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).

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*.

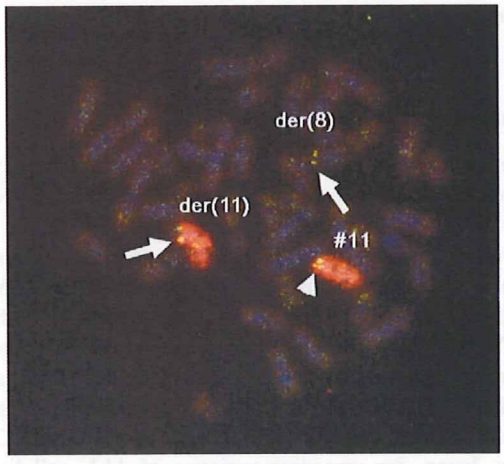

Fig. 1. FISH analysis of *NUP98* rearrangement in a leukemic metaphase. Split signals (arrows) of bacterial artificial chromosome clone PK505 containing *NUP98* were observed on the boundary between painted and unpainted regions of der(11)t(8;11) and der(8)t(8;11). An intact PK505 signal was observed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

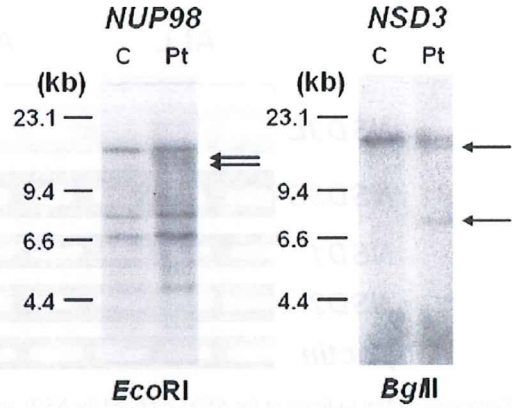

Fig. 2. Southern blotting of the *NUP98* gene with *Eco*RI and the *NSD3* gene with *Bgl*II restriction endonuclease. Arrows indicate rearranged bands. Pt, patient; C, control.

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*

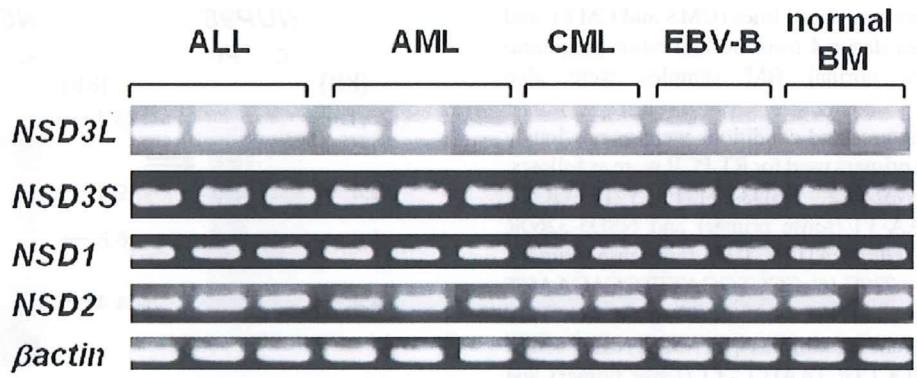

Fig. 3. Expression of two isoforms of the *NSD3* gene and the *NSD1* and *NSD2* genes in acute lymphoblastic, acute myeloid, and chronic myeloid leukemia cell lines, Epstein–Barr virus transformed B lymphocyte (EBV-B) cell lines, and normal healthy bone marrow cells, determined with reverse transcriptase–polymerase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

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ORIGINAL ARTICLE

Possible involvement of RasGRP4 in leukemogenesis

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Abstract It is now conceivable that leukemogenesis requires two types of mutations, class I and class II mutations. We previously established a mouse bone marrow-derived HF6, an IL-3-dependent cell line, that was immortalized by a class II mutation MLL/SEPT6 and can be fully transformed by class I mutations such as FLT3 mutants. To understand the molecular mechanism of leukemogenesis, particularly progression of myelodysplastic syndrome (MDS) to acute leukemia, we made cDNA libraries from the samples of patients and screened them by expression-cloning to detect class I mutations that render HF6 cells factor-independent. We identified RasGRP4, an activator of Ras, as a candidate for class I mutation from three of six patients (MDS/MPD = 1, MDS-RA = 1, MDS/AML = 2, CMMoL/AML = 1 and AML-M2 = 1). To investigate the potential roles of RasGRP4 in leukemogenesis, we tested its in vivo effect in a mouse bone marrow transplantation (BMT) model. C57BL/6J mice transplanted with RasGRP4-transduced primary bone marrow cells died of T cell leukemia, myeloid leukemia, or myeloid leukemia with T cell leukemia. To further examine if the combination of class I and class II mutations accelerated leukemic transformation, we performed a mouse BMT model in which both AML1 mutant (S291fsX300) and RasGRP4 were transduced into bone marrow cells. The double transduction led to early onset of T cell leukemia but not of AML in the transplanted mice when compared to transduction of RasGRP4 alone. Thus, we have identified RasGRP4 as a gene potentially involved in leukemogenesis and suggest that RasGRP4 cooperates with AML1 mutations in T cell leukemogenesis as a class I mutation.

Keywords RasGRP4 · AML1 · Class I mutation · Leukemogenesis · cDNA library

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1 Introduction

Various chromosome translocations and gene mutations were known to participate in leukemogenesis. Recently, it was recognized that multiple gene alterations are required

for leukemogenesis; coexistence of chromosomal translocations and gene mutations are frequently found in the same patient. There are some frequent combinations including c-Kit mutations and AML1/ETO [1–6], c-Kit mutations and inv(16) [1, 5–7], Ras mutations and AML1 point mutations [8, 9], FLT3-ITD and AML1 point mutations [10, 11], FLT3 mutations and PML-RARα [12–16], MLL rearrangement and FLT3-TKD [17], MLL rearrangement and Ras mutations [18], and FLT3-ITD and NPM1 mutations [19]. Interestingly, on the other hand, RAS and FLT3 mutations, which are detected in about 50% of patients with de novo AML, are negatively associated with each other [20, 21]. In mice models, while expression of PML/RARα in transgenic mice caused a nonfatal myeloproliferative syndrome, transplantation of bone marrow cells obtained from PML/RARα transgenic mice retrovirally transduced with FLT3-ITD resulted in development of an APL-like disease in a short latency [22]. Two step leukemogenesis was also suggested by an in vitro culture system of human hematopoietic cells [23]. Based on these findings, leukemia-related mutations are classified into two groups, class I and class II mutations. Class I mutations include activating mutations of tyrosine kinases and a small GTPase Ras or inactivation of apoptosis-related molecule, and these mutations induce cell proliferation or block apoptosis. On the other hand, class II mutations include dominant negative mutations of transcription factors involved in differentiation of hematopoietic cells, such as AML1/ETO, PML/RARa, or constitutively activated mutations of chromosome remodeling factors such as MLL-related fusion genes [24]. Indeed, it has been reported that a combination of class I and II mutations such as PML/RARα plus FLT3-ITD [22], AML1/ETO plus FLT3 mutation [25], AML1/EVI1 plus BCR/ABL [26], MLL/SEPT6 plus FLT3 mutation [27], K-ras plus PML/RARα [28] induced AML in a mouse BMT model, while either class I or II mutation alone led to, myeloproliferative disorders (MPD) or MDS like disease, not leukemia [22–28].

To identify class I mutations from patients with MDS/AML, MPD, or AML, we used retrovirus-mediated expression cloning; cDNA libraries from patients' samples were constructed and retrovirally transfected into an IL-3-dependent myeloid cell line, HF6, immortalized by a class II mutation MLL/SEPT6 [27]. We searched for class I mutations that abrogate IL-3 dependency of HF6 and we identified RasGRP4 as a candidate gene from three different libraries (MDS/MPD = 1, MDS/AML = 1, MDS-RA = 1). In addition, FLT3-ITD was identified in a patient with MDS/AML.

