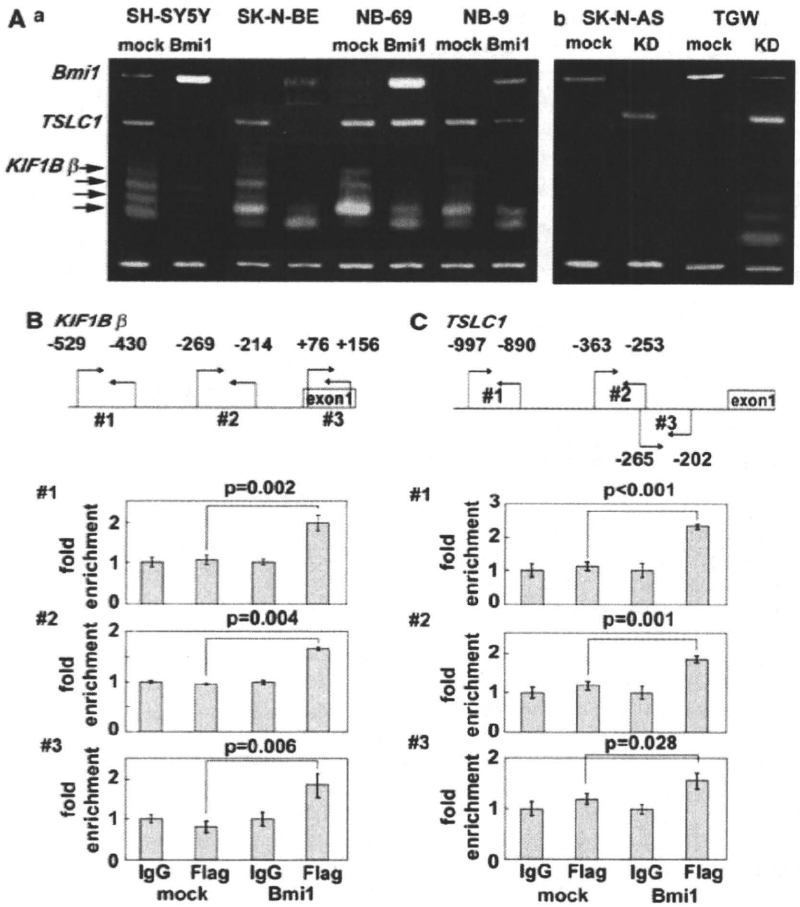

Figure 6 Bmi1 directly binds to TSLC1 and KIF1Bβ promoters and represses transcription in NB cells. (**A**) The indicated NB cell lines were infected with Bmi1-expressing lentivirus (a) and Bmi1-shRNA lentivirus (b), as described in the 'Materials and methods' section. Bmi1 expression modulated by lentivirus infection was examined (top lane of panels). TSLC1 and KIF1Bβ expressions were studied by semi-quantitative RT–PCR assay. The primer sequences are shown in Supplementary Table S1. The results are representative of at least three independent experiments. Arrows indicate alternative splicing products of KIF1Bβ (Munirajan *et al.*, 2008). (**B**: KIF1Bβ and **C**: TSLC1) SK-N-BE cells were infected with FLAG-Bmi1-expressing lentivirus and subjected to quantitative ChIP assay as described in the 'Materials and methods' section. Immunoprecipitation was performed by anti-FLAG (M2) antibody and control mouse IgG. The primers for qPCR analysis were designed using the Primer3 program (Applied Biosystems, Foster City, CA, USA) and locations are indicated in the diagrams. The primer sequences are shown in Supplementary Table S2. The results are presented as fold enrichment and are representative of at least three independent experiments. Error bars represent the s.d. obtained with triplicate samples. Statistical significance was determined by the Mann–Whitney test.

In this paper, we found that MYCN directly binds to the Bmi1 promoter *in vivo* and that binding is enhanced by *MYCN* amplification in NB cell lines and MYCN induction using tetracycline-withdrawal-based gene induction plasmid (Figure 3). MYCN expression correlates with Bmi1 levels both at mRNA and protein levels in NB cell lines (Figures 1A, 2a) and NB tumor samples (Figure 1b). Next, we studied the role of the MYCN binding site and several E2F binding sites in Bmi1 transcriptional regulation using a luciferase expression system (Figure 2). Intriguingly, we found that significantly high luciferase activities of E-box + E2F site promoter (Figure 2c, −196/+53 fragment) and E2F site deletion from this fragment (Figure 2c, −196/−122 fragment) resulted in only a modest reduction of activity. Furthermore, base-deleted mutation to the E-box almost completely suppressed the activity of the deltaE2F fragment (Figure 2c, −196/−122/mut), suggesting the role of MYCN in Bmi1 transcription. MYCN-dependent Bmi1 induction was observed not only in NB cell experiments but also in *in vivo* experiments. The Bmi1 mRNA level was higher in NBs occurring in tyrosine hydroxylase promoter-induced *MYCN* transgenic mice than in ganglions with hyperplasia and normal ganglion (S Kishida and K Kadomatsu, personal communication). Accordingly, these results suggest the important role of MYCN in Bmi1 transcription in NB and further studies will be required to address the exact mechanism of Bmi1 transcriptional regulation by E2F and/or MYCN.

Furthermore, the epigenetic regulation of Bmi1 transcription will be an interesting subject of NB research as we observed considerable effects of Bmi1 on other PRC complex proteins.

Taken together, we found an intriguing MYCN/Bmi1/tumor-suppressor pathway in NB cells. This pathway might have a remarkable impact on NB tumorigenesis and is considered a target for the development of molecular targeted therapy for therapy-resistant NBs.

Materials and methods

Cell culture

Human NB cell lines and QG56 human lung squamous carcinoma cells were obtained from official cell banks and were cultured in RPMI1640 or Dulbecco's modied Eagle's medium (Wako, Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 50 μg/ml penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA) in an incubator with humidified air at 37 °C with 5% CO_2. Tet21/N cells, which are derived from the SH-EP NB cell line, express MYCN under the control of tetracycline (tet-off system) (kindly provided by Dr M Schwab; Lutz *et al.*, 1996). MYCN expression in Tet21/N cells was repressed by 100 ng/ml tetracycline (Sigma-Aldrich) for 48 h before each experiment.

Treatment of cell lines with glial cell line-derived neurotrophic factor, ATRA or TPA

TGW cells were seeded at a density of 1×10^5 cells per 6-cm diameter tissue culture dish in the presence of glial cell line-derived neurotrophic factor (Invitrogen), ATRA (Sigma-Aldrich) or TPA (Nacalai Tesque, Kyoto, Japan) at the concentrations indicated in figure legends, and then the cells were grown for 3 days.

Cell proliferation assay

NB cells were seeded in 96-well plates at a density of 10^3 cells per well in a final volume of 100 μl. The culture was maintained under 5% CO_2 and 10 μl WST-8 labeling solution (Cell counting Kit-8; DOJINDO, Kumamoto, Japan) was added, and the cells were returned to the incubator for 2 h. The absorbance of the formazan product formed was detected at 450 nm in a 96-well spectrophotometric plate reader, according to the manufacturer's protocol.

Western blot analysis

The cells were lysed in a buffer containing 5 mM EDTA, 2 mM Tris–HCl (pH 7.5), 10 mM β-glycerophosphate, 5 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, protease inhibitor cocktail (Nacalai Tesque) and 1% SDS. Western blot analysis was performed as previously reported (Kurata *et al.*, 2008). After transferring to an Immobilon-P membrane (Millipore, Bedford, MA, USA), proteins were reacted with either anti-Bmi1 mouse monoclonal (229F6; Upstate, Charlottesville, VA, USA), anti-MYCN rabbit polyclonal (C-19; Santa Cruz, Santa Cruz, CA, USA) p14 (14P02; Oncogene) mouse, p16 (16P04; Neomarkers/Labvision, Fremont, CA, USA) mouse, anti-β-actin (Sigma-Aldrich) or a monoclonal anti-tubulin (Neomarkers Labvision) antibody. Anti-Ring1b mouse monoclonal antibodies were as described in a previous report (Atsuta *et al.*, 2001).

Immunohistochemistry

A 4-μm thick section of formalin-fixed, paraffin-embedded tissues was stained with hematoxylin and eosin and the adjacent sections were immunostained for Bmi1 using a polyclonal anti-Bmi1 antibody (AP2513c; ABGENT, San Diego, CA, USA). The Bench-Mark XT immunostainer (Ventana Medical Systems, Tucson, AZ, USA) and 3-3′ diaminobenzidine detection kit (Ventana Medical Systems) were used for visualization. Appropriate positive and negative control staining was also performed in parallel for each immunostaining. The tumor samples used in this study were kindly provided from various institutions and hospitals in Japan. Informed consent was obtained at each institution and hospital. All tumors were diagnosed clinically and pathologically as NBs and *MYCN* copy number was determined as previously described (Kurata *et al.*, 2008).

Semi-quantitative RT–PCR

The methods of semi-quantitative RT–PCR analysis were previously described (Kurata *et al.*, 2008). Total cellular RNA to prepare RT–PCR templates was extracted from NB cell lines using Isogen (Nippon Gene K K, Tokyo, Japan), and cDNA was synthesized from 1 μg total RNA templates according to the manufacturer's protocol (RiverTra-Ace-α RT–PCR kit; TOYOBO, Osaka, Japan). Primer sequences are described in Supplementary Table S1.

qPCR analysis for ChIP assay

qPCR analysis was performed using the ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions using SYBR Premix Dimer Eraser (Takara Bio, Ohtsu, Shiga, Japan). The primers for qPCR were designed and synthesized to produce 50–150 bp products. The primer sequence is listed in Supplementary Table S2. Each sample was amplified in triplicate. In Figure 3, the primer set was designed in E-box upstream of Bmi1 (Bmi1 promoter 1). In Figures 3, 6b, primer sets were designed in KIF1Bβ (KIF1B promoter 1, 2, 3) and TSLC1 (TSLC1 promoters 1, 2, 3).

