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Juvenile myelomonocytic leukemia with t(7;11)(p15;p15) and *NUP98-HOXA11* fusion

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The t(7;11)(p15;p15) translocation has been reported as a rare and recurrent chromosomal abnormality in acute myeloid leukemia (AML) patients. The *NUP98-HOXA9* fusion gene with t(7;11)(p15;p15) was identified and revealed to be essential for leukemogenesis and myeloproliferative disease. To date, t(7;11)(p15;p15) with *NUP98-HOXA11* fusion has been reported only in one case of ph-negative chronic myeloid leukemia (CML). Here, we report a case of a 3-year-old girl with juvenile myelomonocytic leukemia (JMML) carrying t(7;11)(p15;p15) abnormality with *NUP98-HOXA11* fusion. AML chemotherapy followed by bone marrow transplantation (BMT) was found to be effective in treating this disorder, and she remains in complete remission for 3 years after BMT. We suggest the possibility that AML chemotherapy might be effective for treating JMML with t(7;11)(p15;p15) abnormality and *NUP98-HOXA11* fusion. Am. J. Hematol. 00:000–000, 2009. © 2009 Wiley-Liss, Inc.

Introduction

Juvenile myelomonocytic leukemia (JMML) is refractory to chemotherapy, and the only curative treatment is hematopoietic stem cell transplantation (HSCT). Although the 5-year probability of event-free survival (EFS) in JMML patients receiving HSCT is approximately 50%, relapse remains the major form of treatment failure and is observed in up to 50% patients [1]. Thus far, somatic mutation in *PTPN11, RAS, or NF1* and monosomy 7 are known to be the essential chromosomal or genetic abnormalities in JMML. The t(7;11)(p15;p15) translocation is reported as a rare and recurrent chromosomal abnormality detected mainly in acute myeloid leukemia (AML) patients [2–5]. *NUP98-HOXA9* fusion involving t(7;11)(p15;p15) was first reported by both Nakamura et al. [6] and Borrow et al. [7]; subsequently, this abnormal fusion has been shown to be critical for leukemogenesis and preleukemic phase [8,9]. In this paper, we report a case of JMML involving t(7;11)(p15;p15) abnormality with *NUP98-HOXA11* fusion. *NUP98-HOXA11* fusion has been reported only in a ph-negative chronic myeloid leukemia (CML) patient [10], and this previous case and our present case indicate that *NUP98-HOXA11* fusion might also be related closely to leukemogenesis and preleukemic phase.

Case Report

A 3-year-old Japanese girl who presented with wheezing was referred to our hospital because of leukocytosis and anemia. Her family history revealed that her grandmother had died of myelodysplastic syndrome (MDS). A physical examination revealed hepatosplenomegaly, but skin eruptions or lymph node swellings were not detected. She had no clinical evidence of autoimmune lymphoproliferative syndrome, neurofibromatosis Type 1, Noonan syndrome, Costello syndrome, or any cardiofaciocutaneous syndromes with germline RAS-pathway mutation. Laboratory data at the time of presentation were as follows: hemoglobin (Hb), 8.2 g/dl; platelets, 165,000/μl; white blood cells, 39,400/μl with 8% monocytoid cells, 13% myelocytes, 8% metamyelocytes, and 1.5% blast cells. Biochemical tests showed normal level of HbF (2%) and low level of neutrophil alkaline phosphatase (score, 150). The serum lysozyme level was elevated (56.8 μl/ml). Infections such as those caused by cytomegalovirus, Epstein-Barr virus, and human herpes virus Type 6 were excluded. Bone marrow aspirate revealed hypercellular marrow with 1% blast cells, and a bilineage myelodysplasia such as macroerythroblasts, Pseudo-Perger-Hüet anomaly, and chromatin clumping in neutrophils were found. Chromosome analysis of the bone marrow showed 46,XX,t(7;11)(p15;p15) in all 20 cells analyzed. Spontaneous growth and hypersensitivity to the granulocyte/macrophage colony-stimulating factor (GM-CSF) were observed in the colony assay. A heterozygous mutation of *NRAS* gene (38G>A, G13D), but not *KRAS*, *HRAS*, or *PTPN11*, was also observed in leukemic cells of the patient. These findings were consistent with the diagnosis of JMML in accordance with the diagnostic criteria of JMML established by the European Working Group of MDS in Childhood [1].

After a 3-week observation period without therapy, her WBC count was 57,600/μl, including 10.5% blast cells; Hb level, 7.2 g/dl; and platelet count, 83,000/μl. Bone marrow pictures revealed hyperplastic cellularity with 3% blast cells. Because of the increasing ratio of blast cells, which were similar to myelocytes, in peripheral blood (PB) and the chromosomal abnormality, which is mainly observed in AML, she was treated with induction chemotherapy (cytarabine, etoposide, and mitoxantrone) on the Japanese Childhood AML Cooperative Study Group Protocol, AML 99 [11]. She achieved complete remission after induction therapy, and the t(7;11)(p15;p15) abnormality disappeared. After two additional courses of intensive chemotherapy (high-dose cytarabine, etoposide, idarubicin and mitoxantrone),

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Conflict of interest: Nothing to report.

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Received for publication 22 October 2008; Revised 24 January 2009; Accepted 26 January 2009

Am. J. Hematol. 00:000–000, 2009.

Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/ajh.21373

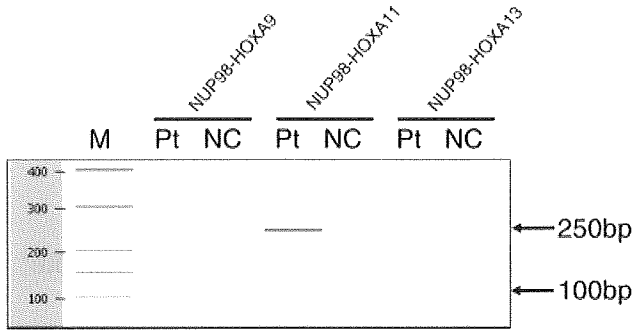

Figure 1. Detection of the NUP98-HOXA11 fusion transcript in the JMML patient by RT-PCR. M, size marker; Pt, patient; NC, normal negative control. The primers used for detection were as follows: NUP98-11S1 and HOXA9-1AS, *NUP98-HOXA9*; NUP98-11S1 and HOXA11-1AS, *NUP98-HOXA11*; and NUP98-11S1 and HOXA13-1AS, *NUP98-HOXA13*.

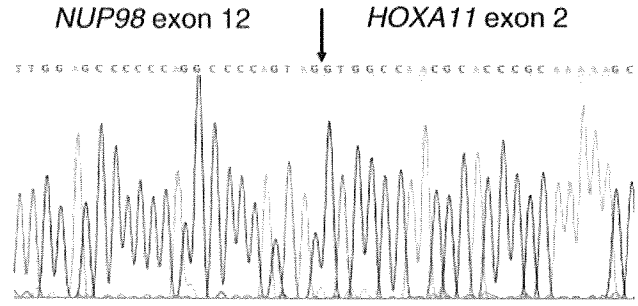

Figure 2. Direct sequencing of the NUP98-HOXA11 fusion transcript junction. Arrow indicates the fusion point. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

she received allogeneic HSCT from a human leukocyte antigen (HLA)-identical sibling donor. The conditioning regimen consisted of total body irradiation (TBI, 6 Gy in 3 fractions over 2 d), fludarabin (35 mg/(m^2 day) for 4 d), melphalan (70 mg/(m^2 day) for 2 d), and cytarabine (3 mg/(m^2 day) for 4 d) combined with the granulocyte colony-stimulating factor (G-CSF). A graft-versus-host disease (GVHD) prophylaxis consisted of a short course of methotrexate alone. A sustained recovery of neutrophils was achieved on day 30. without any severe complications. She developed Grade I acute GVHD of the skin on day 56 and mild chronic GVHD of the skin on day 180; both these conditions were improved by administering topical steroids alone. A temporary pulmonary hypertension was also observed on day 120 and was treated with bosentan. She remains in complete remission for about 3 years after bone marrow transplant (BMT).

Results

To isolate the fusion partner of *NUP98,* we performed reverse transcription (RT)-PCR using antisense primers based on *HOXA9*, *HOXA11*, and *HOXA13* and detected a band of approximately 250 bp and a very faint band of approximately 100 bp when the primers NUP98-11S1 and HOXA11-1AS were used (see Fig. 1). Direct sequence analysis showed that the RT-PCR product was an in-frame fusion transcript of *NUP98-HOXA11* containing exon 12 of the *NUP98* gene fused to exon 2 of the *HOXA11* gene (see Fig. 2).

Discussion

The t(7;11)(p15;p15) translocation with *NUP98-HOXA11* fusion was first reported in a patient with ph-negative CML [10], and our present case is the second case of carrying t(7;11)(p15;p15) aberration with the *NUP98-HOXA11* gene fusion. The t(7;11)(p15;p15) translocation is known as an uncommon and recurrent chromosomal abnormality mainly associated with AML [2–4]. Most cases of this abnormality are observed in the Oriental population, especially in Japanese patients [2]. Thus far, the t(7;11) abnormality with the *NUP98-HOXA9* gene fusion has been reported in AML, MDS, and chronic myelomonocytic leukemia (CMML) patients [3–5], and this abnormal fusion was found to induce a preleukemic phase in a mouse model [8,9]. Iwasaki et al. [9] indicated that approximately 20% of the transgenic mice in which the chimeric *NUP98-HOXA9* fusion cDNA was expressed in promyelocytes progressed to AML after a long latent period, whereas nonleukemic transgenic mice showed an increased G-CSF response and a high self-renewal capacity of myeloid progenitors as compared with wild-type mouse. In our JMML case, hypersensitivity to GM-CSF and spontaneous growth in the colony assay were observed. In addition, it was reported that other factors were required for complete leukemogenesis in *NUP98-HOX* fusion [12,13]. For example, *Meis1* coexpression dramatically induced the development of AML having *NUP98-HOXD13* with a short latency [12]. Slape. C et al. also reported that no *NRAS* or *KRAS* mutations were identified in 12 *NUP98-HOXD13* mice with MDS, whereas three *NRAS* and four *KRAS* mutations were identified in *NUP98-HOXD13* mice with acute nonlymphocytic leukemia (ANLL) and they suggested that the *RAS* mutations occurred as leukemia progression events [13]. These findings suggested that *NUP98-HOX* gene fusion is relevant to preleukemic phase, and it requires additional cofactors for complete leukemogenesis. On the other hand, oncogenic point mutations of *RAS* gene are also found in 20% of JMML patients [14]. In our case, we considered that both *NUP98-HOXA11* fusion and a point mutation of *NRAS* gene were associated with the development of myeloproliferative disorder (MPD), however, the relationship between *NUP98-HOXA11* fusion and a mutation of *NRAS* gene is uncertain.