RasGRP4 belongs to a family of guanine nucleotide-exchange factors (RasGRP1-4) that positively regulate Ras and related small GTPases, and is mainly expressed in myeloid cells and mast cells [29, 30]. RasGRP4 appears to act downstream of the tyrosine kinase receptor c-Kit/CD117 [30]. RasGRP4 is located on 19q13.1 and alterations of this site have been found in several cancers (the "Cancer Chromosomes" at the NCI web site), and was previously isolated by expression cloning from cytogenetically normal AML patients using the focus-forming assay of NIH3T3 cells [29]. In the present study, we isolated RasGRP4 using expression cloning as a gene that fully transforms IL-3-dependent HF6 cells, and investigated the effect of RasGRP4 overexpression in a mouse BMT model and implicated RasGRP4 in leukemogenesis.

2 Materials and methods

2.1 Cell lines and cell culture

A mouse pro-B line Ba/F3 was maintained in RPMI1640/10% fetal bovine serum (FBS) containing 1 ng/ml recombinant mouse IL-3 (obtained from R & D systems). HF6, which had been established by introducing MLL/SEPT6 into mouse bone marrow cells, was maintained in RPMI1640/10% FBS containing 10 ng/ml mouse IL-3 as described [27].

2.2 Screening of cDNA libraries

Complementary cDNA libraries were generated from patients leukemic or MDS cells (MDS/MPD = 1, MDS/AML = 2, CMMoL/AML = 1, MDS-RA = 1, AML-M2 = 1) as described [31]. MDS or leukemic cells of these patients did not harbor recurrent chromosomal translocations involving AML1 or MLL. One patient with AML-M2 did not display t(8;21). The point mutations of AML1 were not screened. Recombinant retroviruses were generated by transient transfection using an ecotropic packaging cell line PLAT-E as described with minor modifications [32]. Bone marrow or peripheral blood samples of patients were taken under the experimental procedure approved by the ethical committees of our institute (approve no. 20-9).

We introduced each cDNA library into two IL-3-dependent cell lines Ba/F3 and HF6. After transduction with the cDNA library, the transduced cells were seeded into 96-well plates in the absence of IL-3, and factor independent clones were isolated. To identify the cDNA that confers factor independency on Ba/F3 or HF6, genomic DNA of the factor independent clones were purified and integrated cDNAs were isolated and sequenced.

2.3 Vector construction

cDNAs for human RasGRP4 were cloned from cDNA libraries of MDS/MPD patients and normal volunteers using PCR primers: 5′-GGAGCTGAGCCCTACTCTTG-3′

(forward), 5′-AGAGTCTGACGGCAGGACTC-3′ (reverse). We used pfu polymerase (Stratagene, La Jolla, CA) to amplify the coding region of human RasGRP4. We subcloned the PCR products into TOPO vector (Invitrogen, San Diego, CA). Then, the EcoRI fragment carrying RasGRP4 was inserted into the EcoRI sites of pMXs vector [32]. RasGRP4 sequences derived from patients and normal volunteers were not identical to those in the data bases as described in result section. We used an AML1 mutant, S291fsX300, identified from case number 27 among MDS/AML patients [33]. This mutant is hereafter referred to as AML1-S291fs. The AML1-S291fs was inserted upstream of the IRES-EGFP cassette of a retrovirus vector pMYs-IG [32] to generate pMYs-AML1-S291fs-IG.

2.4 Expression of RasGRP4 in HF6

To confirm that the isolated RasGRP4 is responsible for factor-independency of HF6, the cells were infected with the retroviruses harboring pMXs-RasGRP4 derived from patients, normal volunteers or an empty vector as a control, and cultured in the absence of IL-3. To investigate the activation of the Ras pathway in the HF6 cells expressing RasGRP4, the transfected cells were lysed in lysis buffer, and lysates were subject to western blot analysis as described with minor modifications [34]. Monoclonal mouse anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody (Sigma) was used for phosphorylated ERK1/2.

2.5 Bone marrow transplantation

Bone marrow mononuclear cells were isolated and cultured as described [35]. The prestimulated cells were infected for 60 h with the retroviruses harboring pMXs-RasGRP4 derived from a patient with MDS/MPD, pMYs-AML1-S291fs-IG or an empty vector as a control, using six well dishes coated with RetroNectin (Takara Bio, Inc.) according to the manufacturer's recommendations. Then, 0.3–1.2 $\times$ 10^6 of infected bone marrow cells (Ly-5.1) were injected through tail vein into C57BL/6 (Ly-5.2) recipient mice (8–12 weeks of age) which had been administered a sublethal dose of 5.25 Gy total-body γ-irradiation (135Cs). Overall survival of the transplanted mice was analyzed using the Kaplan–Meier-method. All animal studies were approved by the Animal Care Committee of the Institute of Medical Science, The University of Tokyo.

2.6 Analysis of the transplanted mice

Engraftment of bone marrow cells was confirmed by measuring the percentage of Ly-5.1-positive and/or GFP positive cells in peripheral blood obtained every 1–2 months after the transplant. After the morbid mice were euthanized, their tissue samples including peripheral blood (PB), bone marrow (BM), spleen, liver, and kidney were analyzed. Circulating blood cells were counted by automatic blood cell counter KX-21 (Sysmex, Kobe, Japan). Morphology of the peripheral blood cells was evaluated by staining of air-dried smears with Hemacolor (Merck). Tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). Cytospin preparations of bone marrow and spleen cells were also stained with Hemacolor. The percentage of blasts, myelocytes, neutrophils, monocytes, lymphocytes, and erythroblasts was estimated by examination of at least 200 cells. To assess whether the leukemic cells were transplantable, 2 $\times$ 10^5–1 $\times$ 10^6 total BM cells including blasts were injected into the tail veins of sublethally irradiated mice. A total of two or three recipient mice were used for each serial transplantation.

2.7 Flow cytometric analysis

Peripheral blood or single-cell suspensions of bone marrow and spleen were stained with the following phycoerythrin (PE)-conjugated monoclonal antibodies: Ly-5.1, Gr-1, CD11b, B220, CD3, CD4, CD8, CD41, c-Kit, Sca-1, CD34, and Ter-119. Then, flow cytometric analysis was performed as described [35].

2.8 RT-PCR

To confirm expression of human RasGRP4, total RNA was extracted from BM cells of transplanted mice using Trizol (Invitrogen, California, USA) and cDNA was prepared with the Superscript II RT kit (Invitrogen, California, USA) and RT-PCR was performed using a 2720 Thermal cycler (Applied Biosystems, Tokyo, Japan). The cDNA was amplified using AmpliTaq Gold (Applied Biosystems by Roche Molecular Systems, Inc., New Jersey. USA). The reaction was subject to one cycle at 95°C for 5 min, 30 cycles of PCR at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. All samples were independently analyzed at least three times. The following primer pairs were used: 5′-ACTGGCTGATGCGACACCC-3′ (forward) and 5′-GAGATGGCACTGTGACACAG-3′ (reverse) for human RasGRP4, 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA -3′ (reverse) for GAPDH.

2.9 Quantitative RT-PCR

To examine expression levels of human RasGRP4 in patients, quantitative RT-PCR was performed. Quantitative RT-PCR was performed using a LightCycler Workflow System (Roche Diagnostics, Mannheim, Germany).