Lentiviral infection

The packaging cell line HEK 293T (4×10^6) was plated and transfected the next day, when 1.5 μg of the transducing vectors containing the gene or shRNA, and 2.0 μg of the packaging vectors (Sigma-Aldrich) were cotransfected by the Fugene6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol. The medium was changed the next day and cells were cultured for another 24 h. Conditioned medium was then collected and cleared of debris by filtering through a 0.45-μm filter (Millipore). Thereafter, 1×10^5 NB cells were seeded in each well of a 6-well plate, and transduced by lentiviral-conditioned media. Transduced cells were analyzed by western blotting and RT–PCR.

Overexpression and knockdown of Bmi1

For the overexpression of Bmi1, FLAG-tagged mBmi1 plasmid was subcloned into lentivirus vector pHR-SIN-DL1. Cells were cultured in RPMI1640 and pooled. The pLKO.1-puromycin-based lentiviral vectors containing five sequence-verified shRNAs targeting human Bmi1 (RefSeq NM_005180) were obtained from the MISSION TRC-Hs 1.0 (Human) shRNA library (Sigma-Aldrich). Virus production, infection and selection were performed according to the manufacturer's protocol. At 1 week post infection, cells

were harvested and knockdown efficiency was assessed by western blotting. We checked Bmi1 knockdown by the five lentivirus-produced shRNAs and used two for experiments.

Luciferase reporter assay

The −1070/+53, −196/+53, −196/−122, −196/−122/mut (E-box sequence CACGTG changed to CA-G-G), −1070/+53 5′-upstream fragments were subcloned into luciferase reporter plasmid pGL4.17 (luc2/Neo) Luciferase Reporter Vector (Promega, Madison, WI, USA).

Tet21/N and SK-N-DZ cells were seeded in a 12-well plate 24 h before transfection at a concentration of 5×10^4 cells per well. Cells were cotransfected with Renilla luciferase reporter plasmid (pRL-TK, 10 ng) and luciferase reporter plasmid with the 5′-upstream region of the Bmi1 gene. The total amount of plasmid DNA per transfection was kept constant (510 ng) with pBlueScript KS+ by Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were lysed and their luciferase activities were measured by the Dual-Luciferase reporter system (Promega). The rey luminescence signal was normalized on the basis of the Renilla luminescence signal.

ChIP assay

ChIP assay was performed as described previously (Orlando *et al*., 1997, Fujimura *et al*., 2006). Cross-linked chromatin prepared from the indicated cells was precipitated with normal mouse IgG (eBioscience, San Diego, CA, USA), monoclonal anti-MYCN antibody (NCMII100; Calbiochem, San Diego, CA, USA) or anti-Flag antibody (M2; Sigma-Aldrich). 'Input' DNA was isolated from the initial lysates of genomic DNA. Species-matched immunoglobulin-immunoprecipitated DNA (IgG), derived from the same volume of the chromatin fraction used for specific antibody immunoprecipitation, was subjected to PCR. Primers used in this study are listed in Supplementary Table S2. Each series of experiments was conducted at least three times.

cDNA microarray experiments

For gene expression profiling, in-house cDNA microarray with 13 440 spots was used. In all, 10 μg each of total RNA were labeled with the CyScribe RNA labeling kit in accordance with the manufacturer's manual (GE healthcare, Little Chalfont, Buckinghamshire, UK), followed by probe purification using the Qiagen MinElute PCR purification kit (Qiagen, Valencia, CA, USA). We used a mixture of RNAs isolated from eight human adult cancer cell lines as a common reference. RNAs from Bmi1-infected SK-N-BE and mock-infected SK-N-BE cells were labeled with Cy3 dye and a reference RNA mixture was labeled with Cy5 dye, mixed, and used as probes together with yeast tRNA and polyA for suppression. Subsequent hybridization and washing were conducted as described previously (Ohira *et al*., 2005). Hybridized microarrays were scanned using the Agilent G2505A confocal laser scanner (Agilent technology, Santa Clara, CA, USA), and fluorescent intensities were quantified using the GenePix Pro microarray analysis software (Axon Instrument, Foster City, CA, USA). The resulting relative expression values for the gene spots were compared between Bmi1-infected and mock-infected SK-N-BE cells

Conflict of interest

The authors declare no conflict of interest.

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Retrospective Analysis of Non-Anaplastic Peripheral T-Cell Lymphoma in Pediatric Patients in Japan

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Background. Reports of non-anaplastic peripheral T-cell lymphoma (PTCL) in pediatric patients are relatively rare. ***Procedure.*** We performed a retrospective analysis in patients with PTCL over an 18-year period (1991–2008). ***Results.*** We could analyze clinical data in 21 patients with non-anaplastic PTCL; 10 were female and 10 male. Median age of onset was 11 years (range: 1–21 years). There were nine patients with PTCL, not otherwise specified (PTCL-NOS); ten with extranodal NK/T-cell lymphoma, nasal type; one with angioimmunoblastic T-cell lymphoma; and one with subcutaneous panniculitis-like T-cell lymphoma. Initial lesions involved cervical lymph nodes in five patients, and the skin in five patients. In five patients, hemophagocytic syndrome (HPS) was the initial clinical feature. There were 12 patients with advanced stage disease (stages III and IV). Chemotherapy and radiation was administered in 18 and 2 patients, respectively. Among the two patients who did not receive chemotherapy and radiation, one patient died while being treated for HPS but another improved spontaneously. Although 5 patients relapsed, 18 of 21 patients remained alive without disease at last follow-up. Five-year overall survival rate was 85.2%. ***Conclusions.*** Generally, the outcome results of conventional chemotherapy for high-risk PTCL are poor in adult patients. However, the excellent results in our study suggest that PTCL of childhood is quite different from that of adulthood. Although this study is first report about PTCL of Asian children, the number of patients was small in this study. Larger studies are needed to confirm these findings. Pediatr Blood Cancer 2010;54:212–215. © 2009 Wiley-Liss, Inc.

Key words: child; peripheral T-cell lymphoma

INTRODUCTION

Peripheral T-cell lymphomas (PTCLs) are a heterogeneous group of rare diseases, usually demonstrating clinical aggressiveness [1]. Because of difficulty and variability in diagnosis, improvements in diagnostic technology, and changing classification systems over time, the interpretation of studies is complicated. In addition, the response to current treatments and long-term outcome are generally poor [2–6]. Reports of non-anaplastic PTCL in pediatric patients are relatively rare [7–11]. Moreover, although geographic variation has been well documented, this may reflect exposure to specific pathogenic viruses, such as Epstein Barr (EB) virus and human T-cell leukemia virus-1 in Asian countries. There are no reports about child PTCL from Asia. We therefore performed a retrospective analysis of patients with PTCL over an 18-year period (1991–2008).

METHODS

We performed this retrospective analysis as the lymphoma committee of the Japan Leukemia and Lymphoma Study Group (JPLSG). Data were obtained from the Japan Association of Childhood Leukemia Study (JACLS), Tokyo Children's Cancer Study Group (TCCSG), Japanese Children's Cancer and Leukemia Study Group (JCCLSG), and Kyushu-Yamaguchi Children's Cancer and Leukemia Study Group (KYCCSG). In the 18-year study period, 55 patients were registered as having PTCL or NK/T lymphoma including blastic NK lymphoma and myeloid/NK lymphoma. Clinical data for 21 patients with non-anaplastic PTCL after excluding 34 patients with blastic NK lymphoma and myeloid/NK lymphoma were analyzed.

Pathologic diagnoses were confirmed by central review in 9 of 21 patients. Central review was performed using WHO classification. For the other 12 children, histopathology was performed at the treating center only and confirmed from a copy of the pathology report. In almost all reports, immunophenotyping such as CD79a, CD20, CD3, CD43, TdT, and MPO was included.

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The presence of an association with EB virus was determined by detection of EB virus genome in white blood cells or plasma, or the detection of this virus in histological material by EB virus encoded small RNA (EBER) in situ hybridization [12].

Statistical Analyses

Analysis of overall survival was performed using the Kaplan–Meier method, with differences compared by log-rank test. Differences between groups were analyzed using a Fisher exact test and a Mann–Whitney *U*-test. Statistical analyses were

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performed using Dr. SPSS II for Windows (release 11.0.1J, SPSS Japan, Inc.).

RESULTS

In the 18-year study period, we were able to analyze clinical data from 21 patients with non-anaplastic PTCL (Table I). Because 1,711 child and adolescent patients with non-Hodgkin lymphoma were registered in the 18-year period, the proportion of NHL classified as PTCL was 1.2%. Of the 21 patients, 10 were male and 11 were female. Median age of onset was 11 years (range: 1–21 years). There were nine patients with PTCL not otherwise specified (PTCL-NOS); ten with extranodal NK/T-cell lymphoma, nasal type; one with angioimmunoblastic T-cell lymphoma; and one with subcutaneous panniculitis-like T-cell lymphoma. Initial lesions involved the cervical lymph nodes in five patients, and the skin in five patients. In five patients, hemophagocytic syndrome (HPS) was the initial clinical feature. With regard to stage of disease at diagnosis, eight patients were at stages I and II, six were at stage III, and six were at stage IV; this information was not available for one patient. Chemotherapy and radiation were administered in 18 and 2 patients, respectively. Two patients received no treatment. Treatment for PTCL was not consistent in this study. Eight patients received a T-cell lymphoma/leukemia regimen, and four received a B cell lymphoma/leukemia regimen. Among the two patients who did not receive chemotherapy and radiation, one patient died while undergoing treatment for HPS and another improved spontaneously. In the latter patient (patient 5), the initial clinical features were fever, cervical lymphadenopathy, and pancytopenia. He was diagnosed with HPS from laboratory data and bone marrow aspiration. Lymph node biopsy revealed PTCL and there was positive staining on EBER in situ hybridization. However, after several days, the fever abated and laboratory data improved. He received no chemotherapy at the request of his parents and remained disease-free at last follow-up, 9 months after onset.

