Grieder, N.C., Morata, G., Affolter, M., and Gehring, W.J. (2009). Spalt major controls the development of the notum and of wing hinge primordia of the *Drosophila melanogaster* wing imaginal disc. Dev. Biol. *329*, 315–326.

Heuser, M., Yun, H., Berg, T., Yung, E., Argiropoulos, B., Kuchenbauer, F., Park, G., Hamwi, I., Palmqvist, L., Lai, C.K., et al. (2011). Cell of origin in AML: susceptibility to MN1-induced transformation is regulated by the MEIS1/AbdB-like HOX protein complex. Cancer Cell *20*, 39–52.

Higuchi, M., O'Brien, D., Kumaravelu, P., Lenny, N., Yeoh, E.J., and Downing, J.R. (2002). Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia. Cancer Cell *1*, 63–74.

Hui, C.C., and Angers, S. (2011). Gli proteins in development and disease. Annu. Rev. Cell Dev. Biol. *27*, 513–537.

Iafrate, A.J., Feuk, L., Rivera, M.N., Listewnik, M.L., Donahoe, P.K., Qi, Y., Scherer, S.W., and Lee, C. (2004). Detection of large-scale variation in the human genome. Nat. Genet. *36*, 949–951.

Ingham, P.W., and McMahon, A.P. (2001). Hedgehog signaling in animal development: paradigms and principles. Genes Dev. *15*, 3059–3087.

Jeanpierre, S., Nicolini, F.E., Kaniewski, B., Dumontet, C., Rimokh, R., Puisieux, A., and Maguer-Satta, V. (2008). BMP4 regulation of human megakaryocytic differentiation is involved in thrombopoietin signaling. Blood *112*, 3154–3163.

Kawada, H., Ito, T., Pharr, P.N., Spyropoulos, D.D., Watson, D.K., and Ogawa, M. (2001). Defective megakaryopoiesis and abnormal erythroid development in Fli-1 gene-targeted mice. Int. J. Hematol. *73*, 463–468.

Kim, Y.S., Kang, H.S., and Jetten, A.M. (2007). The Krüppel-like zinc finger protein Glis2 functions as a negative modulator of the Wnt/beta-catenin signaling pathway. FEBS Lett. *581*, 858–864.

Korchynskyi, O., and ten Dijke, P. (2002). Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. J. Biol. Chem. *277*, 4883–4891.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science *275*, 1784–1787.

Krzywinski, M., Schein, J., Birol, I., Connors, J., Gascoyne, R., Horsman, D., Jones, S.J., and Marra, M.A. (2009). Circos: an information aesthetic for comparative genomics. Genome Res. *19*, 1639–1645.

Lamar, E., Kintner, C., and Goulding, M. (2001). Identification of NKL, a novel Gli-Kruppel zinc-finger protein that promotes neuronal differentiation. Development *128*, 1335–1346.

Lim, Y., and Matsui, W. (2010). Hedgehog signaling in hematopoiesis. Crit. Rev. Eukaryot. Gene Expr. *20*, 129–139.

Lin, M., Wei, L.J., Sellers, W.R., Lieberfarb, M., Wong, W.H., and Li, C. (2004). dChipSNP: significance curve and clustering of SNP-array-based loss-of-heterozygosity data. Bioinformatics *20*, 1233–1240.

Lion, T., Haas, O.A., Harbott, J., Bannier, E., Ritterbach, J., Jankovic, M., Fink, F.M., Stojimirovic, A., Herrmann, J., Riehm, H.J., et al. (1992). The translocation t(1;22)(p13;q13) is a nonrandom marker specifically associated with acute megakaryocytic leukemia in young children. Blood *79*, 3325–3330.

Ma, Z., Morris, S.W., Valentine, V., Li, M., Herbrick, J.A., Cui, X., Bouman, D., Li, Y., Mehta, P.K., Nizetic, D., et al. (2001). Fusion of two novel genes, RBM15 and MKL1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia. Nat. Genet. *28*, 220–221.

Malinge, S., Ragu, C., Della-Valle, V., Pisani, D., Constantinescu, S.N., Perez, C., Villeval, J.L., Reinhardt, D., Landman-Parker, J., Michaux, L., et al. (2008). Activating mutations in human acute megakaryoblastic leukemia. Blood *112*, 4220–4226.

