No *nucleophosmin* mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group

Leukemia (2007) **21**, 1307. doi:10.1038/sj.leu.2404625;
published online 22 February 2007

Acute myeloid leukemia (AML) with normal karyotype had a heterogenous prognosis. In this subgroup, *FLT3*-internal tandem duplication (ITD) was strongly associated with a poor prognosis.[1–3] Recently, it was reported that mutations of *nucleophosmin* (*NPM*) gene occur in 50–60% of adult AML with normal karyotype and were frequently associated with *FLT3*-ITD. In the AML patients with normal karyotype and *FLT3*-ITD, patients with *NPM* gene mutations showed a better prognosis than those without *NPM* gene mutations.[4–6] However, the frequency and clinical impact of *NPM* gene mutations in pediatric AML patients with normal karyotype remained uncertain because there were a few number of reports.[7,8]

We searched for *NPM* gene mutations in 33 (20.9%) of 158 patients with normal karyotype who were treated on Japanese Childhood AML Cooperative protocol, AML 99 (0–15 years old, median 8 years old).[9] We amplified exon 12 of *NPM* gene using the primers; *NPM* cDNA Fow, 5′-AAAGGTGGTTCTCTTCCC AAA-3′ and *NPM* cDNA Rev, 5′-GCATTATAAAAAGGACAGCC AGA-3′ and directly sequenced on a DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems).[9] We could not find any *NPM* gene mutations in this study.

It was reported that the frequency of *NPM* gene mutations in children (<18 years old) was very low (children 1 out of 47 (2.1%) versus adults 32 out of 126 (25.4%), $P<0.001$).[8] Furthermore, they suggested that *NPM* gene mutations were also rarely detected in patients younger than 40 years old (3 (3.5%) out of 85). On the other hand, it was reported that *NPM* mutations were found in seven (27.1%) of 26 pediatric AML patients with normal karyotype from Italy.[7] These seven patients ranged from 5.0–17.9 years old, 10 years old ($n=2$), 11 years old ($n=2$) and 5, 8, 17 years old ($n=1$). Notably, two AML patients with *NPM* gene mutations have been reported in adult Japanese AML study (15 and 16 years old).[5]

We also analyzed *FLT3*-ITD and *RAS* gene alterations in these patients and found *FLT3*-ITD in nine (27.3%), *NRAS* mutation in two (6.1%) and *KRAS* mutation in three (9.1%). The frequencies of these gene alterations were compatible with those of previous reports.[2,3]

We considered that *NPM* gene mutations may be infrequent in Asian pediatric AML patients with normal karyotype, especially less than 15 years old. Frequency of *NPM* gene mutations depends on age and may depend on races. Further larger studies of *NPM* gene analysis are needed to clarify this item.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Cancer Research, a Grant for Clinical Cancer Research and Research on Children and Families from the Ministry of Health, Labor and Welfare of Japan. This work was also supported by Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Research Grant for Gunma Prefectural Hospitals.

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Leukemia Research
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Pro-inflammatory cytokinemia is frequently found in Down syndrome patients with hematological disorders

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Received 30 August 2006; received in revised form 30 August 2006; accepted 9 September 2006
Available online 19 October 2006

Abstract

Down syndrome (DS) patients are frequently complicated with infections, autoimmune phenomena and hematological disorders, including transient abnormal myelopoiesis (TAM) in infancy and acute megakaryoblastic leukaemia (AMKL) in later life. In this study, serum levels of cytokines from 23 TAM and 15 AMKL patients were examined using the highly sensitive microsphere fluorescence system. Statistical differences between DS neonates with or without TAM were found in IL-1β [median 7.0 pg/ml (0.34–271.6) verses 0.05 pg/ml (0.0–2.4), $p=0.034$], TNF-α [8.11 pg/ml (0.1–253.0) verses 0.41 pg/ml (0.1–1.5), $p=0.041$], and IFN-γ [20.0 pg/ml (0.14–406.3) verses 1.5 pg/ml (0.14–5.79), $p=0.036$]. Moreover, abnormal inflammatory cytokinemia was also found in myelodysplastic syndrome (MDS) and AMKL with DS. These abnormal cytokinemia may have a role in the pathophysiology of TAM, MDS and AMKL in DS, especially in liver fibrosis or myelofibrosis.

Keywords: Pro-inflammatory cytokine; Down syndrome; Transient abnormal myelopoiesis; Acute megakaryoblastic leukaemia

1. Introduction

Down syndrome (DS) patients are frequently complicated with infections [1], thyroid dysfunction [2], and autoimmune phenomena [3]. These immunological abnormalities were partially explained by the dysfunction and altered subsets of T-lymphocytes [4–7]. Mental retardation in DS resembles that of Alzheimer's disease, and the neuronal degeneration in DS was partially explained by the existence of abnormal pro-inflammatory cytokines [8–11]. Accelerated aging was observed in DS patients [12]. Immunological abnormalities, including sustained inflammatory cytokinemia, are involved in the various pathophysiology in DS patients.

On the other hand, DS patients may also be highly complicated by various hematological disorders, including transient abnormal myelopoiesis (TAM) or transient myeloproliferative disorder (TMD) in infancy, myelodysplastic syndrome (MDS), and acute myeloid leukaemia (AML), especially acute megakaryoblastic leukaemia (AMKL) within 3 years [13–15]. TAM occurs in about 10% of DS infants, and most cases resolve spontaneously with unknown reasons within a few months after birth [13]. However, recent studies have

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0145-2126/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2006.09.008

revealed that about 20% of TAM patients are life-threatening or fatal cases with respiratory distress or liver failure [16–18]. Our previous report demonstrated that sustained cytokinemia is likely to be involved in the pathophysiology of TAM, and very high serum concentrations might predict a poor outcome [19]. In this study, we assayed several cytokine levels in a larger number of TAM, MDS, and AMKL patients with DS. Our data revealed that abnormal inflammatory cytokinemia existed not only in TAM patients, but also in MDS and AMKL patients, suggesting that abnormal pro-inflammatory cytokinemia contributes to the disease symptoms in TAM and AMKL, including liver fibrosis and myelofibrosis.

2. Patients, materials and methods

Twenty three TAM and 15 AMKL patients with DS were enrolled in this study. All patients had the clinical features of DS and had trisomy 21 in the peripheral blood cultured for 72 h with phytohaemagglutinin. Three TAM patients (13.0%) died within 4 months of birth because of the severe complications, including liver fibrosis, and the remaining 20 TAM patients were alive with or without complications. Sera from 9 DS neonates and 6 DS patients under 2 years old without hematological disorders were used as DS-controls. Sera from 10 neonates without DS were also used as controls. Six patients with less than 20% of blasts in the bone marrow nucleated cells were diagnosed as having myelodysplastic syndrome (MDS) according to the French-American-British (FAB) classification [20]. All patients were enrolled in this study after their parents gave informed consent. The sera were stored at −80 °C until use. No IgM antibodies specific for microorganisms, including rubella, cytomegalovirus, herpes simplex virus, or toxoplasma could be detected in the sera at birth from any of the patients. Maternal infection by human T cell lymphotrophic virus type 1, human immunodeficiency virus, or hepatitis B or C virus was not detected during pregnancy. AMKL was also diagnosed according to the FAB classification, and TAM was diagnosed according to a previous report [13,21].

3. Cytokine assay

Various pro-inflammatory cytokines, including interleukin (IL)-1β, IL-2, IL-6, tumor necrosis factor (TNF)-α, interferon (INF)-γ, granulocyte and macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF), anti-inflammatory cytokines (IL-4, IL-10), and chemokine (IL-8) in the serum were assayed using a highly sensitive microsphere fluorescence system. Fluorescence, a measure of the number of microspheres per unit volume, was measured using the Multiplex Suspension Array System (BioLad Laboratories) [22,23]. The cases that were under the detection limit of this assay system were considered to be 0 pg/ml.

4. Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test, Bonnferroni test, and Pearson's correlation coefficient test. A two-tailed *p*-value less than 0.05 was accepted as indicating significance.

5. Results

5.1. Cytokine/chemokine levels in DS with or without TAM and non-DS healthy neonates

Cytokine and chemokine levels were elevated to some extent in DS neonates with or without TAM compared with nomal healthy neonates (Fig. 1a–c). A statistical difference between DS neonates with or without TAM was found in IL-1β [median 7.0 pg/ml (0.34–271.6) verses 0.05 pg/ml (0.0–2.4), $p = 0.034$], TNF-α [8.11 pg/ml (0.1–253.0) verses 0.41 pg/ml (0.1–1.5), $p = 0.041$], and IFN-γ [20.0 pg/ml (0.14–406.3) verses 1.5 pg/ml (0.14–5.79), $p = 0.036$] according to Bonnferroni analysis. The median serum GM-CSF level in healthy neonates and TAM were 8.00 pg/ml and 132.27 pg/ml ($p = 0.017$), respectively.

