cell lines (Tycko et al., 1991), and the HSB-2 cell line was later shown to carry several activating point mutations in the *LCK* gene (Wright et al., 1994), indicating that overexpression and/or activation of the LCK kinase would lead to cell transformation. On the other hand, the involvement of SRC family members in primary leukemia has not been reported previously. In this study, we showed that the structural abnormality of an SRC-like kinase gene, *FRK*, through translocation with *ETV6* can directly contribute to leukemogenesis through activation of the altered tyrosine kinase. In addition to the analysis of the current case with a t(6;12), we also performed a mutation analysis of the *FRK* gene in 20 hematopoietic cell lines but failed to detect activating mutations or structural abnormalities (data not shown). Thus, it is currently unclear whether FRK could be activated through other mechanisms such as activating mutations or translocations with other partner gene(s), although more intensive analyses may be required.

Two mechanisms could contribute to the constitutive activation of the ETV6/FRK kinase. First, in the ETV6/FRK fusion protein, the SH3 and SH2 domains of FRK are lost or disrupted, respec-

tively. Both the SH2 and SH3 domains are required to maintain the SRC family kinases in an inactive state: the SH2 domain binds to the C-terminal tyrosine residue in a phosphorylation-dependent manner, and the SH3 domain interacts with a short polyproline type II helix located between the SH2 domain and the kinase domain (Schindler et al., 1999; Xu et al., 1999; Young et al., 2001). These intramolecular interactions are believed to lock the molecule in a closed, inactive state, resulting in repression of kinase activity. In this regard, disruption of this closed conformation would activate the SRC family kinases and lead to cell transformation. In fact, some deletions or mutations in either the SH2 or the SH3 domain of SRC have been shown to activate its catalytic and/or transforming activities (Hirai and Varmus, 1990). Thus, the disruption of the SH3 and SH2 domains in ETV6/FRK may contribute to deregulation of kinase activity. Secondly, in the ETV6/FRK fusion protein, the entire PNT domain of ETV6 is fused to the kinase domain of FRK. As is the case with other ETV6/TK fusion proteins (Carroll et al., 1996; Golub et al., 1996; Jousset et al., 1997), the PNT domain would force dimerization of the ETV6/FRK protein and lead to constitutive tyrosine autophosphorylation and activation of the ETV6/FRK kinase.

The downstream signaling pathway mediated by ETV6/FRK still remains to be elucidated. The wild type FRK is expressed primarily in epithelial tissues (Cance et al., 1994), but also weakly in various hematopoietic cell line (data not shown). However, its functions or downstream signaling pathways remain largely unknown, especially in hematopoietic systems. The only known candidate endogenous downstream component of FRK is the SH2-domain adaptor protein SHB. According to recent reports, GTK, a rodent homologue of FRK, induces neurite outgrowth in PC12 cells and insulin stimulated signaling pathways in pancreatic insulin-producing cells via SHB (Anneren et al., 2000; Anneren and Welsh, 2002). In the present study, however, immunoblotting analysis failed to detect expression of the SHB protein in ETV6/FRK-expressing cells (data not shown). Thus, involvement of SHB in transformation by ETV6/FRK remains unclear. We also tested the phosphorylation status of several signaling molecules, including signal transducer and activator of transcription (STAT1, STAT3, STAT5, STAT6, extracellular signal-regulated kinase 1/2 (ERK1/2), P38 mitogen-activated protein kinase (P38 MAPK), phosphatidylinositol 3-kinase (PI3K), and phospholipase C (PLC)-gamma, in ETV6/FRK-expressing cells. However, we failed to detect any aberrant phosphorylation of these molecules in ETV6/FRK-expressing cells in comparison to FRK-expressing cells (data not shown). Future identification of the target substrate of ETV6/FRK might provide a novel insight into the mechanism of ETV6/FRK-induced transformation as well as of wild-type FRK-mediated signal transduction.

Finally, we demonstrated that ETV6/FRK had a dominant-negative effect over ETV6-mediated transcriptional repression. Because ETV6/FRK retains the PNT oligomerization domain of ETV6, ETV6/FRK may interfere with the transcriptional repression activity of ETV6 by heterodimerizing with wild-type ETV6. Our results indicate that ETV6/FRK is a novel oncoprotein with dual functions: deregulated tyrosine kinase activity and a dominant-negative modulation of transcriptional repression by ETV6. Because wild-type ETV6 appears to have tumor-suppressive activity (Romparey et al., 2000), its suppression by ETV6/FRK also could contribute to oncogenesis. It may be possible that ETV6/FRK can contribute to oncogenesis through two independent mechanisms: activation of the ETV6/FRK tyrosine kinase, which would lead to aberrant stimulation of the downstream signaling pathway, and inhibition of the tumor-suppressive functions of ETV6. This model suggests potential strategies for reversion of transformation by ETV6/FRK. Because the kinase-inactive mutant of ETV6/FRK is nontransforming, a specific inhibitor of the SRC family kinases may inhibit transformation by ETV6/FRK. Alternatively, overexpression of wild-type ETV6 also would interfere with the ability of ETV6/FRK to transform cells. Further experiments will explore these possibilities.