Mardis, E.R., Ding, L., Dooling, D.J., Larson, D.E., McLellan, M.D., Chen, K., Koboldt, D.C., Fulton, R.S., Delehaunty, K.D., McGrath, S.D., et al. (2009). Recurring mutations found by sequencing an acute myeloid leukemia genome. N. Engl. J. Med. *361*, 1058–1066.

McCarroll, S.A., Kuruvilla, F.G., Korn, J.M., Cawley, S., Nemesh, J., Wysoker, A., Shapero, M.H., de Bakker, P.I., Maller, J.B., Kirby, A., et al. (2008). Integrated detection and population-genetic analysis of SNPs and copy number variation. Nat. Genet. *40*, 1166–1174.

Mercher, T., Coniat, M.B., Monni, R., Mauchauffe, M., Nguyen Khac, F., Gressin, L., Mugneret, F., Leblanc, T., Dastugue, N., Berger, R., and Bernard, O.A. (2001). Involvement of a human gene related to the *Drosophila* spen gene in the recurrent t(1;22) translocation of acute megakaryocytic leukemia. Proc. Natl. Acad. Sci. USA *98*, 5776–5779.

Mullighan, C.G., Goorha, S., Radtke, I., Miller, C.B., Coustan-Smith, E., Dalton, J.D., Girtman, K., Mathew, S., Ma, J., Pounds, S.B., et al. (2007). Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. Nature *446*, 758–764.

Oki, Y., Kantarjian, H.M., Zhou, X., Cortes, J., Faderl, S., Verstovsek, S., O'Brien, S., Koller, C., Beran, M., Bekele, B.N., et al. (2006). Adult acute megakaryocytic leukemia: an analysis of 37 patients treated at M.D. Anderson Cancer Center. Blood *107*, 880–884.

Olshen, A.B., Venkatraman, E.S., Lucito, R., and Wigler, M. (2004). Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics *5*, 557–572.

Pounds, S., Cheng, C., Mullighan, C., Raimondi, S.C., Shurtleff, S., and Downing, J.R. (2009). Reference alignment of SNP microarray signals for copy number analysis of tumors. Bioinformatics *25*, 315–321.

Radtke, I., Mullighan, C.G., Ishii, M., Su, X., Cheng, J., Ma, J., Ganti, R., Cai, Z., Goorha, S., Pounds, S.B., et al. (2009). Genomic analysis reveals few genetic alterations in pediatric acute myeloid leukemia. Proc. Natl. Acad. Sci. USA *106*, 12944–12949.

Sander, V., Eivers, E., Choi, R.H., and De Robertis, E.M. (2010). *Drosophila* Smad2 opposes Mad signaling during wing vein development. PLoS One *5*, e10383.

Sasaki, H., Hui, C., Nakafuku, M., and Kondoh, H. (1997). A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development *124*, 1313–1322.

Söderberg, S.S., Karlsson, G., and Karlsson, S. (2009). Complex and context dependent regulation of hematopoiesis by TGF-beta superfamily signaling. Ann. N Y Acad. Sci. *1176*, 55–69.

Soneoka, Y., Cannon, P.M., Ramsdale, E.E., Griffiths, J.C., Romano, G., Kingsman, S.M., and Kingsman, A.J. (1995). A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res. *23*, 628–633.

Tabata, T., and Takei, Y. (2004). Morphogens, their identification and regulation. Development *131*, 703–712.

Tallman, M.S., Neuberg, D., Bennett, J.M., Francois, C.J., Paietta, E., Wiernik, P.H., Dewald, G., Cassileth, P.A., Oken, M.M., and Rowe, J.M. (2000). Acute megakaryocytic leukemia: the Eastern Cooperative Oncology Group experience. Blood *96*, 2405–2411.

Van den Wijngaard, A., Pijpers, M.A., Joosten, P.H., Roelofs, J.M., Van zoelen, E.J., and Olijve, W. (1999). Functional characterization of two promoters in the human bone morphogenetic protein-4 gene. J. Bone Miner. Res. *14*, 1432–1441.

Visvader, J.E., Crossley, M., Hill, J., Orkin, S.H., and Adams, J.M. (1995). The C-terminal zinc finger of GATA-1 or GATA-2 is sufficient to induce megakaryocytic differentiation of an early myeloid cell line. Mol. Cell. Biol. *15*, 634–641.