5.2. Cytokine/chemokine levels in DS-AMKL and DS controls

A significant difference between DS children with or without AMKL was observed in IL-1β [median 6.88 pg/ml (0.02–108.5) verses 0.02 pg/ml (0.02–6.76), $p = 0.032$], IL-4 [8.98 pg/ml (4.27–219.97) verses 0.0 pg/ml (4.27–46.42), $p = 0.033$], IL-6 [141.0 pg/ml (7.62–1695.7) verses 18.2 pg/ml (3.21–317.6), $p = 0.0081$], IL-8 [25.79 pg/ml (1.5–247.5) verses 2.02 pg/ml (0.75–18.75), $p = 0.0051$], IFN-γ [34.47 pg/ml (0.14–566.7) verses 1.43 pg/ml (0.14–146.8), $p = 0.042$], and TNF-α [3.13 pg/ml (0.1–573.7) verses 0.10 pg/ml (0.1–27.3), $p = 0.049$] according to the Mann–Whitney *U*-test (Fig. 1d and e). The differences were not significant in other cytokines.

5.3. Cytokine/chemokine levels in DS-MDS and DS controls

The cytokine/chemokine levels are elevated in MDS patients compared with those of DS controls. The difference was statistically significant between DS-MDS and DS controls in IL-1β [median 54.56 pg/ml (48.36–79.26) verses 0.02 pg/ml (0.02–6.76), $p = 0.0037$], in IL-4 [27.80 pg/ml (8.53–60.54) verses 4.27 pg/ml (4.27–46.42), $p = 0.022$], in IL-8 [10.67 pg/ml (9.09–27.12) verses 2.015 pg/ml (0.75–18.75), $p = 0.025$], in IFN-γ [45.98 pg/ml (24.31–879.43) verses 1.43 pg/ml (0.14–146.79), $p = 0.024$], and in TNF-α [112.53 pg/ml (75.31–980.37) verses 0.1 pg/ml (0.1–27.3), $p = 0.0037$] according to the Mann–Whitney *U*-test. The significant difference between DS-MDS and

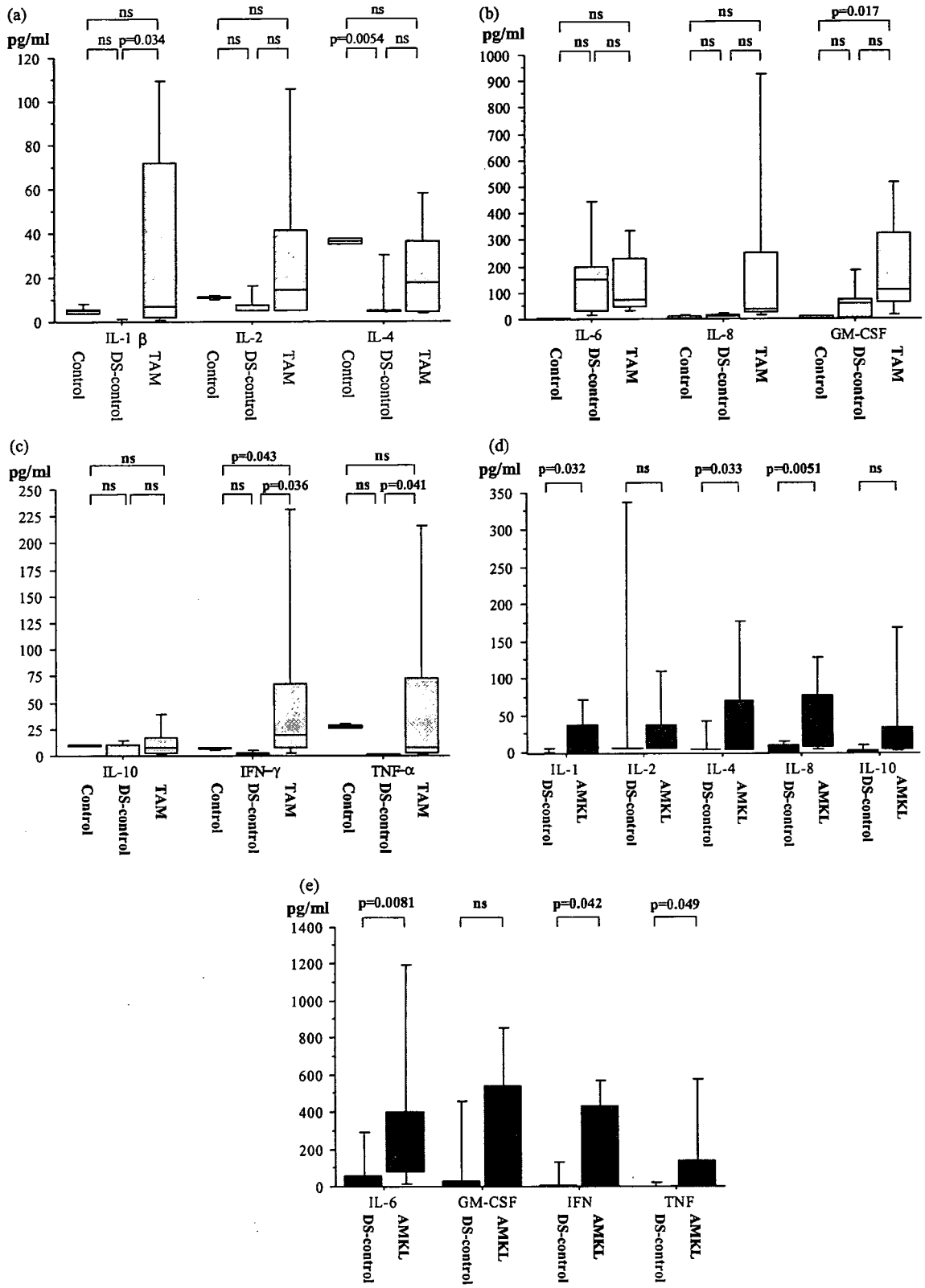

Fig. 1. (a–c) The cytokine levels in the sera of DS neonates with or without TAM and non-DS. A statistical difference between DS neonates with or without TAM was found in IL-1β, TNF-α and IFN-γ. (d and e) The cytokine levels in the sera of DS children with or without AMKL. A significant difference between DS children with or without AMKL was observed in IL-1β, IL-4, IL-6, IL-8, IFN-γ and TNF-α.

DS-AMKL was observed in only IL-1β (median 54.56 pg/ml verses 6.88 pg/ml, $p = 0.035$).

6. Discussion

The production of several abnormal cytokines has been reported to be associated with the pathophysiology of DS [8–11]. For example, IL-1 was overexpressed throughout life in DS and contributed to neuronal degeneration [9,11]. Serum IL-6 levels correlated with the severity of dementia in DS [8]. Overexpression of TNF-α and IFN-γ was observed in the thymus of DS patients [24]. DS patients showed the consistent overexpression of superoxide [SOD]1 on chromosome 21 [25,26]. A high level of inflammatory cytokines may play an important role in several pathophysiologies, including mental retardation [8–11], a high susceptibility to infections [1], and autoimmune phenomena in DS [3]. These abnormal cytokinemia could cause neuronal cell death, via apoptosis [27]. Furthermore, these abnormal cytokinemia were partially explained by the dysfunction of T-lymphocytes in DS patients, but the details remain unknown [4–7].

In contrast, the relationship between inflammatory cytokines and leukemogenesis has been rarely reported so far [28]. High expressions of platelet-derived growth factor and TGF-β1 were observed in blast cells from TAM patients with liver fibrosis [29,30]. Our previous report demonstrated that sustained cytokinemia was found in 4 TAM patients, and very high serum concentrations might predict a poor outcome [19]. Liver fibrosis in fatal TAM was considered to be due to the sustained pro-inflammatory cytokinemia and the imperfect remodeling [28,31,32]. Pro-inflammatory cytokinemia may cause the cellular apoptosis that resulted in the spontaneous regression of TAM blasts. The inflammatory process, which lost its auto regulatory capacity, was frequently found in neurodegeneration [33]. However, DS neonates without TAM also showed the slightly increased levels of inflammatory cytokines in this study, suggesting that these elevated cytokines might be partially due to the existence of heart failure or other complications in these DS neonates without TAM. Furthermore, abnormal cytokine production in amniotic fluid has been reported to be found in DS pregnant women [34,35], suggesting that abnormal cytokine production has begun in utero. DS-TAM was also suggested to develop in utero [36] and hydrops fetalis with DS-TAM was reported [17,37]. What is the cause of this inflammatory cytokinemia in DS-TAM? It was reported that blasts themselves produce several cytokines [30,38]. Moreover, IFN-γ producing T cells are abundant in DS [7]. Further studies are needed to resolve these issues.

Furthermore, we found that inflammatory cytokinemia was also found in DS-MDS and AMKL in this study. The function of inflammatory cytokines, having pro-apoptotic or anti-apoptotic effects, differs according to the situation. IL-1, IL-6, GM-CSF and TNF-α are pro-inflammatory cytokines, but stimulate the growth of megakaryocytes [38–42]. Myeloid leukaemia cells are known to produce cytokines that stimulate their proliferation [43]. GM-CSF induces pro-apoptotic and anti-apoptotic signals in AML [44]. Abnormal inflammatory cytokines might have a role in the stimulation of leukemogenesis in DS patients.

In conclusion, pro-inflammatory cytokinemia is frequently found in DS patients with hematological disorders. Further studies are needed to clarify the role of these immunological disturbances in leukemogenesis with DS.

Acknowledgements

The authors thank Drs. Naoki Sakata, Takashi Kanazawa, Takashi Fukushima, Naoko Kinugawa, Yoshie Tomiyama, Yasuo Horikoshi, Keiko Asami, Kenichi Koike, Kazutoshi Chou, Masahiro Sako, Akira Nishida, and Eturo Itou for providing serum samples and data. The authors also thank Masakazu Yoshizumi and Taisei Ishioka for their technical assistances. Supported in part by a research grant for Gunma Prefectural Hospitals.

