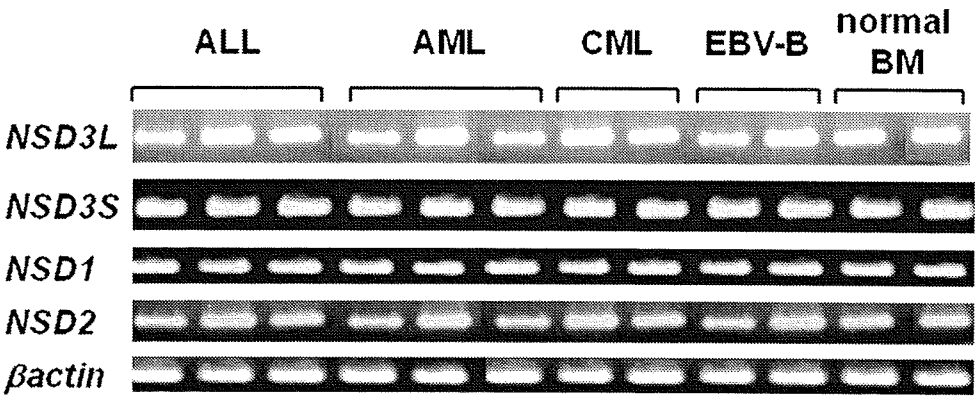

Fig. 3. Expression of two isoforms of the *NSD3* gene and the *NSD1* and *NSD2* genes in acute lymphoblastic, acute myeloid, and chronic myeloid leukemia cell lines, Epstein–Barr virus transformed B lymphocyte (EBV-B) cell lines, and normal healthy bone marrow cells, determined with reverse transcriptase–polymerase 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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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. 84:295–297, 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-

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Conflict of interest: Nothing to report.

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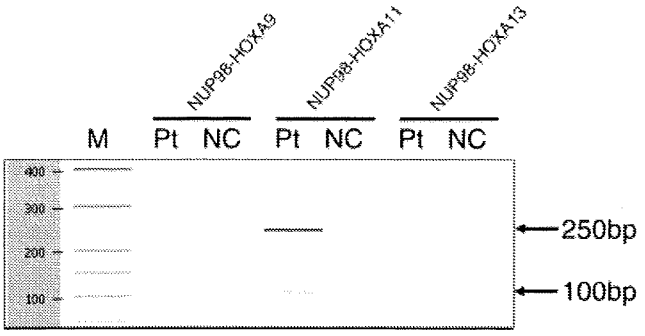

Figure 1. Detection of the NUP98-HOXA11 fusion transcript in the JMML patient by RT-PCR. M, size marker; Pt, patient; NC, normal negative control. The primers used for detection were as follows: NUP98-11S1 and HOXA9-1AS, *NUP98-HOXA9*; NUP98-11S1 and HOXA11-1AS, *NUP98-HOXA11*; and NUP98-11S1 and HOXA13-1AS,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

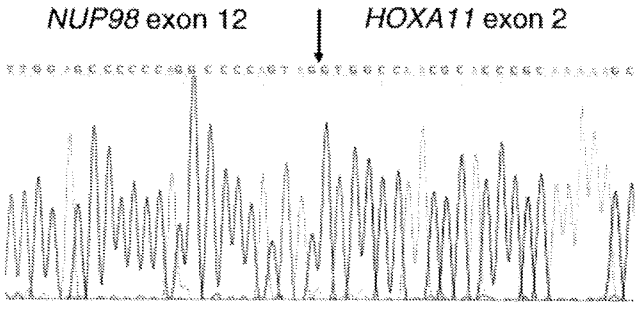

Figure 2. Direct sequencing of the NUP98-HOXA11 fusion transcript junction. Arrow indicates the fusion point. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

dose cytarabine, etoposide, idarubicin and mitoxantrone), 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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CASE REPORT

Monosomies 7p and 12p and *FLT3* internal tandem duplication: possible markers for diagnosis of T/myeloid biphenotypic acute leukemia and its clonal evolution

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Abstract Biphenotypic acute leukemia co-expressing T-lymphoid and myeloid markers is rare, accounting for less than 1% of acute leukemias. However, several clinical characteristics including male predominance, frequent lymphadenopathy and unfavorable outcome have been identified. Recurrence of monosomies 7p and/or 12p in T/myeloid biphenotypic acute leukemia has been reported. We treated a patient with T/myeloid biphenotypic acute leukemia showing clonal chromosomal and genetic abnormalities including dic(7;12)(p11;p11) and Fms-like tyrosine kinase 3 (*FLT3*)-internal tandem duplication. Cytogenetic analysis of both bone marrow and lymph node cells disclosed that the patient's lymph node leukemia cells had chromosomal abnormalities in addition to dic(7;12). Our findings suggest that the leukemia cells of systemic lymphadenopathy had evolved as secondary cells from marrow leukemia cells. The patient was successfully treated with induction chemotherapy for acute myeloid leukemia followed by allogeneic bone marrow transplantation.

Keywords Monosomies · *FLT3*-ITD · Biphenotypic acute leukemia · Stem cell transplantation

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1 Introduction

In the French-American-British classification, acute leukemias are classified as lymphoid or myeloid lineage on the basis of myeloperoxidase (MPO) staining. In a minority of cases, however, blast cells simultaneously express markers for two or three lineages of myeloids and B- and T-lymphoids. According to the World Health Organization (WHO) classification of tumors [1], these cases are classified as biphenotypic acute leukemia, and a diagnostic scoring system for this previously ill-defined entity was proposed by the European group for the immunological classification of leukemias (EGIL) [2]. This system is based on the number and specificity of the lymphoid and myeloid markers expressed on blast cells. The most common immunophenotype of biphenotypic acute leukemia (60–70%) consists of co-expressed B-lymphoid and myeloid markers, while co-expressed T-lymphoid and myeloid markers are less frequent (20–30%) [3–6]. Specific chromosomal and genetic abnormalities and definitive treatment have not yet been reported for T/myeloid biphenotypic acute leukemia. This report concerns a patient with T/myeloid biphenotypic acute leukemia, featuring dic(7;12)(p11;p11) and Fms-like tyrosine kinase 3 (*FLT3*)-internal tandem duplication (ITD) (*FLT3*-ITD) in both marrow and lymph node cells, who was successfully treated with myeloablative stem cell transplantation.