Vokes, S.A., Ji, H., McCuine, S., Tenzen, T., Giles, S., Zhong, S., Longabaugh, W.J., Davidson, E.H., Wong, W.H., and McMahon, A.P. (2007). Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. Development *134*, 1977–1989.

Volanakis, E.J., Williams, R.T., and Sherr, C.J. (2009). Stage-specific Arf tumor suppression in Notch1-induced T-cell acute lymphoblastic leukemia. Blood *114*, 4451–4459.

Wang, G.G., Song, J., Wang, Z., Dormann, H.L., Casadio, F., Li, H., Luo, J.L., Patel, D.J., and Allis, C.D. (2009). Haematopoietic malignancies caused by dysregulation of a chromatin-binding PHD finger. Nature *459*, 847–851.

Wang, L., Gural, A., Sun, X.J., Zhao, X., Perna, F., Huang, G., Hatlen, M.A., Vu, L., Liu, F., Xu, H., et al. (2011). The leukemogenicity of AML1-ETO is dependent on site-specific lysine acetylation. Science *333*, 765–769.

Yu, P.B., Hong, C.C., Sachidanandan, C., Babitt, J.L., Deng, D.Y., Hoyng, S.A., Lin, H.Y., Bloch, K.D., and Peterson, R.T. (2008). Dorsomorphin inhibits BMP signals required for embryogenesis and iron metabolism. Nat. Chem. Biol. *4*, 33–41.

Zhang, J., Wheeler, D.A., Yakub, I., Wei, S., Sood, R., Rowe, W., Liu, P.P., Gibbs, R.A., and Buetow, K.H. (2005). SNPdetector: a software tool for sensitive and accurate SNP detection. PLoS Comput. Biol. *1*, e53.

Zhang, J., Ding, L., Holmfeldt, L., Wu, G., Heatley, S.L., Payne-Turner, D., Easton, J., Chen, X., Wang, J., Rusch, M., et al. (2012). The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature *481*, 157–163.

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ORIGINAL ARTICLE

High *WT1* mRNA expression after induction chemotherapy and *FLT3*-ITD have prognostic impact in pediatric acute myeloid leukemia: a study of the Japanese Childhood AML Cooperative Study Group

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Abstract The prognostic value of *WT1* mRNA expression in pediatric acute myeloid leukemia (AML) remains controversial. A sample of newly diagnosed ($n = 158$) AML patients from the Japanese Childhood AML Cooperative Treatment Protocol, AML 99, were simultaneously analyzed for *WT1* expression, cytogenetic abnormalities and gene alterations (*FLT3*, *KIT*, *MLL*, and *RAS*). *WT1* expression (including more than 2,500 copies/μgRNA) was detected in 122 of the 158 (77.8 %) initial diagnostic AML bone marrow samples (median 45,500 copies/μgRNA). Higher *WT1* expression was detected in French American British (FAB)-M0, M3, M7 and lower expression in M4 and M5. Higher *WT1* expression was detected in AML with inv(16), t(15;17) and Down syndrome and lower in AML with 11q23 abnormalities. Multivariate analyses demonstrated that *FLT3*-internal tandem duplication (ITD), *KIT* mutation, *MLL*-partial tandem duplication were correlated with poor prognosis; however, higher *WT1* expression was not. *FLT3*-ITD was correlated with *WT1* expression and prognosis. Furthermore, 74 *WT1* expression after induction

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chemotherapy was analyzed. Higher *WT1* expression after induction chemotherapy was significantly correlated with M1 or M2/M3 marrow, *FLT3*-ITD and poor prognosis. Multivariate analyses in 74 AML patients revealed that *FLT3*-ITD, *MLL*-PTD, and *KIT* mutations were associated with poor prognosis; however, *NRAS* Mutation, *KRAS* mutation and high *WT1* expression (>10,000 copies/μgRNA) did not show poor prognosis. Our findings suggest that higher *WT1* expression at diagnosis does not correlate with poor prognosis, but that *WT1* expression after induction chemotherapy is considered to be a useful predictor of clinical outcome in pediatric AML.