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Journal of Pediatric Surgery (2007) 42, 2040–2045

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Journal of Pediatric Surgery

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Identification of side population cells (stem-like cell population) in pediatric solid tumor cell lines☆

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Received 6 August 2007; accepted 8 August 2007

Index words:
Cancer stem cell;
Side population;
Neuroblastoma;
Rhabdomyosarcoma;
Ewing's sarcoma

Abstract

Purpose: Recent evidence has supported the *cancer stem cell theory* that cancer contains a small number of cancer stem cells (CSC) as a reservoir of cancer cells. Only the CSC, but not most of the remaining constituent cancer cells, are thought to be responsible for tumorigenesis, progression, and metastasis as well as cancer relapse, suggesting that the CSC should be targeted to eradicate the cancer. Side population (SP) cells isolated by fluorescence-activated cell sorting (FACS) using Hoechst dye are known to be enriched in stem cells in various normal tissues as well as cancers. The authors investigated whether such stem-like SP cells may exist in pediatric solid tumors (PSTs).

Materials and Methods: Sixteen pediatric tumor cell lines including 7 neuroblastomas, 4 rhabdomyosarcomas, and 5 Ewing's sarcomas were used for FACS analysis. Analysis of SP cells based on the exclusion of the DNA binding dye, Hoechst 33342, with and without verapamil using FACS was performed.

Results: One Ewing's sarcoma cell line did not show an SP fraction, and only a small fraction of SP cells (0.12%-14.6%) was detected in the other 15 cell lines. These SP cells were all sensitive to verapamil.

Conclusions: This study suggested that most PSTs would contain a small fraction of SP cells (possible stem-like population). Targeting the CSC will provide a novel treatment strategy to eradicate refractory PSTs.

Stem cells are characterized by the properties of self-renewal and multipotency that are called *stemness*. Normal tissue comprises a hierarchical organization composed of a small fraction of stem cells as a reservoir and their descendants. Recent evidence has shown that cancer may also be maintained by a small fraction of cancer stem cells (CSC) (stem-like cancer cells) that retain the properties

Presented at the 40th annual meeting of the Pacific Association of Pediatric Surgeons, Queenstown, New Zealand, April 15-19, 2007.

☆ This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science and for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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doi:10.1016/j.jpedsurg.2007.08.026

common to normal stem cells [1-4]. It is believed that only the CSC, but not most of the remaining descendants (constituent cancer cells), are responsible for tumorigenesis, progression, metastasis, and relapse after treatments. Recently, the existence of CSC has been proven for several cancers [4-6]. Stem cells have a long life span with the ability to survive in severe environments, to stay quiescent in G_0 phase most of the time, and to self-renew occasionally. In contrast, progenitors and mature cells have a limited life span and divide rapidly. This implies that multiple oncogenic gene abnormalities are more likely to accumulate in stem cells than in progenitors and mature cells. The CSC can generate their descendants, including progenitors and relatively differentiated cancer cells, producing the heterogeneity of the cancer. Furthermore, only the CSC can survive conventional anticancer therapies that target only the rapidly dividing cancer cells, although their descendants (most remaining constituent cancer cells) can be killed. This could be strongly related to treatment failure as well as cancer relapse. Accordingly, the elimination of the CSC is an important goal for eradicating refractory cancers. Despite recent advances in treatment modalities, some refractory pediatric solid tumors (PSTs) remain a challenge for pediatric oncologists. It is possible that targeting the CSC in refractory PSTs may provide a novel treatment strategy to eradicate them completely. The aim of this study was to identify the possible stem-like population in PSTs.

Side population (SP) cells characterized by the efficient efflux of Hoechst 33342 dye are thought to be enriched for stem cells in many normal tissues [7-15]. Recently, SP cells that showed stem cell characteristics were isolated from several cancers [16-20]. Side population cells express various adenosine triphosphate–binding cassette (ABC) transporter family members that are responsible for drug resistance, including ABCG2 (BRCP1) [21]. In this study, the authors investigated the existence of SP cells in PST cell lines by fluorescence-activated cell sorting (FACS) analysis.

1. Materials and methods

1.1. Cell lines

Seven neuroblastoma (NB) cell lines (SK-N-AS, SK-N-DZ, GOTO, LAN1, LAN5, NB16, and NB19), 4 rhabdomyosarcoma (RMS) cell lines (RMS, RD, SCMC-RM-2, and KYM-1), and 5 Ewing's sarcoma (EWS) cell lines (ES-1-OT, UTP-ES-1, SCMC-ES-1, RD-ES, and SK-ES) were cultured in appropriate media and used for FACS analysis [22-24].

1.2. Isolation of side population cells

Pediatric tumor cells were adjusted to 10^6 cells/mL in Hanks' Balanced Salt Solution (HBSS). Hoechst 33342 dye was added to the cell suspension at a final concentration of 5 μg/mL, and the mixture was incubated for 90 minutes at 37°C. As a negative control, verapamil (Sigma, St Louis, Mo), an inhibitor of ABC transporters, was also added to a final concentration of 50 or 500 μmol/L with Hoechst 33342. After Hoechst 33342 staining, the tumor cells were washed by centrifugation, resuspended at 1 to 2 × 10^7 cells/mL in HBSS, and kept on ice until use. Before FACS analysis, propidium iodide solution was added to a final concentration of 1 μg/mL to identify nonviable cells. Fluorescence activated cell sorting analysis and sorting were performed on a dual laser flow cytometer (Becton Dickinson FACS Vantage SE cell sorter, San Jose, CA). The Hoechst dye was excited by the 355-nm ultraviolet laser, and its fluorescence was measured at 2 wavelengths using a 424/44 (Hoechst blue) band-pass filter and a 585/42 (Hoechst red) band-pass filter. Propidium iodide

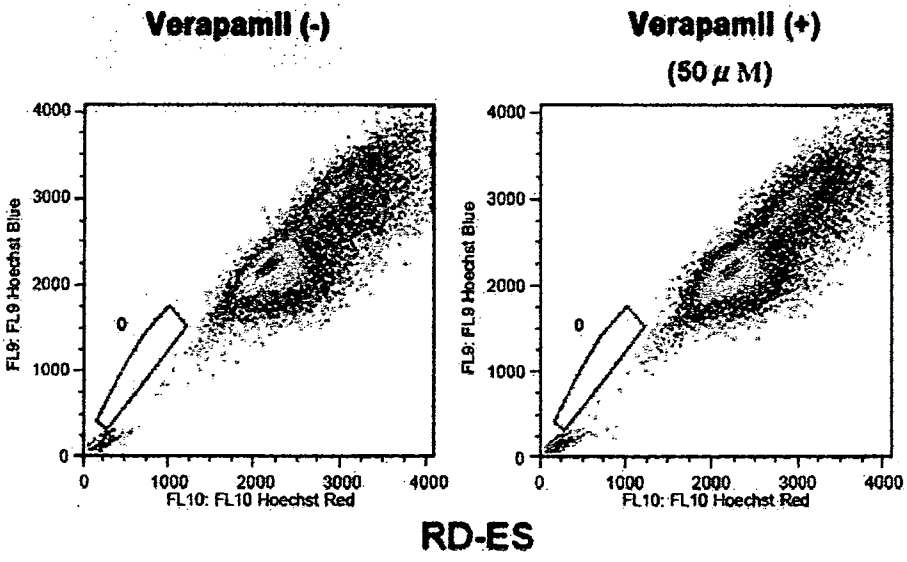

Fig. 1 A EWS cell line, RD-ES, did not show an SP fraction in the absence (left) or presence (right) of verapamil.

fluorescence was excited by 488-nm laser and detected after passing through a 630/22 band-pass filter. Propidium iodide–positive dead cells and debris were excluded.

2. Results

2.1. Side population cells in PST cell lines

One EWS cell line, RD-ES, did not show SP cells in this study (Fig. 1). Side population cells were detected in the remaining 15 cell lines including 7 NB, 4 RMS, and 4 EWS (0.12%-14.6%) (Table 1 and Fig. 2).

2.2. Sensitivity of SP cells to verapamil

The SP fraction cells disappeared upon treatment with 50 μmol/L verapamil in 6 cell lines (GOTO, NB16, SCMC-RM-2, ES-1-OT, SK-ES, and UTP-ES-1) (Table 1, and Fig. 2B and C). In the other 9 cell lines, the SP fraction disappeared in the presence of 500 μmol/L of verapamil (Table 1 and Fig. 2A).

3. Discussion

Macroscopically as well as microscopically, PSTs show a heterogeneous appearance, containing undifferentiated as well as differentiated cells. Furthermore, when the tumor is resected after aggressive chemotherapies and/or radiotherapies, it contains a heterogeneous mixture of tissues, showing not only some viable portions but also other nonviable portions in the same tumor. Certainly, not every cell in the bulk of the tumor behaves in the same manner. How this heterogeneity is generated has been well answered by the *cancer stem cell theory*. Cancer originates from a small fraction of CSC (stem-like cancer cells) with the abilities of self-renewal as well as multipotency as a reservoir. The cancer has a hierarchical organization with only a small number of CSC and a large number of their descendants (constituent cancer cells). Only the CSC are responsible for tumorigenicity, progression, and metastasis in the cancer, whereas their descendants are not. Their descendants will differentiate in various ways, resulting in the heterogeneity of the cancer. Recently, CSC have been isolated from several cancers [4-7]. Normal stem cells and CSC show similar resistance to current therapies, because they both stay in a quiescent state and have a common drug efflux capacity. This means that a small fraction of CSC can survive aggressive therapies, although most remaining constituent cancer cells are responsive to them. This eventually leads to the relapse of the cancer. Accordingly, the real target determining the biological characteristics and the treatment strategy should be the CSC themselves instead of most constituent cancer cells. If this theory is true in the case of PSTs, isolation of the CSC is the first step to better understand their characteristics and to develop novel treatment strategies of eradicating refractory PSTs.

