the gene expression changes were mild, the ectopic expression of C/EBPα and C/EBPε similarly downregulated *Hoxa7* and *Hoxa9*, which are known to be related to chimeric MLL-induced leukemogenesis, (Ayton and Cleary, 2003), and upregulated *Cdkn1α* encoding $p21^{WAF1}$ (data not shown). *Cebpe* was upregulated by the induction of C/EBPα-ER with 4-HT (593 ± 69% at 4-h incubation with 4-HT), but the induction of C/EBPε-ER with 4-HT could not upregulate *Cebpa* (102 ± 2 and 134 ± 7% at 4- and 12-h incubation with 4-HT, respectively).

These results suggested that (i) C/EBPα or C/EBPε-induced monocytic differentiation may be related to the downregulation of *Myc* and upregulation of *Sfpi1*, and (ii) the expression profiles with the ectopic expression of C/EBPα or C/EBPε closely resembled each other, except for upregulation of *Cebpe* mRNA by C/EBPα.

Overexpression of Myc partially antagonized the C/EBPs functions in HF6 cells

As *Myc* was markedly downregulated in HF-6 cells by C/EBPs induction, the ability of Myc to inhibit C/EBPs-induced monocytic differentiation of HF-6 cells was examined. Retroviral vectors expressing the two major isoforms Myc1 and Myc2 (pMYpuro-Myc1 and pMYpuro-Myc2, respectively) were constructed and infected to HF-6 cells to generate HF-6/Myc1 and HF-6/Myc2 cells. Overexpression of Myc isoforms in these cells was confirmed by immunoblotting (Figure 6a). HF-6/Myc1 and HF-6/Myc2 cells had morphologically blastic features similar to the original HF-6 cells (Figure 6b). The expression intensity of Mac-1 and Gr-1 was slightly decreased in these cells, when compared with original HF-6 cells and those with the empty vector (HF-6/pMYpuro) (Figure 6c). The proliferation rates of HF-6/Myc1 and HF-6/Myc2 cells were similar to those of HF-6 cells (data not shown).

C/EBPα-ER or C/EBPε-ER was then infected into HF-6/Myc1 and HF-6/Myc2 cells, and their expression was confirmed with immunoblotting (Figure 6d). Although the intermediate forms of monocytic lineage cells were increased in HF-6/Myc1 cells expressing C/EBPα-ER or C/EBPε-ER, no mature forms were induced in all these cells (Figure 6e). C/EBPα-ER or C/EBPε-ER slightly upregulated the intensities of Mac-1 and Gr-1 in HF-6/Myc1 and HF-6/Myc2 cells, but

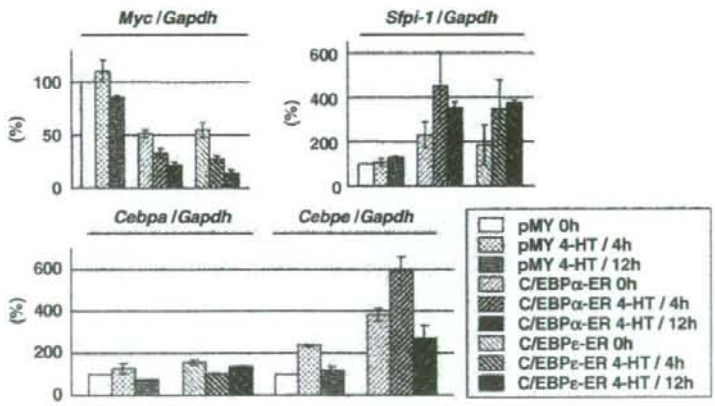
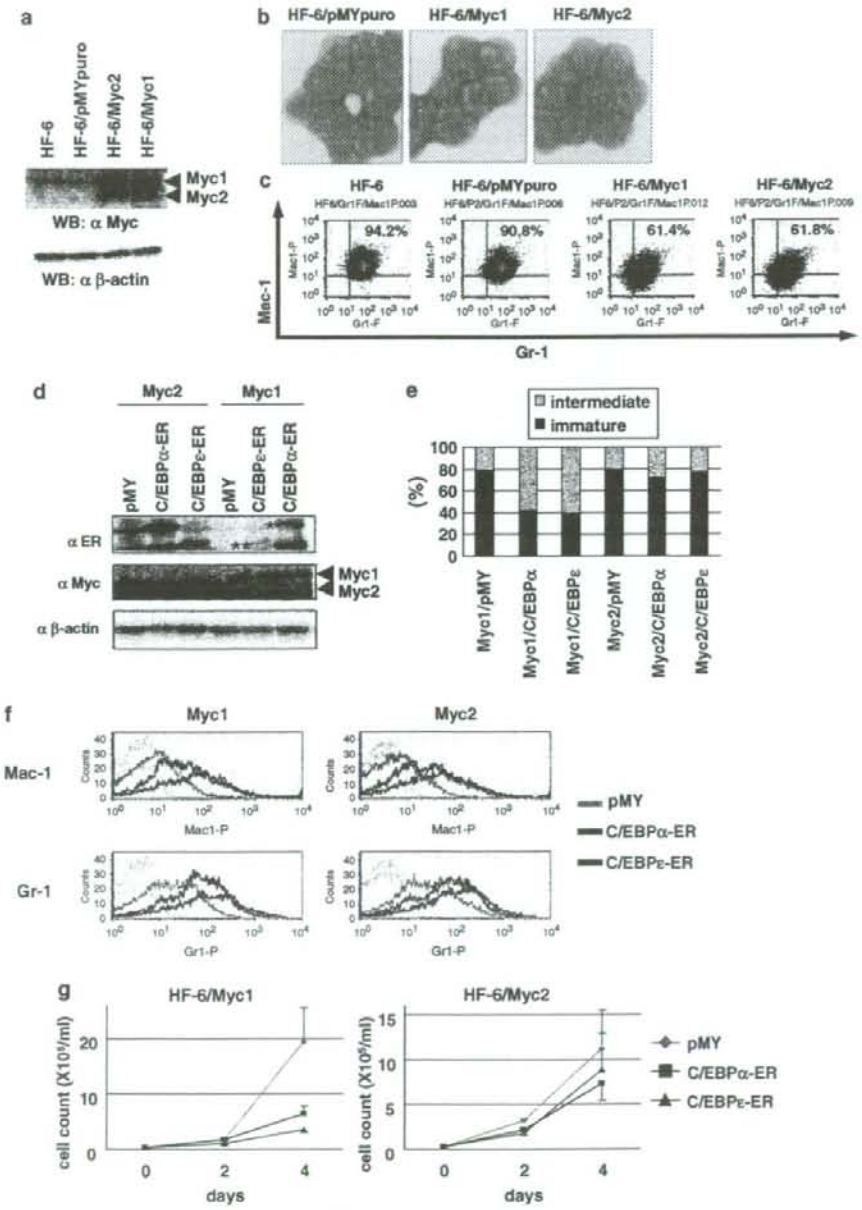


Figure 5 Changes in the mRNA expression of HF-6 cells induced by CCAAT/enhancer binding proteins (C/EBPs) activation. The expression levels of *Myc*, *Sfpi1*, *Cebpa* and *Cebpe* mRNA in HF-6/C/EBPα-ER or HF-6/C/EBPε-ER cells were analysed by quantitative reverse transcription (RT)–PCR. Total RNA was extracted before or after 4 and 12-h incubation with 1 μM of 4-HT. The mRNA content was measured relative to that of murine *Gapdh*. The relative ratios in regard to the data from the HF6/empty vector (pMY) before 4-HT treatment are presented in percentages.