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A Prospective Trial to Evaluate the Safety and Efficacy of Pravastatin for the Treatment of Refractory Chronic Graft-Versus-Host Disease

Akiko Hori,[1] Yoshinobu Kanda,[2,4] Susumu Goyama,[2] Yasushi Onishi,[1] Yukiko Komeno,[2] Kinuko Mitani,[3] Yukiko Kishi,[1] Seishi Ogawa,[2] Osamu Imataki,[1] Shigeru Chiba,[2] Rie Kojima,[1] Tamae Hamaki,[1] Michiyo Sakiyama,[1] Masahiro Kami,[1] Atsushi Makimoto,[1] Ryuji Tanosaki,[1] Yoichi Takaue,[1] and Hisamaru Hirai,[2] for the Japan Hematology and Oncology Clinical Study Group

This prospective study evaluates the safety and efficacy of pravastatin for the treatment of chronic graft-versus-host disease (GVHD). We included 18 patients with refractory chronic GVHD. Oral pravastatin was started at 10 mg/day, and the dose was increased up to 40 mg/day in 4 weeks. This maximum dose was administered over 8 weeks. There were no severe adverse events caused by pravastatin. A clinical response was observed in the skin score in two patients, mouth score in five patients, eye score in two patients, liver score in three patients, platelet count score in one patient, and weight loss in two patients. The overall response rate was 28%. Immunophenotypic analyses showed that T-helper (Th)1 cells were dominant in all but one patient before treatment and that the Th1/Th2 ratio tended to be lower in the responders than in the nonresponders. A randomized controlled trial is warranted to evaluate the efficacy of pravastatin against chronic GVHD.

Keywords: Chronic graft-versus-host disease, pravastatin, treatment.

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Chronic graft-versus-host disease (GVHD) is one of the major complications after allogeneic hematopoietic stem-cell transplantation and develops in 25% to 80% of allogeneic transplant recipients (*1–3*). Corticosteroids and cyclosporine are most widely used to treat chronic GVHD, but they have demonstrated limited efficacy.

Pravastatin is a lipid-lowering agent that inhibits 3-hydroxy-3-methylglutaryl-coenzyme A reductase. Recently, the immunosuppressive effect of statins has been highlighted in both clinical and laboratory studies. Pravastatin reduced the incidence of graft rejection after cardiac and kidney transplantation (*4, 5*). Statins also prevented islet allograft rejection in a mouse model (*6*). Two distinct molecular mechanisms of the immunosuppressive effect of statins have recently been proposed. First, statins suppress the induction of major histocompatibility complex-II expression by interferon-gamma on human endothelial cells and macrophages (*7*). Second, statins selectively inhibit the molecular association between leukocyte function antigen-1 and intercellular adhesion molecule-1 (*8*). With these data, we performed a prospective clinical trial to evaluate the safety and efficacy of pravastatin for the treatment of chronic GVHD.

[1] Hematopoietic Stem Cell Transplant Unit, National Cancer Center Hospital, Tokyo, Japan.
[2] Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Tokyo, Japan.
[3] Department of Hematology, Dokkyo University School of Medicine, Tochigi, Japan.
[4] Address correspondence to: Yoshinobu Kanda, M.D., Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: ycanda-tky@umin.ac.jp.
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Patients aged between 20 and 70 years who had refractory pathologically proven chronic GVHD were eligible for the study. Refractory chronic GVHD was defined as chronic GVHD that was not improved by first-line treatment with corticosteroids at more than 0.5 mg/kg or cyclosporine at a therapeutic blood level for at least 2 weeks, or that showed progression during the tapering of first-line treatment. Patients had to demonstrate good hepatic and renal function as defined by serum bilirubin less than 85.5 μmol/L (5 mg/dL), alanine aminotransferase less than 500 IU/L, and serum creatinine less than 176.8 μmol/L (2.0 mg/dL). Patients with myopathy or who were receiving fibrates were excluded to avoid rhabdomyolysis. All of the patients provided their written informed consent. This study was approved by the institutional review board at each participating institution.

Pravastatin was started orally at 10 mg/day. The dose was increased to 20 mg/day after 2 weeks and finally to 40 mg/day after 2 additional weeks with close monitoring for adverse events. The maximum dose was continued over 8 weeks, unless grade 3 or 4 adverse events attributable to pravastatin were observed. Immunosuppressive agents that were being taken at study entry were continued at the same dose. However, once the dose of these immunosuppressive agents was increased or other immunosuppressive agents were added, the patient was withdrawn from the study and considered a nonresponder.

The incidences and severity of adverse events attributable to pravastatin were evaluated according to the National Cancer Institute Common Toxicity Criteria, Version 2.0. To evaluate the efficacy of pravastatin, chronic GVHD was graded at study entry according to Akpek's prognostic model (*9*). Response was evaluated every 2 weeks for 12 weeks after the initiation of treatment as an intent-to-treat basis. Response in individual organs was defined as follows: A marked response was a change from Akpek's code 2 or 3 to code 1, a

good response was a change from code 3 to code 2, and no response was no change in code or progression. An overall response to treatment was defined as a marked or good response in at least one organ, without progression in any other organs. We planned to include 18 patients with target and lower response rates of 40% and 10% and alpha and beta errors of 5% and 10%, respectively.

The trough blood concentrations of cyclosporine or tacrolimus and the peak plasma concentration of pravastatin were measured every 2 weeks to evaluate interaction between pravastatin and these immunosuppressants. Immunologic changes were evaluated at weeks 2, 4, 8, and 12 by quantification of the CD4/CD8 ratio, the T-helper (Th)1/Th2 ratio, and the expression of human leukocyte antigen-DR on T cells, B cells, and monocytes. Immunologic data were compared between responders and nonresponders using a repeated measures analysis of variance after logarithmic transformation.