Table 1 Side population cells in pediatric solid tumor cell lines and their sensitivity to verapamil

Cell lines	SP cells (%)	Verapamil-induced disappearance of SP fraction (concentration of verapamil, μmol/L)
NB		
SK-N-AS	+ (0.43)	+ (500)
SK-N-DZ	+ (3.26)	+ (500)
GOTO	+ (0.55)	+ (50)
LAN1	+ (0.54)	+ (500)
LAN5	+ (9.88)	+ (500)
NB16	+ (1.76)	+ (50)
NB19	+ (8.71)	+ (500)
RMS		
RMS	+ (0.53)	+ (500)
RD	+ (1.51)	+ (500)
SCMC-RM-2	+ (0.83)	+ (50)
KYM-1	+ (14.6)	+ (500)
EWS		
ES-1-OT	+ (0.33)	+ (50)
UTP-ES-1	+ (5.53)	+ (50)
SCMC-ES-1	+ (0.63)	+ (500)
RD-ES	-	-
SK-ES	+ (0.12)	+ (50)

The CSC in PSTs resembled normal stem cells morphologically as well as immunohistochemically, as found in acute myeloid leukemia, in which CSC were first identified [4]. The CSC are believed to retain properties similar to those of normal stem cells. That means that CSC may be isolated using the same procedures used to isolate tissue stem cells. The major problems in isolating normal stem cells as well as CSC are their rarity and the absence of specific markers for purifying them. Various efforts have been made to isolate CSC from various cancers [4-7]. Cancer stem cells, first identified in acute myeloid leukemia , could reproduce the original cancer with heterogeneous phenotypes and could express the stem cell surface markers $CD34^+$ and $CD38^-$ [4]. In cancers for which specific markers had not been identified, CSC or stem-like cancer cells have been isolated as SP cells that export Hoechst 33342 dye [16-20]. It has been shown that stem cells are enriched in SP cells in various normal tissues, including bone marrow, skeletal muscle, mammary gland, skin, lung, testis, brain, liver, and kidney [7-15]. Recently, SP cells have been isolated as the CSC that possess the stemness characteristics and are responsible for tumorigenesis in several cancers [16-20]. Side population cells are characterized by the rapid efflux of Hoechst 33342 dye via ABC transporters. Side population fractions are known to disappear upon treatment with inhibitors of ABC transporters such as verapamil and rapamycin. A variety of

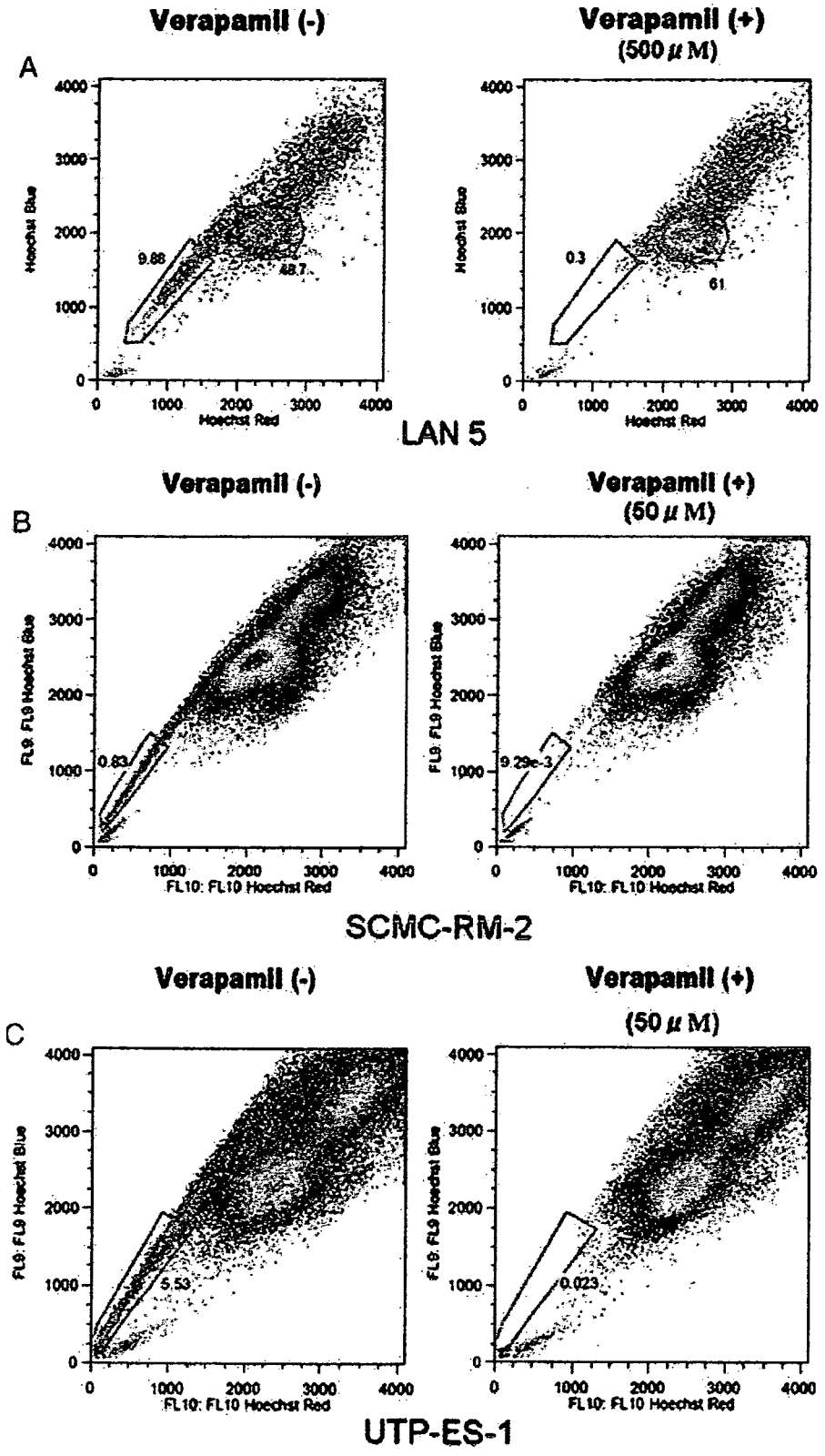

Fig. 2 Side population cells found in PST cell lines. Side population cells identified in a NB cell line, LAN 5 (A, left); a RMS cell line, SCMC-RM-2 (B, left); and a EWS cell line, UTP-ES-1 (C, left). Every SP fraction disappeared with cotreatment of 50 (B and C, right) or 500 μmol/L (A, right) verapamil plus Hoechst 33342.

ABC transporters, including multiple drug resistance protein (MDR1, ABCB1), MDR related protein (MRP1, ABCC1), and breast cancer–resistant protein (BRCP1, ABCG2), have been shown to contribute to the drug resistance in cancers [25-29]. Interestingly, some of these ABC transporters have also been shown to be expressed in various kinds of normal stem cells [21,25-30]. In particular, BRCP1 (ABCG2) is known to contribute to the exclusion of Hoechst 33342 dye in SP cells, which are enriched in stem cells [21]. ABCG2 expressed in normal stem cells and SP cells is believed to play a physiological role in the protection of both of them. This suggests that the SP cells in cancers possess drug resistance because of expression of the same transporters. Transcriptional profiling studies of SP and non-SP cells in several tissues showed that the genes up-regulated in SP cells are implicated in the quiescent status, the maintenance of pluripotency, and the capacity to undergo asymmetric division [31]. Side population cells have also been identified in several tumor cell lines as well as fresh tumor samples, including NB [16-20]. Hirschmann-Jax et al [16] demonstrated that SP cells constituted 0.8% to 51% of the cells in 15 of 23 NB tumor samples as well as 4% to 37% of the cells in 5 NB cell lines. Side population cells in NB were $Gd2^+$, $c\text{-}kit^+$, $CD133^-$, $CD71^-$, and $CD56^{\pm}$ and expressed ABC transporter proteins, ABCG2 and ABCA3, at high levels, supporting the possibility that they were CSC. We have investigated SP cells in PST cell lines, including NB, RMS, and EWS. Unlike fresh tumor samples, cell lines do not contain any contaminating non-CSC such as bone marrow–derived stem cells. Accordingly, the SP cells identified in cell lines are definitely derived from cancer cells. The stem-like population in PSTs may be enriched in SP cells. Our data showed that 15 of 16 PST cell lines contained a small fraction of SP cells (0.12%-14.6%). The percentage of SP fractions is lower than that in the previous study on NB [16]. This may be partly owing to the differences in the gated regions for the SP fraction or in the types of the cell lines used. It is likely that the cells with stemness constitute a smaller fraction. One EWS cell line did not show an SP fraction. Side population formation was blocked by 50 or 500 μmol/L verapamil (an inhibitor of ABC transporters), although the sensitivity of the cell lines varied. The absence of SP cells in the presence of verapamil confirmed the identity of the SP cells as an enriched stem-like population. This also suggested that verapamil-sensitive ABC transporters are involved in the Hoechst dye efflux of these SP cells. Of 15 cell lines, 9 were more resistant to verapamil because verapamil is generally used at a concentration of 50 μmol/L (Table 1). Different mechanisms may be involved in the Hoechst dye efflux activity of these cell lines. Further characterization of the SP cells as candidates of the CSC will be essential to understand the mechanisms of tumorigenesis, progression, metastasis, treatment failure, and tumor relapse in PSTs. Side population analysis should also be done in fresh tumor samples because the cell lines may be a collection of immortalized and rather modified cancer cells after many passages.