2 Case report

A 50-year-old man was admitted to our hospital because of anorexia in June 2004. Physical examination and computed tomography detected systemic lymphadenopathy involving

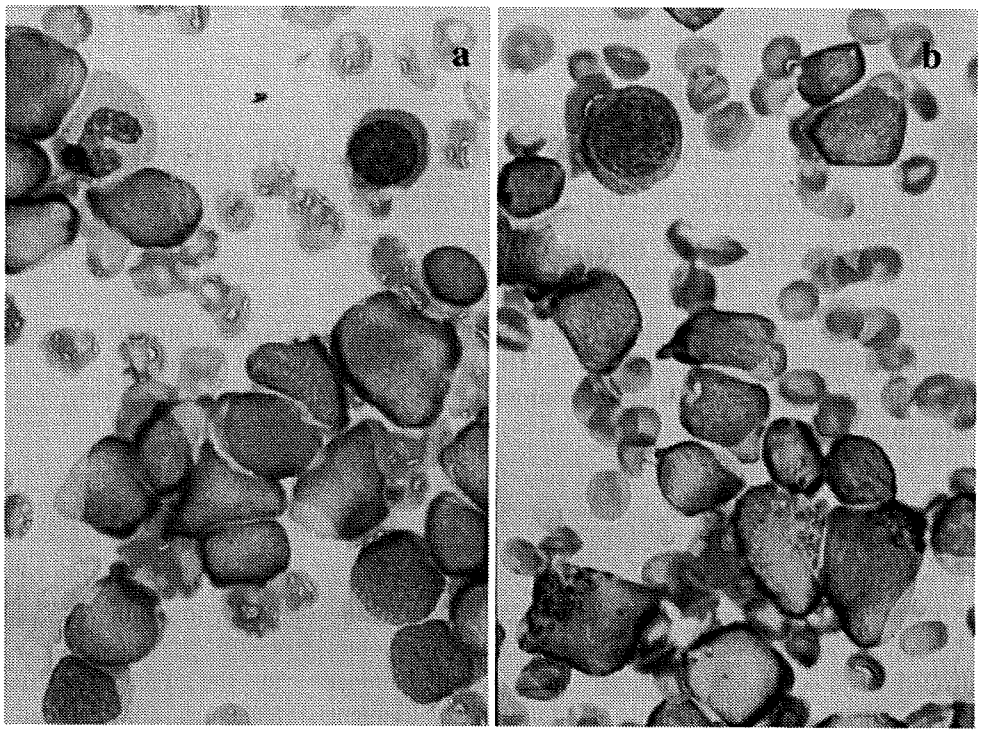

Fig. 1 Bone marrow smears. **a** Blast cells had folded nuclei with dispersed chromatin, prominent nucleoli and abundant blue–gray cytoplasm without azurophilic granules (May-Gruenwald-Giemsa staining). **b** Positivity for myeloperoxidase was detected in 23% of blast cells

the cervical, axillar, mediastinal, mesenteric and inguinal regions but without definite hepatosplenomegaly. The peripheral blood cell count showed 8.3 g/dl Hb, 110 × 10^9/l platelets, 1.0 × 10^9/l WBC, as well as 7% neutrophils, 65% lymphocytes and 28% blasts. The serum lactate dehydrogenase (LDH) level was below the normal upper limit and no coagulopathy was observed. The bone marrow smear stained with May-Gruenwald-Giemsa showed hypercellularity with 97% blast cells featuring folded nuclei with dispersed chromatin, prominent nucleoli and abundant blue-gray cytoplasm without azurophilic granules (Fig. 1a), and 23% of the blast cells were positive for MPO (Fig. 1b). The flow-cytometric (FCM) analysis of bone marrow cells showed positivity for CD5 (42.3%), CD7 (99.0%), CD33 (72.2%), HLA-DR (55.3%), MPO (36.0%), terminal deoxynucleotydil transferase (TdT) (61.9%) and cytoplasmic CD3 (88.4%). Application of the EGIL criteria [2], produced a diagnosis of biphenotypic acute leukemia because the score for both T-lymphoid (cytoplasmic CD3, CD5, TdT, and CD7) and myeloid lineages (MPO and CD33) was higher than 2. Additional FCM analysis showed concomitant expression of both MPO and cytoplasmic CD3 (Fig. 2). Histopathologic examination of the biopsied specimen of the inguinal lymph node showed a dense infiltrate of small malignant cells (Fig. 3a), which were immunohistochemically positive for leukocyte common antigen (LCA) (Fig. 3b), with FCM analysis showing the same biphenotypic features as those of bone marrow cells. The analysis of FCM of lymph node cells showed positivity for CD5 (56.2%), CD7

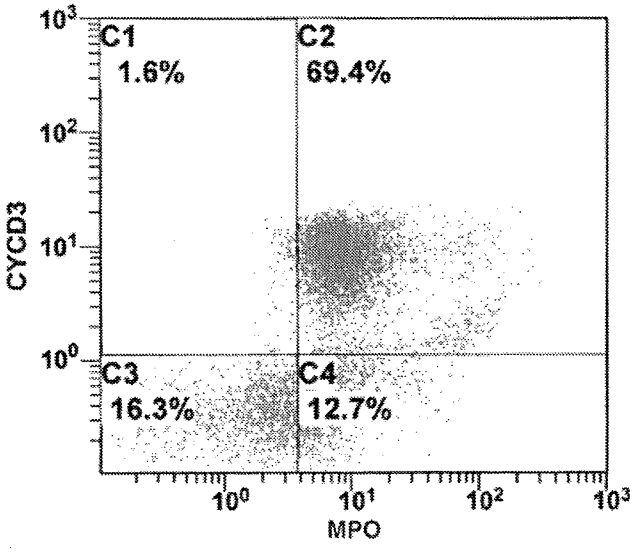

Fig. 2 Flow-cytometric analysis of bone marrow cells. These cells expressed MPO and cytoplasmic CD3 concomittantly

(92.4%), CD33 (70.0%), HLA-DR (42.9%), MPO (34.6%), TdT (53.5%) and cytoplasmic CD3 (69.4%). Cytogenetic analysis of bone marrow cells using G-banding demonstrated 45, XY, dic(7;12)(p11;p11) in two of the 20 analyzed cells, and spectral karyotyping confirmed this alteration (Fig. 4a). The karyotype of the other 18 analyzed cells was 46, XY. On the other hand, nine of the 20 analyzed lymph node cells had 45, XY, dic(7;12)(p11;p11) and five of them had additional chromosomal abnormality, indicating the occurrence of karyotypic evolution from

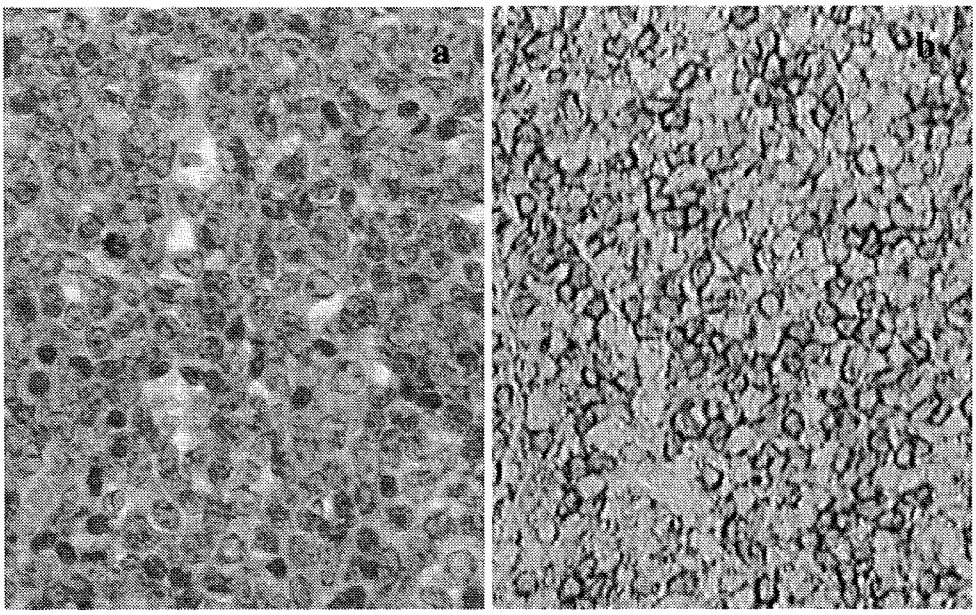

Fig. 3 Histopathologic study of biopsied specimen. **a** Dense infiltration of the lymph node by small malignant cells. **b** Malignant cells of the lymph node were immunohistochemically positive for leukocyte common antigen (LCA)