Eighteen patients with a median age of 44 years (range 20–68 years) were included in the study. There were 14 men and 4 women. The underlying disease was acute myeloblastic leukemia in seven, chronic myeloid leukemia in four, non-Hodgkin's lymphoma in three, acute lymphoblastic leukemia in two, myelodysplastic syndrome in one, and aplastic anemia in one. Thirteen and five patients received grafts from a related or an unrelated donor, respectively. Ten of them demonstrated chronic GVHD of progressive onset. All patients but one demonstrated extensive chronic GVHD before starting pravastatin, and nine patients were receiving prednisolone. The grade of chronic GVHD at study entry according to Akpek's prognostic model is shown in Table 1. Seven patients, 10 patients, and 1 patient were grouped into the low-, intermediate-, and high-risk groups, respectively.

Pravastatin was well tolerated, and no patients developed grade 3 or 4 adverse events attributable to pravastatin. Treatment was discontinued in three patients between 14 and 41 days after starting pravastatin because of unrelated causes, including painful oral chronic GVHD, infection, and interstitial pneumonitis. According to each organ, a response was observed in the skin score in two patients, mouth score in five patients, eye score in two patients, liver score in three patients, and platelet count score in one patient (Table 1). An overall response was seen in five patients (28%). Pravastatin did not act through the interaction with cyclosporine or tacrolimus, because an increase in these blood levels was not observed after the administration of pravastatin (data not shown). The serum pravastatin concentration on day 42 was not different between responders and nonresponders (median 157.5 ng/mL vs. 253.1 ng/mL, $P=0.53$). The serum total cholesterol level significantly decreased from 6.37 mmol/L (standard deviation [SD] 1.79) before treatment to 5.67 mmol/L (SD 1.40, $P=0.0095$) and 4.77 mmol/L (SD 1.99, $P=0.0001$) on days 14 and 84 after starting pravastatin, respectively. The initial cholesterol response (ratio between cholesterol level on day 14 and before treatment) was significantly better in GVHD responders (0.78 vs. 0.95, $P=0.029$).

The Th1/Th2 ratio before the administration of pravastatin was greater than 1.0 in all but one patient. The Th1/Th2 ratio at study entry tended to be lower in responders than in nonresponders and became even lower after pravastatin treatment in responders, but not in nonresponders, although these differences were not statistically significant (Fig. 1, $P=0.22$). The CD4/CD8 ratio and the expression of human leukocyte antigen-DR on T cells, B cells, and monocytes did not change after treatment (data not shown).

This study demonstrated that pravastatin at 40 mg/day can be safely administered in patients with refractory chronic GVHD, including those taking cyclosporine. The overall response of 28% was similar to that with other alternative salvage treatments including tacrolimus, mycophenolate mofetil, thalidomide, and so on (*3*). However, considering the safety profile of pravastatin, it may be worthwhile for patients with chronic GVHD, especially in those with a coexisting infection that precludes severely immunosuppressive treatments. We chose pravastatin among many statins because it is hydrophilic and was considered to be less likely to cause rhabdomyolysis than other lipophilic statins (*10*, *11*). However, atorvastatin, lovastatin, and simvastatin have stronger in vitro immunosuppressive effects than pravastatin, and thus they may also have greater in vivo effects against chronic GVHD (*7*, *8*).

There is some controversy whether human chronic GVHD is a Th1 or Th2 disease. The immunophenotypic analyses in this study clearly showed that Th1 cells were dominant in patients with chronic GVHD. The efficacy of statin against rheumatoid arthritis, a Th1 disease, has been demonstrated clinically (*12*). In a mouse model of chronic and relapsing

TABLE 1. Severity of chronic graft-versus-host disease in each organ and the response to pravastatin

Each organ	Severity code before treatment				Response to treatment				
	1	2	3	NE	Marked	Good	NC	PD	NE
Performance status	16	2	0	0	0	0	18	0	0
Skin and fascia	6	6	4	2	1	1	13	2	1
Mouth	5	11	2	0	3	2	12	1	0
Eye	7	8	3	0	2	0	13	2	1
Liver enzyme	4	5	9	0	2	1	13	2	0
Thrombocytopenia	14	1	3	0	0	1	15	2	0

Overall response		
	Responder	5 (28%)
	Nonresponder	13

NE, not evaluable; NC, no change; PD, progressive disease.

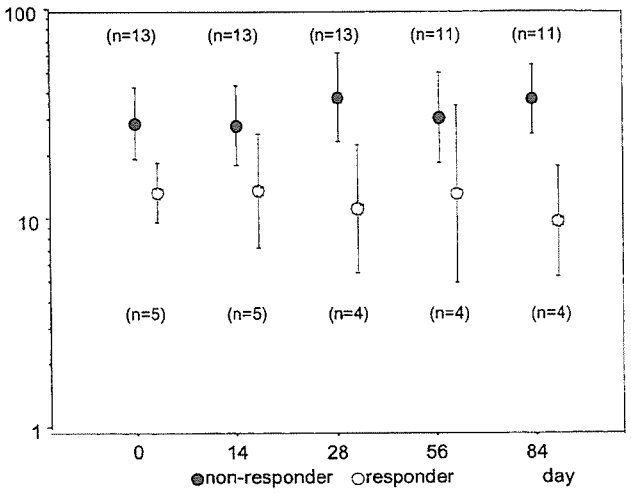

FIGURE 1. Serial changes in the T-helper (Th) 1/Th2 ratio in responders and nonresponders. Data are shown as geometric mean and standard error.

experimental autoimmune encephalomyelitis, oral atorvastatin promoted a Th2 bias and reversed paralysis through the inhibition of STAT4 phosphorylation and the induction of STAT6 phosphorylation (*13*). Although we did not find a statistically significant association between the Th1/Th2 ratio and the response to pravastatin, pravastatin might have ameliorated chronic GVHD by inducing a Th2 shift.