Our study demonstrated that most PSTs contained SP cells (possible stem-like population). Conventional treatments used for PSTs have targeted rapidly growing cancer cells, which are synthesizing DNA. The survival of the CSC in PSTs despite such treatments may be responsible for treatment failure and tumor relapse. The target of future treatments of PSTs should be the CSC themselves. New treatment strategies for targeting the CSC will be required to eradicate the refractory PSTs completely. Although how to selectively attack only the CSC without any influence on the normal tissue stem cells will be a difficult problem, complete removal of the pool of CSC as a reservoir will result in the ability to eradicate refractory PSTs in the near future.

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Pediatr Blood Cancer 2008;50:264–269

Tandem Duplications of *MLL* and *FLT3* Are Correlated With Poor Prognoses in Pediatric Acute Myeloid Leukemia: A Study of the Japanese Childhood AML Cooperative Study Group

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Background. *Mixed-lineage leukemia* (*MLL*)-partial tandem duplication (PTD) is associated with poor prognosis in adult acute myeloid leukemia (AML), but its relationship to pediatric AML is unknown. ***Procedure.*** One hundred fifty-eight newly diagnosed AML patients, including 13 FAB-M3 and 10 Down syndrome (DS) patients, who were treated on the Japanese Childhood AML Cooperative Treatment Protocol AML 99 were analyzed for *MLL*-PTD, as well as internal tandem duplication (ITD) and the kinase domain mutation (D835Mt) in the *FLT3* gene. ***Results.*** We found *MLL*-PTD in 21 (13.3%) of 158 AML patients, but not in FAB-M3 or DS patients. The differences between patients with and without *MLL*-PTD were significant for 3-year overall survival (OS) (56.3% vs. 83.2%, $P=0.018$), disease-free survival (DFS) (41.7% vs. 69.6%, $P=0.010$), and relapse rate (RR) (54.3% vs. 27.6%, $P=0.0085$) of 135 AML patients excluding the FAB-M3 and DS patients. Furthermore, ITD and D835Mt in the *FLT3* gene were found in 17 (12.6%) and 8 (5.9%) of these 135 patients, respectively. The differences between patients with *FLT3*-ITD and the wild-type allele were significant for 3-year OS (35.3% and 84.3%, $P<0.0000001$), DFS (40.0% and 66.9%, $P<0.003$), and RR (52.4% and 30.3%, $P<0.005$). Coduplication of both genes was found in only 3 (1.9%) patients. ***Conclusion.*** AML patients with *FLT3*-ITD, but not D835Mt, showed a poor prognosis. AML patients with *MLL*-PTD were also correlated with poor prognosis in this study. Pediatr Blood Cancer 2008;50:264–269.

Key words: AML; childhood; cytogenetics; *FLT3*; *MLL*; *tandem duplication*

INTRODUCTION

Risk classification of acute myeloid leukemia (AML) patients based on cytogenetic abnormalities has been widely accepted in adult and pediatric AML studies [1–4]. AML patients with t(8;21), inv(16), and t(15;17) have been classified into a low risk (LR) group, those with monosomy 5 and monosomy 7 into a high risk (HR) group, and others into an intermediate risk (IR) group [2–4]. Patients with normal karyotype were classified into the IR group and showed various prognoses. Classification by gene alterations other than karyotypic abnormalities would be preferable for improving the treatment outcome of pediatric AML patients.

Chromosome 11q23 abnormalities involving the *mixed-lineage leukemia* (*MLL*) gene are found in about 5% of adult AML patients and in ~50% of infants with AML [5–7]. *MLL*-partial tandem duplication (PTD) is reported in ~10% of adult AML patients, but in 20–50% of adult AML patients with a normal karyotype and trisomy 11 [8–13]. *MLL*-PTD is associated with a poor prognosis in adult AML patients and a high relapse rate (RR) [10–13]. On the other hand, the prevalence and prognosis of *MLL*-PTD in pediatric AML patients remains obscure, although a relatively high prevalence of *MLL*-PTD has been reported in a few articles [14,15].

Fms-related tyrosine kinase 3 (*FLT3*) is one of the class III receptor tyrosine kinases that is normally expressed in hematopoietic stem cells and early progenitor cells [16,17]. Internal tandem duplication (ITD) of the juxtamembrane domain (JM) of the *FLT3* gene occurs in approximately 30% of adult AML patients [18–20] and in ~20% of pediatric AML patients [21–23]. *FLT3*-ITD is strongly associated with poor prognosis, especially in patients with a normal karyotype [18–23]. Furthermore, ~10% of adult AML patients have an activating loop mutation in the kinase domain specifically, a point mutation in aspartic acid residue at codon 835 (D835Mt). These patients show a poor prognosis [19,20,24]. The prevalence and prognostic significance of *FLT3*-D835Mt in pediatric AML patients are controversial [21,23].

We have previously reported the existence of the coduplication of *MLL* and *FLT3* in pediatric AML patients who had poor prognoses [25]. These results were confirmed in adult patients with a normal karyotype and trisomy 11 [12,13,26,27]. We here performed mutation analysis of both *MLL* and *FLT3* genes in 158 unselected pediatric AML patients treated on the Japanese pediatric AML collaborative treatment protocol AML99. These data suggest that *FLT3*-ITD and *MLL*-PTD are both important markers of poor prognosis in pediatric AML patients.

This article contains Supplementary Material available at http://www.interscience.wiley.com/jpages/1545-5009/suppmat.

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Received 26 October 2006; Accepted 22 June 2007

DOI 10.1002/pbc.21318
Published online 30 August 2007 in Wiley InterScience (www.interscience.wiley.com)

PATIENTS AND METHODS

Patients

The diagnosis of AML was made according to the French–American–British (FAB) classification. Cytogenetic analysis was performed using the G-banding method. Among 318 newly diagnosed de novo AML patients enrolled from January 2000 to December 2002, 158 samples were available for molecular analysis (Table I). Among the 158 patients, there were 13 patients with FAB-M3 and 10 patients with Down syndrome (DS) who were treated with different treatment protocols [28–30]. There were no significant differences between the 135 analyzed patients without FAB-M3 and DS and the 105 non-analyzed patients in terms of age (median 6 years (range: 0–15 years) vs. 6 years (range: 0–15 years)) and initial WBC count (median 24.8×10^9/L (range: 1.65–621.0×10^9/L) vs. 13.8×10^9/L (range: 1.0–489.0×10^9/L, $P = 0.0764$)). Patients who were younger than 2 years old or had an initial WBC count <100,000/μl were treated with the Induction A regimen (etoposide (VP16), cytarabine (CA) and mitoxantrone (MIT), (ECM)). Patients who were older than 2 years old and had an initial WBC count >100,000/μl were treated with the Induction B regimen (VP16, CA and idarubicin (IDA), (ECI)). If patients achieved complete remission (CR), the patients were classified into three risk groups (62 in low, 57 in intermediate and 10 in high) according to the results of cytogenetic analyses or the achievement of CR after initial 2 courses of chemotherapy [28–30] (Supplemental Fig. 1 which has been reported in Blood [30], http://bloodjournal.hematologylibrary.org/cgi/data/2005-08-3408/DC1/2). AML patients with t(8;21) (except for those with WBC counts >50,000/μl) or inv(16)(p11q22) were classified into the LR group. Patients with monosomy 7, 5q-, t(16;21), or Ph1 were classified into the HR group. Patients were treated with additional chemotherapy or allogeneic stem cell transplantation (allo-SCT) in each risk group (Supplemental Fig. 1).

Informed consent was obtained from the patients or patients' parents, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center approved this project.