bone marrow cells (Fig. 4b, c). No clonal T-cell receptor Cβ1 rearrangement was detected by Southern blot analysis. The diagnosis of acute myeloblastic leukemia without maturation (AML M1) was based on the French-American-British classification and that of biphenotypic acute leukemia on the EGIL criteria.

Complete remission (CR) was attained with induction chemotherapy comprising idarubicin and cytosine arabinoside (Ara-C), followed by two courses of consolidation chemotherapies. A myeloablative bone marrow transplantation (BMT) from his HLA-identical sister was performed 6 months after the diagnosis. The conditioning regimen consisted of total body irradiation of 10 Gy (2 × 2.5 Gy on days 6 and 5) and intravenous cyclophosphamide at 60 mg/kg on days 4 and 3. To prevent graft-versus-host disease (GVHD), continuous intravenous infusion of cyclosporin A at 3 mg/kg per day was administered starting on day 1 and intravenous infusion of methotrexate at 10 mg/m^2 on day 1 and at 7 mg/m^2 on days 3 and 6. The total quantity of marrow nuclear cells infused was 1.14 × 10^8/kg recipient weight. Post-transplant hematological recovery monitoring showed that neutrophils had reached ≥0.5 × 10^9/l by day 14 and platelets ≥50 × 10^9/l by day 28. Chimerism evaluated by dual-color fluorescence in situ hybridyzation analysis of sex chromosomes showed that 99.4% of the bone marrow nuclear cells (cut off value 2%) were donor type on day 30. No major regimen-related toxicity was observed. However, Stage 2 [7] intestinal acute GVHD (histologically Grade I and endoscopically Grade 3 according to the grading systems described by Washington et al. and Cruz-Correa et al., respectively [8, 9]) and secondary graft failure (anemia and thrombocytopenia) occurred. The intestinal GVHD was discovered 3 months after the BMT and after 5 months, bicytopenia had improved accompanied by complete chimerism. No recurrence had been noted for 43 months after the BMT.

3 Genomic PCR and nucleotide sequencing

High-molecular-weight DNA, extracted from the patient's lymph node and bone marrow leukemia cells, was subjected to polymerase chain reaction (PCR). The primers used were the previously described 11F and 12R [10]. Nucleotide sequencing of the PCR products was analyzed with the fluorometric method (ABI PRISM 310 Genetic analyzer, Applied Biosystems, Tokyo, Japan). Furthermore, we performed PCR of the leukemia cells of the lymph node and bone marrow using the patient-specific primer FLT3-Hi (sense) 5′-GGGAGTTTCCAAGAGATTATG-3′ and 12R. We also performed the comparative analysis of sensitivity between both primer sets.

4 Results

PCR analysis using primers 11F and 12R detected, in addition to the normal-sized *FLT3* product, a longer-sized product in specimens not of the bone marrow but of the lymph node (Fig. 5a). Sequence analysis of the subcloned PCR product derived from the lymph node leukemia cells demonstrated the presence of ITD within exon 11 of *FLT3* gene (Fig. 5b). To detect this patient-specific *FLT3*-ITD product, we designed the primer FLT3-Hi, which contained a specific sequence of the duplicated *FLT3* gene (Fig. 5b). PCR analysis using primers FLT3-Hi and 12R followed by