Figure 4 CCAAT/enhancer binding proteins (C/EBPs) inhibited the proliferation and induced the monocytic differentiation of HF-6 cells. (**a**) Detection of C/EBPα-ER and C/EBPε-ER in HF-6 cells. They were recognized by anti-ER antibody. Anti-GFP antibody was used for detection of viral integration in HF-6 cells. *: the specific bands, **: nonspecific bands. (**b**) Growth inhibition of HF-6 cells by the induction of C/EBPs activity. The cells were sorted with green fluorescent protein on day 0, and sequentially analysed the proliferation with or without 1 μM of 4-Hydroxytamoxifen (4-HT). pMY: pMY-IRES-GFP, used as a control vector. (**c**) Morphological changes of HF-6 cells by induction of C/EBPs activity. Cytospin slides were prepared on day 1. As a result of C/EBPs expression, the cytoplasm became less basophilic and obtained a number of vacuoles, and the nucleus became indented or lobulated. May–Giemsa staining, magnification: × 400. (**d**) The differentiation count of HF-6 cells expressing pMY, C/EBPα-ER or C/EBPε-ER on day1. They were cultured with or without 4-HT for 24 h. (**e**) The expression of Mac-1 and Gr-1 in HF-6 cells induced by C/EBPs activity. Evaluation was done at sorting (day 0) and 2-day incubation with 1 μM of 4-HT (day 2). Dotted lines: with isotype controls. (**f**) Induction of apoptosis in HF-6 cells by C/EBPs activation. Annexin-positive/propidium iodine (PI)-negative cells were counted as apoptotic cells at sorting and after 18-h incubation with 1 μM of 4-HT.

the intensities were not as high as those observed in HF-6/C/EBPα-ER or HF-6/C/EBPε-ER cells (Figures 4e and 6f). Both of the cells expressing C/EBPα-ER or C/EBPε-ER could proliferate under 4-HT treatment, although the partial growth inhibition was still stronger in HF-6/Myc1 than in HF-6/Myc2 (Figure 6g).

These assays were also performed using proliferating HF-6/Myc1 and HF-6/Myc2 cells introduced with C/EBPα-ER or C/EBPε-ER after 1-week culture. The sustained expression of C/EBPα-ER or C/EBPε-ER in these cells was confirmed using quantitative RT–PCR. These cells showed a blastic appearance, lower intensities of Mac-1 and Gr-1 and increased proliferation



rates, in comparison to the cells immediately after infection. The expression levels of *Myc and Sfpi1* mRNA in HF-6/Myc1 and HF-6/Myc2 cells were equal, regardless of the ectopic expression of C/EBPs. The induction of *Cebpe* mRNA was not observed in HF-6/Myc1 or HF-6/Myc2 cells expressing C/EBPα-ER (data not shown).

Taken together, these data suggested that the ectopic expression of Myc overcame the growth arrest, while also partially inhibiting the monocytic differentiation of HF-6 cells induced by the C/EBPα or C/EBPε activity.

Discussion

This study demonstrated that the induction of C/EBPα or C/EBPε activity by itself could inhibit the cellular growth and induce the monocytic differentiation of myelomonocytic cells with *MLL*-fusion genes. In addition, the downregulation of Myc induced by C/EBPs activity appears to have an important function in their monocytic differentiation.

Many previous studies have shown that C/EBPα and C/EBPε have an important function in granulocytic differentiation. However, this study showed that human and murine myelomonocytic cells with *MLL*-fusion genes ceased their proliferation and were differentiated into the monocytic lineage following treatment with RAs, potent differentiation inducers for the granulocytic lineage, accompanied by the upregulation of C/EBPα and C/EBPε. In addition, the ectopic expression of C/EBPs induced HF-6 cells into monocytes. Consistent with this, C/EBPα and C/EBPε are also reported to induce monocytic differentiation: C/EBPα regulates the monocyte-colony-stimulating factor receptor gene and CD14 (Zhang *et al.*, 1996; Pan *et al.*, 1999), and contributes to monocytic commitment of primary myeloid progenitors by directly activating PU.1 (Wang *et al.*, 2006). C/EBPε also induces the gene expression of monocyte-colony-stimulating factor receptor (Williams *et al.*, 1998), and is required for the development and function of mature macrophages (Tavor *et al.*, 2002). The expression of chemokines MIP-1γ and MCP-3 is defective in macrophages from C/EBPε-deficient mice (Kubota *et al.*, 2000). This study indicates that HF-6 cells are presumably arrested at the monoblastic stage based on both morphological and immunophenotypical analysis. It may be presumed that the differentiation inducers including RAs and C/EBPs are shared in both monocytic and granulocytic differentiation, and that the cell lineage commitment and differentiation is dependent on the developmental level of the cell, rather than the types of differentiation inducers.

This study showed that the induction of *Sfpi1*, the mouse homolog of *PU.1*, was commonly observed in the C/EBPs-induced monocytic differentiation of HF-6 cells. This may be a key step in the differentiation, because PU.1 is crucial for the monocytic development. Although C/EBPα functions against PU.1 and induces granulocytic differentiation in granulocyte–macrophage progenitors (Dahl *et al.*, 2003), there have been several reports that show positive regulation of PU.1 by C/EBPα in other cell components. C/EBPα can bind and activate the PU.1 enhancer (Kummalue and Friedman, 2003). PU.1 mRNA can be upregulated by C/EBPα in the granulocytic differentiation of 32Dcl3 cells (Wang *et al.*, 1999). C/EBPs and PU.1 cooperatively regulate neutrophil esterase promoter and eosinophilic granule gene expression (Oelgeschlager *et al.*, 1996; Gombart *et al.*, 2003). Moreover, C/EBPs, namely C/EBPβ, are upregulated and bind to PU.1 promoter to induce the expression of PU.1 mRNA in ATRA-induced granulocytic differentiation of APL cells (Mueller *et al.*, 2006). These reports as well as the data presented here suggest that C/EBPs induce PU.1, and that C/EBPs and PU.1 function cooperatively in late granulocytic and monocytic differentiation after the granulocyte–macrophage progenitor stage, including myelomonocytic cells with *MLL*-fusion genes.

The gene expression profiles were similar between C/EBPα- and C/EBPε-induced HF-6 cells in this study. This implies a common mechanism in monocytic differentiation of HF-6 cells by C/EBPs. C/EBPε induction by the ectopic expression of C/EBPα-ER suggests that C/EBPε probably have an important function in monocytic differentiation of HF-6 cells. Another possible explanation is that C/EBPα and C/EBPε share the common targets in growth arrest and differentiation. C/EBPα is not only a transcriptional factor, but functions through protein–protein interaction. The target molecules in this interaction include the proteins related to cell cycle progression, such as cyclin-dependent kinase 2 (CDK2) inhibitor p21, pRB, E2F, CDK2 and CDK4 (Schuster and Porse, 2006). C/EBPε downregulates CDK4/6, cyclin D2/A/E, Bcl-2 and Bcl-x (Nakajima *et al.*, 2006), and has a direct interaction with E2F (Gery *et al.*, 2004; Walkley *et al.*, 2004). These interaction and downregulation are supposed to cause cellular growth arrest, thus resulting in the induction of apoptosis as well as monocytic differentiation, as a common pathway. These effects on cellular growth may be more potent than the induction of monocytic

Figure 6 The overexpression of Myc rescued the growth arrest and partially inhibited differentiation of HF6 cell induced by CCAAT/enhancer binding proteins (C/EBPs) activation. (**a**) The detection of the overexpressed Myc1 and Myc2 in HF-6 cells by immunoblotting. (**b**) Morphology of HF-6 cells with indicated viruses. May–Giemsa staining, magnification: ×600. (**c**) Flow cytometric analysis of HF-6/Myc1 and HF-6/Myc2. The intensity of Mac-1 and Gr-1 was slightly decreased in HF-6/Myc1 and HF-6/Myc2. (**d**) Detection of the overexpressed C/EBPs in HF-6/Myc1 and HF-6/Myc2. *: C/EBPα-ER, **: C/EBPε-ER. (**e**) The differentiation count of HF-6/Myc1 and HF-6/Myc2 cells expressing C/EBPα-ER or C/EBPε-ER on day 2. They were cultured with 1 μM of 4-HT. (**f**) Flow cytometric analysis of Mac-1 and Gr-1 in HF-6/pMYpuro, HF-6/Myc1 and HF-6/Myc2 cells expressing C/EBPα-ER or C/EBPε-ER. The analyses were performed on day 2. Dotted lines: with isotype controls. (**g**) The proliferation of HF-6/Myc1 and HF-6/Myc2 expressing C/EBPα-ER or C/EBPε-ER. The cells were cultured with 1 μM of 4-HT.

differentiation as observed in HF-6 cells, when the reinforcement of C/EBPs function is applied as an antileukemic therapy. However, mutated C/EBPα without transcriptional activity induces differentiation of APL cells *in vivo* but mutated C/EBPε cannot (Lee *et al.*, 2006), suggesting that C/EBPα and C/EBPε do not actually share the same molecular pathway of differentiation.