Keywords *WT1* · *FLT3* · AML · Pediatric

Introduction

The risk classification for acute myeloid leukemia (AML) patients according to cytogenetic abnormalities has been widely accepted in pediatric and adult AML [1–4]. AML patients with t(8;21), inv(16) and t(15;17) have been classified into the low risk (LR) group, while those with 5q- and -7 have been categorized into the high risk (HR) group, and others into the intermediate risk (IR) group [2–4]. Detecting chimeric transcripts such as, *PML-RARA* appears to be a good method for detecting minimal residual disease (MRD); however, approximately half of AML patients lack a suitable leukemia-specific marker; thus, there has been considerable interest in developing alternative approaches for MRD detection. One strategy involves the use of flow cytometer to identify and monitor leukemia-associated aberrant phenotypes [5]. Another approach is to detect Wilms tumor 1 (*WT1*) mRNA, which is highly expressed in AML blasts relative to normal PB and BM using a quantitative reverse transcribed-polymerase chain reaction (qRT-PCR) [6–12]. Several researchers have suggested that *WT1* expression is a good prognostic marker; thus, monitoring of the *WT1* mRNA expression is useful for MRD [7–12]. However, the prognostic relevance of *WT1* expression in AML at diagnosis is still controversial. Previous research has suggested that high *WT1* expression at diagnosis correlated with poor prognosis [6–9], while some others did not [10, 11, 15]. Recent work suggests that high *WT1* expression after chemotherapy correlates with poor prognosis [13–15]. This may depend on the uncertain mechanism of *WT1* mRNA expression in AML blasts due to the fact that *WT1* has a role not only for the suppression of tumors, but also for oncogenic potential [16, 17]. Recently, *FLT3*-internal tandem duplication (ITD) [18–21], *MLL*-partial tandem duplication (PTD) [20, 21] and *KIT* gene mutations [22, 23] have been strongly associated with a poor prognosis of adult and pediatric AML. A recent study suggested that *NRAS* gene alterations were associated with poor prognosis, while others did not find the same results [24]. High *WT1* expression was suggested to correlate with *FLT3*-ITD [25], however, the association between *WT1* expression and several gene alterations remains unknown in adult and pediatric AML. Recently, *WT1* gene mutations were frequently found in AML patients with normal karyotypes who showed poor clinical outcome [26, 27].

Thus, the present study aimed to simultaneously investigate *WT1* mRNA expression and examine the association of *WT1* mRNA expression with several gene alterations (*FLT3*, *KIT*, *MLL*, and *RAS*) in a sample of 158 newly diagnosed AML patients. We found that *FLT3*-ITD patients showed higher *WT1* mRNA expression at time of diagnosis as well as after the induction of chemotherapy; however, the prognostic significance of *WT1* mRNA expression was found only after induction chemotherapy. We discuss the usefulness of *WT1* mRNA expression associated with several gene alterations in pediatric AML patients.

Patients and methods

Patients

Patients included 318 newly diagnosed pediatric AML patients [0–15 years old, (240 de novo AML patients, 32 t(15;17)-AML, and 46 Down syndrome related myeloid leukemia (DS-ML)] from the Japanese Childhood AML Cooperative Treatment Protocol, AML 99, recruited between January 2000 and December 2002 [23, 28, 29]. Diagnosis of AML was made according to French–American–British (FAB) classification. Cytogenetic analysis was performed using the G-banding method. Among them, 158 samples at diagnosis were available for molecular analysis including, 13 patients with FAB-M3 and 10 patients with Down syndrome (DS) who were treated with different treatment protocols (see Table 1). Excluding FAB-M3 and DS, there were no significant differences between the analyzed 135 patients and the unanalyzed 105 patients regarding age (median, 6 years [range: 0–15 years] versus 6 years [range: 0–15 years], respectively) and initial WBC count (median, 24.8 $\times$ 10^9/L [range: 1.65–621.0 $\times$ 10^9/L] versus 13.8 $\times$ 10^9/L [range: 1.0–489.0 $\times$ 10^9/L, $p = .0764$], respectively). Also, no significant difference between the analyzed and not-analyzed patients in FAB-M3 and DS was found. The treatment protocol and risk classification were previously reported and described elsewhere [23, 28, 29]. We analyzed *WT1* expression in 85 paired bone marrow patient samples after 1st induction chemotherapy and also tested the blast counts for M1 (blast counts <5 %), M2 (5 % ≤ blast counts <25 %), M3 marrow (>25 %) at 22–74 days (median, 44 days) after the