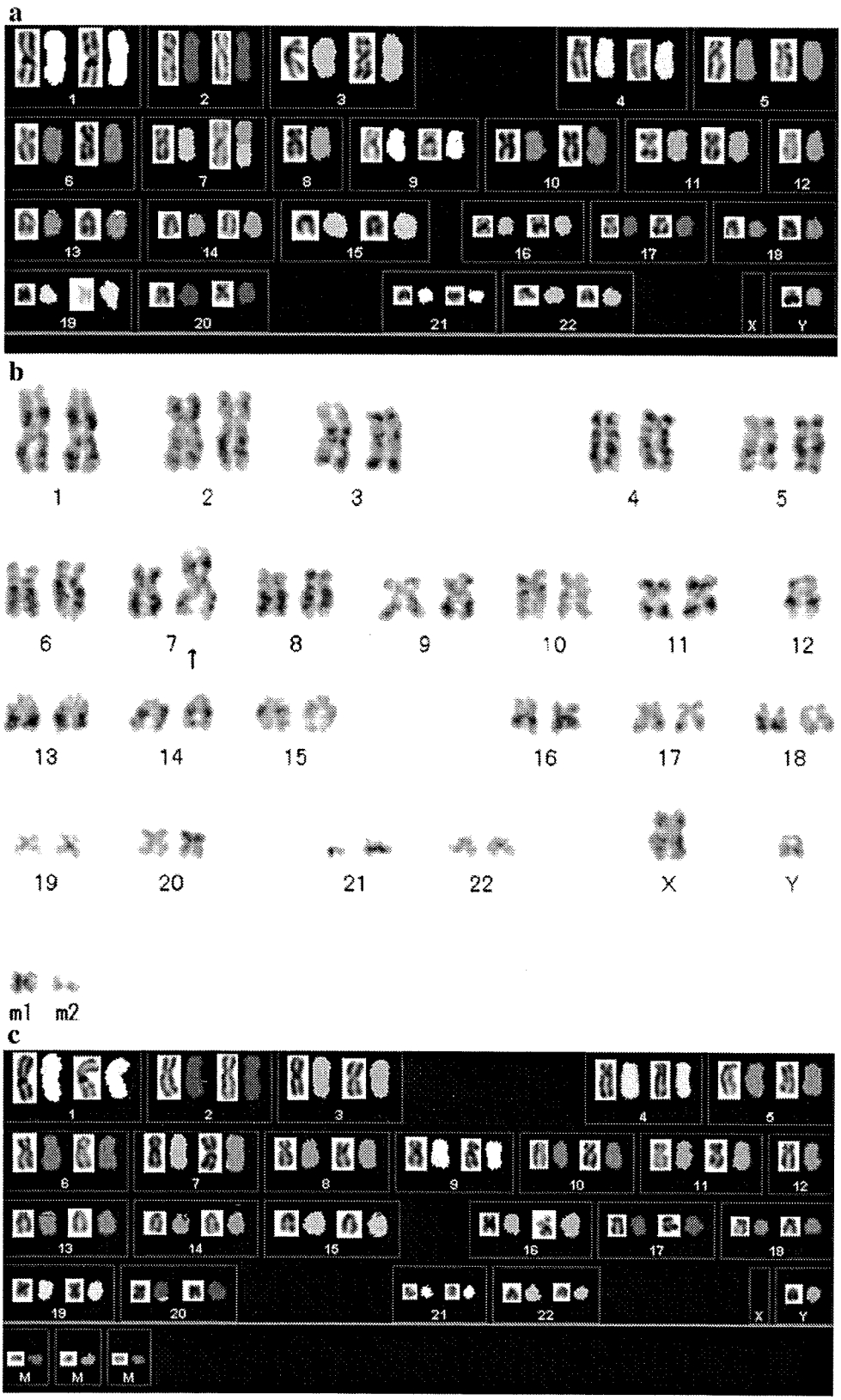

Fig. 4 **a** Bone marrow cytogenetic analysis using spectral karyotyping detected only 45, XY, dic(7;12)(p11;p11) [2/20]. **b**. Cytogenetic analysis of the lymph node using G-banding detected 45, XY, dic(7;12)(p11;p11) [4/20] and 48, XY, dic(7;12)(p11;p11), +mar1, +mar2 [5/20]. **c** Cytogenetic analysis of the lymph node using spectral karyotyping. One of marker chromosomes was derived from chromosome 12

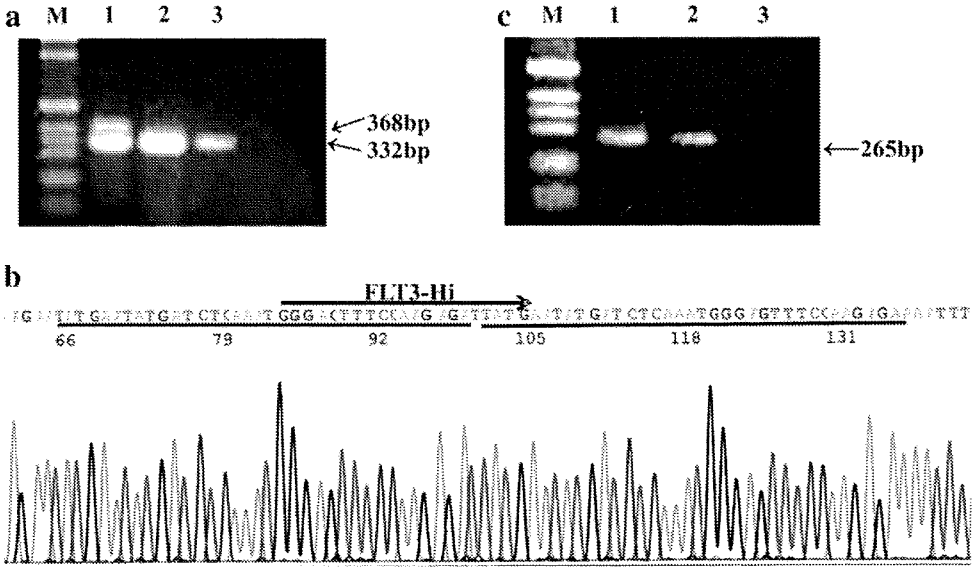

Fig. 5 Detection of *FLT3*-ITD. **a** PCR amplification of genomic DNA using 11F/12R primer combination. *Lanes M, 1, 2* and *3* show results for the marker, lymph node, bone marrow and a normal individual, respectively. **b** Sequence analysis of lymph nodal leukemia cells. The mutant *FLT3* featured duplication of nucleotides no. 85–119 from the start of exon 11 (*underlined text*) with one base (T) insertion. A sequence of FLT3-Hi is shown in this figure. **c** PCR amplification of genomic DNA using FLT3-Hi/12R primer combination. *Lanes M, 1, 2* and *3* show results for the marker, lymph node, bone marrow and a normal individual, respectively

sequencing showed *FLT3*-ITD in the specimens of both the lymph node and the bone marrow (Fig. 5c).

As to sensitivity of the primers, while *FLT3*-ITD in hundredfold dilution magnification of the specimen of the lymph node was detectable with PCR using primers FLT3-Hi and 12R, PCR using primers 11F and 12R failed to detect *FLT3*-ITD in tenfold dilution magnification.

5 Discussion

T/myeloid biphenotypic acute leukemia has not yet been linked to any specific clinical features because of the small number of cases reported in the literature. However, the findings of some studies suggest that the following clinical features may be present (Table 1): (1) The majority of cases are male, (2) patients typically present with lymphadenopathy, (3) laboratory findings show neither elevated LDH nor DIC [11]. The features of our case were thus compatible with those of previously reported cases.

Our patient's leukemia cells of both the lymph node and bone marrow showed chromosomal abnormalities including dic(7;12)(p11;p11). Although there are no rigorous reports on chromosomal and genetic abnormalities of T/myeloid biphenotypic acute leukemia, some chromosomal abnormalities have been described (Table 1), including recurrent observations of monosomies 7p and/or 12p [4, 11–14]. This suggests that T/myeloid biphenotypic acute leukemia may have distinct clinical features including chromosomal abnormalities. Furthermore, we found that leukemia cells of the lymph node feature chromosomal abnormalities in addition to dic(7;12). This cytogenetic evidence means that the cells of systemic lymphadenopathy of biphenotypic acute leukemia are secondary evolved cells from marrow leukemia cells.

As for genetic abnormalities, the leukemia cells of both the lymph node and bone marrow had *FLT3*-ITD, which is usually seen in AML and is very rare in acute lymphoblastic leukemia (ALL) and biphenotypic acute leukemia. To the best of our knowledge, there are only three reports covering two pediatric ALL patients and two pediatric T/myeloid biphenotypic acute leukemia patients with *FLT3*-ITD [15–17]. This is thus the first report about an adult biphenotypic acute leukemia case with *FLT3*-ITD.

The primers 11F and 12R amplified *FLT3* gene segments both with and without ITD, while the patient-specific primer amplified only the *FLT3* gene segment with ITD. The comparative analysis revealed that PCR using the patient-specific primer was more sensitive than the one using primers 11F and 12R. In specimens of the lymph node, *FLT3*-ITD was detected by PCR analysis using both the patient-specific primer and primers 11F and 12R. In specimens of the bone marrow, on the other hand, *FLT3*-ITD was detected only by the PCR using the patient-specific primer. Given that the bone marrow consisted mostly of the leukemia cells, these results suggest that *FLT3*-ITD was presented in only a small proportion of the leukemia cells of the bone marrow, and that the lymph node had a much higher rate of leukemia cells with *FLT3*-ITD than the bone marrow. The genetic analysis disclosed that