The crucial role of Myc in C/EBPs-induced monocytic differentiation of HF-6 cells was also demonstrated in this study. *Myc* induces transformation of hematopoietic cells cooperatively with *MLL-ENL in vitro* (Schreiner *et al.*, 2001). Myc inhibits the expression of C/EBPα by binding its promoter region (Freytag and Geddes, 1992). In contrast, C/EBPα negatively regulates c-Myc through the c-Myc promoter (Johansen *et al.*, 2001). C/EBPε also upregulates the Myc-antagonist Mad1 and downregulates *c-Myc* through repression of E2F1-mediated transcription (Gery *et al.*, 2004; Walkley *et al.*, 2004). Partial inhibition of C/EBPε-induced differentiation by c-Myc is also observed in 32D cells (Nakajima *et al.*, 2006). These observations suggest that Myc contributes to leukemogenesis by *MLL*-fusion genes through inhibition of C/EBPα expression, and that overexpression of C/EBPs downregulates *Myc* and induces differentiation. The present findings that C/EBPs induced monocytic differentiation of HF-6 cells are consistent with the underlying mechanism reported in previous studies.

However, in contrast to Myc, ectopic expression of internal tandem duplication of FLT3 (FLT3-ITD) cannot inhibit C/EBPs-induced monocytic differentiation in HF-6 cells (H Matsushita *et al.*, unpublished data). FLT3-ITD contributes to the cellular proliferation of AML cells through several signal pathways including the JAK/STAT, phosphoinositol 3-kinase and MAPK pathways. These observations suggest that growth arrest and differentiation induction by C/EBPα and C/EBPε are achieved through molecules functioning in the cell cycle rather than signals for cellular proliferation related to FLT3-ITD.

Human AML cells with *MLL*-fusion genes, MV4;11 and MOLM-14 cells, have also been reported to be differentiated by the induction of C/EBPα, however, the committed cell lineage was granulocytic, but not monocytic (Radomska *et al.*, 2006). This difference of the committed cell lineage could be dependent on the methods used for determination of the lineage. They evaluated only nitroblue tetrazolium reduction activity in MV4;11 with C/EBPα, and their granulocytic changes were not typical and definite. It is sometimes difficult to determine whether the lineage is monocytic or granulocytic in the differentiated cells from leukemic cells with conventional methods including cytochemical studies and immunophenotyping, because the differentiation processes share the same mechanisms and phenotypes in various degrees. Especially, these cells are neither normal granulocytes nor monocytes, and may not have the typical features of either of them. The other possibility is that this discrepancy could be due to differences in species or the developmental level, that is, the murine cell line HF-6 cells might be more strictly committed to the monocytic lineage than the human cell lines MV4;11 and MOLM-14 cells.

In summary, myelomonocytic cells with *MLL*-fusion gene were observed to cease the cellular growth and differentiate into monocytes by RA treatment concomitantly with upregulation of C/EBPα and C/EBPε or by induction of C/EBPα or C/EBPε activity. The downregulation of *Myc* is crucial, and C/EBPε may therefore have an important function in C/EBPs-induced differentiation. This is the first report to show the monocytic differentiation of myelomonocytic cells with *MLL*-fusion gene induced by ectopic expression of C/EBPε by itself, as well as C/EBPα. These findings may lead to the development of novel C/EBPs-modulating therapeutic approaches against AML with *MLL*-fusion genes.

Materials and methods

Reagents

All-*trans* RA, 9-*cis* RA and 4-HT (Sigma, St Louis, MO, USA) were resuspended in ethanol. Puromycin and blasticidin-S (Sigma) were diluted in distilled water. All the aliquots were stored at −20 °C.

Cell lines

The THP-1 and Kasumi cells were obtained from RIKEN BioResource Center (Ibaragi, Japan) and American Type Culture Collection (ATCC) (Manassas, VA, USA), respectively. MOLM-14 cells were provided from Cell Biology Institute, Research Center, Hayashibara Biochemical Laboratories (Okayama, Japan). They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 1 U/ml penicillin G, 1 μg/ml streptomycin. HF-6 cells were maintained in the same growth medium supplemented with murine interleukin-3 (10 ng/ml final concentration). The packaging cell line PLAT-E, a generous gift from Dr Toshio Kitamura (Institute of Medical Science, University of Tokyo, Tokyo, Japan), was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 μg/ml puromycin, 10 μg/ml blasticidin-S, 1 U/ml penicillin G and 1 μg/ml streptomycin. All cells were cultured at 37 °C and 5% CO_2.

Patient samples

Three cases of AML samples with 11q23 abnormalities were used (Supplementary Table 1). The AML cells were purified from bone marrow or peripheral blood using Ficoll as mononuclear cells, and were stored in a liquid nitrogen tank. The studies were conducted according to the guidelines of the revised Helsinki protocol, after informed consent from all patients' parents was obtained.

Myeloperoxidase and esterase staining

The Esterase staining kit, Esterase AS-D staining kit and New PO-K staining kit (Muto Pure Chemicals, Tokyo, Japan) were used for α-naphthyl butyrate, naphthol AS-D chloracetate and myeloperoxidase staining, respectively, according to the manufacturer's protocol.

Plasmids

pMY-IRES-GFP/C/EBPα-ER and pMY-IRES-GFP/C/EBPε-ER were generated by ligating either the C/EBPα-ER

or C/EBPε-ER fragment into pMY-IRES-GFP, respectively (Kitamura *et al*., 2003; Fukuchi *et al*., 2006; Nakajima *et al*., 2006). Two murine *Myc* isoforms (582–1946 for Myc1 and 627–1946 for Myc2 in NM_010849, respectively) were amplified by RT–PCR and ligated into pMYpuro to generate pMYpuro-Myc1 and pMYpuro-Myc2 (Kitamura *et al*., 2003).

Transfection, retroviral production and infection
These procedures were described previously (Nakajima and Ihle, 2001).

Assay for cell proliferation and apoptosis
Cellular proliferation and apoptosis were analysed using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) and Annexin V-Biotin Kit (Beckman Coulter, Fullerton, CA, USA), respectively. Streptavidin-allophycocyanin (APC) (BD Pharmingen, San Diego, CA, USA) was used to detect annexin V-biotin in an analysis of apoptosis.

Flow cytometry
The cells were preincubated in phosphate-buffered saline supplemented with 0.2% human γ-globulins (Sigma) for 15 min at 4 °C. They were then incubated with the monoclonal antibodies for 30 min at 4 °C. The applied monoclonal antibodies included anti-PE-conjugated anti-mouse CD117 (c-Kit), CD11b (Mac-1), and Ly-6C (Gr-1), FITC-conjugated anti-mouse CD34, Ly-6A/E (Sca-I) and Gr-1, APC-conjugated anti-human CD34, PE-conjugated anti-human CD11b and FITC-conjugated anti-human CD33 and CD36 (BD Pharmingen). An analysis was performed using FACSCaliber (Becton Dickinson, Franklin Lakes, NJ, USA).

Clonogenic assay
To delete normal hematopoietic stem cells and increase the purity of AML cells, AML samples were sorted out using FACS/Vantage (Becton Dickinson) according to the expression of their surface marker. All of the primary AML samples did not express CD34 (Supplementary Table 1). The purity of AML progenitors was evaluated by a FISH analysis using 5′- and 3′-MLL probe (Supplementary Table 1). A clonogenic assay was performed as described previously (Kawada *et al*., 1999), with or without 10^{-6} M of ATRA or 9-*cis* RA. L-CFU-G, L-CFU-GM and L-CFU-M from primary AML cells were counted on days 11–14.