JMML is a clonal myeloproliferative disorder of early childhood. JMML is defined to possess features of both MDS and MPD, and it is classified into MDS/MPD with ph-negative CML and CMML in the WHO classification [15]. Generally, chemotherapy regimen for AML is thought to be ineffective for JMML, and BMT has been proposed as the only treatment of choice. In our case, we found an increased ratio of blast cells, which were similar to myelocytes, in PB within a short period and chromosomal abnormality of t(7;11)(p15;p15). On the basis of clinical features, we thought this case had a feature of AML and administered AML chemotherapy to the patient, followed by HSCT; the patient achieved complete remission by this treatment strategy. The first reported case of t(7;11)(p15;p15) with *NUP98-HOXA11* gene fusion was a patient with ph-negative CML [10], who developed an acute leukemia phase within a short period and achieved complete remission after treatment with idarubicin and cytarabine, which are used for treating AML. Furthermore, in our case, AML chemotherapy followed by HSCT proved to be effective for treating the disorder. Thus, these two cases indicate that AML chemotherapy may be effective for treating leukemia or MPD with *NUP98-HOXA11* gene fusion.

Materials and Methods

RNA extraction and reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from the bone marrow mononuclear cells at the onset by using the Isogen LS Kit (Wako Nippon Gene,

Osaka, Japan). Four micrograms of total RNA was reverse transcribed to cDNA in a total volume of 33 μl with a random hexamer primer by using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Buckinghamshire, England). Polymerase chain reaction (PCR) was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Tokyo, Japan) by using the reagents recommended by the manufacturer. The primers used were as follows: NUP98-11S1, AGCACCTGG GACTCTTGGAA; HOXA9-1AS, CATTTTCATCCTGCGGTTCTG; HOX A11-1AS, CTCTCGGATCTGGTACTTGGT; HOXA13-1AS, CCT CCTA-TAGGA GCTGGCAT. After 35 rounds of PCR (30 s at 94°C, 30 s at 55°C, 1 min at 72°C), the detection of PCR products was performed with the Agilent 2100 Bioanalyzer and the DNA 1000 Lab Chip kit as described previously [16].

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Short communication

NUP98–NSD3 fusion gene in radiation-associated myelodysplastic syndrome with t(8;11)(p11;p15) and expression pattern of NSD family genes

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Received 9 September 2008; received in revised form 27 November 2008; accepted 15 December 2008

Abstract Chromosomal 11p15 abnormality of therapy-related myelodysplastic syndrome (t-MDS)—acute myeloid leukemia (AML) is rare. *NUP98–NSD3* fusion transcripts have been detected previously in one patient with AML and one patient with t-MDS having t(8;11)(p11;p15). Here we present the case of a 60-year-old man with radiation-associated MDS (r-MDS) carrying chromosome abnormalities, including t(8;11)(p11;p15) and del(1)(p22p32). Fluorescence in situ hybridization analysis demonstrated that the *NUP98* gene at 11p15 was split by the translocation. Southern blot analysis of bone marrow cells showed both rearrangements of *NUP98* and *NSD3* genes. Reverse transcriptase–polymerase chain reaction (RT-PCR) followed by sequence analysis revealed the presence of both *NUP98–NSD3* and *NSD3–NUP98* fusion transcripts. Expression analysis by RT-PCR showed that *NSD3* as well as *NSD1* and *NSD2* was ubiquitously expressed in leukemic cell lines and Epstein–Barr virus transformed B lymphocyte cell lines derived from the normal adult lymphocytes examined. Two isoforms of *NSD3*, *NSD3S* and *NSD3L* (but not *NSD3L2*), were expressed in leukemic cell lines and were fused to *NUP98* in our patient, suggesting that qualitative change of these two isoforms of *NSD3* by fusion with *NUP98* might be related to leukemogenesis, although the function of each isoform of the *NSD3* gene remains unclear.

1. Introduction

Myeloid malignancies with 11p15 translocations are likely to be related to the nucleoporin gene, *NUP98* [1]. These translocations produced fusion genes between *NUP98* and many different partner genes [1]. Four patients with t(8;11)(p11;p15) have been reported previously [2–5], and the four diagnosed with acute myeloid leukemia (AML) or therapy-related myelodysplastic syndrome (t-MDS). The *NUP98–NSD3* fusion gene was identified in only two of these four patients with t(8;11) [4,5].

Therapy-related myelodysplastic syndrome (t-MDS) is considered to be a heterogeneous disorder of pluripotent hematopoietic stem cells that have various findings of bone marrow (BM) failure, often evolve to AML, and have a poor prognosis [6,7]. Although the pathogenesis of t-MDS is unknown, many recurrent chromosomal abnormalities are involved in t-MDS [8,9]. Only 17 patients were identified with 11p15 chromosomal abnormality among 511 patients with t-MDS–AML [10]. In the survey of Japanese childhood t-MDS–AML, 5 of 81 children had 11p15 translocations involving *NUP98* rearrangements [11].

Here we describe the case of a 60-year-old patient with radiation-associated MDS (r-MDS) patient exhibiting translocation t(8;11) and a *NUP98–NSD3* fusion transcript. We also report the expression of NSD family genes *NSD1*, *NSD2*, and *NSD3* in several leukemia and normal Epstein-Barr virus transformed B lymphocyte (EBV-B) cell lines from healthy volunteers.

We note that in the international human gene nomenclature (http://www.genenames.org), *NSD1* is an approved gene symbol, but *NSD2* and *NSD3* are classified as aliases,

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0165-4608/09/$ – see front matter
doi:10.1016/j.cancergencyto.2008.12.008

for *WHSC1* and WHSC1L1, respectively. In the present report, however, for convenience of discussion we continue to use the *NSD* nomenclature for all three genes.

2. Case report

A 60-year-old man was admitted for assessment of anemia. He had been an atomic-bomb survivor in Nagasaki 44 years before. When he was 59 years old, he was diagnosed with sigmoid colon cancer and underwent operative resection. His father died of lung cancer. On examination, blood examination showed a white blood cell count of 6,250/μL with no leukemic blasts, a hemoglobin level of 11.1 g/dL, and a platelet count of 337,500/μL. The BM examination revealed a nuclear cell count of 127,500/μL with no leukemic blasts. He had megakaryocytes with multiseparated nuclei and mature neutrophils with pseudo-Pelger–Hüet anomaly. Conventional chromosomal analysis demonstrated 46,XY,t(8;11)(p11;p15),del(1)(p22p32) in all 20 BM cells examined. He was diagnosed with refractory anemia (RA), but was not treated; he developed AML, 1 year after the diagnosis of RA. Cytogenetic findings in the AML were the same as in the RA. He died of progressive disease 23 months after diagnosis of RA, despite low-dose cytarabine.

2.1. Fluorescence in situ hybridization analysis

The fluorescence in situ hybridization (FISH) analysis of the patient's leukemic cells using bacterial artificial chromosome (BAC) clone PK505 was performed as described previously [12]. We mapped this BAC clone to leukemic cells together with a whole-chromosome painting probe for chromosome 11 (WCP11) (Coatasome 11, digoxigenin-labeled; Oncor, Gaithersburg, MD).

2.2. Southern blot analysis

After obtaining informed consent from the patient, high molecular weight DNA was extracted from BM cells by proteinase K digestion and phenol–chloroform extraction [13]. Ten micrograms of DNA were digested with *Eco*RI and *Bgl*II restriction endonucleases, subjected to electrophoresis on 0.7% agarose gels, transferred to nylon membrane, and hybridized to cDNA probes^{32} P-labeled by the random hexamer method [13]. The probes were an 837-bp *NUP98* cDNA fragment (nucleotide nt 1213 to 2049; GenBank accession no. U41815) and a 512-bp *NSD3* cDNA fragment (nt 929 to 1440; GenBank accession no. AJ295990).

2.3. Reverse transcriptase-polymerase chain reaction and nucleotide sequencing

NUP98–NSD3 chimeric mRNA was detected by reverse transcriptase–polymerase chain reaction (RT-PCR) in essentially the same manner as described previously [14]. Total RNA was extracted from the leukemia cells of the patient using the guanidine thiocyanate–phenol–chloroform method [14]. Total RNA (4 μg) was reverse-transcribed to cDNA, using a cDNA synthesis kit (GE Healthcare Bio-Science, Piscataway, NJ) [14]. The PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems, Tokyo, Japan), using the reagents recommended by the manufacturer.

The primers used for detection of *NUP98–NSD3* fusion transcripts and the reciprocal fusion transcripts were NUP98–S10 (5′-TGGGACTCTTACTGGGCTT-3′) and NSD3-R4 (5′-CTCTCTGGCTGGTTGCTAAA-3′) for *NUP98–NSD3*, and NSD3-S1 (5′-CAAGATCTGAAGAGCG CAAG-3′) and NUP98–R13 (5′-TAGGGTCTGACATCG GATTC-3′) for *NSD3-NUP98*. The PCR amplification was performed with this mixture using a DNA thermal cycler (Applied Biosystems) under the following conditions: initial denaturation at 94°C for 9 minutes, 40 cycles at 96°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by a final elongation at 72°C for 7 minutes.

For detection of *NUP98–NSD3L*, *NUP98–NSD3L2*, and *NUP98–NSD3S* fusions, nested RT-PCR was performed. The primers for first RT-PCR were NUP98–S10 and NSD3L-R (5′-ACCTGGGGTTGCAGATCTCT-3′), NUP983L2-R (5′-AATCTTCCACCTCTGGCAC-3′), NSD 3S-R (5′-ACGGAGCTGTCACTGAATCT-3′), respectively. The primers for second RT-PCR were NUP98–S11 (5′-CCTCTTGGTACAGGAGCCTT-3′) and NSD3-R4. The PCR conditions were as described above. The PCR products were subcloned into pCR2.1 vector (Invitrogen, Carlsbad, CA) and were sequenced by the fluorometric method using the Big Dye terminator cycle sequencing kit (Applied Biosystems).

2.4. Expression of three isoforms of the NSD3 gene and the NSD1 and NSD2 genes by RT-PCR in leukemic cell lines

To analyze the expression pattern of three isoforms of the *NSD3* gene (*NSD3L*, *NSD3L2*, and *NSD3S*) and the family genes *NSD1* and *NSD2* in leukemic cell lines, RT-PCR was performed. In all, 59 cell lines were examined, as follows [14]: 10 B-precursor ALL cell lines (NALM-6, NALM-24, NALM-26, UTP-2, THP-4, RS4;11, SCMC-L10, KOCL-33, KOCL-45, and KOCL-69), 9 B-ALL cell lines (BALM-1, BALM-13, BALM-14, BJAB, DAUDI, RAJI, RAMOS, BAL-KH, and NAMALVA), 9 T-ALL cell lines (RPMI-8402, MOLT-14, THP-6, PEER, H-SB2, HPB-ALL, L-SAK, L-SMY, and KCMC-T), 8 AML cell lines (YNH-1, ML-1, KASUMI-3, KG-1, inv-3, SN-1, NB4, and HEL), 6 acute monocytic leukemic cell lines (THP-1, IMS/M1, CTS, P31/FUJ, MOLM-13, and KOCL-48), 5 chronic myelogenous leukemia cell lines (MOLM-1, MOLM-7, TS9;22, SS9;22, and K-562), 2 acute

megakaryoblastic leukemia cell lines (CMS and CMY), and 10 EBV-B cell lines derived from normal adult peripheral lymphocytes. Five normal BM samples were also examined.