Table 1 Characteristics of reported adult patients with T-biphenotypic leukemia

Age (years)/Sex	FAB	Tumor	Karyotype	Reference
80/F	AML	NS	t(7;22)(q11;p11)	Carbonell F et al. [4]
25/M	AML	NS	Normal	
41/M	ALL	NS	?del(5)(p15), −17, +mar	
20/M	ALL	NS	Normal	
14/M	AML	NS	del(6)(q15;q33),del(11)(q14),**del(12)**(p13)	
33/M	ALL	NS	del(6)(q?)	
43/ND	AML	NS	–	Killick S et al. [12]
14/ND	ALL	NS	del(6q),del(11q14),**del(12p)**	
27/ND	AML	NS	Normal	
25/ND	ALL	NS	add(1q),add(5q),**−7**,del(9q),del(11q)	
44/ND	AML	NS	t(1;7),add(17q),del(9q)	
37/M	ALL	sLN, med	Normal	Rubio MT et al. [11]
52/M	ALL	Med	Normal	
35/M	NS	sLN, med	Normal	
21/M	ALL	sLN	+3,**−7**,**add(12p)**,−15,−19,+21	
26/M	ALL	sLN	–	
50/M	ALL	sLN, med	Normal	
24/M	ALL	sLN, med	Normal	
26/M	ALL	sLN	–	Tiribelli M et al. [13]
60/F	ALL	sLN	**del(7)**, **−12**, +mar	
58/F	ALL	sLN	**del(7)**,del(11),**del(12)**,dup(13)	
32/M	ALL	NS	t(2;6)(q37;p21.3),der(16)t(8?;16)(q11.2;q13)	Owaidah TM et al. [14]
14/M	AML	NS	t(8;14)(p21;q32)	
14/M	ALL	NS	del(5)(q22),del(6)(q22),**del(12)**(p11.2),del(13)(q12q14)	
21/M	AML	NS	Normal	
21/M	AML	NS	Normal	
50/M	AML	sLN, med	**dic(7;12)(p11;p11)**	Present case

FAB French–American–British Classification, *AML* acute myeloid leukemia, *ALL* acute lymphoblastic leukemia, *sLN* superficial lymph nodes, *Med* mediastinal mass, *NS* not specified

FLT3-ITD represents the secondary transformation as previously reported [18], and the leukemia cells of systemic lymphadenopathy were secondary evolved from the original marrow leukemia cells. Although it was difficult to determine the rate of leukemia cells with chromosomal abnormality by G-banding, not only *FLT3*-ITD but also dic(7;12) was detected more frequently in leukemia cells of the lymph node than the bone marrow ([9/20] vs. [2/20], respectively). This result suggested that both dic(7;12) and *FLT3*-ITD were secondary transformation events and part of the clonal evolution.

No regimens of induction and consolidation therapy have been established for patients with biphenotypic acute leukemia. Killick et al. [12] recommended that induction therapy should be administered with either AML or ALL drugs because patients receiving AML or ALL induction therapy alone did not succumb to early death, while induction treatment with combined AML/ALL drugs led to a high rate of early deaths. For T/myeloid biphenotypic acute leukemia, induction chemotherapy with AML using both anthracycline and Ara-C has been recommended based on a higher CR rate than that attained with ALL-type inducution therapy [11].

As for consolidation therapy, Rubio et al. [11] described five T/myeloid biphenotypic acute leukemia patients who had been treated with allogeneic BMT, three of whom were still alive at the last follow-up. Because the prognosis of biphenotypic acute leukemia or AML with *FLT3*-ITD treated with only chemotherapy appears to be unfavorable [11, 19], we decided to treat our patient with induction therapy followed by BMT. Recently, it has been reported that allogeneic stem cell transplantation did not improve overall survival of *FLT3*-ITD patients [20], and the question as to whether a small size of *FLT3*-ITD (<40 nucleotides) is associated with a worse prognosis for AML remains controversial [21, 22]. Although our patient had a

small size of *FLT3*-ITD (35 nucleotides), he remained in CR accompanied by complete chimeric hematopoiesis for 43 months after BMT.

In conclusion, we successfully treated a T/myeloid biphenotypic acute leukemia patient who showed dic(7;12)(p11;p11) and *FLT3*-ITD in both bone marrow and the lymph node. T/myeloid biphenotypic acute leukemia may have distinct clinical characteristics, and is accompanied by systemic lymphadopathy due to leukemia cell infiltration. Allogeneic stem cell transplantation should be considered for consolidation therapy for biphenotypic acute leukemia patients to improve the clinical outcome for this type of leukemia.

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bjh research paper

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

to the time of analysis or first event. Failure to achieve remission, relapse or death that occurred during continuous complete remission were evaluated as events. Overall survival (OS) was defined as the time from diagnosis to death. Multivariate survival analysis was performed using the Cox proportional-hazards model. A P value of less than 0·05 (two-sided) was considered statistically significant. All statistical analyses were performed using STATA 8·1 (STATACORP LP, College Station, TX, USA).

Results

FBXW7 and NOTCH1 mutations in T-ALL and T-NHL patients

FBXW7 and/or *NOTCH1* mutations were found in 22 (40·0%) of 55 T-ALL and 7 (50·0%) of 14 T-NHL patients (Tables I–III). *FBXW7* mutations were found in 8 (14·6%) of 55 T-ALL and 3 (21·4%) of 14 T-NHL patients, and *NOTCH1*

Table I. *FBW7* and *NOTCH1* mutations in T-ALL and T-NHL patients.

	FBXW7 mutation		*NOTCH1* mutation	
Patient no.	Nucleotide*	Amino acid	Nucleotide†	Amino acid
T-ALL 4	–	–	4778T > C	L1593P
T-ALL 5	1662C > T	R505C	4817_4818insGCCCCC	1606delinsLPP
T-ALL 8	1450_1451ins AGCTGTT GTCTCTCATCATATG CCTTCTCAC	435AVVSHHMPSHHfsX	–	–
T-ALL 20	1542C > T	R465C	4847T > A	I1616N
T-ALL 22	–	–	4775_4776insGAC	1592delinsLT
T-ALL 23	–	–	7355_7356insCTGGC	2453WRCTLFCPRKAPPCP RRCHPRWSHPfsX
T-ALL 26	–	–	4818_4819insCTTTATCTC	1606_1607insHYL
T-ALL 30	–	–	4732_4734del	1578delV
T-ALL 31	–	–	4732_4734del	1578delV
T-ALL 32	2029T > C	V627A	–	–
T-ALL 33	–	–	4754T > C	L1585P
T-ALL 34	1543G > A 715_718delinsGAC	R465H 189RPQNIQVPLGLYHV QQHQQLLGTSEQPM AKGNNDAELHLSSHL QASRNGfsX	–	–
T-ALL 35	–	–	7412delinsAG	S2471X
T-ALL 37	–	–	4732-4734del	1578delV
T-ALL 38	1585G > A	R479Q	–	–
T-ALL 41	–	–	4754T > C	L1585P
T-ALL 46	–	–	7318C > T	Q2440X
T-ALL 49	1585G > A	R479Q	4732-4734del	1578delV
T-ALL 50	–	–	7330C > T	Q2444X
T-ALL 65	–	–	4814_4815delinsCCCCCCCCGA CCATAAGCC	1606PPDHKPSVTHTASRfsX
T-ALL 67	1543G > A	R465H	–	–
T-ALL 75	–	–	4818_4822delinsAGCACACCA GCCCAAGC	1606delinsLAHQP
T-NHL 18	–	–	4709_4718del	1570_1573delinsVDK
T-NHL 25	–	–	7541_7542del	2515RVPfsX
T-NHL 54	–	–	4793G>C	R1598P
			7541_7542del	2515RVPfsX
T-NHL 55	1543G > A	R465H	–	–
T-NHL 58	1543G > A	R465H	4845_4847delinsCCCCTCGAA	1615_1617delinsIPSNF
T-NHL 59	–	–	7326_7327insCGCGGAGGTGC	2443RGGACSHWAPAAWRC TLFCPRRAPPCP RRCHPR WSHPfsX
T-NHL 61	2107del	653RVNLFETfsX	7403_7404insGGGGG	2469GGHPRWSHPfsX

*Nucleotide number is according to the GenBank accession number NM_033632.
†Nucleotide number is according to the GenBank accession number NM_017617.