Quantitative RT–PCR
Total RNA was extracted with Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized with SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed with SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio, Shiga, Japan) in LightCycler ST300 (Roche Diagnostics, Indianapolis, IN, USA). The relative levels of gene expression were calculated using standard curves generated by the serial dilution of the PCR products. The mRNA content was measured relative to that of murine *Gapdh*. All the samples were independently analysed at least three times for each gene. The primer pairs are shown in Supplementary Table 2.

Immunoblotting
The procedures were described previously (Nakajima and Ihle, 2001). The applied primary antibodies included rabbit serum against C/EBPα (14AA), C/EBPε (C-22), estrogen receptor-α (ER) (MC-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), green fluorescent protein (Invitrogen) and Myc (Upstate, Lake Placid, NY, USA), and monoclonal antibodies against β-actin (AC-15) (Sigma).

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CASE REPORT

A complex t(1;22;11)(q44;q13;q23) translocation causing *MLL-p300* fusion gene in therapy-related acute myeloid leukemia

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Abstract

The p300 protein shows a striking homology with cyclic-AMP-response-element-binding-protein binding protein (CBP) and both proteins form a family of DNA-binding transcriptional coactivators/histone acetyltransferases. The authors, herein, report a therapy-related acute myeloid leukemia with *MLL-p300* fusion gene. Spectral karyotyping clarified that chromosome 11 is involved in complex t(1;22;11)(q44;q13;q23), and is fused to the chromosome 22, and direct sequencing revealed the fusion of exon 8 of *MLL* and exon 15 of *p300* in this case. This is only the second reported case of leukemia with an *MLL-p300* fusion gene, and the other case with *MLL-p300* was also a therapy-related leukemia. Considering that the *MLL-CBP* fusion gene is also found almost exclusively in therapy-related leukemia, the association of *MLL-p300* and *MLL-CBP* with therapy-related leukemia rather than *de novo* leukemia is thereby suggested.

Key words *MLL*; *p300*; acute myeloid leukemia; therapy-related leukemia; spectral karyotyping

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The 11q23 translocations are frequent in hematological malignancies, occurring in both childhood (approximately 10%) and adult (approximately 5%) acute leukemias, and in most patients with therapy-related leukemia induced by topoisomerase II inhibitors (1). The *MLL* gene is rearranged as a consequence of 11q23 translocation, and at least 60 partner genes for *MLL* have so far been identified (2).

p300, which was originally cloned as a nuclear binding target of the adenovirus E1A oncoprotein, forms a family with cyclic-AMP-response-element-binding-protein (CREB) binding protein (CBP) (3). Both *p300/EP300* and *CBP* encode histone acetyltransferases (HAT) which regulate transcription via chromatin remodeling and are important in the processes of cell proliferation and differentiation (4–6). The mutation and translocation of *p300* and *CBP* genes have been observed in a subset of tumors and hematological malignancies, respectively, thus suggesting the involvement of these genes in the development of human cancer (4–6). We have previously reported the *p300* gene to be fused to the *MLL* gene in a case of therapy-related acute myeloid leukemia (AML) with t(11;22)(q23;q13) (7). Previous studies also revealed that *CBP* forms a fusion gene with *MLL* in AML, and all but one of the reported cases was therapy-related AML (8–12). These results imply that the *MLL-p300* and *MLL-CBP* fusion genes are associated with the development of therapy-related leukemia rather than with *de novo* leukemia. Nevertheless, this association has not been confirmed in *MLL-p300* because no other case with the *MLL-p300* fusion gene has been reported to date. We herein report

a second case of therapy-related AML with *MLL-p300*, which further suggests the association of this fusion gene with therapy-related leukemia.

Case report

A 5-yr-old girl was admitted to our hospital because of a continuous fever and petechiae. She had been diagnosed with neuroblastoma at the age of 2, and treated by an operation followed by 6 months of chemotherapy including pirarubicin, carboplatinum and cyclophosphamide. On admission, she presented with lymphadenopathy but no hepatosplenomegaly. The peripheral blood data were hemoglobin 51 g/L, platelet 7.0×10^9/L and white blood cells 5.1×10^9/L (blasts 2.5%). The diagnosis from the bone marrow aspirate was AML M1 according to the French-American-British classification or AML without maturation according to the WHO classification. Immunophenotyping by CD45 gating revealed the blast cells to be positive for CD4, 7, 13, 19, 33, 34 and human leukocyte antigen (HLA)-DR but negative for CD3, 41 and glycophorin A. She was treated by two courses of chemotherapy followed by cord blood transplantation from an unrelated donor. She is presently alive and free of leukemia and neuroblastoma at 1 yr after transplantation.

Materials and methods

Karyotyping

Regular G-banding karyotyping of the leukemic cells was performed according to previously described protocols. A spectral karyotyping (SKY) was carried out with a SkyPaint kit (Applied Spectral Imaging, Migdal Ha'Emek, Israel) (13). The signal detection was performed according to the manufacturer's instructions.

Southern blot analysis

High-molecular-weight DNA was extracted from the bone marrow samples obtained at the diagnosis of AML, and 10 mg of DNA was analyzed as reported previously (14). A 0.9-kb MLL cDNA probe and restriction enzymes *Bam*HI and *Hind*III were used.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and direct sequence analysis

The total RNA was extracted from the bone marrow samples obtained at the diagnosis of AML, and reverse-transcribed to cDNA as previously described (7). The primers used to amplify the *MLL-p300* fusion transcripts were as follows: MLL-7S: TCCTCAGCACTCTCTCCAA (within exon 7 of *MLL*), MLL-9S: GGTGTTGTCGTCGTTGCAAA (within exon 9 of *MLL*), p300-7.5A: GCTGAAGTACTTGGCTGGTC (within exon 16 of *p300*) and p300-8A: GGCTCCTGATACTGTCCAGT (within exon 18 of *p300*). The *MLL* exon numbering system proposed by Rasio *et al.* (15) was utilized in this study. The positive bands were cut out from the agarose gel and subjected to a direct sequencing analysis using the same primers described above.

Fluorescence *in situ* hybridization (FISH)

A FISH analysis of the *p300* gene was performed according to the previously described protocols using the probes RP11-188A17 and RP11-107O11 (16).

Results

A chromosomal analysis of the blast cells by the G-banding method showed 46,XX,t(1;11)(q44;q23),t(10;17)(q22;q21) in 20 out of the 20 investigated cells (Fig. 1A), and all these abnormalities disappeared in the bone marrow cells at the remission. A Southern blot analysis of the blast DNA revealed rearrangement of the *MLL* gene (data not shown). Because of the complex karyotype, we performed a SKY to confirm the result of the chromosomal analysis. The SKY demonstrated that chromosome 11 was actually involved in complex t(1;22;11)(q44;q13;q23), and the centromeric region of chromosome band 11q23 was fused to the chromosome 22 instead of the chromosome 1 (Fig. 1B). Previously, two genes, *CDCREL1* at 22q11.2 and *p300* at 22q13, were reported to be involved as translocation partners in t(11;22)(q23;q11.2) and t(11;22)(q23;q13), respectively (2). Of these, *p300* was assumed to be closely associated with therapy-related leukemia, because the cases with *MLL-p300* or *MLL-CBP* fusion were mostly therapy-related leukemia, although cases with *MLL-CDCREL1* were not. We therefore conducted RT-PCR in order to detect the *MLL-p300* fusion gene. Two bands (a clear band with slower electrophoresis mobility and a faint band with faster electrophoresis mobility) were detected by PCR with either primer set MLL-7S/p300-7.5A or MLL-7S/p300-8A, although no positive band was detected by the primer sets MLL-9S/p300-7.5A or MLL-9S/p300-8A (Fig. 2A). A direct sequencing of the clear band identified the fusion of exon 8 of *MLL* and exon 15 of *p300* (Fig. 2B). A direct sequencing of the faint band identified the fusion of exon 7 of *MLL* and exon 15 of *p300*, which was considered to be generated by alternative splicing. A FISH using the probes RP11-188A17 and RP11-107O11 confirmed the cleavage of the *p300* gene (Fig. 1C).