The RT-PCR mixtures and conditions were as previously described [13]. The primers used for RT-PCR were as follows: for *NSD3L* and *NSD3L2*, NSD3-2711F (5′-TCTGCCT GCTCTATGGAGAA-3′) (sense primer) and NSD3-3260R (5′-ACCTGGGGTTGCAGATCTCT-3′) (antisense primer); for *NSD3S*, NSD3-1779F (5′-GCCTGGATTTGCAGAAGT GT-3′) (sense primer) and NSD3-2220R (5′-ACGGAGCTGT CACTGAATCT-3′) (antisense primer), for *NSD1*, NSD1-4941F (5′-AACCTGTCATGCCGCTAATCC-3′) (sense primer) and NSD1-5495R (5′-ATCTTATCCTTGCTGCTCACG-3′) (antisense primer); and for *NSD2*, NSD2-2811F (5′-TCAAACC-CAAGGCCGTCAAA-3′) (sense primer) and NSD2-3365R (5′-GACTCTTCCGATCCCTCTGA-3′) (antisense primer).

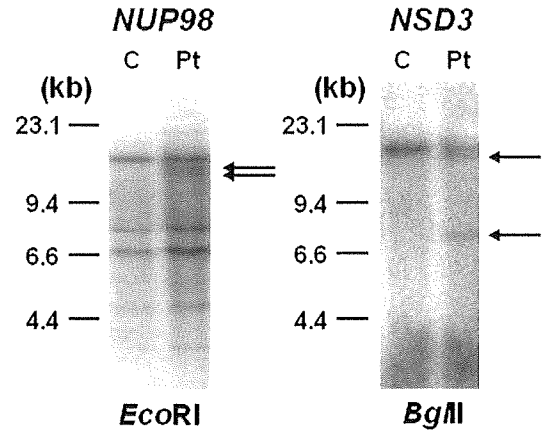

Fig. 2. Southern blotting of the *NUP98* gene with *Eco*RI and the *NSD3* gene with *Bgl*II restriction endonuclease. Arrows indicate rearranged bands. Pt, patient; C, control.

3. Results

Chromosomal abnormalities of the patient's leukemic cells revealed the karyotype as 46,XY,t(8;11)(p11;p15),-del(1)(p22p32), suggesting that the *NUP98* gene located in 11p15 was rearranged. A FISH analysis using the probe containing *NUP98* detected the split signals on both der(11)t(8;11)(p11;p15) and der(8)t(8;11)(p11;p15), in addition to normal chromosome 11 (Fig. 1). To date, the *NSD3* gene on chromosome 8p11 has been reported as a fusion partner gene of *NUP98* in the t(8;11)(p11;p15) anomaly [4].

Southern blot analysis of DNA from leukemic cells of the patient using the *NUP98* probe and the *NSD3* probe showed rearranged bands (Fig. 2). We performed RT-PCR for *NUP98–NSD3* chimeric mRNA and obtained one RT-PCR product of 247 bp. Sequence analysis of the PCR product showed an in-frame fusion transcript of exon 11 of *NUP98* to exon 4 of *NSD3*. Two reciprocal *NSD3–NUP98* transcripts were also detected. Sequence analysis of these PCR products showed that one product was an in-frame fusion transcript of exon 3 of *NSD3* to exon 12 of *NUP98*; the other was an in-frame fusion transcript of exon 3 of *NSD3* to exon 13 of *NUP98*. We also examined which of the *NSD3* isoforms (*NSD3L*, *NSD3S*, and *NSD3L2)* were fused to the *NUP98* gene. We identified two types of chimeric transcripts, *NUP98–NSD3L* and *NUP98–NSD3S*, but not *NUP98–NSD3L2*.

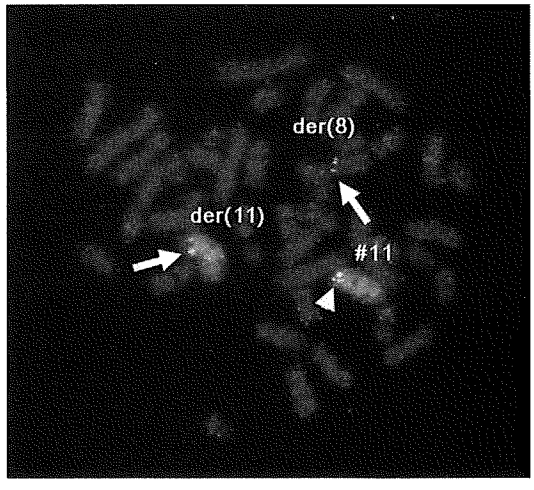

Fig. 1. FISH analysis of *NUP98* rearrangement in a leukemic metaphase. Split signals (arrows) of bacterial artificial chromosome clone PK505 containing *NUP98* were observed on the boundary between painted and unpainted regions of der(11)t(8;11) and der(8)t(8;11). An intact PK505 signal was observed on the normal chromosome 11 (arrowhead).

We next examined the *NSD3* gene and the family gene expression by RT-PCR analysis in 49 leukemic cell lines and 10 EBV-B cell lines (Fig. 3). There are three isoforms of the *NSD3* gene: *NSD3L* (full length), *NSD3L2* (lacking exon 14), and *NSD3S* (from exon 1 to exon 9a, which is completely different from exon 9 of *NSD3L*). There are also two *NSD* family genes (*NSD1* and *NSD2*) in addition to *NSD3*. *NSD1* is located on chromosome region 5q35 and *NSD2* is located on 4p16.3. Two of the three *NSD3* isoforms (i.e., except for *NSD3L2*) were expressed in all leukemic cell lines, the EBV-B cell line, and normal BM cells. The *NSD3L2* isoform was not expressed in any samples examined. *NSD1* and *NSD2* genes were expressed in all samples examined.

4. Discussion

NUP98–NSD3 fusion transcripts have been detected only in a patient with AML and a patient with t-MDS having t(8;11)(p11;p15) [4,5]. All patients reported were diagnosed with adult-onset myeloid malignancies, and had a poor prognosis [4,5]. The present patient died of disease progression. As fusion genes between *NUP98* and isoforms of *NSD* in hematological malignancies, there are two other fusion transcripts; one is the *NUP98–NSD1*

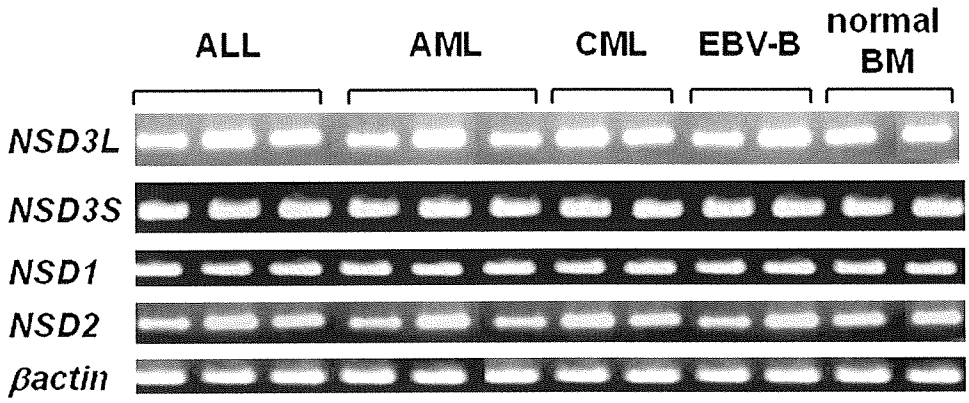

Fig. 3. Expression of two isoforms of the *NSD3* gene and the *NSD1* and *NSD2* genes in acute lymphoblastic, acute myeloid, and chronic myeloid leukemia cell lines, Epstein–Barr virus transformed B lymphocyte (EBV-B) cell lines, and normal healthy bone marrow cells, determined with reverse transcriptase–polymerase chain reaction. β-actin was amplified as an internal control.

fusion gene in t(5;11)(q35;p15) and the other is the *IgH-MMSET* (*NSD2*) fusion gene in t(4;14)(p16.3;q32) [15,16]. In terms of their clinical features, patients carrying the *NUP98–NSD1* fusion gene were similar to those carrying the *NUP98–NSD3* fusion gene. Of reported patients carrying the *NUP98–NSD1* fusion gene, all were diagnosed with myeloid malignancies (6 AML and 1 MDS), and with one exception onset was in childhood [17–21]. In most of these patients, the t(5;11)(q35;p15) translocation could be detected by means of FISH, but not by conventional cytogenetic analysis [18]. Many of the patients had recurrence and died of progressive disease, regardless of stem cell transplantation [17–21]. It is likely, therefore, that *NUP98–NSD* fusion genes are an important prognostic factor in myeloid malignancies.

The fusion protein that is the transcriptional product of the *NUP98–NSD3* fusion gene is predicted to consist of an N-terminal phenylalanine–glycine (FG) repeat motif of *NUP98* and C-terminal PHD finger and SET domain of NSD3. This similar fusion structure is retained in fusion proteins of NUP98–NSD1 and IgH-MMSET (NSD2) [15,16]. The FG repeats in the NUP98 N-terminus are conserved in all *NUP98*-related chimeras, suggesting an important role in leukemogenesis [1]. The NSD family proteins have common regions: PWWP, PHD finger, and SET domain [22,23]. The PHD finger and SET domain of the NSD C-terminus are preserved in NSD-related chimeras [4,15,16].

NUP98–NSD1 induces AML in vivo, sustains self-renewal of myeloid stem cells in vitro, and enforces expression of the HoxA7, HoxA9, HoxA10, and Meis1 proto-oncogenes [24]. Mechanistically, NUP98–NSD1 binds genomic elements adjacent to HoxA7 and HoxA9, maintains histone H3 Lys 36 methylation and histone acetylation, and prevents EZH2-mediated transcriptional repression of the Hox-A locus during differentiation [24]. To clarify the role of NUP98–NSD3 fusion protein, further accumulation of clinical data of t(8;11) patients and functional analysis of this fusion protein are needed.

Expression analysis of normal *NSD* family genes by RT-PCR showed that isoforms *NSD3L* and *NSD3S*, as well as the genes *NSD1* and *NSD2*, were ubiquitously expressed in leukemic cell lines and EBV-B cell lines derived from the normal adult lymphocytes examined. The isoforms *NSD3L* and *NSD3S* were simultaneously expressed in many normal tissues [22]. FISH analysis showed the amplification of *NSD3* in several breast cancer cell lines and primary breast carcinomas [22].

We found coexpression of *NSD3L* and *NSD3S* (but not *NSD3L2*) in all leukemic cell lines examined. We also identified two types of the *NUP98–NSD3* fusion transcript: *NUP98–NSD3S* and *NUP98–NSD3L*. The *NUP98–NSD3L2* fusion transcript was not detected. The *NSD3S* and *NSD3L* genes were fusion partners of *NUP98* and expressed in leukemic cell lines, suggesting that qualitative change of these two isoforms of *NSD3* by fusion with *NUP98* might be related to leukemogenesis although the function of each isoform of the *NSD3* gene remains unclear.