Eleven patients received stem cell transplantation. Of these, two received an autologous peripheral blood stem cell transplant (PBSCT), five received a related bone marrow transplant (BMT), two received a related PBSCT, two received an unrelated cord blood stem cell transplant (CBSCT), and one received an unrelated BMT. Although 5 patients relapsed, 17 of the 21 patients were alive without disease at last follow-up, giving an overall 5-year survival rate of 85.2% (Fig. 1). Causes of death for the three patients who succumbed to their disease were HPS, progression of disease and complications of stem cell transplantation. Ten of the 21 patients had PTCL associated with EB virus. Compared with patients with extranodal NK/T lymphoma, nasal type, those with PTCL-NOS were younger (median 7 years vs. 15.5 years, $P < 0.05$) and had a lower relapse rate (11% vs. 40%). However, gender (male/female; 5/4 vs. 4/6), proportion with advanced stage disease (56% vs. 60%), survival rate (87.5% vs. 80.0%) and association with EB virus (44% vs. 60%) were similar and statistically non-significant differences.

DISCUSSION

Peripheral NK/T-cell neoplasms are an uncommon group of diseases that show distinct racial and geographic variation. The prognostic significance of the T-cell phenotype has been clearly defined in recent studies by using modern lymphoma classification systems. Anaplastic large cell lymphoma, not rare in childhood, is another type of PTCL. Results of conventional chemotherapy for high-risk PTCL are poor compared with those for their aggressive B-cell counterparts in adult patients.

However, although case reports of pediatric PTCL are sometimes seen [7,10,11], large case series are very rare. The only two such case series published are a report from the United Kingdom [8] and the Children's Oncology Group (COG) Study [9]. In the UK series, 25 cases were identified, 44% of children died and 5-year survival rate was 59%. On the other hand, in the 20 patients in the COG series, 5-year survival rate was 90% in patients with localized disease and 50% in those with advanced disease. In the present study, 21 patients with PTCL were identified; these included 9 with PTCL-NOS; 10 with extranodal NK/T-cell lymphoma, nasal type; 1 with angioimmunoblastic T-cell lymphoma; and 1 with subcutaneous panniculitis-like T-cell lymphoma. Surprisingly, although 57% of patients had advanced stage disease and five patients relapsed after chemotherapy, the 5-year survival rate was 85.2%. However, treatment for PTCL was not consistent in this study. Eight patients received a regimen for T-cell lymphoma/leukemia, and four patients received a B cell lymphoma/leukemia regimen. Moreover, in one patient, symptoms improved spontaneously, and this has not previously been reported. Although five patients had relapse, four patients remained disease free at last follow-up and only two patients had undergone stem cell transplantation. Our study suggests that in the present population, PTCL in childhood does not have a poor outcome compared to adult with PTCL. This reason is not clear. However, the role of stem cell transplantation might be important. Stem cell transplantation had been undergone in eight patients with first complete response or partial response, one patient with progressive disease and two patients after relapse. After stem cell transplantation, only two patients died and nine patients are surviving without relapse.

Many cases of extranodal NK/T-cell lymphoma, nasal type were seen in this study compared with previous reports. Moreover, patients with this type of lymphoma were older at initial presentation than those with PTCL-NOS. Extranodal NK/T-cell lymphoma, nasal type is mostly confined to East Asia, and it predominantly occurs in the nasal or paranasal areas and less frequently in the skin. Most of the tumors show NK-cell phenotypes, although T-cell phenotypes are occasionally seen. The EB virus genome can usually be detected in lymphoma cells. Disease was associated with EB virus in 65% of patients with extranodal NK/T-cell lymphoma, nasal type compared with 50% of patients with PTCL-NOS. Suwiwat et al. [13] detected cell-free EBV DNA in 32/38 (84%) of adult PTCL patients, but failed to find EBV in controls. Rates of EB virus were higher in that report than in our study, possibly because Suwiwat et al. examined adults rather than children. However, we found EB virus in three of four patients who had HPS as the initial clinical feature. EB virus associated with HPS is sometimes seen in childhood, and some of these patients might also have PTCL. T-cell lymphoma-associated hemophagocytic syndrome (T-LAHS) has been frequently reported in Asian countries and is considered to have an extremely poor prognosis. Tong et al. [14] retrospectively analyzed the records of 113 patients with aggressive T-cell lymphoma, of which 28 had LAHS. The therapeutic results of chemotherapy alone or in combination with other modalities were discouraging for T-LAHS and the survival time for most patients was no more than 1 year. In the present study, unlike in other reports, three of four patients with HPS remained disease-free at last follow-up.

Pediatr Blood Cancer DOI 10.1002/pbc

TABLE I. Clinical Characteristics and Outcomes for 21 Patients With Peripheral T-Cell Lymphoma

	Age	Gender	Diagnosis	Initial lesion	Stage	Treatment	Response	Relapse	Transplantation	Association of EB virus	Survival time (months)
1	6	M	PTCL-NOS	Liver, spleen	4	JACLS NHL98ER	PR	N	Y	N	68+
2	4	F	PTCL-NOS	HPS	4	ALL (T)	CR	N	N	Y	60+
3	16	M	PTCL-NOS	Cervical	3	BFM NHL-T	PR	N	Y	N	36+
4	5	F	PTCL-NOS	Skin	1	JACLS NHL98T	CR	N	N	N	12+
5	7	M	PTCL-NOS	Cervical, HPS	1	None		N	N	Y	9+
6	9	M	PTCL-NOS	Cervical, spleen	3	TCCSG NHLT01	CR	N	N	ND	57+
7	11	F	PCTL-NOS	Cervical	1	T-LBL	CR	Y	Y	N	12
8	1	F	PTCL-NOS	HPS		VP16 + DEX	CR	N	Y	Y	30+
9	12	M	PTCL-NOS	Submandibular	3	CHOP	PR	N	Y	Y	30+
10	14	F	Subcutaneous panniculitis-like	Skin	2	Steroid	CR	N	Y	N	8+
11	14	M	AITL	Cervical	4	JACLS NHL98T	CR	N	N	N	96+
12	17	M	Extranodal NK/T nasal type	Adrenal grand, HPS	3	None		N	N	N	0
13	14	F	Extranodal NK/T nasal type	Skin	4	93mix	CR	N	Y	N	132+
14	21	F	Extranodal NK/T nasal type	Sinusoidal	4	HLH94	CR	Y	N	Y	30+
15	10	F	Extranodal NK/T nasal type	Orbit, breast	3	DeVIC	PD	Y	Y	N	36+
16	18	F	Extranodal NK/T nasal type	Nasal sinus, kidney, ovary	4	ALL (B)	PD	N	Y	Y	5
17	11	M	Extranodal NK/T nasal type	Skin	3	TCCSG NHL B96-04	CR	N	N	N	107+
18	18	M	Extranodal NK/T nasal type	Nasopharynx	2	Radiation	CR	Y	N	Y	105+
19	8	F	Extranodal NK/T nasal type	Skin	1	CCLSG NHL960LB	CR	N	N	Y	94+
20	10	M	Extranodal NK/T nasal type	Nasal sinus	1	DeVIC + radiation	CR	Y	Y	Y	45+
21	18	F	Extranodal NK/T nasal type	Nasal sinus, HPS	2	CHOP	PR	N	Y	Y	147+

PTCL-NOS, peripheral T-cell lymphoma, not otherwise specified; AITL, angioimmunoblastic T-cell lymphoma; HPS, hemophagocytic syndrome; CR, complete response, PR, partial response; PD, progressive disease; Y, yes; N, no; ND, no data. The drugs contained in remission introduction of each treatment is as follows: JACLS NHL98ER, vincristine (VCR), pirarubicin (THP-ADR), cyclophosphamide (CPM), L-asparaginase (L-asp), dexamethasone (DEX), prednisolone (PSL), JACLS NHL98T, VCR, CPM, adriamycin (ADR), L-asp, PSL, TCCSG NHLT01, VCR, CPM, ADR, L-asp, THPADR, PSL, CHOP: CPM, ADR, VCR, PSL, HLH94: etoposide (VP16), DEX, cyclosporine, DeVIC: DEX, ifosfamide, carboplatinum, VP16, TCCSG NHL B96-04: CPM, VP16, methotrexate (MTX), PSL, CCLSG NHL960LB: CPM, VCR, PRD, ADR, MTX.

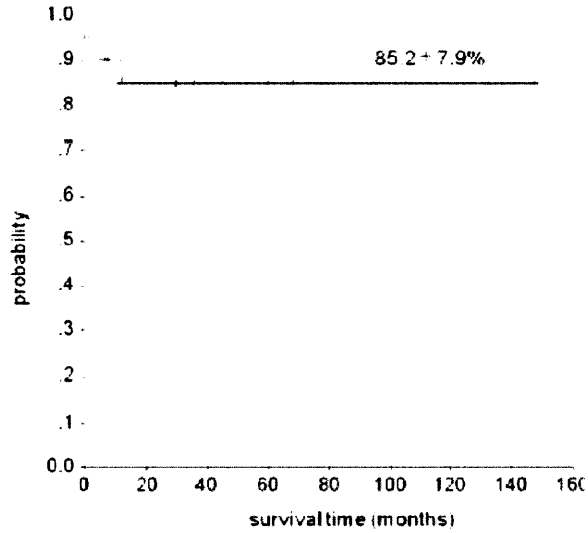

Fig. 1. Survival rate of patients with peripheral T-cell lymphoma. Five-year survival rate was 85.2%.