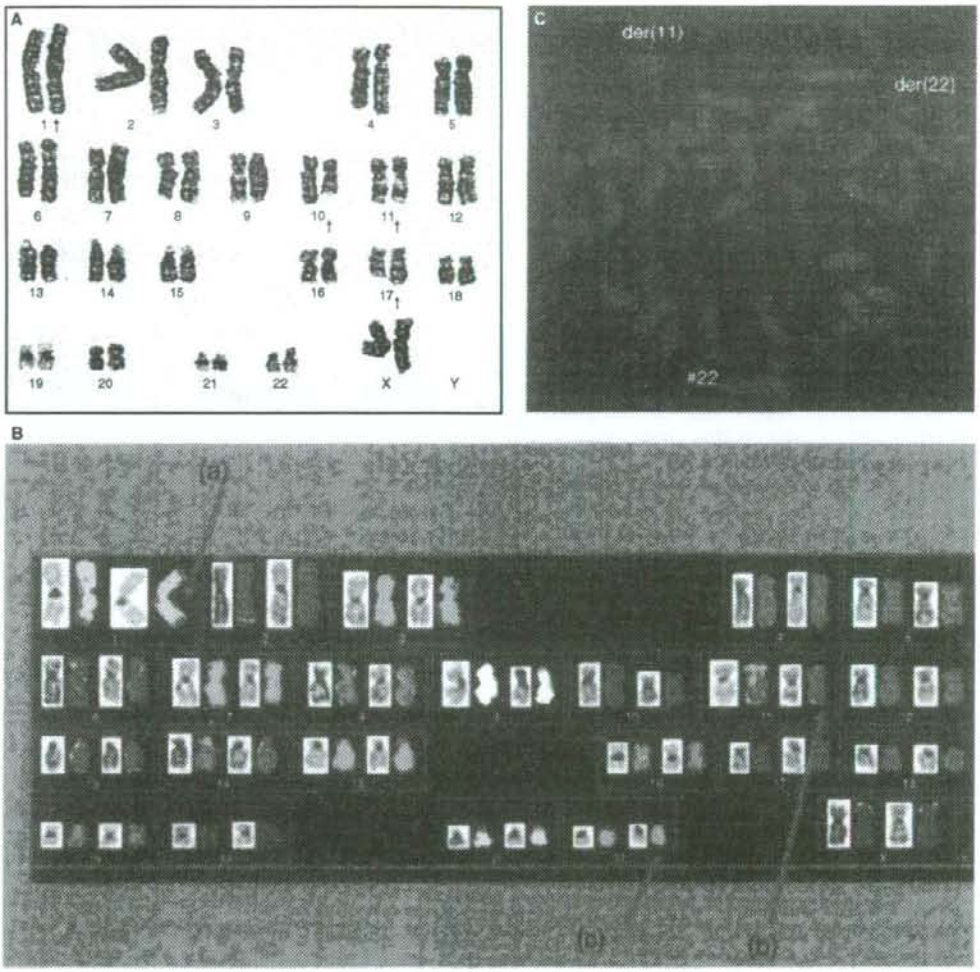

Figure 1 Chromosomal analyses of leukemic cells. (A) Conventional G-banding. Arrows denote the chromosomes assumed to be involved in translocation. (B) Spectral karyotyping. Arrows (a), (b), and (c) indicate der(11q), der(22q), and der(1q), respectively, (C) FISH. Green signals represent the probe RP11-188A17 which binds to the centromeric side of the *p300* gene on chromosome 22. Red signals represent the probe RP11-1078O11 which binds to the telomeric side of the *p300* gene. The split of the both signals, shown as der(11) and der(22), indicates the cleavage of the *p300* gene.

Discussion

This is only the second reported case of leukemia with *MLL-p300* fusion gene. The sole case with *MLL-p300* fusion was previously described by our group, and it was also a therapy-related leukemia following chemotherapy by topoisomerase II inhibitor. In addition, *MLL-CBP* has been detected almost exclusively in cases with therapy-related leukemia following topoisomerase II inhibitor therapy (9). As *p300* and *CBP* represent a striking homology, it is possible that common mechanisms may be involved in the translocation of *p300* or *CBP* to *MLL*. In a previous study, Zhang *et al.* (17) have characterized the genomic breakpoints in six cases of leukemia with *MLL-CBP* fusion. Despite intensive attempts, however, they could not detect any topoisomerase II cleavage sites in the regions surrounding the breakpoints of *MLL* or *CBP*. Several studies analyzing other types of translocation have provided inconsistent results regarding the association of genomic breakpoints and topoisomerase II

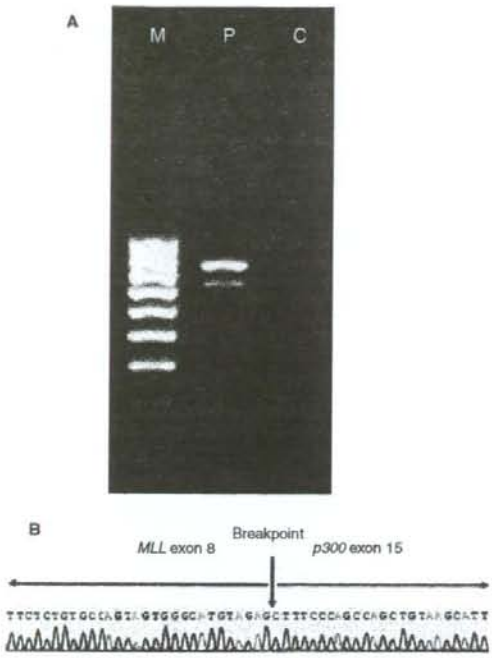

Figure 2 Results of (A) RT-PCR and (B) direct sequencing of the fusion gene. (A) Bone marrow cells of the patient at diagnosis (P) and a negative control (C). M: 1-kb marker. A primer set MLL-7S/p300-8A was used. (B) Direct sequencing of the upper band demonstrated fusion of exon 8 of *MLL* and exon 15 of *p300*.

cleavage sites in therapy-related leukemias (17–19). These results suggest that the association of the genomic breakpoints of translocations in therapy-related leukemias and the topoisomerase II cleavage sites is more complicated than has been assumed. Further accumulation of cases with *MLL-p300* and an analysis of the genomic breakpoints may provide insight regarding this issue.

A schematic presentation of the predicted MLL-p300 fusion protein in this case and the previously reported

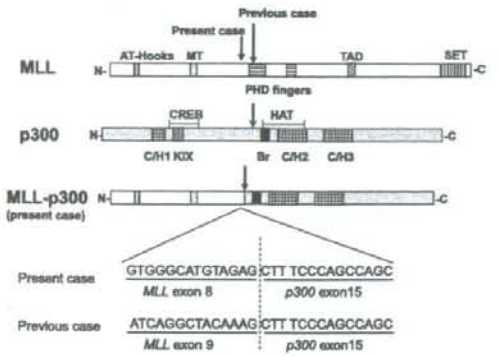

Figure 3 Schematic representation of the predicted MLL-p300 fusion proteins of the present and the previous reported cases. Arrows denote the breakpoints of each gene. MT, methyltransferase; TAD, transactivation domain; CREB, CREB-binding region; HAT, histone acetyltransferase; C/H, cysteine/histidine rich region; Br, bromodomain.

case is shown in Fig. 3. In both cases, the AT-hook, DNA methyltransferase, and a transcriptional repression domain of MLL are retained. However, a part of the plant homeodomain finger domain retained in the chimeric protein in the former case was lacking in the present case, suggesting that this domain is not critical in the development of leukemia in cases with MLL-p300. On the other hand, both chimeric proteins retained the identical part of p300 which includes the HAT domain and the transactivation domain but does not include the CREB-binding region. While MLL-CBP fusion proteins have been reported to retain almost all parts of CBP (9), the CREB-binding region may not be crucial for developing leukemia in cases with MLL-p300 or MLL-CBP fusion. Recent studies suggest that different mechanisms might be involved in the leukemogenesis of MLL fusion proteins, depending on whether the fusion partner is nuclear or cytoplasmic factors (20). The nuclear factors such as CBP and p300 have transcription activity, and this function might be deregulated by fusion with MLL (6). Furthermore, an aberration of other HAT-associated