Acknowledgments

We express our appreciation to Mrs. Shoko Sohma, Hisae Soga, Midori Furui, Mayumi Nagase, and Mayumi Naito for their excellent technical assistance. We thank Dr. Takeyuki Sato, Department of Pediatrics, Chiba University School of Medicine, Japan, for providing AMKL (CMS, CMY) cell lines; Dr. Kanji Sugita, Department of Pediatrics, Yamanashi University School of Medicine, Japan, for providing ALL (KOCL-33, 45, 69) cell lines and AMOL (KOCL-48) cell lines; and Dr. Yoshinobu Matsuo, Hayashibara Biochemical Laboratories, Inc., Fujisaki Cell Center, Japan, for providing varieties of ALL cell lines. Supported by a Grant-in-Aid for Cancer Research, Research on Children and Families, from the Ministry of Health, Labor, and Welfare of Japan, a Grant-in-Aid for Scientific Research (C) and Exploratory Research from

the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This study was also supported by a grant for Research on Children and Families (H18-005) from the Ministry of Health, Labor, and Welfare of Japan.

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Leukemia (2009) 23, 95–103

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ORIGINAL ARTICLE

The key role of stem cell factor/KIT signaling in the proliferation of blast cells from Down syndrome-related leukemia

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Transient leukemia (TL) has been observed in approximately 10% of newborn infants with Down syndrome (DS). Although treatment with cytarabine is effective in high-risk TL cases, approximately 20% of severe patients still suffer early death. In this study, we demonstrate abundant *KIT* expression in all 13 patients with *GATA1* mutations, although no significant difference in expression levels was observed between TL and acute myeloid leukemia. Stem cell factor (SCF) stimulated the proliferation of the TL cells from five patients and treatment with the tyrosine kinase inhibitor imatinib suppressed the proliferation effectively *in vitro*. To investigate the signal cascade, we established the first SCF-dependent, DS-related acute megakaryoblastic leukemia cell line, KPAM1. Withdrawal of SCF or treatment with imatinib induced apoptosis of KPAM1 cells. SCF activated the RAS/MAPK and PI3K/AKT pathways, followed by downregulation of the pro-apoptotic factor BIM and upregulation of the anti-apoptotic factor MCL1. Although we found novel missense mutations of *KIT* in 2 of 14 TL patients, neither mutation led to KIT activation and neither reduced the cytotoxic effects of imatinib. These results suggest the essential role of SCF/KIT signaling in the proliferation of DS-related leukemia and the possibility of therapeutic benefits of imatinib for TL patients.
Leukemia (2009) **23,** 95–103; doi:10.1038/leu.2008.267; published online 2 October 2008
Keywords: Down syndrome; transient leukemia; acute megakaryocytic leukemia; KIT; imatinib; KPAM1

Introduction

Children with Down syndrome (DS) are predisposed to developing leukemia. A leukemoid reaction occurring uniquely in approximately 10% of newborn infants with DS referred to as transient myeloproliferative disorder or transient leukemia (TL). This disorder, in most cases, resolves spontaneously within 3 months after birth. Of all TL patients, approximately 20–30% develop myeloid leukemia of Down syndrome (ML-DS) within 4 years.[1] In comparison with non-DS children with acute myeloid leukemia (AML), ML-DS patients have a better clinical outcome.[2,3] However, approximately 20% of the patients with severe TL are still subject to life threatening or fatal complications.[4,5] Improved treatments for TL are necessary for a better long-term prognosis of DS patients.

Almost all patients with TL and ML-DS have a somatic mutation in *GATA1* in their blast cells. *GATA1* transcription factor is required for the differentiation of erythroid and megakaryocytic cells. Most of the mutations occur in the first coding exon and result in expression of a truncated GATA1 protein (GATA1s).[6] Bourquin *et al.*[7] showed that the gene expression profile in ML-DS is distinctly different from both non-DS-acute megakaryocytic leukemia (AMKL) and AML. Focusing on the gene expression alterations attributed to GATA1s they suggested that in ML-DS, several pro-proliferative genes repressed by GATA1 were aberrantly regulated and one of those was *KIT*. These results indicated the possibility that the loss of repression of the genes by GATA1s contributes to the pathogenesis of ML-DS.

The proto-oncogene *KIT* encodes a transmembrane type III tyrosine kinase, which is the receptor for stem cell factor (SCF). KIT is expressed in approximately 70% of *de novo* AMLs and 95% of relapsed AMLs.[8] Recent reports showed that KIT is also expressed in almost all TL patients.[5,9] However, little is known about the expression level of KIT and the functional roles of SCF/KIT pathway in TL.

In the present study, we show that SCF/KIT signaling has an essential role in the proliferation and survival of blast cells from DS-related leukemia and the possibility of therapeutic benefits of imatinib for severe TL patients.

Materials and methods

Patient samples and cell culture

All clinical samples were obtained with informed consent, and this study was approved by the Ethics Committee of Hirosaki University. Mononuclear cell fractions were obtained by Ficoll-Hypaque separation from peripheral blood of TL patients and bone marrows of acute leukemia patients. Fractionated cells of case TL1, 7, 9, 14 and 15 were resuspended in cell preservation medium CP-1 (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) and kept in a −80 °C freezer until use. These cells were thawed and incubated in Iscove's Modified Dulbecco's medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum overnight at 37 °C in 5% CO_2. After the incubation, cells were used for assays.

Recombinant human SCF, erythropoietin, thrombopoietin, granulocyte-colony stimulating factor, interluekin-3 (IL-3) and murine IL-3 were kindly provided by Kirin Brewery (Tokyo, Japan). Imatinib was kindly provided from Novartis Pharmaceuticals (Basel, Switzerland). U0126, SU6656 and LY294002 were purchased from Calbiochem (San Diego, CA, USA).

K562, HEL and CMK11-5 cell lines were established from patients diagnosed with chronic myelogenous leukemia, erythroleukemia and ML-DS, respectively. KPAM1 cells, derived from ML-DS, were cultured in RPMI1640 supplemented with 10% fetal bovine serum and 50 ng/ml SCF. The murine pro-B cell line Ba/F3 was maintained in RPMI1640 supplemented with 10% fetal bovine serum and 1 ng/ml IL-3.

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Received 21 May 2008; revised 18 July 2008; accepted 25 August 2008; published online 2 October 2008

Case

The KPAM1 cell line was derived from a 3-year-old girl with ML-DS. She was diagnosed with TL at the age of 2 months. Her disease evolved to refractory anemia with excess blasts (RAEB) with monosomy 7 at 1 year of age, then AMKL at 2 years. Her disease was in remission during treatment but relapsed, at which point she received a peripheral blood stem cell transplant from her mother at 2 years and 8 months. Engraftment was prompt, but her disease relapsed soon and she died 165 days after peripheral blood stem cell transplant.

Quantitative real-time polymerase chain reaction analysis

RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories., Hercules, CA, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed with iQ SYBR green supermix kit and the Chromo4 Real-time PCR system (Bio-Rad Laboratories). Primers for qRT-PCR analysis were designed by the Perfect Real Time Support System for Array (Takara Bio., Otsu, Shiga, Japan).

Retroviral vectors and transduction

The full length of *KIT* cDNA was inserted into the retroviral vector pMX-ires-CD8 (kindly provided by Dr Mano, Jichi Medical University). Mutant KIT expression vectors were generated by PCR-mediated mutagenesis. Retroviral infection method was described earlier.[10]

Cell proliferation assay

Cells were grown in appropriate culture media with various concentrations of cytokines or signal transduction inhibitors for 72 h. Viable cells were measured with the Cell Counting Kit 8 (Wako, Tokyo, Japan) according to the manufacturer's instructions.[10]

Western blotting analysis

Cell lysates were transferred to Hybond-P membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). Most of the antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) except anti-KIT, anti-MCL1 antibody (Santa Cruz, San Diego, CA, USA), anti-PARP, anti-BCLX, anti-BAX, anti-BID, anti-BAD antibody (BD Pharmingen, San Diego, CA, USA) and anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA).

Detection of KIT *mutations*

RNA was reverse transcribed and the entire coding regions of *KIT* were amplified. Direct sequencing analysis was performed. The primers used for *KIT* mutation analysis are shown in the Supplementary data (Supplementary Table S1).

Results

Abundant expression of KIT *in TL blast cells*

We analyzed *KIT* expression in TL blast cells to compare the expression with other pediatric leukemic cells by qRT-PCR. Analyzed samples included 5 acute lymphoid leukemias, 19 AML (5 M1, 7 M2, 4 M4 and 3 M7 patients), 13 TL, 4 ML-DS cases and 3 ML-DS cell lines. The relative expression levels normalized to *GAPDH* expression, arbitrarily set at 1000 in TL were distributed from 4.04 to 25.03, averaging 13.42 (Table 1 and Figure 1). ML-DS patient samples expressed *KIT* at lower levels than did TL samples (the average was 8.07, Figure 1a). Although a significant difference in expression levels was not observed between TL and AML, the data demonstrated that *KIT* was uniformly expressed in TL cells at a relatively high level. To investigate the possibility of autocrine mechanisms, we also examined *SCF* expression. qRT-PCR analysis showed that *SCF* mRNA was expressed at extremely low levels in TL cells and there were no significant differences in *SCF* expression among AML, TL and ML-DS (Figure 1b).

Proliferation of TL blast cells is stimulated by SCF and abrogated by imatinib

To investigate the function of KIT in TL cells, we cultivated TL cells with various growth factors including SCF, erythropoietin, thrombopoietin, granulocyte-colony stimulating factor and IL-3. Although TL blast cells did not grow without exogenous cytokines *in vitro* (data not shown), TL cells from all five patients examined proliferated in response to SCF in a dose-dependent manner. Maximal response was observed at the highest concentration examined (200 ng/ml) of SCF (Figure 2a). Three of five TL samples also responded to thrombopoietin but the extent of growth was less than that observed with SCF. These results suggested that SCF/KIT signaling plays key role in the proliferation of TL blast cells.

To confirm the involvement of SCF/KIT signaling in proliferation of TL blast cells, we determined whether a KIT inhibitor, imatinib, would affect growth. Treatment of TL cells with imatinib resulted in a significant decrease in cell proliferation in a dose-dependent manner (Figure 2b). By contrast, imatinib treatment of CMK11-5 cells, which possess an active *JAK3* mutation, had no effect on proliferation.[11]

It was reported that the overexpression of the wild-type FLT3 induced the ligand-independent phosphorylation in infantile acute lymphoid leukemias blasts cells.[12] Therefore, it is very interesting to know if the overexpression of KIT in TL cells results in constitutive autophosphorylation. To study this, KIT phosphorylation was assessed in frozen stocked cells. Western blotting analysis showed that various levels of KIT phosphorylation in all five TL samples examined (Figure 2c). We next examined the KIT activation in TL blast cells after culturing without exogenous SCF. Surprisingly, KIT phosphorylation was induced after overnight culture and remained at the same levels up to 12 h (Figure 2d). To examine the inhibitory effects of imatinib on KIT activation, blast cells were treated with the inhibitor, with or without exogenous SCF. Higher levels of KIT phoshorylation were detected in SCF-stimulated cells compared with unstimulated cells. A total of 1μM of imatinib inhibited KIT phosphorylation although faint signals remained (Figure 2e). These results suggest that the abundant KIT expression in TL blasts might induce the autophosphorylation, although the level of KIT phosphorylation without exogenous SCF is insufficient for proliferation of TL blast cells *in vitro*.