In conclusion, our experience suggests that pravastatin may be safe and effective for the treatment of refractory chronic GVHD. However, a double-blind, randomized, controlled trial is needed to evaluate its true efficacy against refractory chronic GVHD.

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Allografting for older patients

Allogeneic myeloablative transplantation for patients aged 50 years and over

M Yanada[1], N Emi[1], T Naoe[1], H Sakamaki[2], T Iseki[3], N Hirabayashi[4], T Karasuno[5], S Chiba[6], Y Atsuta[7], N Hamajima[7], S Takahashi[3] and S Kato[8]

[1]*Department of Hematology, Nagoya University Graduate School of Medicine, Nagoya, Japan;* [2]*Hematology Division, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan;* [3]*Department of Hematology and Oncology, Institute of Medical Science, University of Tokyo, Tokyo, Japan;* [4]*Department of Hematology, Nagoya Daini Red Cross Hospital, Nagoya, Japan;* [5]*Fifth Department of Internal Medicine, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan;* [6]*Department of Cell Therapy and Transplantation Medicine, University of Tokyo Hospital, Tokyo, Japan;* [7]*Department of Preventive Medicine/ Biostatistics and Medical Decision Making, Nagoya University Graduate School of Medicine, Nagoya, Japan; and* [8]*Department of Cell Transplantation and Regeneration Medicine, Institute of Medical Science, Tokai University School of Medicine, Isehara, Japan*

Summary:

Allogeneic hematopoietic stem cell transplantation (HSCT) has been performed mainly for young patients due to concern about the high incidence of treatment-related mortality (TRM). Recent advances to reduce TRM by using peripheral blood stem cells or nonmyeloablative conditioning regimens have increased the age limit for this procedure, and correctly identifying the indication for transplant is essential for older patients. In this study, we analyzed data from 398 patients aged 50 or over selected from 5147 patients, who received conventional allogeneic HSCT (c-HSCT). Patients aged 50 or older showed inferior outcomes for TRM and overall survival (OS). Mulitivariate analyses confirmed that an age of 50 or over was an independent risk factor for TRM ($P<0.0001$) and OS ($P<0.0001$). Among patients aged 50 or older, increasing age remained an adverse factor for OS ($P=0.0213$). Regimens including total-body irradiation (TBI) correlated with a higher risk of TRM and a lower OS for older patients ($P=0.0095$ and 0.0303, respectively). These findings indicate that allogeneic c-HSCT should be offered to patients over 50 years only if the increased risk of TRM is acceptable, and that a non-TBI regimen is preferable when the transplant is performed.

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Allogeneic hematopoietic stem cell transplantation (HSCT) is now accepted as a curative therapy for patients with hematological malignancies and aplastic anemia. Despite its major potential as a cure, HSCT has been generally limited to younger patients due to concern about the high incidence of treatment-related mortality (TRM) for older patients. Recent advances have contributed to expanding its application. The use of peripheral blood (PB) cells instead of harvested bone marrow (BM) as a source of hematopoietic stem cells has been demonstrated to produce more rapid hematological recovery,[1,3] and has the advantage of a reduced risk of infectious complications during the early post transplant period. Moreover, development of nonmyeloablative transplantation (NST), a novel approach to reduce the intensity of conditioning regimens, has allowed older patients unsuitable for conventional HSCT (c-HSCT) to become transplant candidates.[4–6] These developments have made it necessary to decide on the appropriate option for older patients, especially those over 50 years, not only considering whether allogeneic HSCT should be performed, but also what type of transplant. The NST approach is now being investigated, but the effectiveness and safety of c-HSCT for such patients also needs evaluation. For this purpose, we analyzed data from 5147 patients who underwent allogeneic c-HSCT and whose transplants were reported to the Japan Society for Hematopoietic Cell Transplantation (JSHCT). A total of 398 patients over the age of 50 years were included in this study.

Patients and methods

Patients

The study population consisted of 5147 adult patients, 16 years of age or older, who were reported to the JSHCT as having undergone allogeneic c-HSCT for hematological disorders between January 1991 and December 2001. Patients who had received NST or cord blood transplantation were excluded, as were those who had undergone second or subsequent transplants. Information on surviving patients was updated annually. This study was approved by the Committee for Nationwide Survey Data Management of the JSHCT.