Table II. Association of *NOTCH1* and *FBXW7* mutations with clinical characteristics in 55 T-ALL patients.

	NOTCH1			*FBXW7*			*FBXW7* and/or *NOTCH1*		
	Mutation (+)	Mutation (−)		Mutation (+)	Mutation (−)		Mutation (+)	Mutation (−)	
Patient characteristics	*n* (%)	*n* (%)	*P*	*n* (%)	*n* (%)	*P*	*n* (%)	*n* (%)	*P*
Overall	17	38		8	47		22	33	
Gender									
Male	12 (70·6)	25 (65·8)	0·726	7 (87·5)	30 (63·8)	0·250	16 (72·7)	21 (63·6)	0·481
Female	5 (29·4)	13 (34·2)		1 (12·5)	17 (36·2)		6 (27·3)	12 (36·4)	
Age at diagnosis (years)				3 (37·5)	27 (57·4)	0·295	12 (54·5)	18 (54·5)	1·0
<10	10 (58·8)	20 (52·6)	0·670	5 (62·5)	20 (42·6)		10 (45·5)	15 (45·5)	
≥10	7 (41·2)	18 (47·4)							
Presenting at diagnosis WBC ($x10^9$/l)									
<100	12 (70·6)	18 (47·4)	0·110	17 (89·5)	26 (72·2)	0·238	16 (72·7)	14 (42·4)	0·027
≥100	5 (29·4)	20 (52·6)		5 (9·1)	13 (36·1)		6 (27·3)	19 (57·6)	
Mediastinal involvement									
Yes	12 (70·6)	22 (57·9)	0·371	4 (50·0)	30 (63·8)	0·464	14 (63·6)	20 (60·6)	0·821
No	5 (29·4)	16 (42·1)		4 (50·0)	17 (36·2)		8 (36·4)	13 (39·4)	
T cell immunophenotype									
Pro and Pre	3 (17·6)	5 (13·2)	0·665	0 (0)	8 (17·0)	0·287	3 (13·6)	5 (15·2)	0·164
Cortical	8 (47·1)	14 (36·8)		5 (62·5)	17 (36·2)		12 (54·5)	10 (30·3)	
Mature	6 (35·3)	19 (50·0)		3 (37·5)	22 (46·8)		7 (31·8)	18 (54·5)	
Chromosomal abnormalities*									
No	11 (68·8)	21 (55·3)	0·749	8 (100·0)	24 (52·2)	0·031	16 (76·2)	16 (48·5)	0·172
Yes									
Abnormalities involving TCR locus† (+)	1 (6·3)	5 (13·2)		0 (0·0)	6 (13·0)		4 (19·0)	12 (36·4)	
Abnormalities involving TCR locus (−)	4 (25·0)	12 (31·6)		0 (0·0)	16 (34·8)		1 (4·8)	5 (15·2)	
Relapse									
Yes	0 (0)	10 (26·3)	0·022	1 (12·5)	9 (19·1)	1·0	1 (4·5)	9 (27·3)	0·039
No	17 (100)	28 (73·7)		7 (87·5)	38 (80·9)		21 (95·5)	24 (72·7)	

Pro and Pre ($CD7^+$ and $CD1^-$), Corical ($CD1^+$), Mature ($CD1^+$, $sCD3^+$).
P, χ^2 or Fisher's exact test; TCR, T cell receptor.
*Total $n = 54$.
†Chromosomal abnormalities including 14q11, 7p15, and 7q35.

mutations in 17 (30·9%) of 55 T-ALL and 6 (42·3%) of 14 T-NHL patients. Three (5·4%) T-ALL and two (1·4%) T-NHL patients presented mutations in both *FBXW7* and *NOTCH1* (Table I).

The 12 *FBXW7* mutations detected included nine missense mutations, one 31 bp insertion, one single nucleotide deletion, and one deletion/insertion mutation (Table I). Seven of nine missense mutations were clustered in a 'hot spot' encoding arginines 465 and 479 residues which are highly conserved in the WD40 (tryptophan–aspartic-acid) repeat of FBXW7 (Fig S2A). Of the 12 identified *FBXW7* mutations, one insertion (T-ALL 8), one deletion/insertion (T-ALL 34), and one single nucleotide deletion (T-NHL 61) have not been previously described in T-ALL or other cancers (Fig S2B–D). *FBXW7* missense mutation encoding V627A (T-ALL 32) was also a novel mutation. V627 of FBXW7 is evolutionarily conserved, and V627A was not detected in normal lymphocytes from 20 healthy volunteers. One patient (T-ALL 34) had a *FBXW7* deletion/insertion mutation and a missense mutation that encoded FBXW7 residue R465H (Table I, Fig S2C).

Of the 24 *NOTCH1* mutations detected in 23 cases, 16 (66·7%) were located in sequences encoding the HD domain, 8 (33·3%) in the PEST domain (Table I). In one case (T-NHL 54), mutations were detected in both the HD and PEST domains. Of these 24 mutations, 17 (70·9%) were short in-frame insertion or deletions, 5 (20·8%) were missense mutations, and 2 (8·3%) were nonsense mutations in sequences encoding the HD or PEST domains, respectively. Furthermore, a single nucleotide polymorphism C5097T was observed in the sequence encoding the C-terminal region of the HD domain in 63 (91·3%) of 69 patients, as previously reported for Japanese adult patients with mature T cell malignancies (Shimizu *et al*, 2007).

Table III. Association of *NOTCH1* and *FBXW7* mutations with clinical characteristics in 14 T-NHL patients.