Establishment of a novel SCF-dependent cell line from a female ML-DS patient

Although our study suggested the involvement of SCF/KIT signaling in TL cell proliferation, it was difficult to study the mechanism of signal transduction in TL cells because of the limited numbers of cells and the absence of appropriate cell models. Therefore, we established a growth factor-dependent cell line KPAM1 from ML-DS blast cells.

Table 1 Clinical features, mutation characteristics and relative expression of KIT in TL and ML-DS patients

Case	*Sex*	*Admission*	*Down syndrome*	*WBC*	*Blast %*	*Cell source*	*Outcome*	*GATA1 mutation*	*KIT expression*	*KIT mutation*
TL-1	F	4 days	DS	63900	55	PB	CR	319 C>G (Tyr69stop)	10.03	Wild
TL-2	F	0 days	DS	244000	50–70	PB	Evolved to ML-DS	113 A>G (lost 1st Met)	9.04	Wild
TL-3	F	1 month	Mosaic	34600	48	PB	CR	IVS1to 2nd exon del 148bp (lost 1st Met)	ND	GAT>TAT (D910Y)
TL-4	F	0 days	DS	221000	91	PB	CR	295~305 del 11bp	18.20	ND
TL-5	M	0 days	DS	125700	60	PB	CR	213~220 del 8bp	14.58	Wild
TL-6	F	0 days	DS	220000	86	PB	Early death	202~203 del AG	13.79	Wild
TL-7	M	0 days	DS	166020	93	PB	Early death	265~287 del 23bp	7.81	Wild
TL-8	M	0 days	DS	57600	82	PB	Early death	305~311 GACGCTC>TAGTAGT (Ala65stop)	12.09	Wild
TL-9	M	0 days	DS	473000	93.5	PB	Early death	2nd exon to IVS2 del 218bp	18.71	TCC>CCC (S854P)
TL-10	M	1 day	DS	93300	59.6	PB	Early death	IVS1 GT-AG>GT-AA	25.03	Wild
TL-11	M	28 days	DS	84400	84	PB	CR	342~470 del 129bp	11.92	Wild
TL-12	M	0 days	DS	290800	92	PB	Early death	297 ins GGCACTGGCCTA12bp (Tyr62stop)	ND	Wild
TL-13	M	0 days	DS	206800	68.5	PB	Early death	IVS2 GT-AG>GC-AG	12.52	Wild
TL-14	F	0 days	DS	40000	63	PB	CR	284 ins 5bp, 286 ins 9bp	4.88	Wild
TL-15	F	0 days	Mosaic	119000	94	PB	CR	113 A>G (lost 1st Met)	15.84	Wild
ML-DS-1	M	NA	DS	NA	40 (PB)	BM	NA	113 A>G (lost 1st Met)	17.82	ND
ML-DS-2	M	1 year 3 months	DS	20200	76.6	BM	CR	162~190 del 29bp	4.04	ND
ML-DS-3	F	2 years 1 month	DS	256200	49.5 (PB)	BM	Dead	lost 2nd exon	5.64	ND
ML-DS-4	M	2 years	DS	3000	30	BM	CR	314 ins G	4.39	ND
ML-DS-5	F	1 year 8 months	DS	37400	76	BM	CR	304 ins 29bp	8.43	ND

BM, bone marrow; CR, complete remission; del, deletion; DS, Down syndrome; ins, insertion; F female; M, male; ML-DS, Down syndrome-related acute megakaryocytic leukemia; NA, not available; ND, not done; PB, peripheral blood; qRT-PCR, quantitative real-time polymerase chain reaction; TL, transient leukemia.
TL-2 developed ML-DS after spontaneous remission of TL. This patient was not included in examined ML-DS cases.
Numbers in *GATA1* mutation represent nucleotides of NM002049.
Early death indicates death within 6 months of age.
KIT mRNA was quantified by qRT-PCR and expressed as the relative mRNA level normalized to GAPDH expression arbitrarily set at 1000.

As cytogenetic study of the original blast cells showed: 46,XX,+21,dic(21;21)(p11;p11), KPAM1 had a near tetraploid karyotype. A representative karyotype was given in Supplementary data (Supplementary Figure S1a). The morphology of the cells was shown in Figure 4a. These cells are typical AMKL cells with blebs. Immunophenotypic analysis showed that KPAM1 cells were positive for expression of KIT, and as well as CD13, 33, 71 and glycophorin A (Supplementary Table S2). *GATA1* mutation analysis revealed a deletion of two nucleotides in the first coding exon, which caused a premature stop codon by frame-shifting, and resulted in the expression of GATA1s in this cell line (Supplementary Figure S1b and c).

SCF stimulation of KPAM1 cells activated AKT and ERK

To investigate the growth factor responsiveness of KPAM1, the cells were cultured with erythropoietin, thrombopoietin, granulocyte-colony stimulating factor or IL-3 instead of SCF. KPAM1 cells did not respond to any growth factors examined (Figure 3a). In this respect, KPAM1 resembled TL blasts (Figure 2a). We then investigated downstream pathways implicated in KIT-mediated signal transduction in KPAM1 cells. For this purpose, SCF-stimulated cells were analyzed for phosphorylation of KIT, AKT, ERK1/2 and SRC by western blotting analysis. Phosphorylations of both AKT and ERK were detected simultaneously with KIT activation, whereas the SRC family proteins were not phosphorylated (Figure 3b). Furthermore, addition of imatinib inhibited the phosphorylations of KIT, AKT and ERK in a dose-dependent manner (Figure 3c).

We asked whether these signal pathways were involved in proliferation of KPAM1 cells. For this purpose, various signal transduction inhibitors were added to the culture medium, and cell growth was assessed. Proliferation of KPAM1 cells was significantly reduced by imatinib, PI3K inhibitor LY294002 and MEK inhibitor U0126. However, KPAM1 cells were less sensitive to SRC inhibitor SU6656 than CMK11-5 and K562 cells (Figure 3d). These results suggest the critical roles of PI3K/AKT and RAS/MAPK pathways in the proliferation of KPAM1 cells.

Withdrawal of SCF from KPAM1 cells induces apoptosis

To further investigate the functional role of the SCF/KIT pathway in DS-related leukemia, we studied the consequences of removing SCF from KPAM1's culture medium. Withdrawal of SCF induced cell death accompanied by morphological changes. The fragmentation of nuclei and formation of apoptotic bodies were observed (Figure 4a). Furthermore, we detected

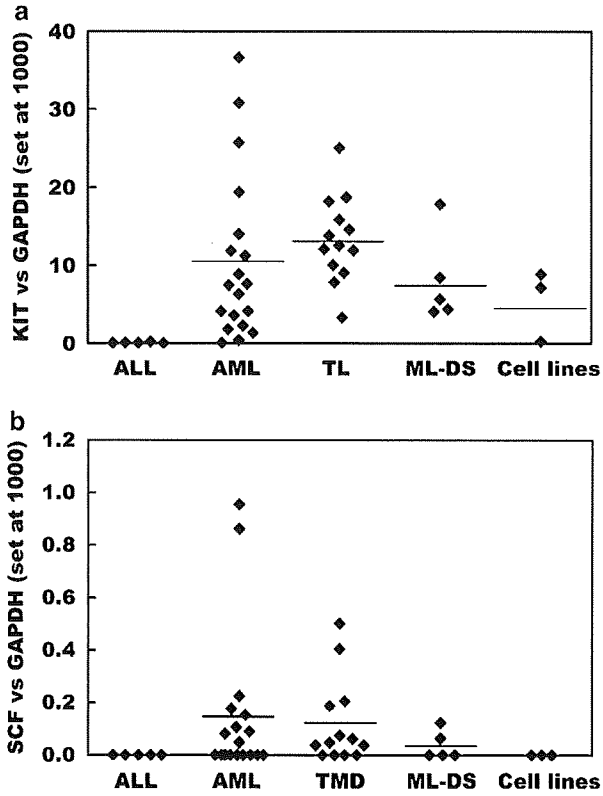

Figure 1 Quantitative real-time PCR analysis of *KIT* and *SCF* expression in cases of pediatric leukemia, TL and ML-DS. (**a**) *KIT* mRNA was quantified as the relative mRNA level normalized to *GAPDH* expression arbitrarily set at 1000. Data are mean values of triplicate analyses. ALL indicates acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; TL, transient leukemia; and ML-DS, Down syndrome-related acute megakaryocytic leukemia. Horizontal bars indicate the average for each group. (**b**) SCF mRNA was quantified as Figure 1a. The expression in promyelocytic leukemia cell line HL-60 as positive control was 0.177 (data not shown). SCF, stem cell factor.

cleavage of PARP, an important marker of caspase 3-mediated apoptosis (Figure 4b). These results suggested that SCF deprivation induced apoptosis in KPAM1 cells.

The relative levels and interactions of anti-apoptotic and pro-apoptotic BCL2 family proteins determine whether a cell survives or undergoes apoptosis.[13] Therefore, we analyzed the expression levels of BCL2 family members, including three anti-apoptotic proteins (BCLX, MCL1 and BCL2) (data not shown), and eight pro-apoptotic members, which included, BIM, BID, BAD, BAX, BOK, BIK, BMF and PUMA (data of latter five not shown). Among the examined proteins, only MCL1 and BIM changed significantly in expression. MCL1 was slightly decreased after 8 h starvation, while BIM was upregulated within 4 h starvation (Figure 4b).

To further understand the effects of SCF/KIT signaling on the expression of BIM and MCL1, we performed western blotting analysis. As shown in Figure 4c, a band shift of BIM was observed immediately and transiently after SCF stimulation. The shifted bands are reportedly due to phosphorylation of BIM, and the phosphorylation promotes proteasome-dependent degradation.[14] The results showed that imatinib and U0126 efficiently reduced the shift (Figure 4d). These results suggested that the RAS/MAPK pathway participated in the regulation of BIM protein downstream of SCF/KIT signaling in this cell line. With regard to MCL1, upregulation was observed after 2 h SCF stimulation (Figure 4c). The upregulation of MCL1 was effectively inhibited by treatment with imatinib, U0126 or LY294002 (Figure 4e), suggesting that the expression of MCL1 is regulated by not only the RAS/MAPK pathway but also the PI3K/AKT pathway. These results suggest that the inhibition of SCF/KIT signaling induces apoptosis in this cell line accompanied by up- and downregulation of BIM and MCL1, respectively.