The findings of the present study differ from those of past reports of PTCL that included adults and children. However, the present study examined only a small number of patients. Larger studies are needed to confirm these findings.

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Short communication

FLT3-internal tandem duplication in a pediatric patient with t(8;21) acute myeloid leukemia

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Abstract Patients diagnosed with t(8;21)-acute myeloid leukemia (AML) are currently considered to have good prognoses, but about half of these patients relapse. *FLT3*-internal tandem duplication (ITD) is generally thought to be strongly associated with poor prognosis in AML, but is rarely reported in patients with t(8;21)-AML. Expression of the neural cell-adhesion molecule (CD56) is also associated with a significantly shorter complete remission duration and survival in patients with t(8;21)-AML. Patients with t(8;21)-AML expressing CD56 have been reported to exhibit a higher incidence of granulocytic sarcoma (GS), and t(8;21)-AML with GS results in a less favorable prognosis than AML with this translocation alone. Here, we report on a 15-year-old girl with t(8;21)-AML having both CD56 expression and *FLT3*-ITD. This patient underwent unrelated donor bone marrow transplantation and achieved complete remission, but thereafter presented with obstructive jaundice caused by GS compression of the common bile duct without bone marrow invasion at relapse. Autopsy revealed multiple nodules of the stomach membrane and invasion into the head of the pancreas. For earlier detection of relapse, we suggest that it would be useful to examine existence of GS in CD56-positive t(8;21)-AML patients at diagnosis and hematologic remission. Even though t(8;21)-AML is less likely to co-occur with *FLT3*-ITD in pediatric patients, this report suggests that prognostic factors, including *FLT3* and *KIT* genes and the surface marker CD56, should be analyzed in these patients.

1. Introduction

Even though patients with t(8;21)-acute myeloid leukemia (AML) are thought to have a good prognosis, it is reported that 50% relapse and have poor prognosis [1,2]. High-presenting leukocyte count, neural cell-adhesion molecule (CD56) expression, and extramedullary disease has been reported to be associated with a poor prognosis in t(8;21)-AML [1,3]. *FLT3*-internal tandem duplication (ITD) is also considered to be strongly associated with a poor prognosis in adult and pediatric AML patients [4], but is rarely reported in patients with t(8;21)-AML [5,6]. Patients with t(8;21)-AML expressing CD56 have been reported to have a high incidence of granulocytic sarcoma (GS). However, the correlation of *FLT3*-ITD, CD56 expression, and GS with the prognosis of t(8;21)-AML remains unclear. We report on a patient with t(8;21)-AML having CD56 expression and *FLT3*-ITD, who also showed obstructive jaundice caused by GS compressing the bile ducts at relapse and had a poor prognosis.

2. Materials and methods

2.1. Case report

A 15-year-old girl presenting with a persistent fever was admitted to our hospital with anemia and general fatigue. Upon admission, the patient's face was pale and she

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demonstrated no signs of cervical lymphadenopathy or hepatosplenomegaly. Blood tests revealed a white blood cell (WBC) count of 12,700/μL containing 54.5% blasts, a hemoglobin concentration of 5.7g/dL, and a platelet count of 2.2×10^4/μL. Bone marrow examination revealed 82.6% blasts that were positive for peroxidase staining (100%), as well as both nonspecific (21%) and specific (53%) esterase staining. A diagnosis of AML-M2 was made according to the morphologic and immunophenotypic criteria of French–American–British (FAB) classification. Immunophenotypic analysis of the CD45 dim cells showed the presence of CD13 (74.6%), CD33 (48%), CD34 (96.5%), CD38 (99.8%), HLA-DR (94.6%), CD56 (27.3%), and CD19 (51.2%). Cytogenetic analysis of the bone marrow cells demonstrated the 46, XX, t(8;21)(q22;q22), del(9)(q13q22) in 20 bone marrow cells. The *AML1/RUNX1-MTG8/ETO/RUNX1T1* fusion transcript was also detected at a concentration of 34×10^6 copies/μg RNA by real-time quantitative reverse-transcriptase polymerase chain reaction (RQ-RT-PCR).

The patient was treated in accordance with the low-risk treatment protocol of the Japanese Childhood AML Cooperative Study Group Protocol AML99 [7] due to the presence of t(8;21) and a WBC of less than 5×10^4/μl. Treatment consisted of etoposide, cytosine arabinoside, and mitoxantrone for induction therapy. After induction therapy, she achieved complete remission, and cytogenetic analysis revealed normal results in 20 bone marrow cells. After five courses of treatment, she remained in complete remission, although the *RUNX1-RUNX1T1* transcript was still detected at a concentration of 1.8×10^3 copies/μg RNA. Two months later, bone marrow analysis showed relapse and chemotherapy was continued. Finally, she could not achieve a second remission before allogeneic transplantation, and *RUNX1-RUNX1T1* transcript was 6.3×10^6 copies/μg RNA from bone marrow. Seven months after diagnosis, she underwent an unrelated-donor bone marrow transplant with the following conditioning regimen and acute graft-versus-host disease (GVHD) prophylaxis: busulfan and cyclophosphamide administration, as well as total lymph node irradiation followed by FK506 and short-term methotrexate administration. On day 15, she developed grade IIa GVHD with diffuse pruritic erythroderma, which improved after treatment with methylprednisolone. On day 25, her skin condition worsened and she reported severe abdominal pain and diarrhea. Lower GI endoscopy was performed and the biopsy revealed GVHD. The *RUNX1-RUNX1T1* fusion transcript of bone marrow cells was not detected. The volume of bloody diarrhea was almost 4 liters/day at its peak. She received steroid pulse therapy, octreotide acetate, and low-dose antithymocyte globulin, with her condition gradually improving after 2 months. During hematologic remission, ultrasound resonance of the abdomen did not show GS, although gastro-intestinal endoscopy was not performed at that time. On day 102, diarrhea symptoms returned and steroid pulse therapy and mycophenolate mofetil treatment were initiated. On day 238, she presented with jaundice and the *RUNX1-RUNX1T1* transcript was elevated up to 4.3×10^6 copies/μg RNA without bone marrow involvement. Computed tomography scanning disclosed thickening of the common bile duct wall and obstruction. Ultrasound resonance of the abdomen also showed obstructive findings (Fig. 1). She died on the day of the scheduled endoscopic retrograde biliary drainage, most likely due to septic shock caused by biliary tract infection, 2 years

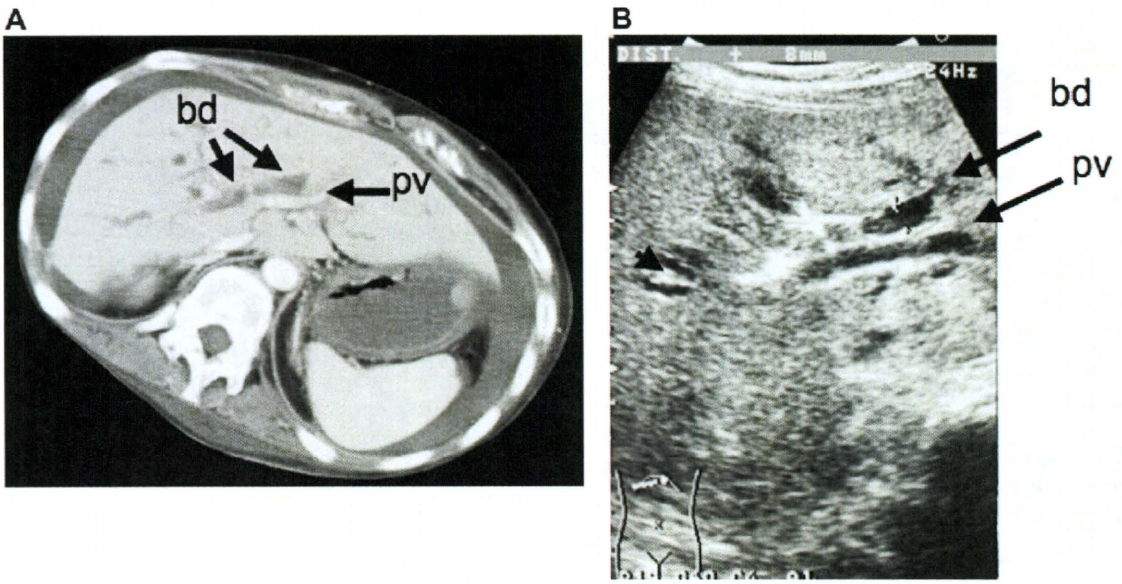

Fig. 1. (A) Enhanced computed tomography of the abdomen showing dilatation of the intrahepatic bile duct (bd) and obstruction of portal vein (pv). (B) Ultrasonography of abdomen showing dilatated intrahepatic bile duct (bd) and its accompanying hepatic portal vein (pv) with "parallel channel sign" of biliary obstruction.

after AML was diagnosed. The autopsy revealed multiple GS of the common bile duct, the head of the pancreas, and six nodes in the stomach mucosa that were formed by GS. The junction of the bile duct, pancreatic duct, and duodenum was also compressed by leukemic cells invading the tissues and causing obstructive jaundice (Fig. 2).