Complementary DNAs derived from bone marrow cells of leukemia or MDS patients as well as normal bone marrow cells were amplified using a SYBR Premix EX Taq (TAKARA). The reaction was subject to one cycle at 95°C for 30 s, 45 cycles of PCR at 95°C for 5 s, 55°C for 10 s, and 72°C for 10 s. All samples were independently analyzed at least three times. The primer pairs for human RasGRP4 and GAPDH were the same as described above. The samples from the patients were obtained under written consents which had been approved by the local ethical committee of each institute or hospital.

2.10 Bubble PCR

Genomic DNA was extracted from BM or spleen cells of transplanted mice and digested with EcoRI, and then the fragments were used for Bubble PCR to identify the integration sites of the retroviruses as described [35]. We confirmed inverse repeat sequence "GGGGGTCTTTCA" as a marker of junction between genomic DNA and retrovirus sequence.

3 Results

3.1 RasGRP4 induces factor-independent growth of HF6

In the screening of cDNA libraries, some wells gave rise to cell growth in the absence of IL-3 from HF6 but not from BaF3 cells. The factor-independent clones were isolated and the cDNAs integrated in the genome DNA were sequenced using PCR. FLT3-ITD was identified in one MDS/AML patient. In addition, RasGRP4 was identified from three different libraries (MDS/MPD = 1, MDS/AML = 1, and MDS-RA = 1). We introduced the isolated RasGRP4 into HF6 to confirm that RasGRP4 was responsible for autonomous growth of HF6 cells (Fig. 1a). In the sequence of RasGRP4 derived from the MDS/MPD, MDS/AML and MDS-RA patients, we found several different amino acids that compared with the sequences in two databases GenBank (accession number AF448437) and GenBank (accession number AY048119) (Table 1). Therefore, we introduced RasGRP4 derived from a patient and two Japanese normal volunteers (normal 1 and 2 in Table 1) into HF6 cells to examine if RasGRP4 from normal volunteers also gives rise to factor-independency. As a result, RasGRP4 from normal volunteers also induced factor-independent growth of HF6, indicating that overexpression of RasGRP4 by itself induced transformation of the cells, independent of some mutations in the amino acid sequence of RasGRP4. While several gene alterations were observed in the samples of patients, we focused on E468K because this change was observed only in a patient with MDS/MPD but not in the sequence derived from the two databases and two normal volunteers (Table 1). However, we did not find any functional importance of the alteration at codon 468 that changes a glutamic acid to a lysine. Moreover, SNPs of this gene are not correlated with lymphoma and leukemia (Y. Nakamura, unpublished results).

To assess the RasGRP4-mediated Ras activation, we examined phosphorylation of ERK1/2 using HF6-cells-transduced RasGRP4. Stimulation with IL-3 induced much stronger phosphorylation of ERK1/2 in the HF6 cells expressing RasGRP4 when compared with parent HF6 cells (Fig. 1b, lanes 6–8). Although we did not observe enhanced phosphorylation of ERK1/2 in the cells overexpressing RasGRP4 without IL-3 (Fig. 1b, lane 5), we assume that non-detectable enhancement of ERK1/2 was

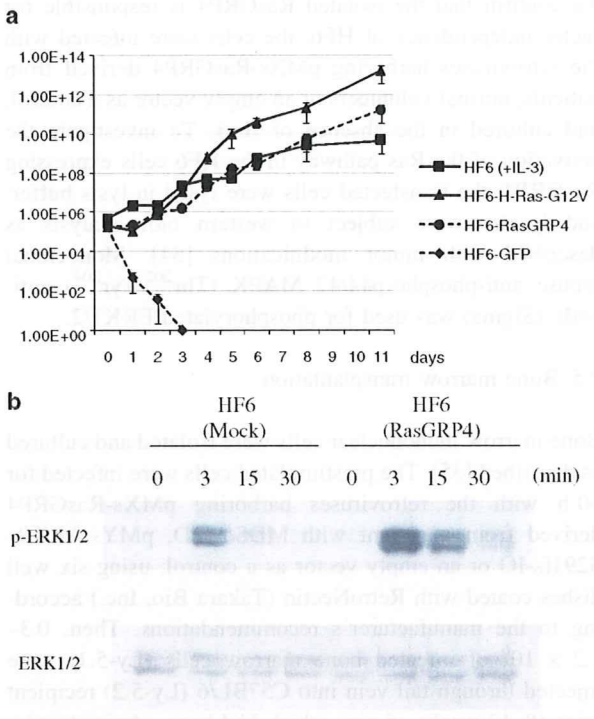

Fig. 1 RasGRP4 conferred factor independency on HF6. **a** HF6 cells expressing the H-Ras-G12V, RasGRP4 and GFP vector were deprived of IL-3, and cells were counted by trypan blue exclusion. The parental HF6 cells in the presence of IL-3 (10 ng/mL) were counted as same. **b** Stimulation with IL-3 induced strong phosphorylation of ERK1/2 in the HF6 cells expressing RasGRP4. Phosphorylation of ERK1/2 (pERK1/2) was examined in HF6 cells transfected with RasGRP4 or empty by western blot analysis using anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) Ab. Loading amount was estimated by re-probing immunoblots with Abs specific for ERK1/2. The transfected HF6 cells were washed with PBS twice and cultured in RPMI1640/10% FBS without IL-3 for 4 h. Then, some of cells were collected and lysed (*lanes 1 and 5*). The remained cells were stimulated with IL-3 (100 ng/mL) for the indicated period and collected and lysed (*lanes 2–4*, and *6–8*)

Table 1 Polymorphism of RasGRP4

Position of amino acid	AF448437	AY048119	Patient 1 (MDS/MPD)	Patient 2 (MDS/AML)	Patient 3 (MDS-RA)	Normal 1	Normal 2
18	T	T	T	I	T	T	I
120	Q	L	Q	Q	Q	Q	Q
261	R	C	R	R	R	R	R
468	E	E	K	E	E	E	E
541	H	H	H	H	H	H	Y
671	L	P	P	L	L	L	L

enough to induce factor-independent growth of HF6. Only weak activation of the signaling molecule, even non-detectable in biochemical experiments, sometimes induces autonomous cell growth.

3.2 RasGRP4 induced myeloid leukemia and T cell leukemia in mice

We further examined if overexpression of RasGRP4 induced leukemia in a mouse BMT model. We confirmed expression of human RasGRP4 in BM cells of transplanted mice by RT-PCR (Fig. 2a). Transduction of RasGRP4 (E468K) induced myeloid and/or T cell leukemia with various phenotypes, and the transplanted mice died within 2–8 months after the transplantation (Fig. 2b). For example, a mouse (ID 402) died of T cell leukemia with thymoma (weight of thymus was 1,416 mg) and hepatosplenomegaly on day 252 after the transplantation. Leukemic cells showed a CD4- and CD8-double-positive phenotype (Fig. 3). One other mouse developed a similar disease (ID 401). Unfortunately, this mouse died on day 224 before we found out. Therefore, we could only confirm hepatosplenomegaly and a giant thymoma after the death. Two mice (ID 407 and 408) died of AML with hepatosplenomegaly on days 47 and 66 after the transplantation. Severe leukocytosis, anemia and thrombocytopenia were observed in a mouse (ID 408), but severe pancytopenia was observed in the other mouse (ID 407). Leukemic cells of the mouse (ID 408) in bone marrow and thymus uniformly expressed Gr1, CD11b, and B220 on their surfaces (Fig. 3). Four of the transplanted mice (ID 403, 404, 405 and 406) developed both myeloid and T cell leukemia with hepatosplenomegaly, and in some cases, thymoma (ID 404, 405 and 406). In the mouse ID 404, both myeloid and T cell leukemia cells were observed in the bone marrow, while peripheral blood was occupied with myeloid leukemia and thymus was occupied with T cell leukemia (Figs. 3, 4). In summary, two mice died of AML after a short latency (days 47 and 66), two mice died of T cell leukemia after a long latency (days 224 and 252), and four mice died of AML and T cell leukemia (days 76, 83, 129, and 248). The