Correspondence: Dr M Yanada, Department of Hematology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan; E-mail: myanada@med.nagoya-u.ac.jp
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Statistical analysis

The primary end point of the analyses was to assess the influences of patient age on TRM and overall survival (OS). The secondary end point was to assess these effects on relapse, the development of grade II–IV acute graft-versus-host disease (GVHD) and extensive chronic GVHD. Whole-population analyses compared patients aged 50 years or older to those younger than 50. The analyses of the subgroup including only patients aged over 50 were performed using age as a continuous variable. OS was defined as time from the day of transplant to death or last follow-up. Relapse was defined as hematological recurrence, and only those with malignant diseases were evaluated. Patients never achieving complete remission (CR) after transplant were considered to have had a recurrence on day 0. TRM was defined as death while in continuous CR. Acute and chronic GVHD were evaluated according to standard criteria.[7,8] Those who died before engraftment were excluded from the analysis of acute GVHD, and those who died before day 100 were excluded from the analysis of chronic GVHD. The Cox proportional hazards regression model was used to evaluate the independent effect of age, sex, years of transplant, disease status, donor type, graft source, conditioning regimen and GVHD prophylaxis. Hazard ratio (HR) was calculated in conjunction with a 95% confidence interval (CI). For disease status, those with hematological malignancies in CR at the time of transplant, those in chronic phase of chronic myeloid leukemia (CML), those with refractory anemia (RA) or refractory anemia with ringed sideroblasts (RARS) of myelodysplastic syndrome (MDS), and all those with nonmalignant diseases were defined as being at standard risk, while those in other situations were defined as being at advanced risk. All donors except for human leukocyte antigen (HLA)-identical siblings were classified as alternative donors. For assessment of the effect of graft source, patients receiving both BM and PB stem cells (2% of the entire population) were excluded. Patients receiving GVHD prophylaxis other than cyclosporine (CSP)- or tacrolimus (FK506)-based regimens (3% of the entire population) were excluded from assessment of the effects of GVHD prophylaxis. Distributions of variables between the two age groups were compared by using the χ^2 test. Kaplan–Meier survival analyses were performed to estimate probability of OS, relapse and TRM. For relapse and TRM, each was used as a censored event for the other. Differences between groups were compared by means of the log-rank test. Stat View 5.0 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Patient characteristics

Patient characteristics are summarized in Table 1. The median age for the entire population was 34 years (range: 16–67). In all, 398 (8%) patients were 50 years of age or older (including 16 patients aged 60 years or older, and 77 patients aged 55–59 years), and 4749 (92%) patients were younger than 50 years. The two populations differed in the distributions of diagnosis, disease status, graft source, donor type and use of methotrexate (MTX). In the older group, the frequency of MDS and non-Hodgkin's lymphoma (NHL) was comparatively high, while that of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) was low. Older patients were more likely to be at advanced risk, to receive PB stem cells, to receive donations from HLA-identical siblings, and to receive no MTX. The distributions of sex, conditioning regimen (total-body irradiation [TBI] regimen *vs* non-TBI regimen) and GVHD prophylaxis (CSP- *vs* FK506-based regimen) were similar for the two groups. As shown in Figure 1, the proportion of older patients undergoing allogeneic c-HSCT has increased over time.

Transplant outcome

The median follow-up period for surviving patients was 1208 days. TRM was significantly higher for the older group than for the younger group (log-rank, $P<0.0001$; Figure 2a). The estimated probabilities were 16.5 *vs* 11.8% at 100 days, and 34.7 *vs* 22.7% at 1 year, respectively. The relapse rate was also higher for the older group than for the younger group, with respective probabilities of 36.4% and 29.7% at 4 years (log-rank, $P=0.0042$; Figure 2b). Consequently, age at transplant strongly correlated with survival (log-rank, $P<0.0001$; Figure 2c). The probability of OS at 4 years was 35.6% for patients 50 years or older, and 53.3% for those under 50 years.

Risk factor analysis

Nine pretransplant parameters were selected to be included for the subsequent multivariate analyses, ie, age group, sex, year of transplant, disease status, donor type, graft source, conditioning regimen and GVHD prophylaxis (CSP- *vs* FK506-based regimen and MTX *vs* no MTX). When the risk of GVHD was analyzed as times to events, patients aged 50 or older tended to be at greater risk of developing grade II–IV acute GVHD and extensive chronic GVHD ($P=0.0886$ and 0.0844, respectively). The results of multivariate analyses for outcome are shown in Table 2. Although the age group had no effect on risk of relapse ($P=0.3165$), being 50 or older was associated with an increased risk of TRM ($P<0.0001$), and had a significantly negative impact on OS ($P<0.0001$). In addition to patient age, transplants performed in the early years, advanced risk disease, donation from alternative donors and omission of MTX were identified as adverse factors for both TRM and OS. Male sex and a CSP-based regimen also correlated with an increased risk of TRM. The risk of relapse was significantly higher among those with advanced risk disease and among those receiving transplants from HLA-identical siblings.

Analysis of the subgroup consisting of patients aged 50 or older

To identify the risk factors and their effect on transplant outcome in older patients, subgroup analyses were