Table 1 Genetic alterations and *WT1* mRNA expression in AML subgroup according to FAB classification

FAB classification	No. of patients	*FLT3*-ITD	*FLT3*-D835Mt	*MLL*-PTD	*KIT* -Mt	*NRAS* -Mt	*KRAS* -Mt	*WT1* expression (median) (copies/μgRNA)*	
M0	6	1	0	1	0	2	0	64,750	(2,400–190,000)
M1	24	4	2	7	0	1	2	23,000	(0–390,000)
M2	46	4	2	5	8	4	4	23,000	(85–680,000)
M3	13	3	3	0	0	0	0	140,000	(450–290,000)
M4	22	1	1	4	3	2	3	9,200	(50–150,000)
M5	25	5	3	3	0	2	5	9,600	(0–440,000)
M6	1	0	0	0	0	0	0	670	–
M7	9	1	0	1	0	0	0	36,000	(0–980,000)
M7 with DS	10	0	0	0	1	0	1	63,500	(140–630,000)
Unclassified	2	1	0	0	0	0	0	27,000	(14,000–40,000)
Total	158	20	11	21	12	11	15	23,500	(0–980,000)
	(%)	12.7	7.0	13.3	7.6	7.0	9.5		

ITD internal tandem duplication, *PTD* partial tandem duplication, *Mt* mutation, *DS* Down syndrome

* The difference of median *WT1* mRNA expression in each subgroups according to FAB classification was statistically significant ($p = 0.0011$)

initiation of induction chemotherapy. The evaluation for complete remission (CR) was conducted after two consecutive induction chemotherapy cycles. We also analyzed *WT1* expression at the time point after three consecutive chemotherapy cycles ($n = 56$) and the completion of therapy ($n = 47$).

Next, the 28 bone marrow samples from the healthy individuals who were the donors for bone marrow transplantation aged 7–52 years old from Nagoya University Hospital were also analyzed. Informed consent was obtained from the patients or their care-givers, according to guidelines based on the tenets of the revised Helsinki protocol. The institutional review board of Gunma Children's Medical Center and Nagoya University Hospital approved the present study.

Detection of *WT1* mRNA expression by qRT-PCR

Total RNA extracted from the bone marrow samples was reverse transcribed to cDNA with a cDNA Synthesis Kit (Amersham Bioscience, Tokyo, Japan). *WT1* expression was measured using real time PCR system (ABI 7700, Applied Biosystems). A set of primers used in the present study were as follows, *WT1* forward primer located in exon 6 (5′-GAT AAC CAC ACA ACG CCC ATC-3′) and reverse primer located in exon 6 and 7 (5′-CAC ACG TCG CAC ATC CTG AAT-3′) [12]. The TaqMan probe (5′-ACA CCG TGC GTG TGT ATT CTG TAT TGG-3′) was designed to hybridize at the sense strand of exon 6 and labeled with FAM as reporter dye at the 5′ end and with the quencher dye carboxy-teramethyl-rhodamin (TAMRA) at the 3′ end terminus. As endogenous mRNA control ubiquitous gene *GAPDH* mRNA was simultaneously quantified using a set of primers [forward primer (5′-GAA GGT GAA GGT CGG AGT C-3′), reverse primer (5′-GAA GAT GGT GAT GGG ATT TC-3′)] and TaqMan probe (5′-CAA GCT TCC CGT TCT CAG CC-3′). The *WT1* expression was corrected by the each *GAPDH* mRNA expression and multiplied by 2.7×10^7 (copies/μgRNA), because 1.0 μg RNA contains 2.7×10^7 GAPDH. *WT1* mRNA expression was examined in 158 individuals from the diagnostic bone marrow samples, 85 paired patient samples after the 1st induction chemotherapy, 56 samples after three consecutive chemotherapy sessions, and 47 samples during the finalization of therapy.