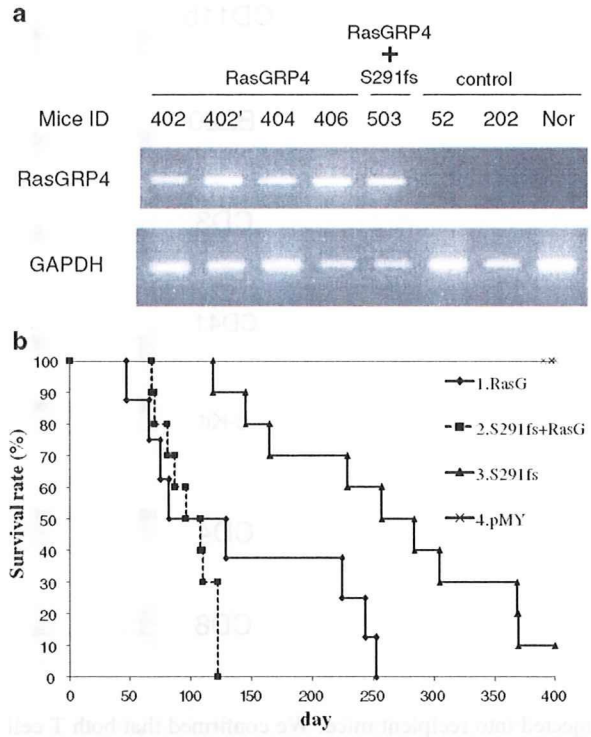

Fig. 2 Co-transduction of RasGRP4 and AML1-S291fs led to early onset of leukemia. **a** Expression of retrovirally introduced RasGRP4 in BM cells. Total RNA from BM cells of transplanted mice were extracted, and the derived cDNAs were subjected to RT-PCR. Mice IDs were shown on the top of the panel. ID 402′ is a second recipient of ID 402. Controls are AML1-S291fs (ID 52), empty vector (ID 202), and normal mouse (Nor). **b** Kaplan–Meier analysis for the survival of mice transplanted with RasGRP4, AML1-S291fs, and double-transduced BM cells. Average survival of RasGRP4 alone (139.8 days) was not significantly different when compared with double transduced mice (101.5 days) ($P = 0.223$, log rank test). Average survival of the double transduced mice (101.5 days) was significantly shorter than that of AML1-S291fs-transduced mice (263.6 days) ($P = 0.00003$, log rank test). RasGRP4 ($n = 8$), AML1-S291fs ($n = 10$), RasGRP4 + AML1-S291fs ($n = 11$), mock ($n = 16$) transduced bone marrow cells were transplanted to mice

details of individual mice are shown in Table 2 and Fig. 5. To assess whether the leukemic cells were transplantable, 2×10^5–1×10^6 total BM cells including blasts were

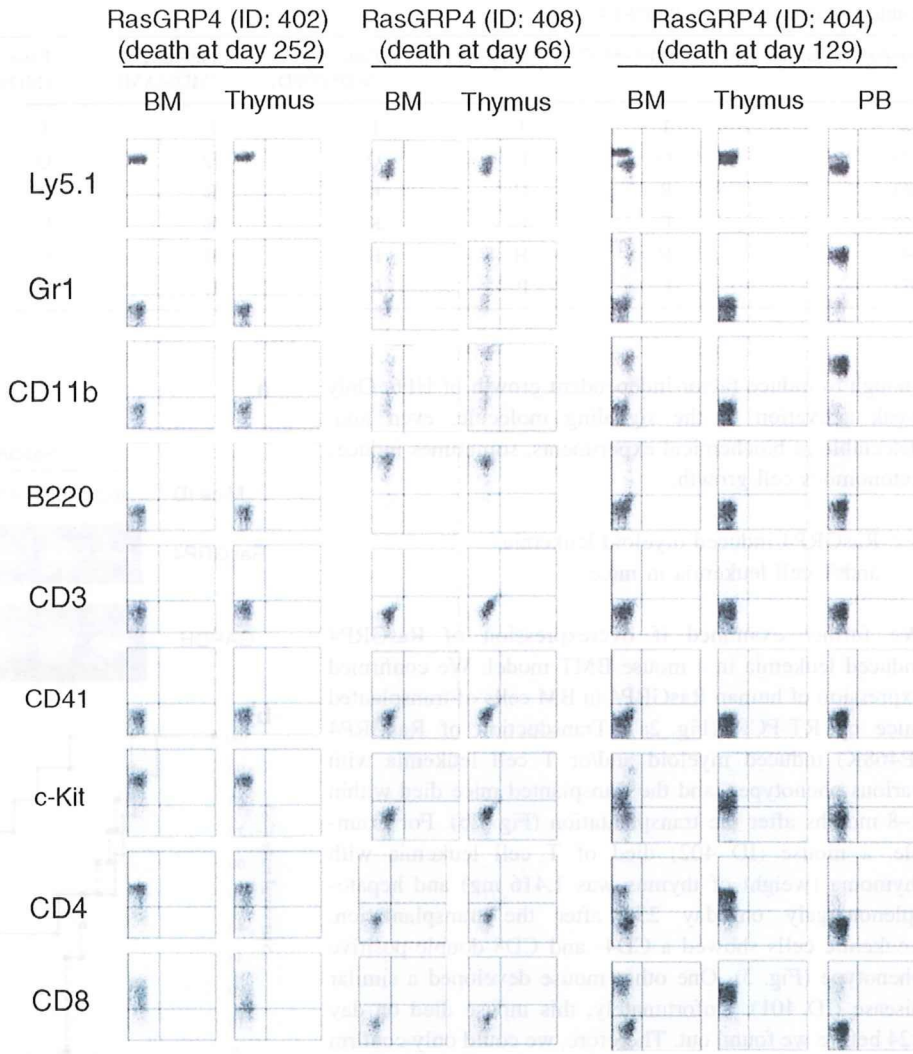

Fig. 3 RasGRP4 induced T cell leukemia and myeloid leukemia in the BMT model. The *dot plots* show Ly5.1, Gr-1, CD11b, B220, CD3, CD41, c-kit, CD4, or CD8 expression detected by corresponding PE-conjugated mAb

injected into recipient mice. We confirmed that both T cell leukemia and myeloid leukemia cells were serially transplantable although the phenotypes slightly changed after the serial transplantation (Supplemental Fig. 1).

3.3 Different integration sites were identified from T cell or myeloid leukemia cells derived from an individual mouse

To examine if the T cell and myeloid leukemia cells were derived from different clones or the same clone, we identified the integration sites in genomic DNA samples of thymus, peripheral blood or bone marrow cells. As shown in Table 3, different integration sites were identified from T cell and myeloid leukemia cells derived from an individual mouse, suggesting that T cell and myeloid leukemic cells were derived from different clones.

The integration near the Samsn1 gene was found twice in ID 405 and ID 406. These mice were transplanted on the same day. The integration site was identical among these leukemic cells indicating that leukemic cells of the two cases were derived from a single hematopoietic progenitor. This result suggests that the integration induced expansion of the transduced stem cells during the 3-day-culture period before the transplantation. Indeed, the mice with the integration at Samsn1 site developed AML with the same phenotype (CD11b positive) and similar latencies (83 and 76 days). On the other hand, different T lineage clones grew up in thymus and developed thymoma.