Table 1 Clinical and biological features of the two cases with *MLL-p300*

	Sex	Age (yr)	Former malignancy	Topoisomerase dose	Latency[1] (month)	Subtype of leukemia	Immunophenotype	Cytogenetics	Fusion gene
Previous case (7)	M	9	Non-Hodgkin lymphoma	Etoposide 5200 mg/m^2	67	AML M1	CD7, 33	48,XY,+8,+8, t(11;22)(q23;q13)	*MLL* exon 9/*p300* exon 15
Present case	F	5	Neuroblastoma	Pirarubicine 300 mg/m^2	36	AML M2	CD4, 7, 13, 19, 33, 34	46,XX,t(1;22;11)(q44;q13;q23)[2], t(10;17)(q22;q21)	*MLL* exon 7 and 8/*p300* exon 15

AML, acute myeloid leukemia.
[1]Latency between the onset of the former malignancy and the secondary leukemia.
[2]Revealed by spectral karyotyping.

genes is also known to be involved in the development of leukemia. For example, *MOZ* or *MORF* is involved in translocation of AML in the forms of *MOZ-CBP*, *MOZ-p300*, *MOZ-TIF2* and *MORF-CBP* (19, 21–23). *TIF2* is also a HAT-associated gene, and all of these translocations retain the HAT domain in involved genes. These results suggest an association of activated transcription by HAT with leukemogenesis in these fusion transcripts. A further functional analysis of these fusion proteins will give insight into the mechanism of leukemogenesis in these cases (Table 1).

SKY was useful for revealing cryptic t(11;22)(q23;q13) in this case. Performing SKY for blast cells is thereby recommended unless a known translocation is detected by conventional G-banding after a diagnosis of leukemia has been made. However, SKY is not always sufficiently informative. In cases where one gene is found to be rearranged but the translocation partner gene is undetectable, other PCR-based methods, such as panhandle PCR or bubble PCR, are also known to be useful in revealing the translocation partner gene (24, 25). The clarification of a cryptic translocation and a fusion gene using these techniques will provide further information regarding the phenotype of leukemia, while it is also helpful in clinical settings, such as when used for the detection of minimal residual disease.

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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.

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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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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.

diluted 1:200) and PDGFRβ (SantaCruz, sc-6252, diluted 1:200) were used. We also analyzed the expression of KIT and PDGFRβ in 6 ganglioneuroma samples (Table III). GIST specimens were used for the positive controls. The evaluation of immunohistochemistry was performed by two independent observers (AS and JH). We evaluated the complete cytoplasm and membrane staining in more than 30% of cells as positive, and cytoplasm or membrane staining in less than 30% of cells as negative. We considered that the positive specimens showed the expression of the protein.

Statistical Analysis

Statistical analysis was performed using Statview software (SAS). The χ^2-test was used to correlate the categorical variables. The prognostic significance of the clinical variables was assessed by using Cox proportional hazards model. For all analyses, the *P* values were 2-tailed, and a *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Expression and Mutation Analysis of *KIT*

KIT mRNA expression was found in 22 (91.7%) of 24 cell lines with RT-PCR (Supplemental Table I). All cell lines predominantly showed a 12 bp (GGTAACAACAAA) deleted product (GNNK$^-$ isoform) at the end of the extra cellular domain (exon 9) compared to the wild-type of *KIT* (Supplemental Fig. 1) [27]. We could not find any activating mutations as previously reported in GIST and AML [10,11]. Two single nucleotide polymorphisms (SNPs) were found [541aa, A > C of 1642 bp in exon 10 (Reference SNP (refSNP) Cluster Report: rs 3822214 by NCBI) in SCMC-N4 and SKNSH, 862aa, G > C of 2,607 bp in exon 18 (rs 3733542) in SJNB-5 and SKNSH]. A silent mutation was also found (I798I, ATC > ATT of 2,414 bp in exon 17 in SJNB-8). All cell lines, except for one, expressed *SCF*. Both soluble and membranous bound forms of *KIT* mRNA were found.

KIT expression was detected in 32 (80.0%) of 40 tumor samples by RT-PCR (Table I) and 23 (60.5%) of 38 paraffin sections of tumor samples by immunohistochemistry (Table II). The expression of mRNA and protein was measured in ten patients using both RT-PCR and immunohistochemistry. The expression of *KIT* mRNA and protein was associated with NB patients under 1 year ($P = 0.0016$, 0.0074, respectively) and inversely associated with *MYCN* amplification ($P = 0.0006$, 0.017, respectively) and Stages 3 or 4 NB patients over 1 year old ($P < 0.0001$, 0.014, respectively). *KIT* mRNA expression was significantly associated with *TRKA* mRNA expression ($P < 0.001$). Multivariate analysis showed the coefficient of correlation between *KIT* mRNA and *TRKA* mRNA was 0.627 (0.398–0.785, $P < 0.001$) and between KIT mRNA and survival was 0.665 (0.446–0.809, $P < 0.001$). The KIT protein expression was found in two of four differentiating NB samples and five of six samples of ganglioneuroma (Table III). The difference of expression rate of KIT protein between neuroblastoma (NB) and ganglioneuroma or between differentiating and poorly differentiated NB were not statistically significant.

Expression and Mutation Analysis of *PDGFRs*

PDGFRα mRNA was detected in all cell lines and tumor samples by RT-PCR (Supplemental Table I). As for the mutation of *PDGFRα*, no activating mutations were found. Three SNPs were found (567aa A > G of 1,849 bp in exon12 (rs 1873778) in SJNB4, 603aa G > A of 1957 bp in exon13 (rs 10028020) in SJNB4, NB16, NB69, LAN2, and SKNSH, 824aa C > T of 2,620 bp in exon 18 (rs 2228230) in SJNB-5, SJNB-8, NB-19, LAN-1, LAN-5, and SKNSH). Silent mutation was found in GOTO (V533V, GTG > GTA of 1,747 bp in exon 11). PDGFRα protein was strongly expressed in almost all tumor samples by immunohistochemistry.

PDGFRβ mRNA was expressed in 14 (58%) of 24 cell lines and 29 (73%) of 40 NB samples using RT-PCR (Tables I and II). *PDGFRβ* was expressed in 24 (63%) of 38 tumor samples by immunohistochemistry (Table III). The expression of *PDGFRβ* mRNA and protein was associated with NB patients under 1 year ($P = 0.0014$ and 0.019, respectively) and inversely associated with *MYCN* amplification ($P = 0.0198$ and 0.0052, respectively), advanced stage patients one year old and over ($P = 0.0046$ and 0.0011, respectively). The correlation between *PDGFRβ* and *TRKA* mRNA expression was significant ($P = 0.0003$). Multivariate analysis showed the coefficient of correlation between *PDGFRβ* mRNA and *TRKA* mRNA was 0.574 (0.320–0.751, $P < 0.001$) and between *PDGFRβ* mRNA and survival was 0.525 (0.256–0.719, $P = 0.004$). The correlation between PDGFRβ protein expression and a favorable histology was also significant ($P = 0.0021$). The

TABLE III. Correlation of KIT and PDGFRβ Expression to Histopathology of NB According to INPC System

INPC system	Number of patients	KIT (%)	PDGFRβ (%)
Neuroblastoma (Schwannian stroma-poor)			
Undifferentiated	1	0	0
Differentiating	4	2 (50)	4 (100)
Poorly differentiated	27	17 (63)	16 (59.3)
Ganglioneuroblastoma			
Intermixed (Schwannian stroma-rich)	4	2 (50)	4 (100)
Nodular	2	2 (100)	1 (50)
Total	38	23 (60.5)	25 (65.8)
Ganglioneuroma (Schwannian stroma-dominant)	6	5 (83.3)	6 (100)

The difference of expression rate of KIT or PDGFRβ protein between neuroblastoma and ganglioneuroma was not statistically significant. The difference of expression rate of KIT or PDGFRβ protein between differentiating and poorly differentiated neuroblast.

expression of PDGFRβ was found in all four differentiating NB samples and all five ganglineuroblastoma samples (Table III). The difference of expression rate of PDGFRβ protein between NB and ganglioneuroma or between differentiating and poorly differentiated NB were not statistically significant.