2.2. Analysis of FLT3 and KIT genes

RNA extracted from bone marrow cells at diagnosis was reverse-transcribed to cDNA, and alterations in the *FLT3* and *KIT* genes were examined according to previous reports [7]. Mutations of *FLT3*-D835/I836 were examined by restriction fragment length polymorphism PCR, and *FLT3*-ITD was analyzed by RT-PCR. Mutational analysis of exons 8–11 and exons 17–18 of the *KIT* gene was performed with RT-PCR followed by direct sequencing. In this patient, a 48-base pair (bp) *FLT3*-ITD was identified, while *KIT* or *FLT3* mutations did not show at diagnosis and at the first relapse.

This study was approved by the institute's ethics committee, and written informed consent was obtained from our patient's parents.

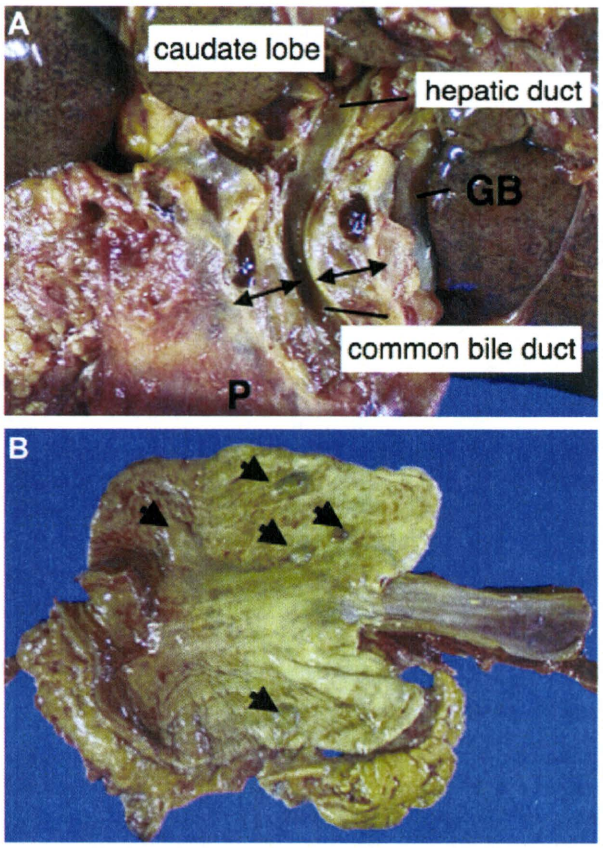

Fig. 2. (A) Granulocytic sarcoma invasion into the junction of gall bladder, duodenum, and common bile duct. P, pancreas; GB, gall bladder. (B) Multiple granulocytic sarcomas in stomach (indicated by arrows).

3. Results and discussion

t(8;21)-AML is a heterogeneous disease in terms of its clinical and biologic features, as well as its clinical outcome. *KIT* mutations are identified in 12–47% of patients with t(8;21) and are strongly associated with a poor prognosis in pediatric t(8;21)-AML [7,8]. In contrast, *FLT3*-ITD of AML is present in approximately 15% of pediatric and 25% of adult patients [4,5,9]. *FLT3*-ITD was demonstrated in only 2 (4.6%) of 46 patients with t(8;21)-AML entered in the study of the Japanese Childhood AML Cooperative Study Group [7] similar to the previous study [1]. This case of 1 of 2 patients with t(8;21)-AML carrying *FLT3*-ITD is thought to be strongly associated with a poor prognosis in AML, but is rarely reported in patients with t(8;21)-AML [1,7].

It has been shown recently that in pediatric AML, patients with ITD longer than 48 bp had a worse relapse-free survival rate (19 vs. 51%, $P = 0.035$), while the presence of more than one ITD was not clinically significant. Physical characteristics, including the length of *FLT3*-ITD, may influence *FLT3* activation state by altering its structure and may impact response to therapy [10]. In this patient, the length of the *FLT3*-ITD was 48 bp, while the position of the ITD within this sequence has been reported to be associated with poor prognosis. Prognostic factors other than *FLT3*-ITD were also thought to influence the prognosis of this patient.

The analysis of immunosurface markers, such as CD56 expression, on leukemic blasts may also determine the risk of developing extramedullary relapse. The expression of CD56 may also be correlated to the predisposition of t(8;21)-AML to develop GS [11]. CD56 expression in t(8;21)-AML was associated with significantly shorter complete remission duration and survival [3]. Although some studies suggest that the presence of GS adversely affects the prognosis of patients with t(8;21)-AML [1], other reports have indicated that GS does not influence the outcome [12]. Extramedullary leukemia does not appear to be a sufficient explanation for the adverse treatment outcome of t(8;21)-AML with CD56 expression and, thus, additional prognostic factors must be investigated. The high relapse rate of t(8;21)-AML patients with leukemia cells expressing CD56 could possibly be explained by an association between CD56 expression and drug resistance [13]. Clonal evolution of leukemic blasts with CD56 expression has been observed in AML patients who were originally CD56 negative after relapse [14,15]. CD56 expression has also been shown to be related to relapse and metastasis [16]. In this study, we could not examine CD56 expression in leukemic cells of GS. The association of GS, CD56 expression, and *FLT3*-ITD in t(8;21)-AML, all of which are considered to be poor prognosis factors, currently remains unknown. It is possible that CD56 positivity of leukemic blasts might be related to GS, while CD56 expression and *FLT3*-ITD remain independent.

While GS tends to occur most commonly in the skin, lymph nodes, spine, and small intestine, it has also been

Table 1
AML presenting with obstructive jaundice and granulocytic sarcoma

Patient no.	Age/sex	FAB/karyotype	CD56 expression	Onset of jaundice	AML	Site of GS	Outcome	Reference
1	73/M	NA	NA	Concurrent	Diagnosis	Head of pancreas	D	[25]
2	51/M	NA	NA	Precedent	1CR	Gallbladder wall, bile duct, pretropancreatic lymph node	D	[20]
3	48/F	NA	(−)	Precedent	2CR	Gallbladder wall	D	[21]
4	37/F	NA	NA	Concurrent	3CR	Head of pancreas	D	[26]
5	31/M	NA	(−)	Precedent	Diagnosis	Head of pancreas	CR (NA)	[27]
6	61/F	NA/trisomy 8,13	NA	Concurrent	Diagnosis	Head of pancreas	D (10 mo)	[27]
7	75/F	NA	NA	Precedent	Diagnosis	Bile duct	D (1 mo)	[28]
8	84/M	M0	NA	Precedent	Diagnosis	Gallbladder wall	D	[29]
9	36/M	NA/46XY	NA	Concurrent	Diagnosis	Bile duct	CR (12 mo)	[18]
10	51/F	M2	NA	NA	Relapse	Head of pancreas	NA	[20]
11	47/M	M2	NA	Concurrent	1CR	Common bile duct	CR (NA)	[30]
12	20/F	M2/t(8;21)	NA	Precedent	Diagnosis	Bile duct, ovary	CR (3 mo)	[31]
13	64/M	M2/t(8;21)	NA	Precedent	1CR	Head of pancreas	D (4 mo)	[32]
14	55/M	M2/trisomy 21	NA	Concurrent	Diagnosis	Common bile duct	CR (8 mo)	[33]
15	60/M	M4	NA	Concurrent	Diagnosis	Liver, colon	NA	[34]
16	49/M	M2/t(8;21)	(+)	Precedent	1CR	Paraspinal	NA	[22]
17	36/F	M4/46XX	NA	Precedent	Diagnosis	Head of pancreas	CR (7 mo)	[19]
18	17/M	M2/t(8;21)	(+)	Precedent	2CR	Bile duct	D	This patient

Abbreviations: NA, not available; D, dead; CR, complete remission.

described in a variety of other organs [17]. The association of obstructive jaundice with AML has been reported infrequently. While jaundice caused by GS compression of the bile ducts as a primary manifestation of AML is well known in some cases [18–20], it appears to be very uncommon as the first manifestation of relapse [21]. Our patient exhibited periods of nausea, vomiting, and abdominal pain before the development of jaundice. It appears likely that we need to pay more attention to these symptoms and further examine the possibility of GS in the abdomen at relapse in AML rather than intestinal GVHD. The increase in hyperbilirubinemia and liver transferase, however, occurred very rapidly. Table 1 shows AML patients with obstructive jaundice that have been reported to date. In some of these patients, GS may have occurred before bone marrow invasion. One report (patient no. 16) was the patient with t(8;21)-AML with CD56 expression and paraspinal GS without bone marrow involvement, who relapsed 8 months after successful induction chemotherapy [22]. We are unable to identify the status of both *FLT3* gene and CD56 expression in patients with t(8;21)-AML with obstructive jaundice and GS in the literature.

Our patient was considered to have a good prognosis due to her clinical characteristics of t(8;21)-AML, including a low WBC count and absence of extramedullary myeloid tumor symptoms at diagnosis. However, her disease was multidrug-resistant and she relapsed despite severe GVHD after an unrelated bone marrow transplant. We hypothesized that AML clones in this patient could not survive in the bone marrow due to a graft-versus-leukemia (GVL) effect, but those that escaped from a GVL effect had managed to survive and grow in the common bile duct. Different GVL effects both inside and outside of the bone marrow may depend on the quantity and/or activity of effector cells in those areas [23].

In this case report especially, when the minimal residual disease was detected without bone marrow recurrence in morphology after allogeneic transplantation, one should infer not only hematologic recurrence, but also the existence of GS, presenting as a soft-tissue mass, and examine any site of the body. Therefore, for earlier detection of relapse, we suggest that it would be useful to examine the existence of GS in CD56-positive t(8;21)-AML patients at diagnosis and hematologic remission.