3.4 RasGRP4 cooperates with an AML1 mutant in leukemogenesis

RasGRP4 appears to function downstream of the tyrosine kinase receptor c-Kit/CD117 [30]. High expression of c-kit

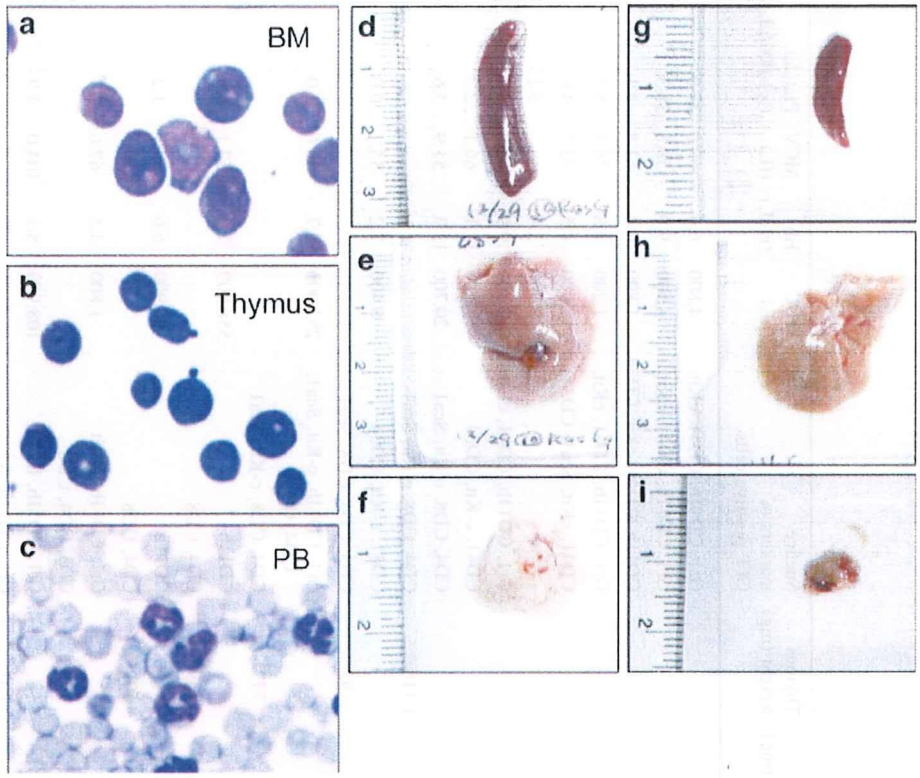

Fig. 4 RasGRP4 induced both of T cell leukemia and myeloid leukemia in the same mouse. Giemsa-stained cells derived from **a** bone marrow, **b** thymus, and **c** peripheral blood obtained from mouse ID 404. Macroscopic findings of **d** spleen, **e** liver, **f** thymus from mice ID 404; *left* **g** spleen, **h** liver, **i** thymus from normal mice; *right* are shown. Images (**a**, **b**, **c**) were obtained with a BH51 microscope and DP12 camera (Olympus, Tokyo, Japan); objective lens, UPlanFl (Olympus); ×1,000

has been found in 60–80% of AML [36] and higher expression is observed in 81.3% of patients with t(8;21) when compared with the patients with other leukemias [2]. Niimi et al. [9] reported that MDS/AML arising from AML1/RUNX1 mutations frequently involves receptor tyrosine kinase (RTK)-RAS signaling pathway activation. We have recently demonstrated that bone marrow cells transduced with AML1 mutants induced MDS-like symptoms after a long latency [35]. Therefore, we also tested if the combination of RasGRP4 and AML1-S291fs, one of the AML1 mutants, induced rapid leukemic transformation in a BMT model. As a result, co-transduction of RasGRP4 and AML1-S291fs led to early death in the transplanted mice (average 101.5 days, $n = 11$) than the expression of RasGRP4 alone (average 139.8 days, $n = 8$) (Fig. 2b). We diagnosed the double-transfected disease mice as T cell leukemia because of enlarged thymus, hepatosplenomegaly, and expansion of blast expressing CD3, CD4, and CD8 in bone marrow, peripheral blood, and thymus (Fig. 6). The onset of T cell leukemia was significantly earlier in the RasGRP4 + AML1 mutant (average 102.7 days, $n = 9$) than RasGRP4 alone (average 238 days, $n = 2$). On the other hand, onset of AML was not significantly changed between RasGRP4 + AML1 mutant (average 96 days, $n = 2$) and RasGRP4 alone (average 56.5 days, $n = 2$) transplanted mice.

3.5 RasGRP4 was overexpressed in some patients with hematological malignancies

We examined expression levels of RasGRP4 in patients with myeloid or T lineage hematological malignancies. As shown in Fig. 7, cells from some patients (T-ALL, AML-M1, MDS-RAEB, MDS-RA, CMMoL) overexpressed RasGRP4.

4 Discussions

We identified RasGRP4 from patients' cDNA libraries as a gene that renders IL-3-dependent HF6 cells factor independent when expressed at high levels via retrovirus-mediated gene transfer. Although we did not find any gain-of-function mutation of RasGRP4 in three patients from whom we identified cDNA for RasGRP4, and we detected high expression of RasGARP4 in only one out of the three patients, it is possible that overexpression or activating mutations are found in patients with malignant diseases including leukemia and MDS. Thus, RasGRP4 is a candidate gene for class I mutations. In addition to RasGRP4, we also identified FLT3-ITD from a patient with MDS/AML, thus showing the feasibility of our functional cloning strategy. The HF6 cells were immortalized by expression

Table 2 Hematological data of the transplanted mice

Gene	Mice ID	GFP (%)	Ly5.1 of BM (%)	Ly5.1 of Thymus (%)	Period from BMT (day)	Spleen weight (mg)	Liver weight (mg)	Thymus weight (mg)	Suefice markers of GFP positive cells	WBC (μL)	Hb (g/dL)	MCV (fL)	Platelet (×1,000/μL)
S291fs	51	27.4	39.8	–	368	153	1,236	–	Gr1, CD11b, CD41, cKit	4,800	6.6	60.0	27.0
S291fs	52	47.8	83.0	–	256	90	1,568	–	Gr1, CD11b, CD41, cKit	4,500	9.9	59.8	38.3
S291fs	54	29.8	45.3	–	304	338	1,812	–	CD11b, CD41, cKit, CD34	1,800	10.4	61.3	4.1
S291fs	55	75.2	83.1	–	165	166	1,574	–	Gr1, CD11b, CD41, cKit,	1,200	4.8	76.4	5.7
S291fs	56	54.9	85.2	–	118	73	1,318	–	CD41, cKit, Sca1, CD34,	2,900	4.5	72.7	4.6
S291fs	57	–	–	–	145	181	1,249	–	–	5,700	2.4		5.1
S291fs	58	72.7	56.5	–	228	225	1,582	–	Gr1, CD11b, CD41, cKit	2,100	9.9	62.7	26.7
S291fs	60	76.3		–	369	186	1,682	–	CD41, c-Kit, CD34	7,500	11.1	66.1	22.2
RasGRP4	402	–	96.5		252	318	1,975		CD4, CD8, c-Kit, Sca1	20,700	12.3	51.9	3.6
				98.9				1,416	CD4, CD8, c-Kit, Sca1				
RasGRP4	403	–	93.5		248	696	2,138		CD41, c-Kit, CD34	8,400	7.2	55.1	0.0
				85.9				92	CD3, CD4, CD8, Sca1				
RasGRP4	404	–	93.9		129	714	2,557		Gr1, CD11b, c-Kit, Sca1, CD4, CD8	282,000	11.7	56.6	3.0
				99.7				777	CD4, CD8, c-Kit, Sca1				
RasGRP4	405	–	91.3		83	1,184	3,699		CD11b	558,900	7.2	57.1	9.9
				98.3				139	CD4, CD8				
RasGRP4	406	–	94.8		76	1,348	2,678		CD11b	96,900	6.6	59.7	1.2
				99.5				609	CD4, CD8				
RasGRP4	407	–	32.0		47	670	4,795		Gr1, CD11b, c-Kit	1,800	4.5	67.6	2.7
				17.1				95	CD3, CD4, CD8				
RasGRP4	408	–	95.7		66	1,110	4,155		Gr1, CD11b, B220	108,900	5.1	104.0	3.9
				93.5				202	Gr1, CD11b, B220				
S291fs + RasGRP4	503	92.1	92.1		96	1,180	6,065		CD3, CD4, CD8	33,600	10.3	64.5	9.3
		95.0		95.0				112	CD3, CD4, CD8				
S291fs + RasGRP4	507	–	–		68	302	–		CD3, CD4, CD8				
				–				805	CD3, CD4, CD8				
S291fs + RasGRP4	513	75.8	80.7		130	544	2,784		CD3, CD8	59,500	10.7	68.8	2.2
		83.8		90.9				155	CD3, CD8				