Analyses of *FLT3*, *KIT*, *MLL* and *RAS* genes

Mutational analysis for internal tandem duplication (ITD) within the JM domain and D835 mutation (D835Mt) within the TK2 domain of *FLT3* were performed as previously described [30, 31]. The mutation analysis of kinase domain, extracellular domain and transmembrane domain for the *KIT* gene was performed with RT-PCR followed by direct sequencing as previously reported [23]. *MLL*-partial tandem duplication (PTD) was examined by simple first round RT-PCR with 35 cycles using the primer pair 6.1 (located on exon 9) and E3AS (located on exon 4) as previously described [21, 32, 33]. The amplified products were purified and directly sequenced to confirm the *MLL*-PTD.

Exons 2 and 3 of the *NRAS* and *KRAS* genes were amplified by RT-PCR and directly sequenced using primer pairs for *NRAS* as previously reported. [34]

Statistical analysis

Estimates of the survival distributions were performed using the Kaplan–Meier method. Differences were

compared using a log-rank test. Overall survival (OS) was defined as the time from diagnosis to death (owing to any cause), or to the last follow-up. Event-free survival (EFS) was defined as the time from diagnosis to the date of relapse and death. Patients with Down syndrome ($n = 10$) or FAB-M3 ($n = 13$) were treated using different protocols, thus, we analyzed the 5-year OS and EFS in 135 patients except for patients with DS and FAB-M3. Mann–Whitney U tests were performed to detect the statistical differences between two groups and the Krusukal–Wallis test was performed to detect differences among several groups. The prognostic significance of the clinical variables was assessed using a Cox proportional hazards model. These statistical analyses were performed with the statistical software StatView (Abacus Concepts, Inc.). For all analyses, the p values were two-tailed, and a p of less than 0.05 ($p < .05$) was considered to be statistically significant.

Results

WT1 mRNA expression in normal bone marrow samples

We analyzed *WT1* mRNA expression in 28 RNA samples extracted from mononuclear cells of normal bone marrow samples and found that it ranges from 123 to 3,725 copies/μgRNA (median, 1,200 copies/μgRNA, see supplemental Figure 1). We determined the cut-off value to be 2,500 copies/μgRNA, because the value for the 90th percentile was 2,519 copies/ugRNA. Alternatively, the cut-off value for peripheral blood was 50 copies/μgRNA while using the same method [12]. Additionally, we set the *WT1* mRNA expression for bone marrow samples more than 2,500 copies/μgRNA as *WT1* expression positive.

WT1 mRNA expression at diagnosis, gene alterations and clinical outcomes

The *WT1* expression at time of diagnosis in 122 (77.8 %) of the 158 AML patients demonstrated to have more than the cut-off value (2,500 copies/ugRNA, see supplemental Figure 1) and was markedly elevated (median, 45,500 copies/μgRNA). No association was found between *WT1* expression and sex, age or initial WBC count. Complete remission (CR) was obtained in 149 of the 158 patients (94.3 %) after two consecutive induction chemotherapy cycles; however, the median *WT1* expression at time of diagnosis was not statistically different in patients with or without CR. The *WT1* expression in each FAB subgroup was statistically different (p=0.0011), (see Table 1). High *WT1* mRNA expression was found in FAB-M0, M3, M7, whereas low levels of expression were found in M4 and M5 ($p < 0.0001$). The median *WT1* mRNA expression in each karyotypic subgroup is presented in Table 2. Higher *WT1* mRNA expression was found in t(15;17), inv(16) and in patients with Down syndrome, whereas lower expression of *WT1* was found in AML patients with an 11q23 abnormality. In particular, more than 90 % of t(15;17), inv(16) and Down syndrome samples presented with a *WT1*-positive (>2,500 copies/μgRNA); whereas more than half (55 %) of AML patients with 11q23 abnormalities were *WT1* negative.

WT1 expression and prognosis were not different between patients with and without *FLT3*-ITD in 13 t(15;17) AML patients. In FAB-M7, there was no difference in *WT1* expression in DS and non-DS patients.