Table 1 Patient characteristics

	Total $n=5147$	*Age* $\geqslant 50$ $n=398$ (8)	*Age* <50 $n=4749$ (92)	*P-value*
Age				
Median	34	52	32	
Range	16–67	50–67	16–49	
Sex				$P=0.4761$
Male	3160 (61)	251 (63)	2909 (61)	
Female	1987 (39)	147 (37)	1840 (39)	
Disease				$P<0.0001$
AML	1427 (28)	91 (23)	1336 (28)	
ALL	1054 (20)	58 (14)	996 (21)	
CML	1334 (26)	94 (24)	1240 (26)	
MDS	539 (10)	82 (21)	457 (10)	
NHL	351 (7)	45 (11)	306 (6)	
AA	297 (6)	10 (3)	287 (6)	
Others	145 (3)	18 (4)	127 (3)	
Disease status				$P<0.0001$
Standard risk	3638 (71)	220 (55)	3418 (72)	
Advanced risk	1509 (29)	178 (45)	1331 (28)	
Graft source				$P<0.0001$[a]
BM	4569 (89)	292 (74)	4277 (90)	
PB	484 (9)	83 (21)	401 (9)	
BM + PB	81 (2)	18 (5)	63 (1)	
Donor type				$P<0.0001$
HLA-identical sibling	3419 (67)	316 (80)	3103 (66)	
Alternative donor	1704 (33)	78 (20)	1626 (34)	
TBI regimen				$P=0.6820$
Y	3385 (66)	245 (62)	3140 (66)	
N	1753 (34)	152 (38)	1601 (34)	
GVHD prophylaxis				$P=0.8692$[b]
CSP-based regimen	4549 (89)	343 (87)	4206 (89)	
FK506-based regimen	437 (8)	32 (8)	405 (9)	
Others	135 (3)	21 (5)	114 (2)	
MTX				P = 0.0049
Y	4746 (93)	353 (89)	4393 (93)	
N	375 (7)	43 (11)	332 (7)	
Year of transplant[c]				$P<0.0001$
1991–1996	2505 (49)	72 (18)	2433 (51)	
1997–2001	2642 (51)	326 (82)	2316 (49)	

AML = acute myeloid leukemia; ALL = acute lymphoblastic leukemia; CML = chronic myeloid leukemia; NHL = non-Hodgkin's lymphoma; AA = aplastic anemia; BM = bone marrow; PB = peripheral blood; HLA = human leukocyte antigen; TBI = total-body irradiation; GVHD = graft-versus-host disease; CSP = cyclosporine; FK506 = tacrolimus; MTX = methotrexate.
Values in parentheses are percentages.
[a]Compared between BM and PB. [b]Compared between CSP and FK506. [c]Details are shown in Figure 1.

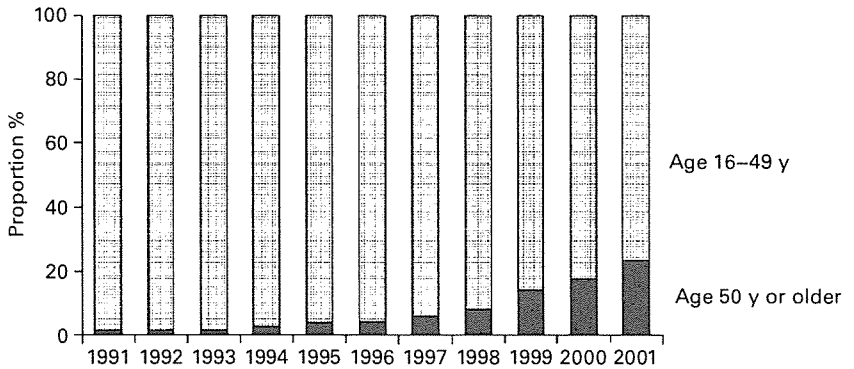

Figure 1 The proportion of patients aged 50 or older undergoing allogeneic myeloablative transplantation. The proportion of patients aged 50 or older among those who received allogeneic conventional transplantation has increased over time between 1991 and 2001.

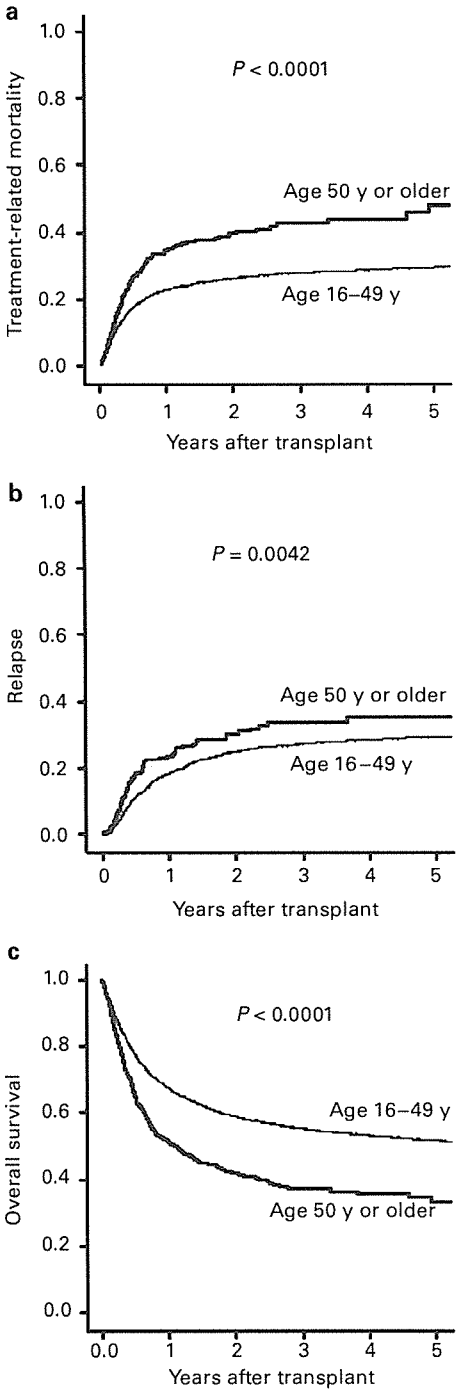

Figure 2 Treatment-related mortality, relapse and overall survival by age group. (**a**) The probability of treatment-related mortality was significantly higher for patients 50 years old or older (log-rank, $P<0.0001$). (**b**) Relapse rate was also higher for the older group (log-rank, $P=0.0042$), but the difference was not significant in multivariate analysis ($P=0.3165$). (**c**) Age at transplant was strongly associated with overall survival (log-rank, $P<0.0001$).