Detection of *MLL*-PTD

Total RNA (4 μg) extracted from the bone marrow or peripheral blood samples at diagnosis was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *MLL*-PTD was examined by simple first round reverse transcriptase-polymerase chain reaction (RT-PCR) with 35 cycles using the primer pair 6.1 (located on exon 9) and E3AS (located on exon 4), according to the conditions previously reported [10,25,31]. We did not use the nested RT-PCR method because a previous report suggested that the *MLL*-PTD transcripts were highly detected in the healthy controls [31]. We used the CTS cell line as a positive control for *MLL*-PTD and water as a negative control for RT-PCR analysis

TABLE I. Clinical Characteristics of Patients With *MLL* or *FLT3* Gene Alterations

	All patients	*MLL*-PTD	*FLT3*-ITD	*FLT3*-D835Mt
Age, median (year)	6 (0–15)	10 (2–15)	9 (2–15)	11 (2–14)
WBC count, median ($\times 10^9$/L)	20.7 (1.0–620.0)	31.4 (3.6–343.4)	33.2 (3.0–620.0)	45.0 (3.3–440.0)
Male/female	89/69	12/9	8/12	7/4
FAB classification				
M0	6	1	1	0
M1	24	7(2[a])	4(2[a])	2
M2	46	5	4	2
M3	13	0	3	3
M4	22	4(1[a])	1(1[a])	1
M5	25	3	5	3
M6	1	0	0	0
M7	19	1	1	0
Unclassified	2	0	1	0
Karyotypic abnormalities				
Normal	33	8(2[a])	9(2[a])	2
t(8;21)	46	4	2	1
11q23 abnormalities	20	5	0	1
t(15;17)[b]	13	0	3	3
inv(16)	7	0	0	2
DS[b]	10	0	0	0
Others[c]	27	4(1[a])	5(1[a])	2
Unknown	2	0	1	0
Total	158	21	20	11
Risk group				
Low	62	4	2	3
Intermediate	57	13(2[a])	8(2[a])	4
High	10	3	2	0
Non-CR	6	1(1[a])	5(1[a])	1
Total	135	21	17	8

[a]Cases who showed *MLL*-PTD and *FLT3*-ITD simultaneously; [b]DS—Down syndrome, patients with FAB-M3 or DS were treated with the different protocol; [c]others contain −7, +8 or complex karyotypes.

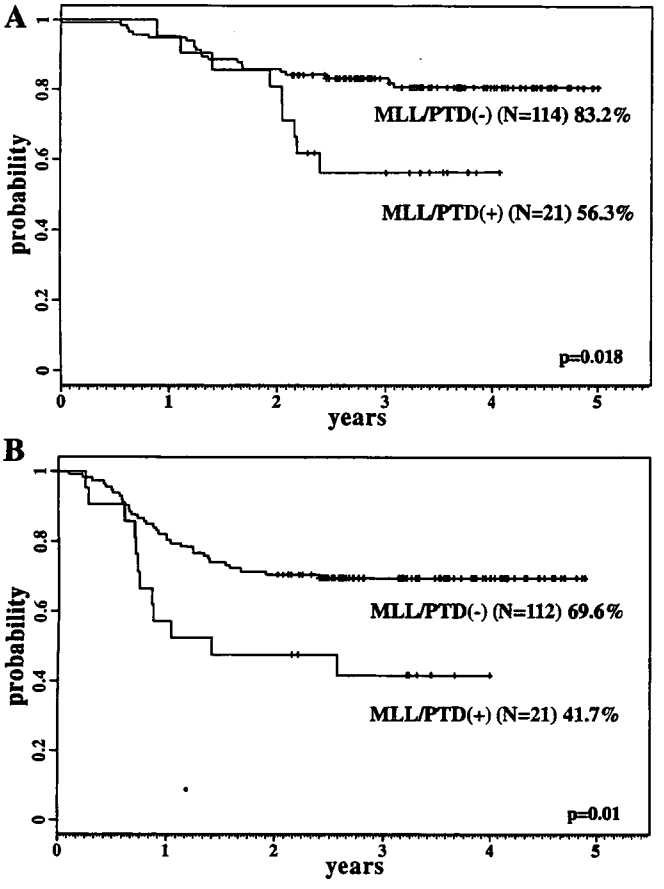

Fig. 1. Probabilities of 3-year OS (**A**) and 3-year DFS (**B**) in 135 AML patients excluding those with FAB-M3 and Down syndrome. Kaplan–Meier method estimates for patients with and without *MLL*-PTD are shown. The difference in patient numbers between OS and DFS resulted from the death of two patients during induction therapy.

[32,33]. Furthermore, we analyzed *MLL*-PTD in 10 normal bone marrow samples. Five microliter of the PCR products were electrophoresed in a 3% agarose gel. The amplified products were purified and directly sequenced.

Detection of *FLT3*-ITD and D835Mt

Using 1 μl of the cDNA, PCR amplification was performed for the JM and tyrosine kinase domain of the *FLT3* gene. The PCR procedure has been reported previously using primer pairs R5, R6, and 17F, TKR [30,34,35]. If a longer size product was found, the product was cut from the gel, purified with a QIAquick gel extraction kit (Qiagen, Chatsworth, CA), and directly sequenced on a DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). D835Mt was confirmed using *Eco*RV digestion and followed by direct sequencing as previously reported [24,30,34,35].

Statistical Analysis

Estimation of the survival distributions was performed using the Kaplan–Meier method and the differences were compared using the log-rank test. Disease-free survival (DFS) was defined as the time from diagnosis until the date of relapse. Overall survival (OS) was defined as the time from diagnosis until death owing to any cause or the last follow-up. Statistical difference analysis was performed using the χ^2 test. The prognostic significance of the clinical variables was assessed by using Cox proportional hazards model. These statistical analyses were performed with statistical software R. For all analyses, the *P*-values were two-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

MLL-PTD

MLL-PTD was found in 21 (13.3%) of 158 pediatric AML patients (Table I). One type of fusion transcript (exon 9 and exon 3) was found in 10 patients, and the other type (exon 10 and exon 3) was found in 11 patients. Only one patient showed both fusion transcripts corresponding to alternatively spliced exons 10 and 11 to exon 3 (Supplemental Fig. 2). Furthermore, 10 normal bone marrow samples did not show *MLL*-PTD transcripts. *MLL*-PTD was frequently found in FAB-M1, M4 and patients with normal karyotype or 11q23 abnormalities (Table I). *MLL*-PTD was not found in FAB-M3 and DS patients. Patients with trisomy 11 were not found in this study. Remarkably, more than half of the patients with *MLL*-PTD were classified into the IR group (13 of 21 (61.9%)). The median age of patients with *MLL*-PTD was 10 years old (2–15) and no patients with *MLL*-PTD under 2 years old were found. Excluding the FAB-M3 and DS patients, the statistical differences in the clinical outcome between patients with and without *MLL*-PTD were significant for 3-year OS (56.3% vs. 83.2%, $P = 0.018$), DFS (41.7% vs. 69.6%, $P = 0.01$), and RR (54.3% vs. 27.6%, $P = 0.0085$) (Fig. 1). Allo-SCT was performed in 18 (85.7%) of 21 *MLL*-PTD patients, and 9 (50.0%) of them have been alive for a median of 42.0 months. The three patients without allo-SCT are all alive. Notably, six of the eight patients who received allo-SCT in the 1st CR and three of four patients who received allo-SCT in the 2nd CR are still alive.

FLT3-ITD and D835Mt

FLT3-ITD was found in 20 (12.7%) of 158 patients (Table I). All patients except for one showed both *FLT3*-ITD and *FLT3*-WT transcripts by RT-PCR. Half of the *FLT3*-ITD consisted of an in-frame tandem repeat of exon 11 (12–147 bp). The other half of *FLT3*-ITD showed insertions of 1–15 bp between the duplicated regions. *FLT3*-D835Mt was found in 11 (7.0%) of 158 patients. D835Mt consisted of D835Y (seven patients), D835V (two patients) and D835H (two patients). Differences in the median age of patients with *FLT3*-ITD, D835Mt, and the wild-type gene (WT) were not statistically significant (9, 11, and 5 years old, respectively). All patients with *FLT3*-ITD or D835Mt were older than 2 years old. The difference in the median initial WBC count between patients with *FLT3*-ITD and WT was significant ($P = 0.014$). Excluding FAB-M3 and DS patients, the differences between AML patients with *FLT3*-ITD, D835Mt, and WT were significant for the 3-year OS (35.3%, 100% and 84.3%, $P < 0.0000001$), DFS (40.0%, 87.5%, and 66.9%, $P < 0.003$), and RR (52.4%, 11.8% and 30.3%, $P < 0.005$) (Fig. 2). *FLT3*-ITD was found in five (83.3%) of six patients who did not

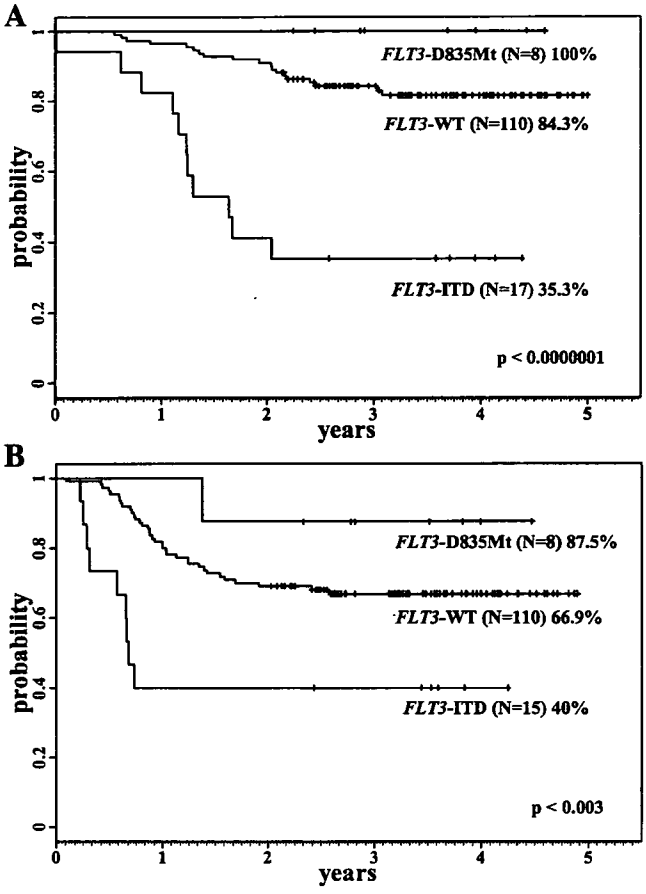

Fig. 2. Probabilities of 3-year OS (**A**) and 3-year DFS (**B**) in 135 AML patients, excluding those with FAB-M3 and Down syndrome. The Kaplan–Meier method for patients with *FLT3*-ITD, D835Mt, and WT is shown. The difference in patient numbers between OS and DFS resulted from the death of two patients during induction therapy.

attain CR. Allo-SCT was performed in 12 (70.6%) of 17 *FLT3*-ITD patients; of whom 4 (33.3%) were still alive for a median 43.5 months. The remaining eight patients died. Furthermore, four of seven patients who received allo-SCT in the 1st CR are still alive. Three of five patients without allo-SCT are also alive.