Finally, recent reports have demonstrated that both the mutations of *KIT*, *FLT3*, *JAK2*, and *RAS* genes, and the secondary chromosome aberrations of del(9q) related to a loss of *TLE1* and *TLE4* genes occur in addition to t(8;21)(q22;q22) [8]. Although non-Caucasian patients with t(8;21) having del (9q) exhibited longer survival compared with patients with t(8;21) alone and with other cytogenetic abnormalities, there were no differences in the long-term survival observed among Caucasian patients [24]. Additional chromosome aberrations of del(9q) remain as unknown prognostic factors in Japanese patients. We were unable to analyze the *RAS* and *JAK2* genes in this patient. Even though pediatric patients with t(8;21)-AML are unlikely to have *FLT3*-ITD, this report suggests that molecular prognostic factors, including *FLT3* and *KIT* genes, as well as surface marker CD56, should be analyzed.

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High frequencies of simultaneous *FLT3*-ITD, *WT1* and *KIT* mutations in hematological malignancies with *NUP98*-fusion genes

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Acute myeloid leukemia (AML) is heterogeneous in clinical features and molecular pathogenesis. Cooperating alterations of several genes, including oncogenes or tumor suppressor genes, lead to AML development.[1] AML leukemogenesis is thought to require at least two different types of genetic change: class I mutations, which confer a proliferative or survival advantage; and class II mutations, which block myeloid differentiation and provide self-renewability.[1] In hematological malignancies with 11p15 translocations, the nucleoporin (*NUP*) *98* gene is reportedly fused to various partner genes, often including homeobox genes, such as *HOXA9, A11, A13, C11, C13, D11, D13* and *PMX1*.[2] With respect to the oncogenic mechanism of NUP98-HOX fusion proteins, a previous study using a murine bone marrow transplantation assay revealed that NUP98-HOXA9, -HOXD13 and -PMX1 fusion proteins induce myelodysplastic syndrome (MDS) or myeloproliferative neoplasm (MPN), which progress to AML.[2] This latency period indicates that additional genetic events might be required for leukemic transformation. Therefore, we examined somatic mutations of the *FLT3, KIT, WT1, RUNX1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes, which are prevalent in AML, in leukemia patients with *NUP98* fusion genes. This study was approved by local ethical committee.

Sixteen patients with chromosomal 11p15 translocations included nine with *NUP98-HOXA9*, two with *NUP98-HOXA13*, two with *NUP98-HOXA11* and one each with *NUP98-HOXC11, NUP98-HOXD11, NUP98-HOXD13* or *NUP98-NSD3* (Table 1). The partner gene fused to *NUP98* could not be detected in one patient with t(4;11)(q21;p15); however, fluorescent *in situ* hybridization analysis using a probe containing *NUP98* showed split signals (data not shown). No patients had any additional chromosomal abnormality except for chromosomal 11p15 translocations (Supplementary data). Two patients with t(7;11)(p15;p15) had double *NUP98* fusion transcripts: patient (PN) 13 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA13* fusions, and PN14 had simultaneous *NUP98-HOXA9* and *NUP98-HOXA11* fusions. In all, 15 of the 16 patients with *NUP98*-related hematological malignancies were diagnosed as having myeloid malignancies, and the other patient (PN16) were initially diagnosed as having T-cell non-Hodgkin's lymphoma with t(4;11)(q21;p15), and transformed into acute myelomonocytic leukemia with the same t(4;11) (lineage switch). Patients with myeloid malignancies consisted of 10 patients with AML, 2 patients with MDS and 3 patients with MPN.

We examined the internal tandem duplications (ITDs) and tyrosine kinase domain (TKD) mutations of the *FLT3* gene in 16 patients, and detected ITDs in nine (56.3%) patients, and TKD mutations in none (Table 1, Figure 1a). The incidence of *FLT3*-ITD in our study was much higher than that in an AML cohort reported previously (12–35%).[1] A high frequency of *FLT3*-ITD was previously reported in 30–35% of AML patients with either normal karyotype or with t(15;17)(q21;q11) resulting in *PML-RARA*, and in 70% of AML patients with t(6;9)(p23;q34) resulting in *DEK-CAN/NUP214*.[1] Interestingly, both *NUP98* and *NUP214* encode a part of the nucleoporin complex. The general activation effects on reporters of the DEK-CAN/NUP214 fusion protein are specific for myeloid cells.[3] Moreover, in murine bone marrow transplantation assays, NUP98-related fusion proteins such as NUP98-HOXA9, -HOXD13 and -PMX1 induced MDS or MPN, which progressed to AML.[2] These results demonstrate that the nucleoporin-related proteins share a common ability for myeloid differentiation. Furthermore, the very tight correlation between *nucleoporin*-related fusion genes and *FLT3*-ITD suggest that *FLT3*-ITD may contribute to the myeloid leukemogenesis involved in *nucleoporin*-related fusions.

We further examined mutations of the *KIT, WT1, AML1, CEBPA, NPM1, NRAS, KRAS* and *MLL* genes,[4] which are prevalent in AML. *KIT, NRAS* and *KRAS* mutations were found in four (25.0%), three (18.8%) and two (12.5%) patients, respectively (Table 1, Figure 1b). *WT1* aberrations were found in eight patients (50.0%; Table 1, Figure 1c). No mutations were found in the other four genes (*RUNX1, CEBPA, NPM1* and *MLL*). The mutations in *KIT* were all missense mutations including Val399Ile, Met541Leu and Asp816Val, and all mutations of *NRAS* and *KRAS* were Gly13Asp. All of *KIT, NRAS* and *KRAS* mutations were heterozygous. The aberrations in *WT1* comprised a frameshift insertion of exon 7 in four patients, missense mutation of exon 9 in one, deletion of exon 5 in one and deletion of the whole cording region in two. Frameshift and

Table 1 Clinical features and additional mutations of patients with *NUP98*-related leukemias

PN	Age	Sex	Disease	WBC at diagnosis	Karyotype	Fusion partner gene of NUP98	CR	Relapse	Therapy	Prognosis	FLT3	KIT	WT1	NRAS	KRAS
PN1	14	M	AML-M1	12 500	t(11;12)	*HOXC11*	yes	yes	Chemo+SCT	Death	ITD	Val399Ile	del	WT	WT
PN2	12	F	AML-M2	133 100	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT
PN3	13	M	AML-M2	460 000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN4	13	F	AML-M2	147 000	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Alive	WT	WT	WT	WT	WT
PN5	15	M	AML-M2	22 700	t(7;11)	*HOXA9*	yes	no	Chemo+SCT	Alive	WT	WT	WT	WT	Gly13Asp
PN6	57	M	AML-M2	252 000	t(7;11)	*HOXA13*	yes	yes	Chemo	Death	ITD	WT	WT	WT	WT
PN7	38	M	AML-M2	6400	t(7;11)	*HOXA9*	yes	yes	Chemo+SCT	Death	ITD	Asp816Val	ins4bpfsX	WT	WT
PN8	15	M	AML-M4	187 900	t(2;11)	*HOXD11*	yes	no	Chemo+SCT	Alive	WT	WT	ins4bpfsX	WT	Gly13Asp
PN9	56	M	AML-M4	204 500	t(7;11)	*HOXA9*	yes	yes	Chemo	Lost to follow-up	ITD	WT	WT	WT	WT
PN10	62	M	AML-M4	6500	t(2;11)	*HOXD13*	yes	no	Chemo	Alive	ITD	WT	WT	WT	WT
PN11	60	M	RA	6250	t(8;11)	*NSD3*	no	ND	Chemo	Death	ITD	Met541Leu	ins4bpfsX	WT	WT
PN12	69	F	RAEB	2500	t(7;11)	*HOXA9*	no	ND	Chemo	Death	WT	WT	WT	WT	WT
PN13	45	M	CMML	29 800	t(7;11)	*HOXA9/HOXA13*	yes	yes	Chemo	Death	ITD	WT	Arg250Trp	WT	WT
PN14	58	F	CML(Ph-)	11 200	t(7;11)	*HOXA9/HOXA11*	yes	no	Chemo	Alive	ITD	WT	del	WT	WT
PN15	3	F	JMML	39 400	t(7;11)	*HOXA11*	yes	no	Chemo+SCT	Alive	WT	WT	del exon5	Gly13Asp	WT
PN16	51	F	T-NHL	2600	t(4;11)	undetermined	yes	yes	Chemo+SCT	Death	WT	WT	WT	Gly13Asp	WT

Abbreviations: AML, acute myeloid leukemia; Chemo, chemotherapy; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; CR, complete remission; del, deletion; F, female; JMML, Juvenile myelomonocystic leukemia; M, male; ND, not determined; Ph-, Philadelphia chromosome; PN, patient number; RA, refractory anemia; RAEB-t, refractory anemia with excess of blasts in transformation; SCT, stem cell transplantation; T-NHL, T-cell non-Hodgkin's lymphoma; WBC, white blood cell; WT, wild type.
t(11;12), t(11;12)(p15;q13); t(2;11), t(2;11)(q31;p15); t(4;11), t(4;11)(q21;p15); t(7;11), t(7;11)(p15;p15); t(8;11), t(8;11; p11; p15).