performed for the 398 patients aged 50 or over (Table 3). As described in *Patients and methods*, age at transplant was included in the Cox proportional hazards regression model as a continuous variable. Multivariate analysis demonstrated that increased age remained as an influence on OS for this older population ($P=0.0213$). Advanced risk disease increased the risk of relapse ($P<0.0001$), and correlated with inferior OS ($P<0.0001$). Transplants from alternative donors, omission of MTX and TBI-containing regimen were also independent risks for TRM ($P=0.0429$, 0.0175 and 0.0095, respectively). Kaplan–Meier curves based on the type of conditioning regimen are shown in Figure 3. TBI-containing regimens were associated with a higher probability of TRM without improvement of relapse rate, thus resulting in a significantly lower probability of OS (log-rank, $P=0.0256$ for TRM, $P=0.6448$ for relapse, and $P=0.0255$ for OS). The influence on TRM and OS was of statistical significance in multivariate analysis ($P=0.0095$ for TRM and $P=0.0303$ for OS).

Discussion

Allogeneic HSCT has been preferentially used for younger patients primarily due to concern about the high incidence of treatment-related morbidity and mortality. Generally, advanced age is considered to be a poor prognostic factor following HSCT. The major reasons for this are (1) underlying medical complications or organ dysfunction, (2) delay in drug metabolism potentiating the toxicity of conditioning regimens, and (3) a high incidence of GVHD as reported previously.[9,12] Although the upper age limit for allogeneic HSCT has been set at around 50 years in many institutions, recent advances in HSCT have raised this limit. The use of PB instead of BM as a stem cell source has been demonstrated to reduce the duration of neutropenia,[1,3] which is especially beneficial for older patients exposed to a greater risk of life-threatening infection. Another major advance is the development of NST. The reduced toxicity of conditioning regimens has made it possible for this procedure to be used for older patients ineligible for c-HSCT. Published studies of NST have reported its use for patients in their 60s and even 70s.[4,6] Changes in recent years have expanded the range of treatment options, thus making assessment of c-HSCT for older patients an urgent necessity. In this study, we evaluated 398 older patients who underwent allogeneic c-HSCT and compared their outcomes with those for 4749 patients less than 50 years old. To our knowledge, this is the largest analytical study performed to date of allogeneic HSCT for patients aged 50 or older.

This study demonstrated that age 50 or more was strongly associated with a higher risk of TRM and inferior OS. Univariate analysis showed the effect of age on the incidence of relapse to be statistically significant, but multivariate analysis did not. This is attributable to the fact that advanced risk disease and transplantation from HLA-identical siblings, both of which were independent adverse factors for relapse, were more common among older patients. A study by the International Bone Marrow Transplant Registry (IBMTR) analyzed a total of 2180 leukemia patients, including 80 patients aged 50 or older, who had received allogeneic HSCT from HLA-identical siblings.[13] Patients were divided into four age groups and

Table 2 Hazard ratios and 95% confidence intervals of risk factors with statistical significance in multivariate analysis

	HR (95% CI)	*P-value*	*Adverse factor*
Treatment-related mortality			
Donor type	2.38 (2.10–2.69)	$P<0.0001$	Alternative donor
Disease status	1.97 (1.74–2.23)	$P<0.0001$	Advanced risk
Age	1.70 (1.38–2.09)	$P<0.0001$	Age $\geqslant 50$
Year of transplant	1.05 (1.02–1.07)[a]	$P=0.0003$	Early years
MTX	1.48 (1.18–1.86)	$P=0.0003$	No MTX
Sex	1.15 (1.02–1.30)	$P=0.0216$	Male
GVHD prophylaxis	1.26 (1.03–1.55)	$P=0.0286$	CSP-based regimen
Relapse			
Disease status	3.82 (3.35–4.34)	$P<0.0001$	Advanced risk
Donor type	1.18 (1.01–1.38)	$P=0.0341$	HLA-identical sibling
Overall survival			
Disease status	2.72 (2.49–2.97)	$P<0.0001$	Advanced risk
Donor type	1.59 (1.45–1.75)	$P<0.0001$	Alternative donor
Age	1.51 (1.30–1.77)	$P<0.0001$	Age $\geqslant 50$
Year of transplant	1.04 (1.02–1.06)[a]	$P<0.0001$	Early years
MTX	1.24 (1.04–1.47)	$P=0.0161$	No MTX

HR = hazard ratio; 95% CI = 95% confidence interval; MTX = methotrexate; GVHD = graft-versus-host disease; CSP = cyclosporine.
HR corresponds to risk of death or relapse.
[a]Modeled as a continuous variable; hazard ratio is shown per year.