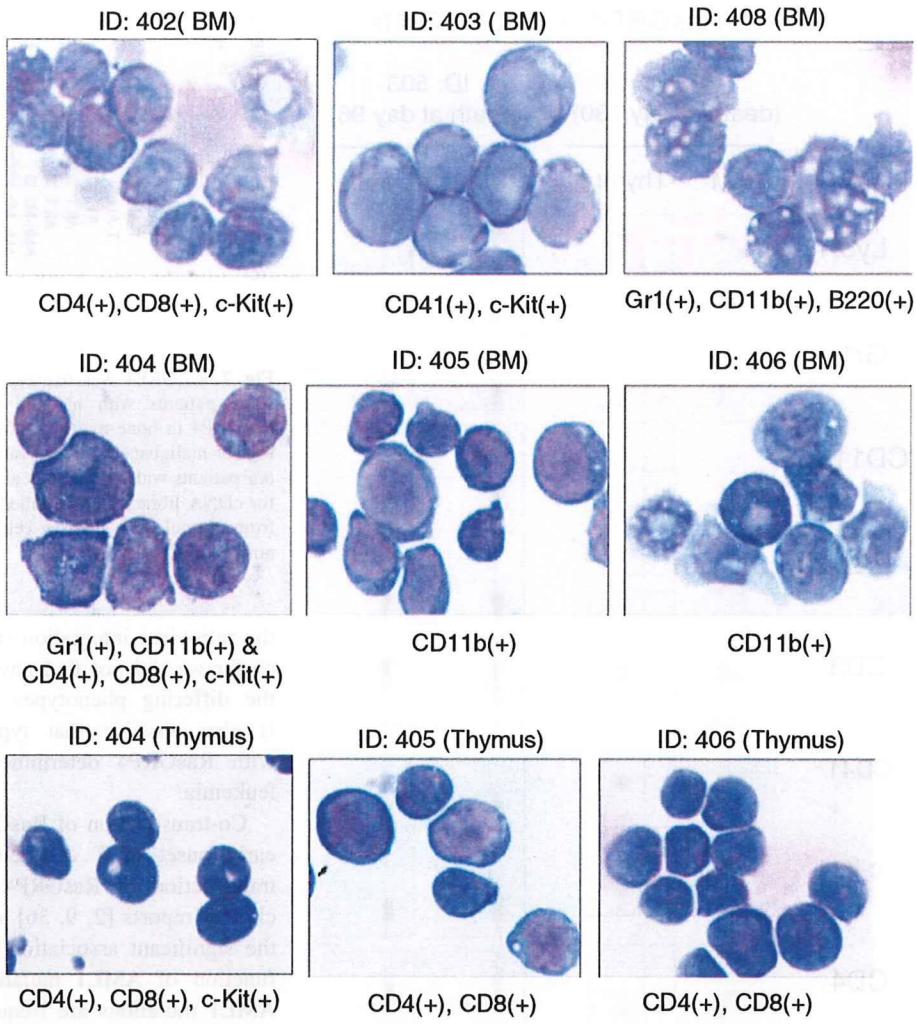

Fig. 5 Morphology of leukemic cells induced by RasGRP4. Giemsa staining photos of the leukemic cells are shown. Mice IDs were shown at *top of the panel*. Surface expression proteins were shown at bottom of the panel. Images were obtained with a BH51 microscope and DP12 camera (Olympus, Tokyo, Japan); objective lens, UPlanFl (Olympus); magnification, ×1,000

Table 3 Retroviral integration sites in the transplanted mice

Mice ID	Sample	Chr. number	Nearest gene	Gene ID	Distance to gene (start or end)	Location	Forward or reverse orientation	RTCGD hits
404	Thymus	10	Bcr	110279	Disrupt CDS	Intron 8	F	0
404	PB	15	Trio	223435	Disrupt CDS	Intron 9	F	3
405	Thymus	14	LOC100042147	100042147	12,962 bp	3′	R	0
405	BM	16	Samsn1	67742	95,998 bp	5′	R	2
406	Thymus	18	LOC100042131	100042131	Disrupt CDS	Exon 2	F	0
406	BM	16	Samsn1	67742	95,998 bp	5′	R	2

of MLL-SEPT6, and can be transformed by oncogenic Ras and Ras-related signals (manuscript in preparation). Therefore, HF6 is a suitable cell line for identification of Ras mutations as well as mutations of Ras-related signaling molecules. On the other hand, Ba/F3 cells can be transformed by STAT5 activation. In addition to these two cell lines, we have developed several other IL-3-dependent bone marrow-derived cell lines immortalized by class II mutations or related molecules (unpublished results). Because these IL-3-dependent cell lines have different signaling profiles, they would be applicable for identification of mutations in a variety of signaling molecules, providing a versatile system for functional cloning of oncogenic mutations.

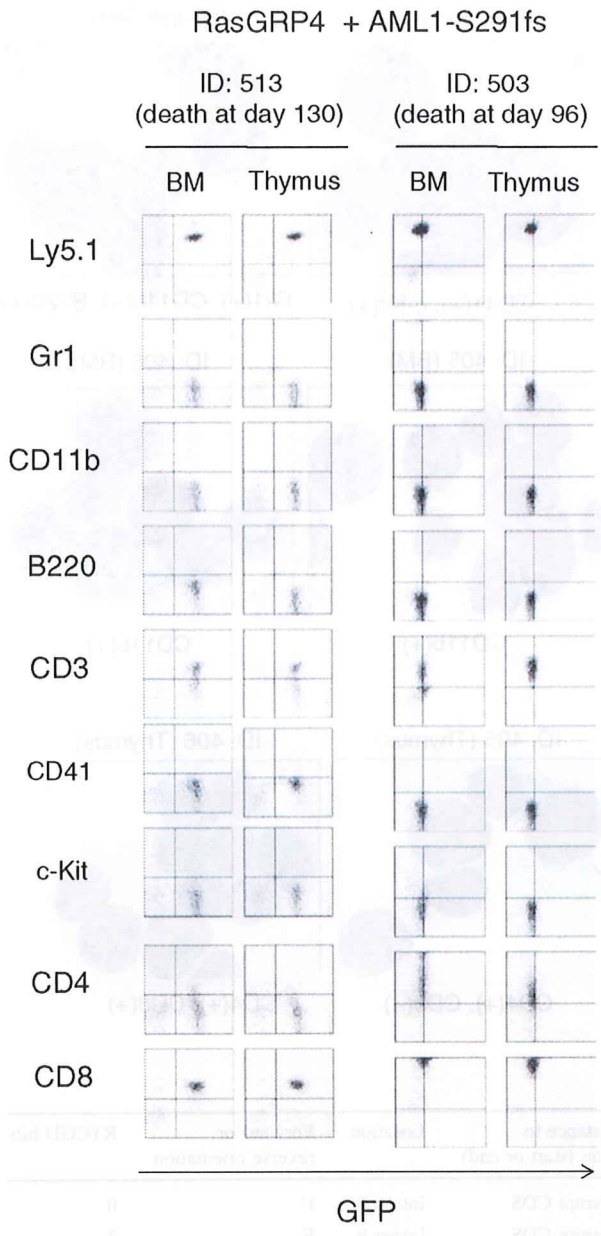

Fig. 6 RasGRP4 and AML1-S291fs induced T cell leukemia in the BMT model. The *dot plots* show Ly5.1, Gr-1, CD11b, B220, CD3, CD41, c-kit, CD4, or CD8 expression detected by corresponding PE-conjugated mAb