KIT *gain-of-function mutations were not present in TL cases*

Mutations in *KIT* are important for tumor growth and progression in a variety of neoplasms, including mast cell diseases, gastrointestinal stromal tumor and AML. A previous report showed that no mutations were detected in 14 ML-DS and 4 TL patients.[15] However, the screening of *KIT* mutation was restricted to one of the hot spots, exon 17. To examine whether mutations in *KIT* are involved in the etiology or aberrant proliferation of TL blast cells, we screened the entire coding region of *KIT* in 14 TL patients. In our screening, two had novel substitutions: patient TL9 had a S854P change and the other patient, TL3, had a D910Y change. Both substitutions were located in the distal kinase domain of KIT protein (Figure 5a). To determine whether these substitutions were polymorphisms or mutations, we performed direct sequence analysis of exons 18 and 20 of the *KIT* gene using genomic DNA from 50 unrelated controls. Identical substitutions were not detected (data not shown).

To investigate whether or not these mutations involved gain-of-function, we first generated murine IL-3-dependent cell line Ba/F3 expressing either KIT^{S854P} or KIT^{D910Y} As shown in Figure 5b, expression of either KIT mutation did not confer factor-independent cell growth to Ba/F3 cells. Although Ba/F3 cells expressing KIT^{D910Y} proliferated in response to SCF in a dose-dependent manner, KIT^{S854P} cells did not grow. This result suggested that KIT^{S854P} was a loss-of-function mutation.

Next, we transduced the mutant KIT expression vectors to KPAM1 cells. As positive controls, we used KIT^{D816V} active mutant. As shown in Figure 5c, neither exogenous expression of wild-type KIT, KIT^{S854P} nor KIT^{D910Y} conferred SCF-independent growth to KPAM1 cells, except for the KIT^{D816V} mutation. The growth of KPAM1 cells expressing loss-of-function mutation KIT^{S854P} was slightly repressed in comparison with other transduced cells at more than 50 ng/ml of SCF stimulation. However, the conversion of optical densities into cell densities according to the standard curves indicated that the dominant negative effect of KIT^{S854P} was extremely weak or absent (Supplementary Figure S2).

A number of imatinib-resistant *KIT* mutations including KIT^{D816V} have been reported.[16] To examine whether the mutant KIT proteins found in TL patients affected growth inhibition by imatinib, cell proliferation assay was performed with various concentrations of imatinib. As shown in Figure 5d, all assessed cell lines except the cell line expressing KIT^{D816V} were significantly reduced in growth by imatinib in a dose-dependent manner. These results suggested that the *KIT* mutations identified in TL did not induce imatinib resistance.

Discussion

In this paper, we showed that SCF alone could support the growth of TL blast cells. Abnormalities in KIT expression and function are assumed to play important roles in the pathogenesis of several human cancers. Two principal mechanisms of KIT dysregulation in human malignancies have been described. One

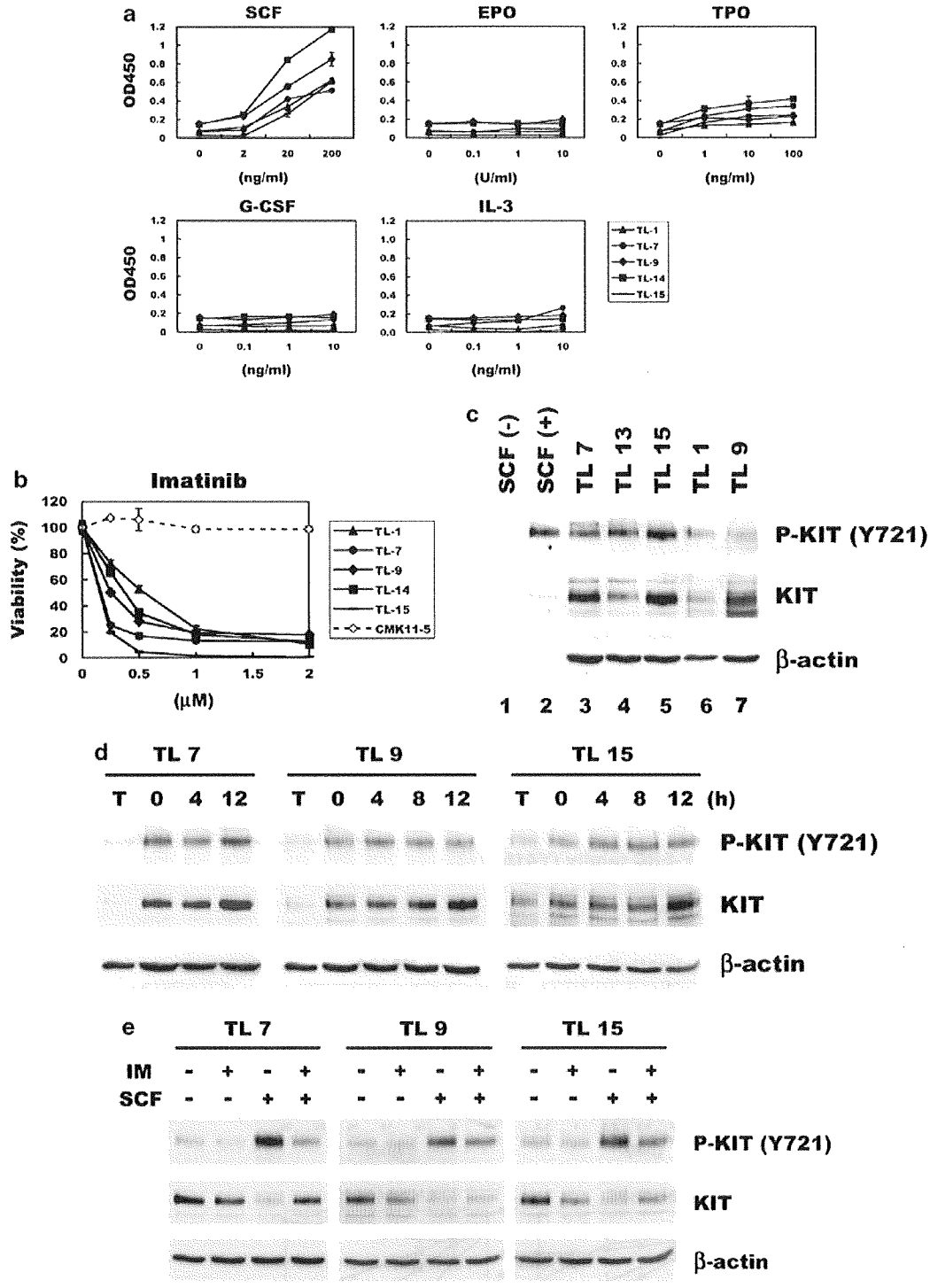

Figure 2 TL blast cells' responsiveness to growth factors and imatinib. (**a**) Cells were plated in liquid culture with the indicated concentrations of various growth factors. After 72 h incubation, viable cells were measured by Cell Counting Kit 8. (**b**) In the presence of 50 ng/ml SCF, TL blast cells were cultured with the indicated concentrations of imatinib for 72 h. Data are mean values ± s.d. in triplicate analyses. The relative viabilities of cells were calculated as percentages of control (no inhibitor). (**c**) A total of 60 μg of cell lysates of frozen stocked TL cells were prepared for detection of phosphoryrated KIT. Lanes 1 and 2 indicate 1 μg of cell lysates of CMK11-5 not stimulated or stimulated by 100 ng/ml of SCF, respectively. (**d**) Stocked TL blast cells were thawed (indicated by T) and pre-incubated overnight. Washed cells were cultured without exogenous SCF for 0, 4, 8 and 12 h. (**e**) Pre-incubated blast cells were treated with 1 μM of imatinib, with or without 100 ng/ml of SCF for 4 h. SCF, stem cell factor; TL, transient leukemia.

mechanism is gain-of-function mutation of *KIT* and the other is autocrine or paracrine production of SCF. We found 2 novel *KIT* missense mutations out of 14 TL patients. However, neither mutation was an activating mutation, suggesting that these mutations were 'passengers' and did not contribute to leukemogenesis in DS. It has been proposed that TL originates from