Excluding 13 FAB-M3 and 10 DS patients, 102 out of 135 patients (75.6 %) were *WT1* positive at the time of diagnosis. Multivariate analyses in 135 AML patients revealed that *FLT3*-ITD, *MLL*-PTD and *KIT* mutations were associated with poor prognosis, however, *FLT3*-D835 mutation (Mt), *N-RAS* Mt, *K-RAS* Mt and high *WT1* expression (>100,000 copies/μgRNA) did not show poor prognosis in patients in 5-year OS and EFS (see Tables 3, 4). Specifically, *FLT3*-ITD was found in 20 (12.7 %) and *FLT3*-D835Mt was found in 11 (7.0 %) of the 158 patients. The median *WT1* mRNA expression of patients with *FLT3*-ITD, D835Mt and wild type *FLT3* was 89,500 copies/μgRNA (330–330,000 copies/μgRNA), 85,000 copies/μgRNA (0–250,000 copies/μgRNA) and 18,500 copies/μgRNA (0-980,000 copies/μgRNA), respectively. This difference was statistically significant ($p = 0.0025$); however, our data suggested that only *FLT3*-ITD indicated poor prognosis [5-year overall survival for *FLT3*-ITD, D835Mt and wild type were 35.3, 100 and 84.3 %, respectively ($p < 0.0000001$)] [21]. For additional genetic alterations, *MLL*-PTD was found in 21 (13.3 %) out of 158 patients, the median *WT1* expression was not different in patients with (median, 21,000 copies/μgRNA) or without *MLL*-PTD (median, 28,000 copies/μgRNA), however, the prognosis was quite different within both subgroups [5y OS 56.3% in *MLL*-PTD (+) and 83.2 % in *MLL*-PTD(-)] [21]. *KIT* gene mutation (Mt) was found in 12 (7.6 %) out of 158 patients. The median *WT1* expression was not different in patients with (13,500 copies/μgRNA) or without *KIT*-Mt (32,500 copies/μgRNA). Interestingly, the median *WT1* expression in t(8;21)-AML patients with or without *KIT*-Mt was statistically different [3,300 copies/μgRNA (85-55,000 copies/μgRNA) when compared with 30,000 copies/μgRNA (200-680,000 copies/μgRNA)] ($p = 0.020$). Mutation in codons 12, 13 or 61 of the *NRAS* gene was found in 11 (7.0 %) out of 158 patients, the median *WT1* expression was not different in patients with (36,000 copies/μgRNA) or without *NRAS*-Mt (23,000 copies/μgRNA). Mutation in codons 12, 13 or 61 of the *KRAS* gene in 15 (9.5 %) out of

Table 2 Genetic alterations and *WT1* mRNA expression in AML subgroup according to karyotypic abnormalities

Karyotypic abnormalities	Number of patients	*FLT3*-ITD	*FLT3*-D835Mt	*MLL*-PTD	*KIT*	*NRAS*	*KRAS*	*WT1* mRNA expression (copies/μgRNA)	
Normal	33	9	2	8	2	2	3	18,000	(73–280,000)
t(8;21)	46	2	1	4	8	4	5	23,500	(85–680,000)
inv(16)	7	0	2	0	1	2	0	56,000	(8,700–220,000)
11q23 abnormalities	20	0	1	5	0	1	4	720	(0–150,000)
t(15;17)	13	3	3	0	0	0	0	140,000	(450–290,000)
Down syndrome	10	0	0	0	1	0	1	63,500	(140–360,000)
Others[a]	29	6	2	4	0	2	2	23,000	(0–980,000)
Total	158	20	11	21	12	11	15	32,000	(0–980,000)
	(%)	12.7	7.0	13.3	7.6	7.0	9.5		

The median value of *WT1* mRNA expression is shown and the difference was statistically significant ($p = 0.0003$)

[a] The subgroup of other karyotypic abnormalities included 2 patients with unknown karyotype

Table 3 Prognostic factors for overall survival in 135 AML patients except for FAB-M3 and Down syndrome

Variable	*P* value	Odds ratio	95% CI
FLT3-ITD	<0.0001	6.767	2.844–16.101
FLT3-D835Mt	n.d.	n.d.	n.d.
MLL-PTD	0.0443	2.229	1.021–4.870
KIT-Mt	0.0148	3.661	1.290–10.395
NRAS-Mt	0.3538	1.789	0.523–6.122
KRAS-Mt	0.1313	2.407	0.769–7.537
WT1>100,000 copies/μgRNA	0.8733	0.931	0.386–2.244

Table 4 Prognostic factors for Event Free Survival in 135 AML patients except for FAB-M3 or Down syndrome

Variable	*P* value	Odds ratio	95% CI
FLT3-ITD	0.0015	3.455	1.607–7.430
FLT3-D835Mt	0.3770	0.404	0.054–3.019
MLL-PTD	0.0213	2.177	1.123–4.221
KIT-Mt	0.0013	3.816	1.686–8.635
NRAS-Mt	0.7613	0.832	0.254–2.726
KRAS-Mt	0.1849	1.852	0.745–4.607
WT1>100,000 copies/μgRNA	0.9169	0.960	0.447–2.063

158 patients, the median *WT1* expression was not different with (23,000 copies/μgRNA) or without *KRAS*-Mt (24,000 copies/μgRNA).