Expression and Mutation Analysis of *FLT3*

FLT3 mRNA expression was detected in 19 (79.2%) of 24 cell lines and in 32 (80%) of 40 tumor samples by RT-PCR (Table I and Supplemental Table I). No ITDs or kinase domain mutations were observed in any cell lines. *FLT3* expression was associated with NB patients under 1 year ($P = 0.0177$) and *TRKA* expression ($P < 0.0001$; Table I). Inverse correlations were observed for *MYCN* amplification ($P = 0.0019$) and advanced stage patients over one year old ($P = 0.0011$). FLT3 protein expression was not examined.

Expression and Mutation Analysis of *RET*

RET expression was detected in 22 (91.6%) of 24 cell lines and in 17 (42.5%) of 40 tumor samples by RT-PCR (Table I and Supplemental Table I). However, no mutations were found in this study. We identified SNPs (691aa or 769aa) of the *RET* gene. *RET* expression was not associated with any clinical findings (Table I). Furthermore, we examined the expression of both isoforms RET51 and RET9. There were no correlations between the RET isoforms and the clinical findings.

Expression and Mutation Analysis of *TRKA*

TRKA expression was detected in 7 (29.2%) of 24 cell lines and in 28 (70.0%) of 40 tumor samples by RT-PCR (Table I and Supplemental Table I). *TRKA* expression was associated with NB in patients under age 1 year ($P = 0.0006$) and with good prognosis (Table I). We examined the expression of the TRKA isoform, but did not detect isoform III in any cell lines or tumor samples [20]. On the other hand, we found another novel isoform (deletion of exons 7–9) in 6 (25%) in 24 cell lines (SJNB-2, SJNB-6, NB1, TGW, SKNSH, SCMC-N4) with the coexpression of isoforms I or II, which we referred to as isoform IV in this article (Supplemental Fig. 2). However, we could not find this isoform IV in any of 40 tumor samples.

DISCUSSION

The aberrant expression of KIT and SCF has been reported in several solid tumors, such as small cell lung cancer [28], gynecological tumors [29], and breast cancer [30]. However, *KIT* mutations are rarely reported in other cancers [31–33] except for GIST [10] and the core-binding factor AML [11]. An autocrine or paracrine loop of KIT and SCF has been hypothesized in NB cell proliferation [34]. Moreover, the GNNK$^-$ isoform of *KIT* has been shown to be predominantly expressed in varieties of tumors, such as AML and germ cell tumor [35,36], and the GNNK$^-$ isoform has a growth advantage compared with the GNNK$^+$ isoform and phosphorylates downstream signals, such as MAP and STAT kinases [27]. In this study, KIT expression was associated with NB patients under 1 year of age and good prognosis as previously reported [7]. The GNNK$^-$ isoform was predominantly expressed in NB patients. An inverse correlation between KIT expression and *MYCN* amplification was observed and it supported the observation of Krams et al. [7]. On the other hand, KIT expression has been reported to be associated with a poor prognosis and with *MYCN* amplification in NB [4,9]. These different results may due to the differences of experimental method, race or the number of patients analyzed. Moreover, the loss of KIT expression has also been reported in advanced cancer, including breast cancer [32], melanoma [37], thyroid cancer [38], and ovarian cancer [39]. The loss of KIT expression may be associated with NB tumor progression.

PDGFRs and their ligands, PDGFA and PDGFB, have an important role not only in embryogenesis, but also in the progression of some tumors, suggesting the presence of an autocrine or paracrine mechanism [40,41]. PDGFRs can become potent oncoproteins when they are overexpressed or mutated [40–42]. The intensive expression of PDGFRα protein was detected in this study, suggesting that expressed PDGFRα may be the therapeutic target for the kinase inhibitor, imatinib. On the other hand, the expression pattern of PDGFRβ was associated with good clinical outcome in NB similar to KIT. PDGFRβ has been considered to have oncogenic potential compared to PDGFRα [14].

FLT3 expression was associated with a good clinical outcome of NB in our study. Our results may provide the evidence that neuroectodermal and hematopoietic cells share common regulatory pathways, as previously reported [15]. It was reported that the RET and TRKA pathways collaborate to regulate NB differentiation [16], but RET expression was not associated with TRKA expression or any clinical parameters in present study. We could not find the alternative spliced variant form of TRKA, TRKAIII, which was reported to have the oncogenic potential [20]. We found another new isoform (deletion of exons 7–9) in 6 (25%) of 24 cell lines. Further study is needed to clarify the function of this new isoform.

In conclusion, our data suggest that the loss of expression of several RTKs may be related to disease progression and poor clinical outcome in NB.

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Short communication

Transient abnormal myelopoiesis in a Down syndrome newborn followed by acute myeloid leukemia: identification of the same chromosomal abnormality in both stages

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Abstract A transient abnormal myelopoiesis was observed in a newborn with Down syndrome. Cytogenetic study revealed multiple oligoclonal abnormalities: 47,XY,inv(6)(p23q21),+21c[3]/47,XY, der(7)t(1;7)(q25;p15),+21c[1]/47,XY,del(13)(q?),+21c[1]/47,XY,+21c[15]. Ten months after the patient achieved remission, the transient abnormal myelopoiesis evolved to an acute megakaryoblastic leukemia. Cytogenetic study revealed only a single clonal abnormality, 47,XY,der(7)t(1;7)(q25;p15),+21c, identical to one of the structural changes seen at birth. Sequence analysis of the *GATA1* gene revealed a deletion–insertion mutation within the exon 2 introducing a stop codon after Arg 64. It may be that the der(7)t(1;7)(q25;p15) abnormality played some selective role in the development of acute megakaryoblastic leukemia in this patient. To our knowledge, the present case is unique in demonstrating a subclone with der(7)t(1;7)(q25;p15) evolving to acute leukemia.

1. Introduction

Approximately 10% of patients with Down syndrome are born with transient abnormal myelopoiesis (TAM) [1,2]. Of these cases, ~20% recur as acute megakaryoblastic leukemia (AMKL) [3]. Acquired mutations in *GATA1* in the leukemic blasts are detected in virtually all of these cases [4–7], but the second hit for the full expression of AMKL is still a matter of discussion [6,7]. GATA1 is a transcription factor that regulates megakaryocytic differentiation, and the mutations observed are considered to cause accumulation of poorly differentiated megakaryocytic precursors [4]. Strikingly, *GATA1* is located on chromosome X; therefore, genetic interaction of *GATA1* with one or more genes on other chromosomes presumably contributes to the development of AMKL in Down syndrome.

Acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) in Down syndrome often demonstrate chromosomal abnormalities in addition to the constitutional trisomy 21 [8–12]. These include both numerical and structural abnormalities, mostly complete or partial trisomy of a specific chromosome; reciprocal translocations are relatively rare. The role of these chromosomal translocations in developing leukemia in Down syndrome is also unknown. Here, we report the case of a Down syndrome patient who developed AMKL showing der(7)t(1;7)(q25;p15) following TAM at birth.

2. Case report

This Down syndrome patient was a boy born at 39 weeks gestational age, weighing 3,235 g. Thrombocytopenia was revealed after birth. At 5 days after birth, he was diagnosed as having TAM. Initial white blood cell count was 13,200/μL with 7% blasts, hemoglobin was 15.0 g/dL, and platelet count was 52,000/μL. Chromosomal analysis of bone marrow cells at diagnosis revealed the

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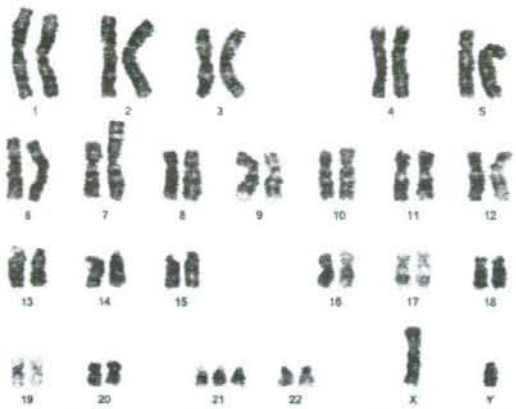

Fig. 1. Karyogram of the blast in leukemic phase showing 47,XY,der(7)t(1;7)(q25;p15),+21c.

karyotype 47,XY,inv(6)(p23q21),+21c[3]/47,XY,der(7)t(1;7)(q25;p15),+21c[1]/47,XY,del(13)(q?),+21c[1]/47,XY,+21c[15]/. Although he was not treated, his white blood cell count gradually decreased within 2 months, and at 7 months after birth his platelet count spontaneously recovered to within the normal range (although he suffered from thrombocytopenia due to an unknown viral infection). At 10 months after birth, the blasts increased suddenly.