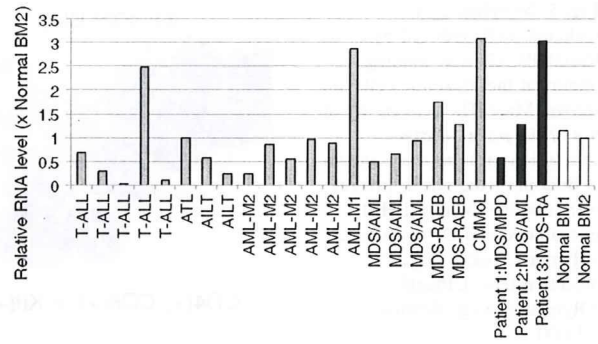

Fig. 7 RasGRP4 was overexpressed in a patient with T-ALL and some patients with myeloid malignancies. Expression levels of RasGRP4 in bone marrow cells derived from patients with hematological malignancies were evaluated by quantitative RT-PCR. *Gray bar* patients with hematological malignancies, *black bar* patients used for cDNA library and identified RasGRP4, *white bar* normal. RNAs from normal bone marrow cells served as a control (RNA level of normal BM2 = 1)

Overexpression of RasGRP4-induced T cell leukemia and/or myeloid leukemia in a mouse BMT model. We found that four of eight mice developed both types of leukemia and two mice died of AML after a short latency, while others died of T cell leukemia after a long latency when transplanted with RasGRP4 alone. At present, it is not clear what determines the different phenotypes of leukemia induced by RasGRP4. Although the retrovirus integration site should modify the outcome, so far we did not find any integration that could explain the differing phenotypes of leukemia. Alternatively, it is also possible that types of progenitors transduced with RasGRP4 determine the different phenotypes of leukemia.

Co-transduction of RasGRP4 and AML1-S291fs led to early onset of T cell leukemia as compared with the transduction of RasGRP4 alone. Putting together with clinical reports [2, 9, 36] and our results, we can suggest the significant association of Ras signaling pathway and function of AML1 mutation in leukemogenesis. While AML1 mutations are frequently associated with myeloid leukemia in human patients, they seemed to shorten the latency of T cell leukemia induced by forced expression of RasGRP4 in mouse BMT model. Intriguingly, while RasGRP4 induced c-Kit+/CD3−/CD4+/CD8+ T cell leukemia, combination of RasGRP4 and AML1-S291fs developed more mature T cell leukemia (c-Kit−/CD3+/CD8+/CD4− or CD4+). The reason for this difference is elusive at present. Although we need more cases of BMT mice for confirmation of this difference, AML1-S291fs may also play some roles to induce T cell differentiation in addition to its overall dominant effects on AML1 transcription. In the clinical cases, AML1-LAF4 [37] and AML1-FGA7 [38] were associated with T-ALL, although most of AML1 translocations are associated with myeloid leukemia. Because AML1 is important for transcription of TCR and silencing of CD4, it is possible that AML1-S291fs inhibited the normal ontogeny of T cells, thus accelerating leukemogenic process caused by RasGRP4 in a mice BMT model as a class II mutation that disturbs T cell ontogeny.

Reuther et al. previously isolated RasGRP4 by focus-forming assay of NIH3T3 cells from a patient with AML. This AML-derived RasGRP4 contained a point mutation at codon 620 that changes glutamic acid to lysine at the carboxyl terminus of the protein [29]. However, they found no significant difference in the ability of the AML-derived point mutated RasGRP4 (E620K) or wild-type RasGRP4 (GenBank™ accession number AF448437) in activation of Ras proteins. In our study, we found a gene alteration that induces an amino acid substitution from glutamic acid to lysine at 468 position of RasGRP4 in a patient with MDS/MPD. However, we did not detect a functional difference between RasGRP4 harboring an E468K substitution and RasGRP4 derived from normal volunteers in the ability to abrogate IL-3-dependency of HF6 cells. Moreover, SNPs of this gene are not correlated with lymphoma and leukemia (Y. Nakamura, unpublished results). These results indicate that the sequence difference simply represents a polymorphism or a neutral mutation and has no significant meaning in inducing leukemia. At present, it is not clear whether the sequence alterations in RasGRP4 gene are derived from germ line or somatic mutations.

We found overexpression of RasGRP4 in a patient with T-ALL but it is difficult to conclude the association of RasGRP4 with T-ALL. We also found overexpression of RasGRP4 in some patients with AML-M1, MDS-RAEB, MDS-RA, and CMMoL. The current results suggest that RasGRP4 plays important roles in leukemogenesis in some patients. A clinical study using a large number of patients' samples is required to fully understand the association of RasGRP4 with leukemogenesis.

In summary, we identified RasGRP4 as a candidate gene of class I mutations by our expression cloning strategy based on retrovirus-mediated gene transfer [31, 32, 39]. Although we did not find significant mutations in RasGRP4 derived from patients, overexpression of RasGRP4 confers factor independency on an IL-3 dependent cell line and induced T cell leukemia and myeloid leukemia in a mouse BMT model. Our results indicate possible involvement of RasGRP4 in leukemogenesis.

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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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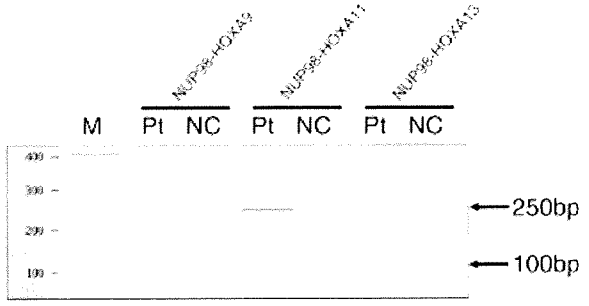

Figure 1. Detection of the NUP98-HOXA11 fusion transcript in the JMML patient by RT-PCR. M, size marker; Pt, patient; NC, normal negative control. The primers used for detection were as follows: NUP98-11S1 and HOXA9-1AS, *NUP98-HOXA9*; NUP98-11S1 and HOXA11-1AS, *NUP98-HOXA11*; and NUP98-11S1 and HOXA13-1AS,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

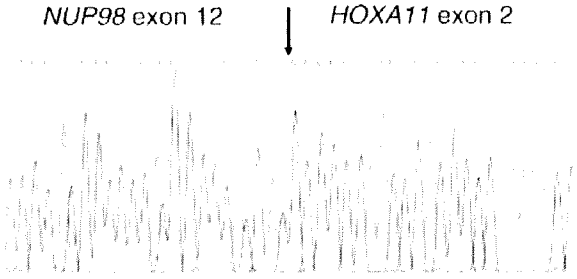

Figure 2. Direct sequencing of the NUP98-HOXA11 fusion transcript junction. Arrow indicates the fusion point. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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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FBXW7 and *NOTCH1* mutations in childhood T cell acute lymphoblastic leukaemia and T cell non-Hodgkin lymphoma