Table 3 Hazard ratios and 95% confidence intervals of risk factors for patients aged 50 or older with statistical significance in multivariate analysis

	HR (95% CI)	*P-value*	*Adverse factor*
Treatment-related mortality			
Conditioning regimen	1.80 (1.15–2.80)	$P=0.0095$	TBI
MTX	2.23 (1.15–4.31)	$P=0.0175$	No MTX
Donor type	1.62 (1.02–2.60)	$P=0.0429$	Alternative donor
Relapse			
Disease status	2.53 (1.59–4.03)	$P< 0.0001$	Advanced risk
Overall survival			
Disease status	1.84 (1.38–2.46)	$P< 0.0001$	Advanced risk
Age	1.06 (1.01–1.11)[a]	$P=0.0213$	Older age
Conditioning regimen	1.41 (1.03–1.93)	$P=0.0303$	TBI

HR = hazard ratio; 95% CI = 95% confidence interval; TBI = total-body irradiation; MTX = methotrexate; HR corresponds to risk of death or relapse.
[a]Modeled as a continuous variable; hazard ratio is shown per year.

those with advanced leukemia and aged 45 or older showed a slightly higher risk of TRM, but no difference in leukemia-free survival was observed among age groups. Another study of a large series was that reported by the European Group for Bone Marrow Transplantation (EBMT), which compared 192 patients over 40 with 1119 younger patients.[14] Only those with AML and aged 45 or older were at higher risk of TRM but without any difference in terms of OS. These data as well as ours should be interpreted with caution because patients actually undergoing allogeneic HSCT are thought to be highly selected. However, in our study covering the largest number of patients to date, it should be emphasized that even highly selected patients showed poorer prognosis. It is in accordance with previously reported findings that the incidence of GVHD was higher in older patients,[9 12] which almost reached statistical significance in our study. The higher incidence of GVHD has been proposed as one of the causes contributing to the higher risk of TRM.

Among patients aged over 50 years, increasing age remained an adverse factor for OS. As expected, advanced risk disease was associated with an inferior outcome in terms of relapse and OS. Notably, patients receiving a TBI-containing regimen were at higher risk of TRM and demonstrated a lower probability of OS. Although radiation is effective as part of the conditioning regimen, no conclusion has been reached as to whether a TBI-containing regimen is superior or inferior to a non-TBI regimen.[15,16] Two of five randomized controlled trials showed the superiority of TBI-regimens for survival.[17,18] but no difference was observed in the other three trials.[19 21] It should be remembered that previous trials have not included older patients according to each eligibility criterion. In the study reported here, TBI-regimens were

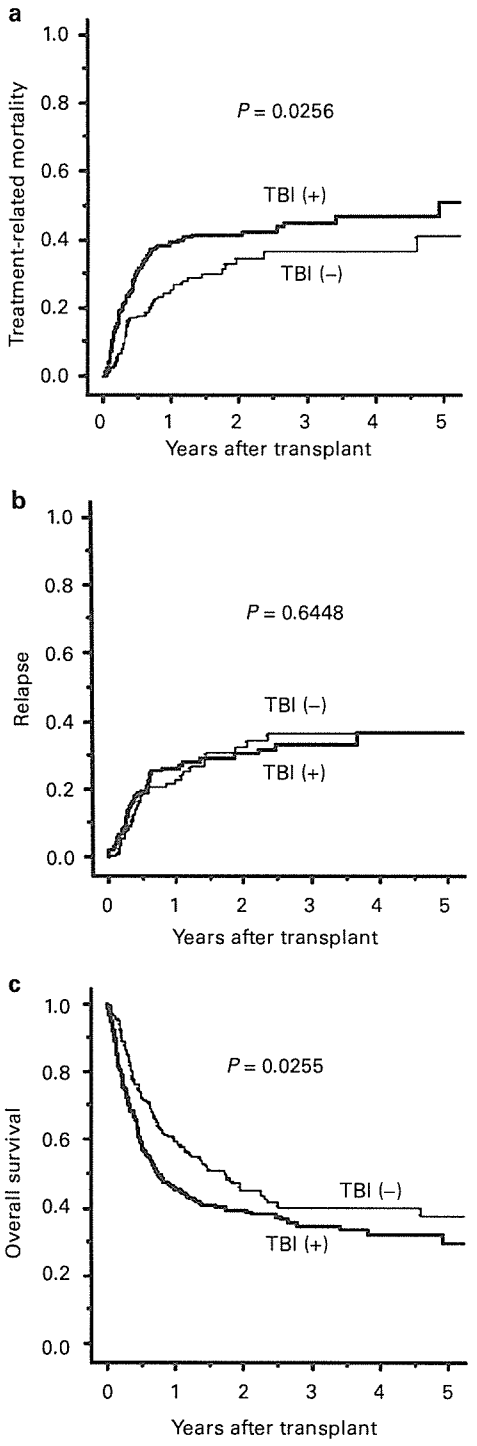

Figure 3 Treatment-related mortality, relapse and overall survival by conditioning regimen for patients aged 50 or older. TBI-containing regimens were associated with (**a**) higher incidence of TRM, (**b**) with no improvement of relapse rate (log-rank, $P = 0.0256$ and 0.6448, respectively). (**c**) Patients aged 50 or older receiving a TBI-containing regimen showed poorer survival (log-rank, $P = 0.0255$).

found to be more toxic and to worsen OS for patients over 50 years, while the detrimental effect of TBI was not demonstrated among younger patients.

In summary, patient age at transplantation is a strong indicator for outcome after allogeneic c-HSCT. This treatment for patients aged 50 or older is associated with a higher risk of TRM and with a lower OS than for those under 50 years. Furthermore, conditioning regimens including TBI enhance negative effects. It is therefore suggested that allogeneic c-HSCT should be offered to patients 50 years of age or older only if the higher risk of TRM is acceptable. When the transplant is performed, a non-TBI regimen is preferable. Prospective comparative analyses of c-HSCT and NST are also needed for this population group.

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