FLT3-ITD and D835Mt were found in 3 (23.1%) of 13 patients with FAB-M3. Both alterations of the *FLT3* gene did not influence the clinical outcome of FAB-M3 patients. Furthermore, these *FLT3* alterations were not found in DS patients.

Coduplication of the *MLL* and *FLT3* Genes

Coduplication of the *MLL* and *FLT3* genes were found in only 3 (1.9%) of 158 patients (Table I). Two patients had normal karyotype and one patient had +8. All three patients received allo-SCT, and two of them died because of disease progression.

Multivariate Analysis of Clinical Outcome

Multivariate analysis of *FLT3*-ITD, *MLL*-PTD, M1 marrow after induction therapy and initial high WBC count (more than 100×10^9/L) was carried out for 3-year OS and DFS data from 135 AML patients. Only *FLT3*-ITD was significant for 3-year OS (hazard ratio 8.4, 95% confidence interval (95% CI) 3.2–21.8, $P < 0.0001$). For 3-year DFS, *FLT3*-ITD, and M1 marrow after induction therapy were significant (hazard ratio 3.6 and 3.1, 95% CI 1.7–7.6 and 1.4–7.0, $P < 0.001$ and 0.007). Moreover, multivariate analysis was performed for 108 patients excluding those who received allo-SCT in 1st CR. Only *FLT3*-ITD was significant for 3-year OS (hazard ratio 16.0, 95% CI 4.7–54.7, $P < 0.00001$) (Table II). On the other hand, *MLL*-PTD was significant for 3-year DFS (hazard ratio 3.2, 95% CI 1.3–7.7, $P < 0.01$) (Table III).

DISCUSSION

In this study, *MLL*-PTD was found in 21 (15.6%) of 135 pediatric AML patients excluding those with FAB-M3 and DS. We used the simple first round RT-PCR method and not the nested RT-PCR method to minimize the possibility of detecting false positive *MLL*-PTD transcripts. *MLL*-PTD in pediatric AML has been reported at a relatively high frequency in a small number of patients: 2 (20%) of 10 patients [14] and 5 (9.4%) of 53 patients [15]. These data are compatible with our results. However, Shih et al. [36] have recently reported that *MLL*-PTD was rarely found in pediatric AML patients (one of 123, 0.8%). The difference of these frequencies in pediatric AML remains unknown but it may be partially due to the patient's age; although the median age of 16 patients with *MLL* rearrangements, including one *MLL*-PTD, is 1.3 years (1 day to 5.5 years) in the paper by Shih et al. [36], that of 21 patients with *MLL*-PTD is 10 years (2–15 years), and 17 of 21 patients with *MLL*-PTD is more than 6 years old in our study.

Patients with *MLL*-PTD showed a poor prognosis, a short duration of remission, and a high RR, as previously reported for adult AML patients [10–14,26]. Multivariate analysis suggested that *MLL*-PTD was a marker of poor prognosis for 3-year DFS, but not for 3-year OS, in AML patients excluding those who received allo-SCT in 1st CR in this study. This result may be explained by the effectiveness of allo-SCT in 2nd CR for patients with *MLL*-PTD. Indeed, four patients received allo-SCT in 2nd CR, and three of these patients are still alive.

Regarding karyotypic abnormalities, our results also confirmed that *MLL*-PTD was frequently found in AML patients with a normal karyotype as reported for adult patients [10–14]. Interestingly, *MLL*-PTD was found in AML patients with 11q23 translocations in this study. Moreover, *MLL*-PTD was also found in AML patients with

TABLE II. Prognostic Factors for 3-year Overall Survival in 108 AML Patients Treated on AML99 Protocol, Excluding Those Who Received Allo-SCT in 1st CR

Variable	*P*-values	Hazard ratio	95% CI
FLT3-ITD	<0.00001	16.0	4.7–54.7
MLL-PTD	0.25	2.1	0.6–7.4
M1 marrow after induction therapy	0.092	5.3	0.8–37.3
WBC > 100×10^9/L	0.14	0.19	0.02–1.7

TABLE III. Prognostic Factors for 3 Year Disease-Free Survival in 108 AML Patients Treated on AML99 Protocol, Excluding Those Who Received Allo-SCT in 1st CR

Variable	*P*-values	Hazard ratio	95% CI
FLT3-ITD	<0.0001	7.7	2.9–20.6
MLL-PTD	0.0099	3.2	1.3–7.7
M1 marrow after induction therapy	0.028	9.3	2.1–40.1
WBC > 100×10^9/L	0.013	3.1	1.3–7.5

t(8;21), which has not previously been reported in adult AML [10–14,26]. Unfortunately, we could not analyze DNA because genomic samples were not available. Two of the 4 t(8;21)-AML patients with *MLL*-PTD were also found to have *KIT* mutations in our previous study [30], suggesting that some patients possibly had genetic instability. We must await further studies to clarify these issues.

As for *FLT3* gene, multivariate analysis also strongly suggested that *FLT3*-ITD was an independent marker of poor prognosis in pediatric AML as previously reported [18,20,22]. D835Mt did not represent a poor prognosis in this study, confirming a previous report of pediatric AML [21], although D835Mt has been reported to be associated with poor prognosis in adult AML [18–20,24]. The difference between adult and pediatric AML remains unknown.

The coduplication of both genes was found in 3 (1.9%) of 158 patients in this study, which is compatible with previous reports (4 (1.6%) of 250 and 16 (1.7%) of 956 adult AML patients) [12,26]. The mechanism of formation of *MLL*-PTD and *FLT3*-ITD remains unknown. *MLL* and *FLT3* loci demonstrate similar susceptibilities to agents that modify chromatin configuration, including topoisomerase II inhibitors [27]. We conclude that the coduplication of *MLL* and *FLT3* genes is rare in pediatric AML as well as adult AML.

There was no definitive result as to the effectiveness of allo-SCT for the pediatric patients with *MLL*-PTD or *FLT3*-ITD. In this study, the majority of patients received allo-SCT due to the protocol agreement or relapse (18 (85.7%) of 21 *MLL*-PTD and 12 (70.6%) of 17 *FLT3*-ITD). Eight *MLL*-PTD patients and seven *FLT3*-ITD patients received allo-SCT in the 1st CR. Although similar results for 3-year DFS were found in patients with *MLL*-PTD (41.7%) and *FLT3*-ITD (40.0%), there was a difference in the 3-year OS between *MLL*-PTD (56.3%) and *FLT3*-ITD (35.3%) ($P = 0.024$). This difference was possibly due to the effectiveness of allo-SCT for the patients with *MLL*-PTD rather than those with *FLT3*-ITD as a lack of effectiveness of allo-SCT has been recently reported for patients with *FLT3*-ITD [37].

ACKNOWLEDGMENT

We express our appreciation to all the doctors for their participation in the Japanese Childhood AML Cooperative Study Group. This study was supported in part by a Grant-in-Aid for Cancer Research and a grant for Clinical Cancer Research and Research on Children and Families from the Ministry of Health, Labor and Welfare of Japan, and by a Research grant for Gunma Prefectural Hospitals, and also supported by Kawano Masanori Memorial Foundation for Promotion of Pediatrics. Supported also by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Pediatr Blood Cancer 2008;50:213–217

Expression of KIT and PDGFR Is Associated With a Good Prognosis in Neuroblastoma

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Background. The clinical outcome of neuroblastoma (NB) depends on age, stage, and *MYCN* amplification. Receptor tyrosine kinases (RTKs) promote cell growth, migration, and metastasis in cancer cells, including NB. However, the correlation of the expression profile of RTKs with prognosis in NB remains controversial. ***Procedure.*** Expression and mutation analysis of *KIT, PDGFR, FLT3, RET,* and *TRKA* mRNAs were performed in 24 NB cell lines and 40 tumor samples using RT-PCR followed by direct sequencing. Immunohistochemical analysis of KIT and PDGFR protein expression was also examined in 38 paraffin sections of NB tumor samples. ***Results.*** The expression of *KIT, PDGFRβ,* and *FLT3* mRNA was associated with NB in patients under 1 year ($P < 0.02$) and *TRKA* expression ($P < 0.001$). The loss of expression of these kinases was associated with *MYCN* amplification ($P < 0.02$) and advanced stages of disease in patients over 1 year of age ($P < 0.005$). *PDGFRα* mRNA expression was detected in all cell lines and tumor samples, and *RET* mRNA expression was not associated with any clinical parameters. Immunohistochemistry results showed the similar findings. We did not find any activating mutations in *KIT, PDGFR, FLT3,* or *RET.* Notably, the GNNK$^-$ isoform of *KIT* was predominant in all cell lines and clinical samples. ***Conclusion.*** Expression of *KIT, PDGFRβ,* and *FLT3* was associated with a good prognosis in NB. The loss of expression of these RTKs might correlate to the disease progression of NB. Pediatr Blood Cancer 2008;50:213–217. © 2007 Wiley-Liss, Inc.