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Summary

Mutation analysis of *FBXW7* and *NOTCH1* genes was performed in 55 T cell acute lymphoblastic leukaemia (T-ALL) and 14 T cell non-Hodgkin lymphoma (T-NHL) patients who were treated on the Japan Association of Childhood Leukaemia Study (JACLS) protocols ALL-97 and NHL-98. *FBXW7* and/or *NOTCH1* mutations were found in 22 (40·0%) of 55 T-ALL and 7 (50·0%) of 14 T-NHL patients. *FBXW7* mutations were found in 8 (14·6%) of 55 T-ALL and 3 (21·4%) of 14 T-NHL patients, and *NOTCH1* mutations in 17 (30·9%) of 55 T-ALL and 6 (42·9%) of 14 T-NHL patients. Three (5·4%) T-ALL and two (1·4%) T-NHL patients had mutations in both *FBXW7* and *NOTCH1*. *FBXW7* mutations included one insertion, one deletion, one deletion/insertion and nine missense mutations. *NOTCH1* mutations were detected in the heterodimerization domain (HD) in 15 cases, in the PEST domain in seven cases, and in both the HD and PEST domains in one case. Five-year event-free survival and overall survival for patients with *FBXW7* and/or *NOTCH1* mutations were 95·5% (95% CI, 71·9–99·4%) and 100% respectively, suggesting that T-ALL patients with *FBXW7* and/or *NOTCH1* mutation represent a good prognosis compared to those without *FBXW7* and/or *NOTCH1* mutations (63·6%, $P = 0·007$ and 78·8%, $P = 0·023$, respectively).

Keywords: ALL, childhood, prognostic factors, genetic analysis, T cells, molecular diagnosis.

The outcomes of paediatric T cell acute lymphoblastic leukaemia (T-ALL) have improved in recent years as a result of intensified therapies, with 5-year relapse-free survival rates in the range of about 60–85% (Gaynon *et al*, 2000; Maloney *et al*, 2000; Moghrabi *et al*, 2007; Pui *et al*, 2004), which are relatively low compared to those of B-precursor ALL. A stringent assessment of the risk of relapse is critical in determining which patients need to receive more effective therapy. In T-ALL, it has been reported that the abnormal expression of *TLX1* (*HOX11*) is associated with a favourable prognosis, although the prognostic significance of this finding has yet to be determined (Ferrando *et al*, 2004; Ferrando *et al*, 2002). On the other hand, a few reports have suggested that microarray analysis could distinguish high-risk cases in T-ALL (Ferrando & Look 2003; Winter *et al*, 2007).

Recently, activating mutations of *NOTCH1* gene have been reported in more than half of T-ALL cases (Weng *et al*, 2004). *NOTCH1*, previously termed *TAN1*, was discovered as a

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partner gene in T-ALL with a *t*(7;9)(q34;q34·3), and was found in <1% of T-ALLs (Ellisen *et al*, 1991). A good clinical outcome for T-ALL patients with *NOTCH1* mutations was reported in the paediatric ALL-BFM 2000 study (Breit *et al*, 2006). On the contrary, other papers reported that *NOTCH1* mutations were not associated with good clinical outcome in T-ALL (van Grotel *et al*, 2008; Zhu *et al*, 2006). Thus, clinical significance of *NOTCH1* mutation in T-ALL still remains controversial.

F-box and WD40 domain protein 7 (FBXW7; previously termed FBW7, CDC4, or Archipelago), is also considered a candidate prognostic factor in T-ALL. FBXW7 was originally isolated as a Lin12/NOTCH-negative regulator in Caenorhabditis elegans (Hubbard *et al*, 1997), and plays a critical role in intracellular NOTCH1 degradation which depends on an intact NOTCH1 PEST domain (Fryer *et al*, 2004; Tetzlaff *et al*, 2004). Recently, it was reported that the *FBXW7* gene was mutated in various tumours including breast, ovarian, and endometrial cancers and T-ALL cell lines (Moberg *et al*, 2001).

In this study, we analyzed the frequencies and clinical significance of *FBXW7* and *NOTCH1* mutations in paediatric T-ALL and T cell non-Hodgkin lymphoma (T-NHL). *FBXW7* as well as *NOTCH1* was found to be frequently mutated in paediatric T-ALL and T-NHL. We firstly described that mutations of either *FBXW7* or *NOTCH1* genes, rather than *FBXW7* or *NOTCH1* alone, were associated with good clinical outcome in T-ALL.

Methods

Patients and treatments

All children with T-ALL or T-NHL, aged under 15 years were enrolled into the Japan Association of Childhood Leukaemia Study (JACLS) protocol ALL-97 between 1997–2001 and JACLS trial NHL-T98 between 1998–2002 (Oda *et al*, 2006) (Fig S1). All T-NHL patients were pathologically diagnosed as having lymphoblastic lymphoma. Patients who failed to obtain complete remission (CR) with risk adapted induction chemotherapy were scheduled to undergo F-protocol at 6 weeks following the start of their initial induction chemotherapy. Samples from 55 newly diagnosed T-ALL and 14 T-NHL patients were examined in this study. At the time of diagnosis, bone marrow (BM) and/or peripheral blood (PB) cells were obtained from T-ALL patients and lymph nodes and/or pleural effusions were obtained from T-NHL patients. T-lineage immunophenotypic subtype was defined as simultaneous expression of two or more T-lineage associated molecules including CD2, CD3, CD5, CD7, and CD8, on at least 20% of lymphoblasts. T-ALL was characterized by definition as the presence of more than 25% bone marrow involvement of lymphoblasts. Cytogenetic studies were performed on 60 patients by using regular G-banding method. Advanced stage (stages 3 and 4) T-NHL patients were enrolled in this protocol, and the histopathology of specimens was reviewed by central pathology reviewers. A total of 69 patients were included in the present study; 49 were male and 20 female; 55 were children diagnosed with T-ALL (median age of 9·5 years; range: 2·0–15·0 years) and 14 with T-NHL (median age of 11·0 years; range: 3·7–15·0 years). The basic clinical and immunological characteristics of this patient subgroup did not differ from those of the entire group. The two-year treatment regimen consisted of induction therapy (vincristine sulfate, high dose-methotrexate, cytarabine, prednisone, L-asparaginase), five drug consolidation therapy A and B including high doses of L-asparaginase, and maintenance therapy with block-rotated treatment using the drugs listed above. Informed consent was obtained from the patients or their parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Centre approved this project.

DNA and RNA preparation

DNA and RNA were prepared from samples of BM, PB, lymph nodes, and pleural effusions containing tumour cells of patients with primary T-ALL and T-NHL, by using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA).

Detection of FBXW7 and NOTCH1 mutations

Mutation detection was performed by polymerase chain reaction (PCR)-based denaturing high-performance liquid chromatography (dHPLC) using a WAVE DNA fragment analysis system (Transgenomic, Omaha, NE, USA) equipped with a DNASep HT cartridge (Weng *et al*, 2004). The PCR products of positive cases detected by PCR-based dHPLC were purified using the QIAquick PCR Purification Kit (Qiagen). Sequencing by means of fluorescent-dye chemistry was performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) (Shimada *et al*, 2006; Taketani *et al*, 2004). For further confirmation of insertion and deletion mutations, the purified PCR products were subcloned using a TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and then sequenced (Taketani *et al*, 2004). *FBXW7* mutations were screened from exons 2 to 12 using primers described previously (Cassia *et al*, 2003). *NOTCH1* mutations in the N-terminal region and the C-terminal region of the HD domain (exons 26 and 27), the transcriptional activation domain (TAD) (exon 34), and the PEST domain (exon 34) were screened by using primers described previously (Weng *et al*, 2004).

Statistical analyses

Proportional differences between groups were analyzed by chi-squared or Fisher's exact tests. The Kaplan–Meier method was used to estimate survival rates. Differences in prognosis between groups were evaluated using the log-rank test. Event-free survival (EFS) was measured from the time of diagnosis