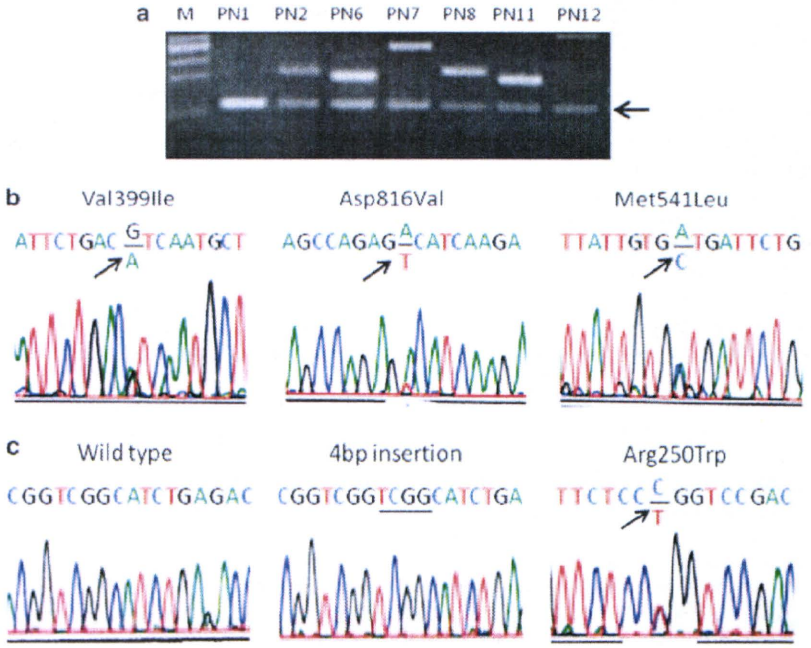

Figure 1 *FLT3*-ITD, *KIT* and *RAS* mutations, and *WT1* aberrations. (**a**) Identification of *FLT3*-ITD by reverse transcription PCR. M, size marker; arrow indicates wild-type allele. (**b**) *KIT* mutations. All figures show the sequence of PCR products. (**c**) *WT1* aberrations. Left panel shows wild type of *WT1* exon 7. Middle panel shows 4-bp insertion in exon 7 of *WT1*. Right panel shows *WT1* missense mutation. Left and middle panels show the sequence of each plasmid subclone, and right panel shows that of PCR products.

missense mutation of *WT1* are heterozygous, whereas deletion was homozygous. *FLT3*-ITD, *KIT* and *RAS* mutations reportedly confer cellular proliferative abilities.[1] In our study, 14 patients (88%) had at least one mutation involved in cellular proliferation (*FLT3*, *KIT* or *RAS*). Recently, Chou *et al.*[5] reported that the *NUP98-HOXA9* fusion is strongly associated with *KRAS* and *WT1* mutations. *Nras* and *Kras* mutations were frequently found in AML developed in transgenic mice expressing *NUP98-HOXD13*.[6] These results indicate that *NUP98*-related leukemias have a high frequency of mutations involved in growth advantage.

Interestingly, five of the six patients with *WT1* aberrations had *FLT3*-ITD, and three of the five patients with both *FLT3*-ITD and *WT1* aberrations had a *KIT* mutation, although the simultaneous *FLT3*-ITD and *KIT* mutations are reportedly very rare[1]. These results suggest that the *NUP98*-related leukemias share a distinct molecular subgroup in leukemias. In addition, all four patients with *KIT* mutations had both *FLT3*-ITD ($P=0.04$) and *WT1* aberrations ($P=0.03$), whereas all five patients with *RAS* mutations did not have *FLT3*-ITD. In all, 14 (88%) of the 16 patients had either *FLT3*-ITD or *RAS* mutations, but they were mutually exclusive as described in previous papers.[1] These

suggest the distinct molecular basis between *NUP98*-related leukemias having *FLT3*-ITD and those having *RAS* mutations.

The relationships between clinical features and gene mutations were described in Table 1. In our study, male patients were more likely than female patients to have *FLT3*-ITD ($P=0.01$) and patients with *FLT3*-ITD have leukocytosis ($P=0.08$) more than those without *FLT3*-ITD. Patients with *RAS* mutations were significantly younger than those without the mutations (median age of 15 vs 56 years; $P=0.04$). In total, 9 (64.3%) of the 14 patients who achieved complete remission relapsed, and 9 (60.0%) of the 15 patients whose data were available died, although they were treated by different protocols (Table 1). All three patients who had both *FLT3*-ITD and *KIT* mutations, and five (83.3%) of the six patients who had both *FLT3*-ITD and *WT1* aberrations, died. Many studies have shown that *FLT3*-ITD is related to a poor prognosis in AML patients,[1] and that *KIT* mutations are associated with a worse outcome in CBF-leukemia patients.[1] *WT1* mutations are also reported to be a poor prognostic factor in adult AML patients with normal karyotypes.[7] These results suggest that simultaneous occurrence of *FLT3*-ITD, *KIT* mutations and *WT1* aberrations in *NUP98*-related leukemia may be associated with poor prognosis.

FLT3-ITD, *KIT* and *RAS* mutations lead to constitutive activation of downstream pathway, resulting in acquirement of a proliferative advantage.[1] In a mouse model, *FLT3*-ITD alone does not induce AML, and *RAS* mutations can induce myeloid leukemia with distinct leukemogenic strengths and phenotypes.[1] *NUP98*-related fusions alone require long periods of time to induce AML, although these fusions induce MDS or MPN by impaired myeloid differentiation.[2] Cooperation between BCR-ABL (which enhances proliferation) and NUP98-fusion (which inhibits differentiation) lead to CML blast crisis.[2] Moreover, the *WT1* mutations were clustered within the DNA binding domain, and were subsequently considered to impair the ability of DNA to bind to target genes associated with apoptosis, cell cycle or cellular proliferation.[8] These results suggest that a high frequency of cell proliferation gene mutations may contribute to leukemogenesis in *NUP98*-related leukemia, and that simultaneous occurrence of *FLT3-ITD* and *WT1* aberrations may have an important role in the clinical outcome of *NUP98*-related leukemia.

Conflict of interest

The authors declare no conflict of interest.

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BRIEF REPORT
NOTCH1 Mutation in a Female With Myeloid/NK Cell Precursor Acute Leukemia

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A 6-year-old Japanese female was diagnosed as having myeloid/NK cell precursor acute leukemia (MNKL) using immunocytochemical analysis. The patient was treated by cord blood transplantation from an HLA 1-locus mismatched unrelated donor after chemotherapy comprising cytosine arabinoside, idarubicin, etoposide, and L-asparaginase. We detected a nonsense mutation, C7412A, resulting in S2471X, where X is a terminal codon, in the PEST domain of NOTCH1 in this patient. The presence of the NOTCH1 activating mutation in MNKL might suggest a possible role in the leukemogenesis of MNKL. Pediatr Blood Cancer. 2010;55:1406–1409. © 2010 Wiley-Liss, Inc.

Key words: activating mutation; cord blood transplantation; L-asparaginase; myeloid/NK cell precursor acute leukemia; NOTCH1

INTRODUCTION

Myeloid/NK cell precursor acute leukemia (MNKL) was initially identified as the leukemia of natural killer (NK) cells, with the coexpression of both myeloid and NK cell precursors [1]. This disease is considered a myeloid antigen-positive T/NK cell precursor acute leukemia originating from bipotential T/NK progenitor cells [2]. No standard therapy for MNKL has yet been established because this is a rare disease that develops both in children and in adults.

Notch signaling regulates normal pre-T cell development [3] and activating mutations in NOTCH1 appear to be the most common acquired genetic lesion found in human T-cell acute lymphoblastic leukemia (T-ALL) [4]. It has been reported that the presence of activating NOTCH1 mutations was significantly correlated with a favorable prognosis in pediatric T-ALL [4]. Recently, some groups have reported on the analysis of the NOTCH1 mutation in other hematologic malignancies [5–7] and suggested that NOTCH1 might be involved in leukemogenesis associated with various forms of leukemia/lymphoma, rather than only with T-ALL. We report a case of MNKL with NOTCH1 mutation.

PATIENTS AND METHODS

A 6-year-old Japanese female was admitted to our hospital with intermittent fever and leukocytosis. Hepatosplenomegaly and bleeding were not observed. Laboratory testing revealed leukocytosis (58,800/μl with 92% immature cells) and an elevated serum LDH level (596 U/L). Bone marrow aspiration revealed 75.2% of the cells were lymphoblasts (Supplemental Fig. 1). Surface marker analysis showed that the leukemic blasts in the bone marrow were positive for CD7 (93.2%), CD33 (90.3%), CD34 (89.2%), CD56 (58.7%), CD244 (95.2%), and HLA-DR (21.4%), but negative for surface CD3, CD13, CD117, CD11a, and CD18 were not tested. Chromosomal analysis of bone marrow cells revealed 46, XX, add(1)(p32), inv(1)(p36q32), del(4)(q?), del(11)(q?), add(12)(p13), and del(16)(q12). The patient was diagnosed as having MNKL and subsequently treated with induction chemotherapy for acute myeloid leukemia (AML) [8]. Chemotherapy consisted of etoposide (VP-16, 150 mg/m^2) for 5 days, cytosine arabinoside (Ara-C, 200 mg/m^2) for 7 days, mitoxantrone hydrochloride (Mit, 5 mg/m^2) for 5 days and intrathecal methotrexate (MTX). However, the patient did not achieve complete remission. Since it was reported that administration of L-asparaginase was sometimes effective in leukemia patients with a low expression of asparaginase synthetase (AS) [9], we determined that chemotherapy including L-asparaginase (5,000 U/m^2/day) for 5 days was administered. Nine days after treatment with L-asparaginase, the normal counterpart of the peripheral blood increased and the patient achieved complete remission and absence of the abnormal karyotype initially detected (Fig. 1).