Key words: *FLT3; KIT;* neuroblastoma; *PDGFR;* receptor tyrosine kinase

INTRODUCTION

The receptor tyrosine kinases (RTKs) play an important role in the growth, migration, metastasis and angiogenesis in varieties of malignancies [1–3]. KIT is one of the type III RTKs and is well known to have roles not only in hematopoiesis, but also in germ cell and melanocyte development and differentiation as well as in neuroectodermal tumor cells [1–9]. Recently, KIT expression in NB has been reported to be associated with a poor prognosis with MYCN amplification [4,9]. On the other hand, another report suggested that KIT expression was associated with a good prognosis [7]. Moreover, a tyrosine kinase inhibitor, imatinib, has been shown to have an inhibitory effect for NB cell growth in vitro and in vivo [4–6]; however, imatinib was suggested not to inhibit the stem cell factor (SCF)/KIT pathway in NB cells [6]. Therefore, the therapeutic mechanism of imatinib in NB remains undetermined. *KIT* mutations have been frequently found in gastrointestinal stromal tumor (GIST) [10] and a subtype of acute myeloid leukemia (AML) [11], but not in NB [8]. The platelet derived growth factor receptor (PDGFR)-α has important roles in the development of neural crest-derived cells [12]. *PDGFRα* mutation has been frequently found in GIST [13]. PDGFRβ is overexpressed in metastatic medulloblastoma, and has been considered to have a more oncogenic potential than *PDGFRα* [14]. The roles of *PDGFRα* and *PDGFRβ* remain to be elucidated in NB.

FLT3 and RET have been reported to have roles in proliferation and differentiation in NB [15,16]. Although *FLT3*-internal tandem duplication (ITD) is a poor prognostic factor in AML [17], *FLT3*-ITD or kinase domain mutations have not yet been reported in NB. The RET receptor signal pathway is functional in most NB [16,18]. *RET* gene mutations have been identified in multiple endocrine tumors [19]. The expression of TRKA has been associated with good clinical outcome in NB. On the other hand, Tacconelli et al. [20] reported that the alternative spliced isoform III of TRKA has oncogenic potential. Therefore, we performed expression and mutation analysis of these 5 RTK (*KIT, PDGFRs, FLT3, RET,* and *TRKA*) genes in 24 NB cell lines and 40 clinical specimens.

Here we described that the expression of *KIT, PDGFRβ,* and *FLT3* is associated with NB in patients under 1 year of age and with a good prognosis. The loss of expression of these RTKs may be associated with NB disease progression.

MATERIALS AND METHODS

Cell Lines and Clinical Samples

Twenty-four NB cell lines were examined in this study (Supplemental Table I). RNAs were extracted from 40 frozen tumor samples using a QIAGEN RNA extraction kit (Qiagen, Chatsworth, CA), which were obtained before chemotherapy from January 2001 to December 2005. Twenty of these samples were taken from patients under 1 of age, and they received surgical resection and chemotherapy. All patients except for one are alive. Five patients were stages I or II and over 1 year of age and received surgical resection and chemotherapy and were alive. Fifteen patients had advanced stage and were over 1 year of age; they received surgical resection, radiation therapy, and intensive chemotherapy including autologous-SCT [21] (Table I); however, five patients (33.3%) died due to the disease progression after autologous-SCT. Informed consent was obtained from parents. The institutional review board of Gunma Children's Medical Center approved this project.

This article contains Supplementary Material available at http://www.interscience.wiley.com/jpages/1545-5009/suppmat.

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Grant sponsor: Ministry of Health, Labor, and Welfare of Japan.

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Received 18 August 2006; Accepted 16 May 2007

DOI 10.1002/pbc.21288
Published online 16 October 2007 in Wiley InterScience (www.interscience.wiley.com)

TABLE I. Expression of *KIT, PDGFRβ, FLT3, RET* in 40 Clinical NB Samples by RT-PCR

	No. of patients	*KIT* (%)	*PDGFRβ* (%)	*FLT3* (%)	*RET* (%)
Age					
<1 year	20	20 (100)	19 (95)	19 (95)	8 (40)
>1 year	20	12 (60)	10 (50)	13 (65)	9 (45)
		($P=0.0016$)	($P=0.0014$)	($P=0.0177$)	ns
MYCN status					
>5 copies	6	2 (33.3)	2 (33.3)	2 (33.3)	1 (16.7)
1 copy	34	30 (88.2)	27 (79.4)	30 (88.2)	16 (47.1)
		($P=0.0006$)	($P=0.0198$)	($P=0.0019$)	ns
Clinical stage					
III, IV, and over 1 year old	15	7 (46.7)	7 (46.7)	8 (53.3)	4 (26.7)
I, II, IVs at any age	25	25 (100)	22 (88)	24 (96)	13 (52)
		($P<0.0001$)	($P=0.0046$)	($P=0.0011$)	ns
TRKA					
Positive	28	27 (96.4)	25 (89.3)	27 (96.4)	12 (42.9)
Negative	12	5 (41.7)	4 (33.3)	5 (41.7)	5 (41.7)
		($P<0.0001$)	($P=0.0003$)	($P<0.0001$)	ns
Total	40	32 (80)	29 (72.5)	32 (80)	17 (42.5)

P-value is analyzed for the correlation between RTK expression and age, *MYCN* amplification, clinical stage, and TRKA expression, respectively. ns represents not significant.

Expression and Mutation Analysis of *KIT* and *PDGFR*

The procedure was reported previously. Briefly, a total of 4 μg of RNA was reverse transcribed to cDNA. Using 1 μl of the cDNA, polymerase chain reaction (PCR) was performed using primer pairs for extracellular (EC), juxtamembrane (JM), transmembrane (TM), and the second tyrosine kinase (TK2) domains of *KIT* and *PDGFR* using an ABI 2700 thermal cycler (Applied Biosystems, Tokyo, Japan; Supplemental Table II) [11,22]. If the PCR-product was found as the estimated size and confirmed by sequencing directly, we evaluated it as positive expression of mRNA.

Mutation analyses of *KIT* and *PDGFR* in 24 NB cell lines were performed by direct sequencing using an ABI prism 310 sequence analyzer (Applied Biosystems). The mRNA expression of each ligand (*SCF, PDGFA*, and *PDGFB*) was also analyzed by RT-PCR.

Expression and Mutation Analysis of *FLT3*

Using 1 μl of the cDNA, PCR amplification was performed for the JM or TK2 domain of the *FLT3* gene. The PCR procedure has been reported previously using primer pairs R5, R6 and 17F, TKR [23]. If more than two bands were found, the amplified products were cut from the gel, purified with a QIAquick gel extraction kit (Qiagen) and directly sequenced.

Expression and Mutation Analysis of *RET*

Using 1 μl of the cDNA, PCR amplification was performed for the TM and TK domain of the *RET* gene. PCR was performed using previously reported condition and primer pairs RET-TM(+) and RET-TK2(−) [24]. *RET* isoforms, RET9 and RET51, were analyzed as previously reported [25].

Expression and Mutation Analysis of *TRKA*

TRKA mRNA expression was analyzed using newly designed primer pairs, TRKA-F and TRKA-R (Supplemental Table II). This primer pair could distinguish the alternative spliced form I (deleted exon 9), II (no-deletion), and III (exons 6, 7, and 9) [24] by the forward primer in exon 5 and reverse primer in exon 10.

Protein Expression Analysis

Paraffin sections were obtained from 38 NB samples (Table II). Eight samples were classified as advanced stage and older than 1 year old. Ten RNAs and ten paraffin sections were obtained from the same patients. The expression of KIT, PDGFRα and PDGFRβ proteins was analyzed using the avidin–biotin–peroxidase complex method on paraffin sections [26]. Antibodies of KIT (DAKO, A4502, diluted 1:80), PDGFRα (SantaCruz, CA, USA, sc-338,

TABLE II. Expression of KIT and PDGFRβ in NB Tumor Specimens by Immunohistochemistry

	Number of patients	KIT (%)	PDGFRβ (%)
Age			
<1 year	27	20 (74)	20 (74)
>1 year	11	3 (27.3)	4 (36.4)
		($P=0.0074$)	($P=0.019$)
MYCN status			
>5 copies	6	1 (16.2)	0
1 copy	32	22 (68.8)	24 (75)
		($P=0.017$)	($P=0.0052$)
Clinical stage			
III, IV, and over 1 year old	8	2 (25)	1 (12.5)
I, II, IVs in any age	30	21 (70)	22 (73.3)
		($P=0.014$)	($P=0.0011$)
Shimada's Histology			
Favorable	27	19 (70.4)	21 (77.8)
Unfavorable	11	4 (36.4)	3 (27.3)
		($P=0.052$)	($P=0.0021$)
Total	38	23 (60.5)	24 (63.2)

P-value is analyzed for the correlation between each RTK expression and age, *MYCN* gene amplification, clinical stages, and histology.