	NOTCH1			*FBXW7*			*FBXW7* and/or *NOTCH1*		
	Mutation (+)	Mutation (−)		Mutation (+)	Mutation (−)		Mutation (+)	Mutation (−)	
Patient characteristics	*n* (%)	*n* (%)	*P*	*n* (%)	*n* (%)	*P*	*n* (%)	*n* (%)	*P*
Overall	6	8		3	11		7	7	
Gender									
Male	5 (83·3)	7 (87·5)	1·0	2 (66·7)	10 (90·9)	0·396	6 (85·7)	6 (85·7)	1·0
Female	1 (16·7)	1 (12·5)		1 (33·3)	1 (9·1)		1 (14·3)	1 (14·3)	
Age at diagnosis (years)									
<10	4 (66·7)	2 (25·0)	0·277	2 (66·7)	4 (36·4)	0·538	4 (57·1)	2 (28·6)	0·592
≥10	2 (33·3)	6 (75·0)		1 (33·3)	7 (63·6)		3 (42·9)	5 (71·4)	
Mediastinal involvement									
Yes	0 (0·0)	1 (12·5)	1·0	0 (0·0)	1 (9·1)	1·0	0 (0·0)	1 (14·3)	1·0
No	6 (100·0)	7 (87·5)		3 (100·0)	10 (90·9)		7 (100·0)	6 (85·7)	
T cell immunophenotype									
Pro and Pre	0 (0·0)	0 (0·0)	1·0	0 (0·0)	0 (0·0)	1·0	0 (0·0)	0 (0·0)	1·0
Cortical	2 (33·3)	2 (28·6)		1 (33·3)	3 (30·0)		2 (28·6)	2 (33·3)	
Mature	4 (66·7)	5 (71·4)		2 (66·7)	7 (70·0)		5 (71·4)	4 (66·7)	
Chromosomal abnormalities*									
No	4 (66·7)	3 (42·9)	0·755	3 (100·0)	4 (40·0)	0·217	5 (71·4)	2 (33·3)	0·470
Yes									
Abnormalities involving TCR locus† (+)	1 (16·7)	1 (14·3)		0 (0·0)	2 (20·0)		1 (14·3)	1 (16·7)	
Abnormalities involving TCR locus (−)	1 (16·7)	3 (42·9)		0 (0·0)	4 (40·0)		1 (14·3)	3 (50·0)	
Relapse									
Yes	0 (0·0)	2 (25·0)	0·473	0 (0·0)	2 (18·2)	1·0	0 (0·0)	1 (14·3)	1·0
No	6 (100·0)	6 (75·0)		3 (100·0)	9 (81·8)		7 (100·0)	6 (85·7)	

Pro and Pre ($CD7^+$ and $CD1^-$), Corical ($CD1^+$), Mature ($CD1^+$, $sCD3^+$).

P, χ^2 or Fisher's exact test; TCR, T cell receptor.

*Total $n = 13$.

†Chromosomal abnormalities including 14q11, 7p15, or 7q35.

Clinical characteristics of FBXW7 and NOTCH1 mutations

The clinical and biological characteristics of the patients in this study are shown in Tables II and III. *FBXW7* and/or *NOTCH1* mutations were associated only with white blood cell (WBC) counts. *FBXW7* and/or *NOTCH1* mutations, but not *FBXW7* or *NOTCH1* alone, were found more frequently in T-ALL patients with low WBC count, $<10 \times 10^9$/l, than in those with higher WBC count, $>10 \times 10^9$/l ($P = 0{\cdot}027$). *FBXW7* mutations, but not *NOTCH1* mutations, were negatively associated with chromosome abnormalities in both T-ALL and T-NHL. All T-ALL and T-NHL patients having *FBXW7* mutation lacked a chromosome abnormality (100% vs. 52·2%, $P = 0{\cdot}031$ in T-ALL and 100% vs. 40·0%, $P = 0{\cdot}217$ in T-NHL).

Prognostic significance of FBXW7 and NOTCH1 mutations

We next analyzed the correlation between *FBXW7* and/or *NOTCH1* mutations and clinical outcome. T-ALL patients with *NOTCH1* mutation had a better clinical outcome than those without *NOTCH1* mutation {100% vs. 65·8% [95% confidence interval (CI), 48·5–78·5%]; $P = 0{\cdot}008$ for 5-year EFS and 100% vs. 81·6% [95% CI, 65·2–90·8%]; $P = 0{\cdot}065$ for 5-year OS, respectively} (Fig S3), while the prognostic difference between patients with and without *FBXW7* mutation was not significant [87·5% (95% CI, 38·7–98·1%) vs. 74·5% (95% CI, 59·4–84·6%); $P = 0{\cdot}400$ for 5-year EFS and 100% vs. 85·1% (95% CI, 71·3–92·6%); $P = 0{\cdot}259$ for 5-year OS, respectively] (Fig S4). The 5-year EFS and OS for T-ALL patients with *FBXW7* and/or *NOTCH1* mutations were extremely high, suggesting a good prognosis for patients with *FBXW7/NOTCH1* mutation compared to those without [95·5% (95% CI, 71·9–99·4%) vs. 63·6% (95% CI, 45·0–77·5%); $P = 0{\cdot}007$ and 100% vs. 78·8% (95% CI, 60·6–89·3%); $P = 0{\cdot}023$, respectively] (Fig 1). Notably, all three patients with both *FBXW7* and *NOTCH1* mutations were alive without relapse.

Multivariate analysis of prognostic factors adjusted for gender, age at diagnosis, and WBC count presented at diagnosis revealed that *FBXW7* and/or *NOTCH1* mutation status, risk group for treatment, and chromosomal abnormalities retained

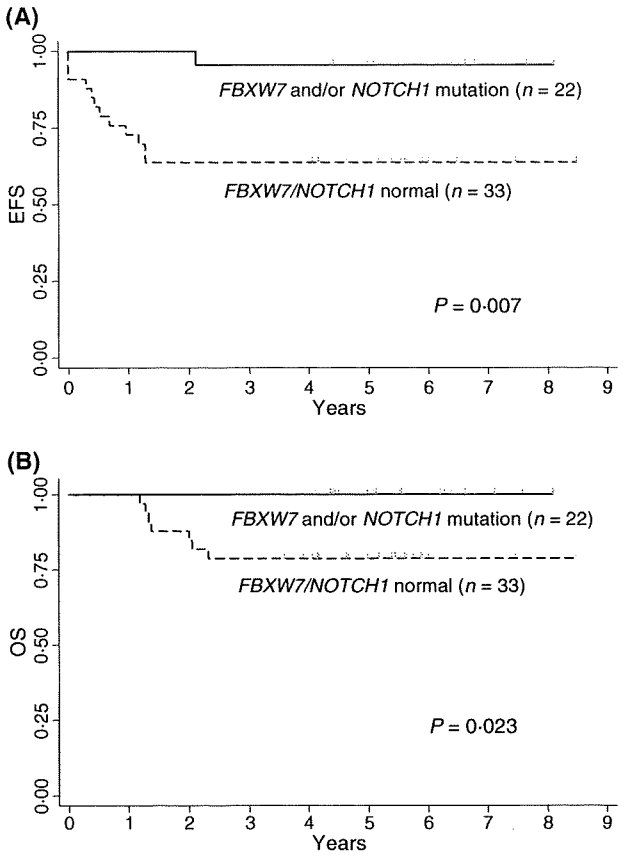

Fig 1. Kaplan–Meier estimate of (A) event-free survival and (B) overall survival of T-ALL patients with or without *FBXW7* and/or *NOTCH1* mutation.

their significant effects on EFS (Table IV). On the other hand, multivariate analysis adjusted for *NOTCH1* and/or *FBXW7* mutation status, risk group for treatment, and chromosomal abnormalities, in addition to gender, age at diagnosis, and WBC count presented at diagnosis, revealed that none of them retained EFS significance (Table IV).