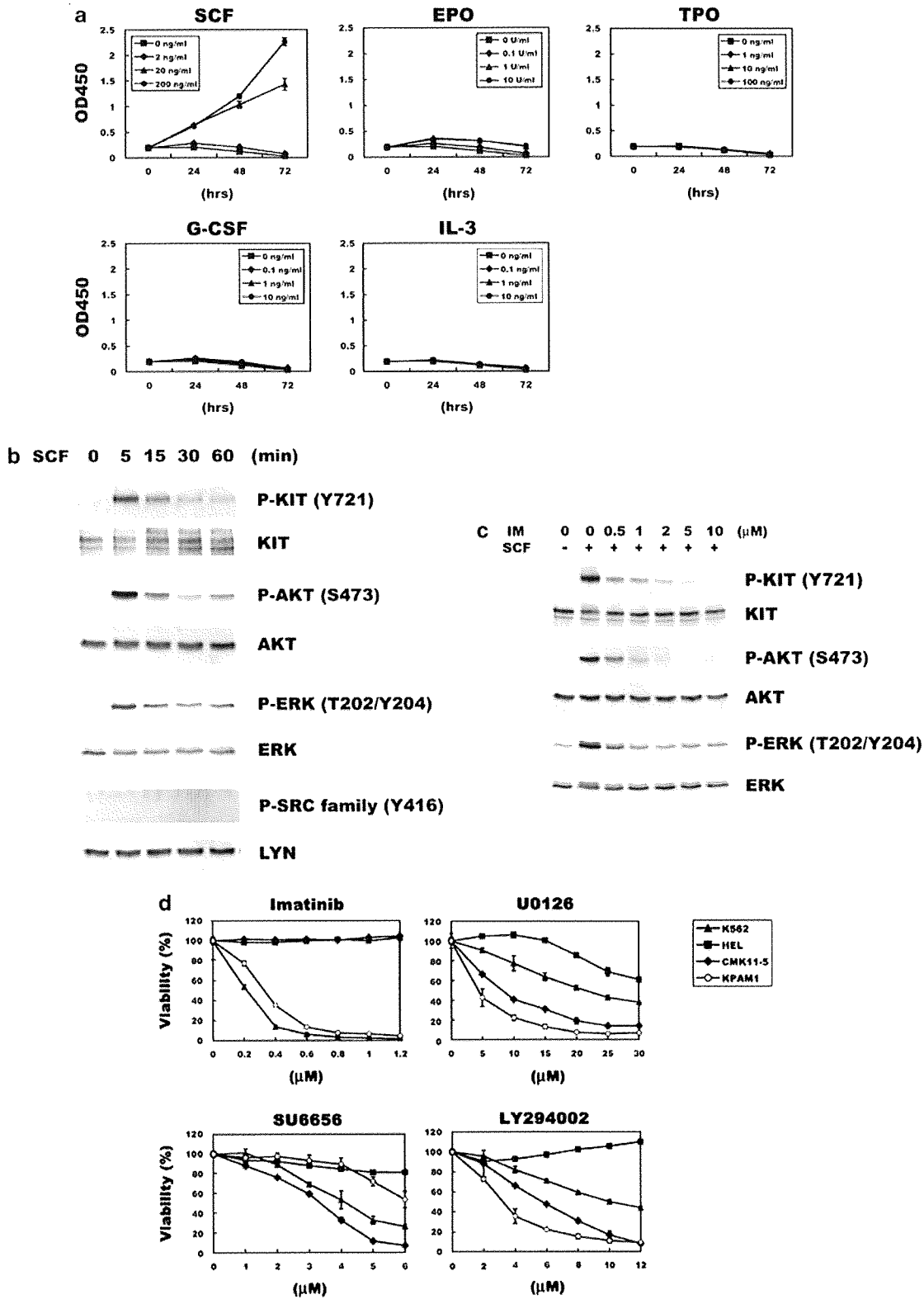

Figure 3 AKT and ERK signal pathways were activated in SCF-stimulated KPAM1 cells. (**a**) KPAM1 cells were cultured with the indicated concentrations of growth factors for 72 h. (**b**) After removal of SCF and FBS for 4 h, 50 ng/ml SCF was added and cells were solubilized at the indicated times. Proteins were immunoblotted by indicated antibodies. (**c**) Starved KPAM1 cells were treated with the inhibitors for 30 min prior to stimulation and with 50 ng/ml SCF for 10 min. Immunoblot analysis was performed as described above. (**d**) KPAM1 cells were cultured with the indicated concentrations of signal transduction inhibitors. After 72 h, viable cell numbers were measured spectrophotometrically. Data are mean values ± s.d. of triplicate analyses from three independent trials. The relative viabilities of cells in the presence of the indicated concentrations of inhibitor were calculated as Figure 2b. FBS, fetal bovine serum; SCF, stem cell factor; EPO, erythropoietin; TPO, thrombopoietin; G-CSF, granulocyte-colony stimulating factor; IL-3, interluekin-3.

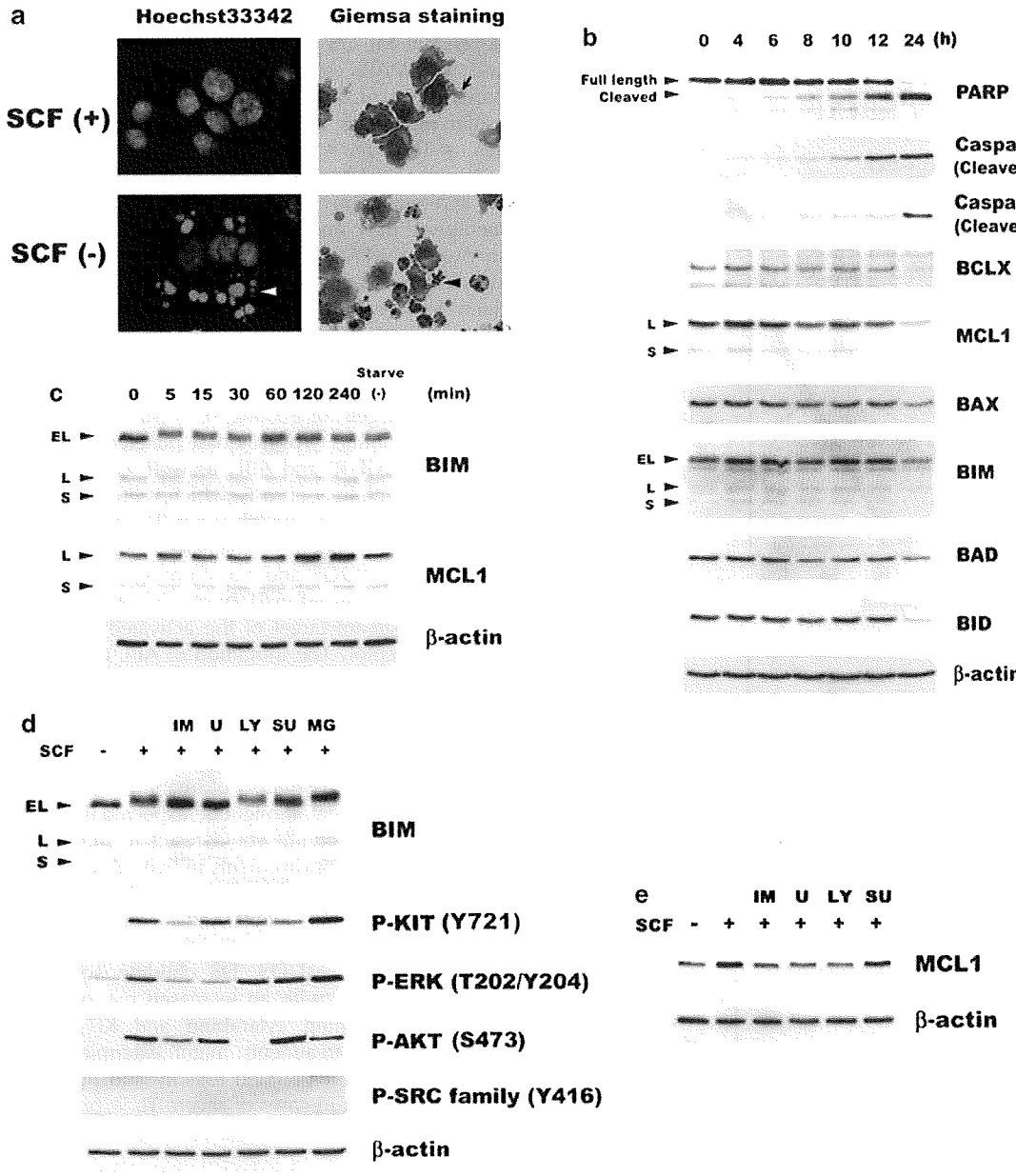

Figure 4 Induction of apoptosis by withdrawal of SCF. (**a**) KPAM1 cells were cultured in the presence (upper) or absence (lower) of SCF for 24 h and were prepared by Cytospin and stained by Giemsa (right) or Hoechst33342 (left). The arrow and the arrowhead indicate cytoplasmic blebs and nuclear fragmentation in KPAM1 cells, respectively. (**b**) KPAM1 cells were cultured without SCF up to 24 h and harvested. The blots were probed with the indicated antibodies. EL indicates extra long form; L, long form; and S, short form. (**c**) Sequential changes of the migration of BIM and the expression of MCL1 after SCF stimulation were detected by western blot analysis. (**d**) KPAM1 cells were SCF-starved for 4 h and then incubated with 1 μM imatinib, 20 μM U0126, 10 μM LY294002, 5 μM SU6656 or 30 μM MG132 for 30 min prior to SCF (50 ng/ml) stimulation. Cells were prepared 10 min after the stimulation. Western blotting was performed as described above. (**e**) After 8 h incubation with SCF and inhibitors as in Figure 4d, cell lysate was prepared. Western blotting was performed for MCL1. IM, imatinib; U, U0126; LY, LY294002; SU, SU6656; or MG, MG132; SCF, stem cell factor.

the fetal liver.[17] Interestingly, high levels of SCF expression have been observed in human fetal liver within a restricted time period.[18] Furthermore, a recent report showed the transient hyperproliferation of fetal hepatic megakaryocytic progenitors in GATA1s knock-in mouse.[19] Our present data, together with these findings, suggest that paracrine activation of the SCF/KIT pathway might play very important roles in the expansion of TL cells in the fetal liver.

We showed that *KIT* was uniformly expressed in TL cells at a relatively high level, although *KIT* expression in TL was not higher than in AML in general. Recently, Kindler *et al.*[20] reported that imatinib is effective only in small subgroup of AML patients without activating *KIT* mutations. It is conceivable that the therapeutic potential of KIT inhibitor is primarily determined by dependency of the survival or growth of leukemic cells to SCF/KIT signaling. The proliferative response of primary AML cells to SCF is very heterogenous and is not correlated with the number of KIT.[21] In contrast to AML, all TL cells examined proliferated well in response to SCF (Figure 2a). Therefore, KIT will be a good candidate as a therapeutic target in TL.

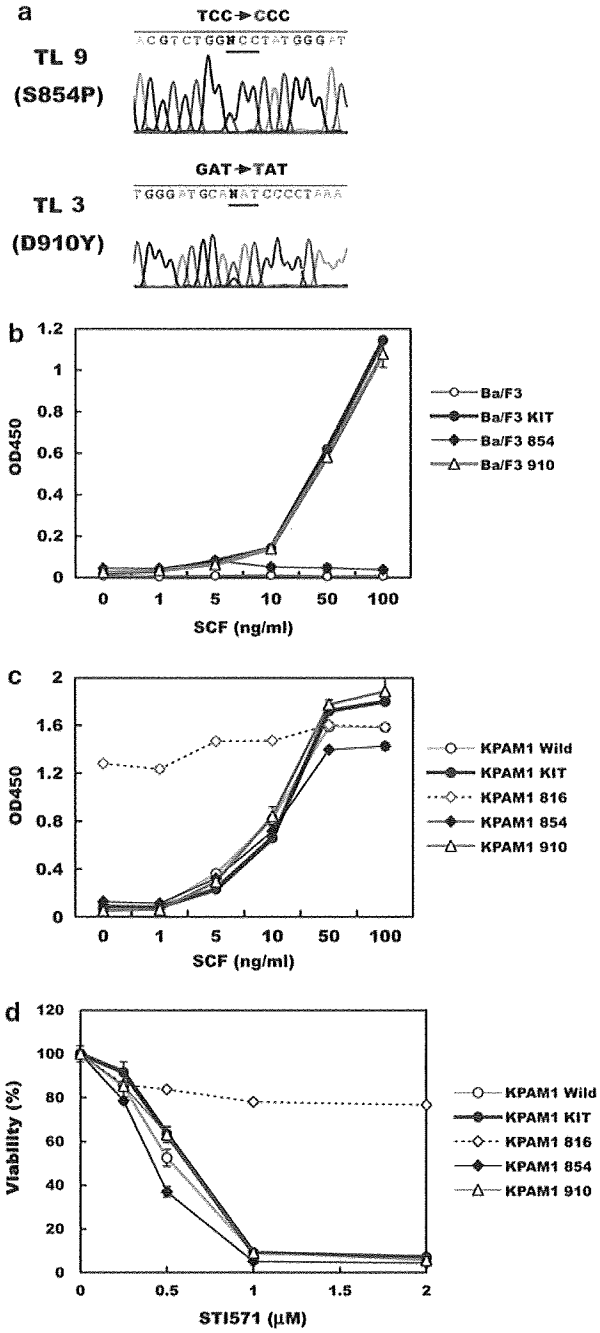

Figure 5 *KIT* mutations isolated from TL patients. (**a**) *KIT* was directly sequenced in 14 TL patients using cDNA. Two mutations including D910Y (patient TL3) and S854P (patient TL9) were identified. (**b**) Ba/F3 cells expressing exogenous KIT were cultured with various concentrations of SCF without IL-3. After 72 h, viable cell numbers were measured spectrophotometrically. Data are mean values ± s.d. in triplicate analyses from three independent trials. (**c**) KPAM1 cells expressing exogenous KIT were assessed as described in Figure 5b. (**d**) KPAM1 cells expressing mutant KIT were incubated in the indicated concentration of imatinib. After 72 h culture, viable cell numbers were measured as described in Figure 5b. The relative viabilities of the cells in the presence of the indicated concentrations of imatinib were calculated as Figure 2b. Data are mean values ± s.d. in triplicate analyses from three independent trials. TL, transient leukemia; SCF, stem cell factor; IL-3, interluekin-3.