Persistent thrombocytopenia was noted after platelet transfusions twice weekly. A bone marrow aspirate showed excessive myelofibrosis. Immunophenotyping of peripheral blasts showed $CD7^+$, $CD13^+$, $CD33^+$, $CD38^+$, $CD41^+$, $CD42b^+$, $CD56^+$, $CD157^+$, and $HLA\text{-}DR^+$. Chromosome analysis showed a der(7)t(1;7)(q25;p15) abnormality, in addition to the constitutional trisomy 21 (Fig. 1). Spectral karyotyping further confirmed the presence of the t(1;7) translocation, expressed as 47,XY,der(7)t(1;7)(q25;p15),+21c (Fig. 2), resulting in partial trisomy of 1q.

We analyzed the *GATA1* mutation in the peripheral blood sample, after written informed consent was obtained from his parents. Genomic DNA was extracted, and then polymerase chain reaction (PCR) was performed. Subcloning and nucleotide sequencing of PCR products were performed as described previously [6]. Sequence analysis of *GATA1* gene revealed a deletion–insertion mutation within exon 2, introducing premature stop codon after Arg 64 (Fig. 3).

A trephine biopsy revealed the presence of a typical megakaryocyte proliferation and prominent fibrosis. The final diagnosis of AMKL led to the initiation of combination therapy of pirarubicin HCl (25 mg/m^2 per day for 2 days), cytosine arabinoside (100 mg/m^2 per day for 7 days), and etoposide (150 mg/m^2 per day for 3 days) [13]. Complete remission was achieved after two courses of the therapy. Continuation of the consolidation therapy was uneventful, and six cycles of the same regimen were completed. As of writing, the patient had been in continuous complete remission without marked side effects for 5 years after initiation of the therapy.

3. Discussion

Recent collaborative studies on AML in Down syndrome children have established that the most frequent cytogenetic abnormality in AML or MDS in Down syndrome is trisomy 8 or partial trisomy of 1q [2,14–17]. Because AML and MDS with Down syndrome have distinct biologic and clinical features, the identification of Down syndrome patients with a mild or normal phenotype in the AML/MDS population is of fundamental importance for clinical diagnosis and management. Partial trisomy of 1q

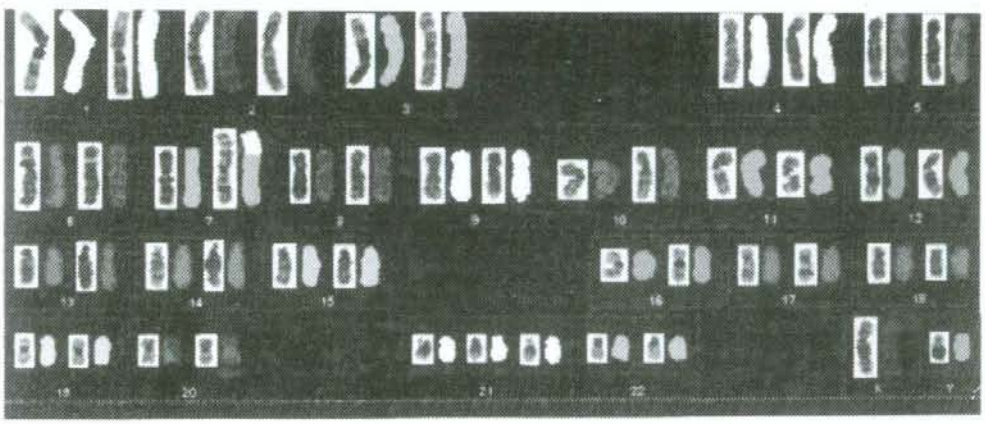

Fig. 2. Spectral karyotyping showing 47,XY,der(7)t(1;7)(q25;p15),+21c.

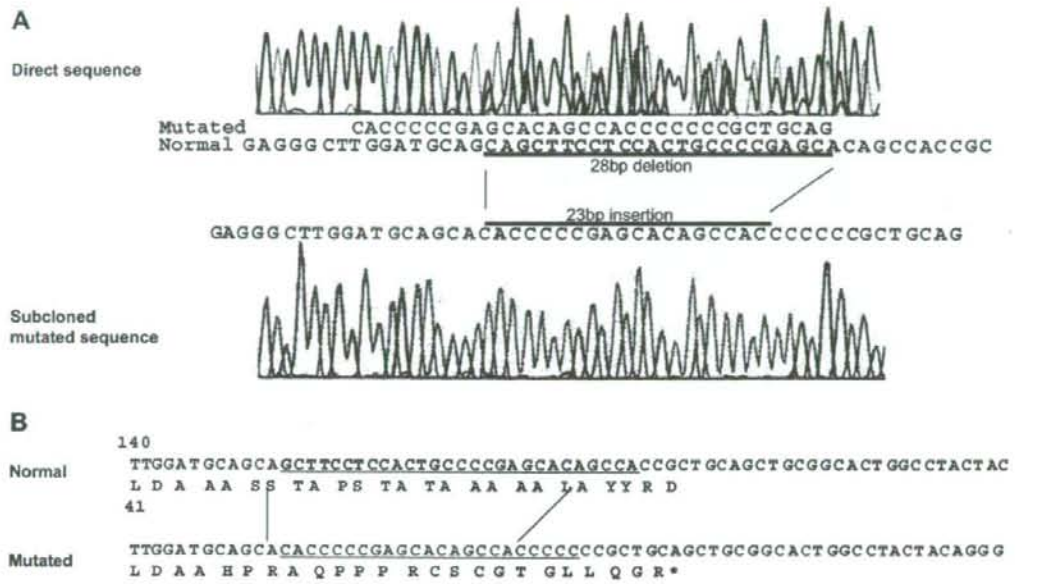

Fig. 3. Mutational analysis of the *GATA1* gene. (A) Sequence analysis was performed directly and using subcloned polymerase chain reaction product and showed a deletion–insertion mutation (28-bp deletion and 23-bp insertion) between 152 and 179 within exon 2. (B) This deletion–insertion mutation introduced a premature stop codon after Arg64. Numbers represent nucleotides from the 5′ end of exon 2.

has been reported by several authors and appears to represent a nonrandom chromosomal abnormality in patients with MDS/AML and Down syndrome [14,17]. Partial trisomy of 7q [8] or monosomy 1 [18,19] have also been reported. Unbalanced translocation t(1;7) in childhood myelodysplasia has been reported [20]. It is also possible that the t(1;7) played some role in the development of the MDS [21]. The mechanism of formation of the der(7)t(1;7) and its role in leukemogenesis are still unclear. Given that der(7)t(1;7) results in partial trisomy of 1q and partial monosomy of 7q, the increased dosage of the oncogenes located at 1q or the loss of the tumor suppressor genes located at 7q (or both factors) may be implicated in leukemogenesis of MDS and AML with der(7)t(1;7).

Cases of TAM usually have no karyotypic abnormality [1], but AMKL is associated with chromosomal abnormalities, including 8 trisomy and 19 trisomy [2]. Rare TAM cases have had chromosome abnormalities that were also observed in developing AMKL [22]. As for the *GATA1* gene, the deletion–insertion mutations within exon 2 in our patient have been reported previously in only two cases of TAM [5,7]. Reciprocal translocations are rare in TAM with Down syndrome. In the present case, a der(7)t(1;7) with partial trisomy of 1q, which is among the most frequently observed abnormalities in Down syndrome, might contribute to evolution to acute leukemia. The present report contributes insight into the mechanism of leukemic transformation from TAM in Down syndrome.

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