No matched donor was available, and consequently, it was decided to perform a cord blood transplantation (CBT) from an unrelated donor. Consolidation therapy VP-16 (100 mg/m^2) for 3 days, Ara-C (3 g/m^2 × 2/day) for 3 days, idarubicin (10 mg/m^2), and L-asparaginase (25,000 U/m^2) for 5 days was administered from day −52 to −41. After the consolidation therapy, CBT was performed using an HLA 1-locus mismatched unrelated donor. The conditioning regimen consisted of total body irradiation (TBI, 12 Gy total dose given from day −9 to day −6), VP-16 (1,200 mg/kg on day −5) and cyclophosphamide (1,200 mg/kg × 2 from day −4 to day −3). Graft-versus-host disease (GVHD) prophylaxis consisted of tacrolimus was employed. Donor cell dose was 3 × 10^5 CD34 cells/kg. Granulocyte colony-stimulating factor was administered for 20 days after transplantation. The patient suffered several transplantation-related complications, including Grade 1 acute GVHD (mucositis, skin rash, fever, and diarrhea), which were detected on day 21 and were controlled with prednisolone. Hematological reconstitution included a WBC count of more than 1,000/μl, a neutrophil count of more than 500/μl and a platelet count of more than 50,000/μl on day 20 for WBC and neutrophils and day 32

Additional Supporting Information may be found in the online version of this article.

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Conflict of interest: Nothing to declare.

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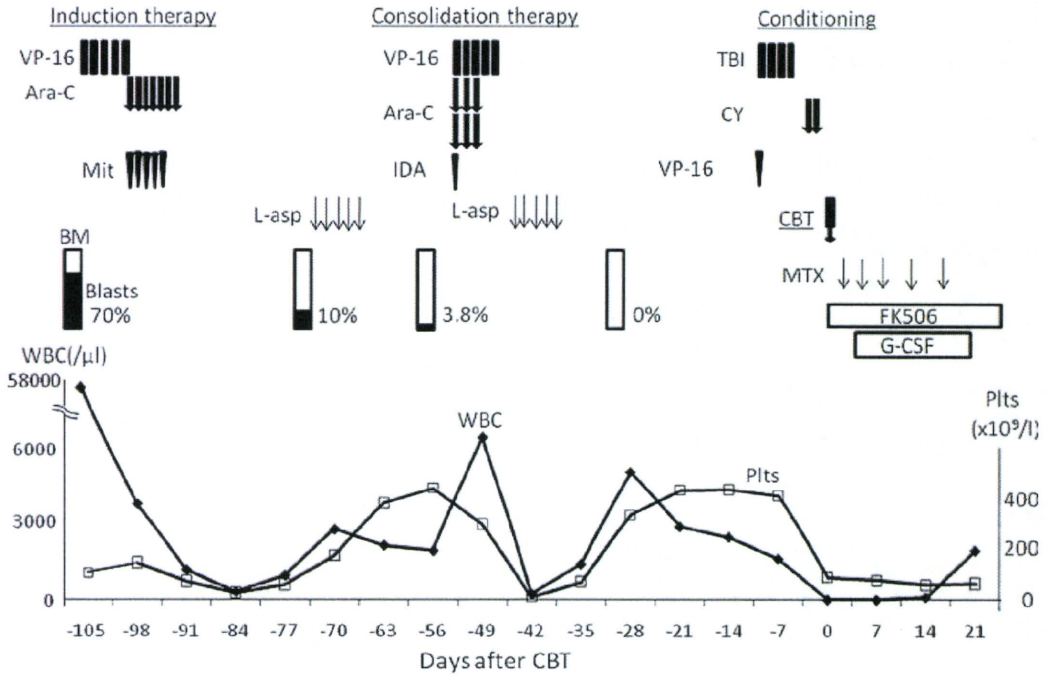

Fig. 1. Clinical course of the patient. Note that bone marrow blasts were significantly decreased after the administration of L-asparaginase (L-asp) chemotherapy. VP-16 indicates etoposide; Ara-C, cytarabine; TBI, total body irradiation; CY, cyclophosphamide; Mit, mitoxantrone; IDA, idarubicin; MTX, methotrexate; BM, bone marrow; FK506, tacrolimus; G-CSF, granulocyte-colony stimulating factor; WBC, white blood cell count; Plts, platelets; CBT, cord blood transplantation.

for platelets after transplantation, respectively. Cytogenetic analysis of a bone marrow sample on day 62 showed 46, XY in 99.8% of metaphases indicating the donor type. The patient was discharged on day 134 after CBT and showed no sign of infectious diseases, and no evidence of relapse. Hematological remission, with a Karnofsky score of 100%, has continued for more than 3 years and 10 months after transplantation.

Genomic DNA was prepared from a bone marrow sample of the patient. Site of NOTCH1 activating mutations in T-ALL cases, located in exons 26 and 27, which encode the N-terminal and C-terminal regions of the heterodimerization domain (HD), and in exon 34, which encodes the PEST domain and transcriptional-activation domain were analyzed [10]. We detected a nonsense mutation in MNKL, C7412A, resulting in S2471X, where X is a terminal codon, in the PEST domain of NOTCH1 (Fig. 2).

DISCUSSION

NOTCH1 is thought to play an important role in normal hematopoiesis, where it has been implicated in the maintenance of the hematopoietic stem cell niche [11], hematopoietic stem cell self-renewal [12] and the determination of lymphoid progenitor cell fate [13]. Aberrant activation of the NOTCH1 signaling pathway induces the transformation of T-cell progenitors and is broadly involved in the pathogenesis of human T-ALL [14].

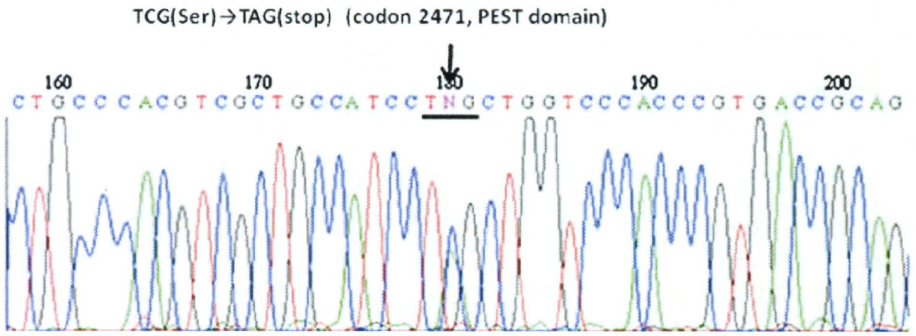

Fig. 2. Chromatogram of sequencing with fluorescent-dye chemistry for unfractionated genomic DNA from a myeloid/NK-cell precursor acute leukemia patient with heterozygous C-to-A mutation (resulting in S2471X, where X is a terminal codon) in exon 34 of NOTCH1.

Weng et al. [10] have shown that the enforced NOTCH1 pathway is an effective inducer of T-ALL in mice. They also reported that more than 50% of human T-ALL cases possess activating mutations in the extracellular HD and in the terminal PEST domain of NOTCH1 [10]. These mutations were not observed in the healthy control populations or in patients with precursor B cell-ALL, indicating that they are characteristic for T-ALL [4,10]. It has been reported that small molecule γ-secretase inhibitors, which can effectively block the activation of NOTCH1 signaling in vitro and in vivo, may have antileukemic effects and are being tested to assess their efficacy in the treatment of T-ALL [15].

Breit et al. [4] reported that the presence of NOTCH1 mutations was significantly correlated with a more rapid early treatment response and a favorable long-term outcome in children with T-ALL. On the contrary, other groups have reported that NOTCH1 mutations were not associated with good clinical outcome in T-ALL [16,17]. Thus, the clinical significance of NOTCH1 mutation in T-ALL still remains controversial.

With respect to other hematologic malignancies, it has been reported that the high frequencies of NOTCH1 mutations in T cell non-Hodgkin lymphoma (T-NHL), as well as in T-ALL, are associated with the good prognosis of T-NHL although this association was not found to be statistically significant [18]. Shimizu et al. [5] reported that the NOTCH1 mutation occurred infrequently in mature T-cell leukemia/lymphoma and that NOTCH1 might be involved in leukemogenesis. A case of an aggressive Langerhans cell histiocytosis with activating NOTCH1 mutations following T-ALL was also reported [6].

There are only limited studies regarding NOTCH1 mutation in AML. It has been reported that activating NOTCH1 mutations can be found in rare cases of AML and in leukemia cases with lineage infidelity, suggesting that these mutations might occur in a leukemic stem cell that precedes both myeloid and T-lineage commitment [7]. Chen et al. [19] reported that down-regulation of NOTCH-1 expression decreases PU.1/M-CSF receptor expression and disrupts the NOTCH-1/PU.1 complex, which may impede the PU.1-mediated myeloid signaling and contribute to the leukomogenesis of AML. To confirm the contribution of activation NOTCH1 mutation in MNKL leukemogenesis, further analysis of NOTCH1 expression level and interaction with its downstream targets are needed. The role of NOTCH1 signaling in leukemogenesis has not been well-established in other hematologic malignancies, in contrast with the prevalent role of NOTCH1 activation in T-ALL [5–7].

Neoplasms of NK-cell origin have not been clearly identified because the developmental pathway of normal NK cells is not well understood [1]. It has been suggested that MNKL could conceivably originate from CD7 + CD33 + CD34+ stage I and stage II T/NK/dendritic cell tripotential progenitors, but the possibility that this disease is of true myeloid cell origin cannot be ruled out [20]. The effect of NOTCH signals on NK cell development is less certain, but the presence of the NOTCH1 activating mutation in MNKL might suggest a possible role in the leukemogenesis of MNKL. It may also provide new insight into the pathogenesis of NK cell malignancies.

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