Following stimulation of KIT with SCF, multiple downstream signal transduction components are activated. However, the pivotal downstream cascades were unclear in both TL and ML-DS blast cells. In this report, we established the first SCF-dependent ML-DS cell line, and demonstrated that PI3K/AKT and RAS/MAPK signaling pathways had essential roles in proliferation of the cell line. This novel cell line may be a useful model for the identification of therapeutic molecules against TL and/or ML-DS.

Apoptosis-related molecules are common targets in therapeutic approaches to neoplasms. We showed that BCL2 family members, BIM and MCL1, are involved in apoptosis and survival of KPAM1 cells. Our results suggest that SCF/KIT signaling promotes the survival of KAPM1 cells by (1) protecting them from BIM-mediated apoptosis and (2) inducing anti-apoptotic effects mediated by MCL1. Identification of the molecules inducing upregulation of BIM or downregulation of MCL1 might prove useful in the identification of novel molecular targets for treating TL or ML-DS.

Imatinib inhibits tyrosine kinase platelet-derived growth factor receptor (PDGF-R) and ABL, as well as KIT. TL is sometimes associated with liver fibrosis, which is life threatening and often fatal.[4,5] Transforming growth factor-β1 and PDGF were reported to be key mediators in fibrogenesis.[22] Recently, we and others reported that PDGF and transforming growth factor-β1 were abundantly expressed in TL blasts.[23,24] ABL has been shown to be a downstream target of the activated transforming growth factor-β receptor. The inhibition of ABL activity with imatinib ameliorated renal fibrosis in an experimental model.[25] PDGF is the most potent proliferating stimulus for hepatic stellate cells. Inhibition of the function of PDGF/PDGF-R attenuated the development of experimental liver fibrosis.[26] Yoshiji *et al.*[27] demonstrated the usefulness of imatinib in suppression of liver fibrosis development. It might be expected that the treatment of TL cases with imatinib would result in inhibition of TL blast growth and also improvements in liver fibrosis.

Recently, Klusmann *et al.*[5] showed that low-dose cytarabine treatment had a beneficial effect on high-risk TL cases. However, the treatment was insufficient to prevent the progression from TL to ML-DS. DS children suffer high rates of treatment toxicity, with an increased risk of treatment-related death.[2,3] Combining cytarabine and KIT-targeting tyrosine kinase inhibitor treatments might exterminate the TL clone without serious drug-related toxicity and provide a beneficial therapeutic effect in cases of severe TL.

Conclusion

SCF/KIT signaling stimulated TL blast proliferation. The treatment of TL blasts with imatinib suppressed the proliferation effectively *in vitro*. We established the first SCF-dependent ML-DS cell line, KPAM1, and elucidated signal cascades downstream from SCF/KIT signaling which promoted survival of this cell line. These results suggest the essential role of SCF/KIT signaling in DS-related leukemia, and the possibility of therapeutic benefits from KIT-targeting tyrosine kinase inhibitor treatment of severe TL patients.

Acknowledgements

We thank Dr K Kageyama (Hirosaki University Graduate School of Medicine) for helpful discussion. We are grateful to Dr K Kawakami, S Morinaga, M Migita, K Kato, H Kanegane, M Miura, T Nakano, T Taga, A Shimada, D Hasegawa for clinical samples. XG is supported by a fellowship from the Ministry of Education, Science, Sports and Culture. This work was supported by

Grants-in-Aid for Scientific Research and Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture, and Grants from Ministry of Health Labour and Welfare.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

Ex vivo expanded cord blood CD4 T lymphocytes exhibit a distinct expression profile of cytokine-related genes from those of peripheral blood origin

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doi:10.1111/j.1365-2567.2009.03122.x
Received 1 September 2008; revised 30 March 2009; accepted 15 April 2009.
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Summary

With an increase in the importance of umbilical cord blood (CB) as an alternative source of haematopoietic progenitors for allogenic transplantation, donor lymphocyte infusion (DLI) with donor CB-derived activated $CD4^+$ T cells in the unrelated CB transplantation setting is expected to be of increased usefulness as a direct approach for improving post-transplant immune function. To clarify the characteristics of activated $CD4^+$ T cells derived from CB, we investigated their mRNA expression profiles and compared them with those of peripheral blood (PB)-derived activated $CD4^+$ T cells. Based on the results of a DNA microarray analysis and quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR), a relatively high level of forkhead box protein 3 (Foxp3) gene expression and a relatively low level of interleukin (IL)-17 gene expression were revealed to be significant features of the gene expression profile of CB-derived activated $CD4^+$ T cells. Flow cytometric analysis further revealed protein expression of Foxp3 in a portion of CB-derived activated $CD4^+$ T cells. The low level of retinoic acid receptor-related orphan receptor γ isoform t (RORγt) gene expression in CB-derived activated $CD4^+$ T cells was speculated to be responsible for the low level of IL-17 gene expression. Our data indicate a difference in gene expression between $CD4^+$ T cells from CB and those from PB. The findings of Foxp3 expression, a characteristic of regulatory T cells, and a low level of IL-17 gene expression suggest that CB-derived $CD4^+$ T cells may be a more appropriate source for DLI.

Keywords: CD4; cord blood; donor lymphocyte infusion; forkhead box protein 3; interleukin 17; T cell

Abbreviations: BIM, BCL2-like 11; CB, cord blood; CTLA-4, cytotoxic T-lymphocyte antigen-4; CDKN, cyclin-dependent kinase inhibitor; DLI, donor lymphocyte infusion; Foxp3, forkhead box protein 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte–macrophage colony-stimulating factor; GVHD, graft-versus-host disease; GVL, graft-versus-leukaemia; HSCT, haematopoietic stem cell transplantation; ICOS, inducible T-cell co-stimulator; IFNG, interferon γ; IL, interleukin; PB, peripheral blood; RORγt, retinoic acid receptor-related orphan receptor γ isoform t; RT, reverse transcriptase; TCR, T-cell receptor; Th, T helper cell; Treg, regulatory T cell.

Introduction

Donor lymphocyte infusion (DLI) is a direct and useful approach for improving post-transplant immune function. DLI has been shown to exert a graft-versus-leukaemia (GVL) effect and has emerged as an effective strategy for the treatment of patients with leukaemia, especially chronic myelogenous leukaemia, who have relapsed after unrelated haematopoietic stem cell transplantation (HSCT).[1] In addition, DLI has been successfully used for some life-threatening viral infections, including Epstein–Barr virus and cytomegalovirus infections after HSCT.[2]

Although DLI frequently results in significant acute and/or chronic graft-versus-host disease (GVHD), several groups have demonstrated that depletion of CD8 T cells from DLIs efficiently reduces the incidence and severity of GVHD while maintaining GVL activity.[3,4] Therefore, selective CD4 DLI is expected to provide an effective and low-toxicity therapeutic strategy for improving post-transplant immune function. Actually, selective CD4 DLI based on a recently established method for *ex vivo* T-cell expansion using anti-CD3 monoclonal antibody and interleukin (IL)-2 is now becoming established as a routine therapeutic means of resolving post-transplant immunological problems in Japan.[5]

The importance of umbilical cord blood (CB) as an alternative source of haematopoietic progenitors for allogenic transplantation, mainly in patients lacking a human leucocyte antigen (HLA)-matched marrow donor, has increased in recent years. Because of the naïve nature of CB lymphocytes, the incidence and severity of GVHD are reduced in comparison with the allogenic transplant setting. In addition, CB is rich in primitive $CD16^-$ $CD56^+$ natural killer (NK) cells, which possess significant proliferative and cytotoxic capacities, and so have a substantial GVL effect.[6]

In contrast, a major disadvantage of CB transplantation is the low yield of stem cells, resulting in higher rates of engraftment failure and slower engraftment compared with bone marrow transplantation. In addition, it was generally thought to be difficult to perform DLI after CB transplantation using donor peripheral blood (PB), with the exception of transplantations from siblings. However, the above-described method for the *ex vivo* expansion of activated T cells can produce a sufficient amount of cells for therapy using the CB cell residues in an infused bag, which has solved this problem and made it possible to perform DLI with donor CB-derived activated $CD4^+$ T cells in the unrelated CB transplantation setting.[5] It has also been reported that CB-derived T cells can be expanded *ex vivo* while retaining the naïve and/or central memory phenotype and polyclonal T-cell receptor (TCR) diversity,[7] and thus potential utilization for adoptive cellular immunotherapy post-CB transplantation has been suggested.[8]

There are functional differences between CB and PB lymphocytes, although the details remain unclear. In an attempt to clarify the differences in characteristics between activated $CD4^+$ T cells derived from CB and those derived from PB, we investigated gene expression profiles. In this paper we present evidence that CB-derived $CD4^+$ T cells are distinct from PB-derived $CD4^+$ T cells in terms of gene expression.

Materials and methods

Cell culture and preparation

CB was distributed by the Tokyo Cord Blood Bank (Tokyo, Japan). The CB was originally collected and stored for stem cell transplantation. Stocks that were inappropriate for transplantation because they contained too few cells were distributed for research use with informed consent, with the permission of the ethics committee of the bank. In addition, all of the experiments in this study using distributed CB were performed with the approval of the local ethics committee. The mononuclear cells were isolated by Ficoll-Paque centrifugation and cultured in the presence of an anti-CD3 monoclonal antibody and interleukin (IL)-2 using TLY Culture Kit 25 (Lymphotec Inc., Tokyo, Japan) as described previously.[5] Although several different methods for T-cell stimulation have been reported, this method is currently being used clinically in Japan. Thus we selected this method in this study. After 14 days of culture, $CD4^+$ cells were isolated using a magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. As a control, mononuclear cells isolated from the peripheral blood of healthy volunteers were similar examined.

Polymerase chain reaction (PCR)

Total RNA was extracted from cells using an RNeasy kit (Qiagen, Valencia, CA) and reverse-transcribed using a First-Strand cDNA synthesis kit (GE Healthcare Bio-Science Corp., Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions. Using cDNA synthesized from 150 ng of total RNA as a template for one amplification, real-time reverse transcriptase (RT)-PCR was performed using SYBR® Green PCR master mix, TaqMan® Universal PCR master mix and TaqMan® gene expression assays (Applied Biosystems, Foster City, CA), and an inventoried assay carried out on an ABI PRISM® 7900HT sequence detection system (Applied Biosystems) according to the instructions provided. Either the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene or the β-actin gene was used as an internal control for normalization. The sequences of gene-specific primers for real-time RT-PCR are listed in Table 1.

DNA microarray analysis

The microarray analysis was performed as previously described.[9] Total RNA isolated from cells was reverse-