In T-NHL, patients with *NOTCH1* and/or *FBXW7* mutation also had a good prognosis, although the differences in 5-year EFS and OS for patients with and without *NOTCH1* and/or *FBXW7* mutations were not significant [EFS, 85·7% (95% CI, 33·4–97·9%) vs. 57·1% (95% CI, 17·2–83·7%), $P = 0·313$; OS, 85·7% (95% CI, 33·4–97·9%) vs. 53·6% (95% CI, 13·2–82·5%), $P = 0·286$].

Discussion

In this study, we found 14·6% *FBXW7* mutations and 30·9% *NOTCH1* mutations in T-ALL patients, and 21·4% *FBXW7* mutations and 42·3% *NOTCH1* mutations in T-NHL patients. Frequencies of *FBXW7* and *NOTCH1* mutations in T-ALL in this study were similar to those in other recent studies (8·6–30·8% for *FBXW7* mutations, and 30·8–70·8% for *NOTCH1* mutations) (Akhoondi *et al*, 2007; Lee *et al*, 2005; Malyukova *et al*, 2007; Mansour *et al*, 2006; O'Neil *et al*, 2007; Thompson *et al*, 2007; van Grotel *et al*, 2008). This is the first report describing high frequencies of *FBXW7* and *NOTCH1* mutations in T-NHL as well as in T-ALL. The types of mutations identified were similar in T-ALL and T-NHL patients (Table I), although it was previously reported that gene expression profiling revealed intrinsic differences between T-ALL and T-NHL (Raetz *et al*, 2006).

Our results demonstrated that *FBXW7* and/or *NOTCH1* mutations as well as *NOTCH1* mutations alone had a good prognosis in T-ALL patients. The *P* value regarding the significant difference in prognosis for patients with *FBXW7* and/or *NOTCH1* status ($P = 0·007$ for EFS) was less than for those with *NOTCH1* status alone ($P = 0·008$), although the difference in prognosis for *FBXW7* status alone was not significant ($P = 0·397$). All T-ALL and T-NHL patients with

Table IV. Multivariate analysis of effects of *FBXW7* and/or *NOTCH1* mutations on EFS in 55 T-ALL patients.

	Crude HR		Adjusted HR1*		Adjusted HR2†	
	(95% CI)	*P*‡	(95% CI)	*P*‡	(95% CI)	*P*‡
FBXW7 and/or *NOTCH1* mutation						
Negative	1·00§		1·00§		1·00§	
Positive	0·10 (0·01–0·78)	0·028	0·10 (0·01–0·77)	0·027	0·24 (0·05–1·13)	0·071
Chromosomal abnormalities						
No	1·00§		1·00§		1·00§	
Yes						
Abnormalities involving TCR locus (+)	5·99 (1·55–23·22)	0·010	6·04 (1·54–23·70)	0·010	6·41 (1·35–30·58)	0·020
Abnormalities involving TCR locus (−)	7·63 (1·53–38·11)	0·013	10·80 (2·03–57·57)	0·005	3·22 (0·89–11·67)	0·076

HR, hazard ratio; CI, confidence interval.

*Adjusted for sex, age at diagnosis and presenting white blood cell count (categorical: see Table I).

†Adjusted for sex, age at diagnosis, presenting white blood cell count, *FBXW7* and/or *NOTCH1* mutations category, determined risk and chromosomal abnormalities) (categorical: see Table I).

‡*P*, X^2 test.

§Reference category.

FBXW7 mutations, with the exception of one T-ALL patient, have survived without relapse. One patient (T-ALL 38) had an isolated CNS relapse; however, the patient had survived 2 years after the relapse episode.

The paediatric ALL-BFM 2000 study reported the good clinical outcome for T-ALL patients with *NOTCH1* mutations (Breit *et al*, 2006), however, other two reports described results that were not compatible with this (van Grotel *et al*, 2008; Zhu *et al*, 2006). One possible explanation for this discrepancy of prognostic impact is the different treatment protocols used; the survival rates reported in other papers were apparently lower [28·8% 3-year relapse free survival (Zhu *et al*, 2006) and 65% 5-year disease-free survival (van Grotel *et al*, 2008)] for T-ALL patients with *NOTCH1* mutation than those of the ALL-BFM 2000 study (90% relapse-free survival) and our study (100% 5-year EFS). On the other hand, there was no statistically significant impact of *NOTCH1* mutations on prognosis in T-NHL patients, perhaps because the number of T-NHL patients was small in this study. Further study of T-NHL patients is needed to clarify the association of *FBXW7* and *NOTCH1* mutations with T-NHL prognosis.

Four novel mutations were found, and two of the four, V627A in T-ALL 32 and a frame shift mutation at codon 653 in T-NHL 61, were positioned outside of a 'hot spot' region. Codon 627 is localized in the seventh β-propeller blade (β-PB7) of FBXW7 (Orlicky *et al*, 2003), and a R689W mutation in the β-PB8 was also reported in T-ALL cases (Malyukova *et al*, 2007). C-terminal truncation of FBXW7 observed in T-NHL 61 was also reported in an endometrial tumour (nonsense mutation of codon 658) (Akhoondi *et al*, 2007), and these mutations result in the absence of a portion of β-PB7 and all of β-PB8. These findings suggested that a structural change of any β-propeller blades may have similar effects on FBXW7 function. Furthermore, it was also demonstrated that *Fbxw7* deficiency in adult haematopoietic cells leads to T-ALL in mice (Matsuoka *et al*, 2008), suggesting that inactivation of FBXW7 plays a critical role in T-ALL leukaemogenesis.

Chromosomal abnormalities of the *TLX3* (5q35) and *TLX1* (10q24) locus have been reported to be associated with poor and good outcome (van Grotel *et al*, 2008). In this study, chromosomal abnormalities involving the *TLX1* locus were found in one patient and chromosomal abnormalities involving the breakpoint at 5q35·1 (*TLX3*) were not found in any patients. *t*(10;11)(q13;q14) [*PICALM-MLLT10* (previously termed *CALM-AF10*)] was not found. The prognostic significance of these cytogenetic abnormalities was not clear because the number of patients was small.

Notably, *FBXW7* mutations were only observed in T-ALL and T-NHL patients lacking chromosomal abnormalities. *FBXW7* is considered to be a haplo-insufficient tumour suppressor gene (Mao *et al*, 2004). Inactivation of FBXW7 has been reported to cause chromosomal instability in karyotypically stable colorectal cancer cells, resulting in a striking phenotype associated with micronuclei and chromosomal instability (Rajagopalan *et al*, 2004). On the contrary, *FBXW7* mutation has been reported to lack association with chromosomal instability in colorectal cancer (Kemp *et al*, 2005), which was compatible with the present results for T-ALL. Further studies are needed to clarify this issue.

In conclusion, *FBXW7* and *NOTCH1* are functionally related each other, and the mutations of either *FBXW7* or *NOTCH1* genes rather than *FBXW7* or *NOTCH1* alone were associated with good clinical outcome in T-ALL, suggesting that the status of both *FBXW7* and *NOTCH1*, rather than *FBXW7* or *NOTCH1* alone, is a useful prognostic factor in T-ALL.

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