In patients with normal karyotype, the median *WT1* expression in patients with and without *FLT3*-ITD were 65,000 copies/μgRNA (73–280,000 copies/μgRNA 9 *FLT3*-ITD and 2 *FLT3*-D835Mt) and 7,150 copies/μgRNA (330–240,000 copies/μgRNA, 22 *FLT3* wild type), respectively, which was a statistically significant ($p = 0.023$).

In AML with 11q23 abnormalities, the median *WT1* expression was quite low (median, 720 copies/μgRNA) and more than half (55 %) of AML patients with 11q23 abnormalities were *WT1* negative. There were significant differences among each of the subgroups with t(9;11) ($n = 8$; median = 35 copies/μgRNA); t(6;11) ($n = 3$; 81,000 copies/μgRNA); and other variants ($n = 9$; 780 copies/μgRNA) ($p = 0.0139$). Furthermore, there were significant differences in their clinical outcomes, with 5-year OS rates nearly to 100 % in t(9;11), 0 % in t(6;11) and 77.8 % in other variants, respectively.

WT1 mRNA expression after 1st induction therapy

We also evaluated the *WT1* expression and blast counts in 85 bone marrow samples including 5 t(15;17) and 6 DS patients after 1st induction chemotherapy. There were no significant differences between the analyzed 85 patients and unanalyzed 73 patients regarding age (median 6 vs. 7-years old, respectively), WBC count (median 20,200 vs. 21,400/μl, respectively), or initial *WT1* expression level (median 21,000 vs. 32,000 copies/μg RNA, respectively).

In 5 t(15;17)-AML patients examined, *WT1* expression decreased by a log of 1–3 after induction chemotherapy except for one patient who showed 32,000 copies/μgRNA after induction chemotherapy. This patient died after relapse. In all six DS patients analyzed, *WT1* expression decreased by a log of 2–3 and all were cut-off value after induction chemotherapy. All these patients were alive without relapse.

Excluding 5 t(15;17) and 6 DS patients, 58 out of 74 patients (78.4 %) were *WT1* positive at the time of diagnosis (median, 18,000 copies/μgRNA) and 11 out of 74 patients (14.9 %) remained *WT1* positive after induction chemotherapy (median, 215 copies/μgRNA) (see

supplemental Figure 2). Specifically, *WT1* expression decreased after the 1st round of chemotherapy at a rate of 1–4 log(s) in 53 out of 57 *WT1*-positive patients at the time of diagnosis (93.0 %) [1 log reduction in 15 of 57 (26.3 %), 2 log reduction in 22 of 57 (38.6 %), 3 log reduction in 12 of 57 (21.0 %) and 4 log reduction in 4 of 57 (7.0 %)]. Furthermore, 10 of 57 (17.5 %) patients still showed *WT1* positive after the 1st round of chemotherapy. A total of 16 out of 17 patients with *WT1* negative at the time of diagnosis remained *WT1* negative after induction chemotherapy, and only one patient whose *WT1* expression increased at a rate of 1 log could not achieve complete remission and died. There was a statistically difference in 5-year OS between the *WT1*-positive subgroup (54.5 %, $n = 11$) and the *WT1* negative subgroup (79.4 %, $n = 63$) ($p = 0.036$) (see Fig. 1). The difference between the subgroup with *WT1* $\geq$10,000 copies/μgRNA (25 %, $n = 4$), and *WT1* <10,000 copies/μgRNA (77.1 %, $n = 70$) after induction chemotherapy was significant ($p = 0.0018$).

There was also a statistical difference in 5-year OS between M1 marrow (79.1 %, $n = 67$) and M2/M3 marrow (42.9 %, $n = 7$) ($p = 0.013$). The *WT1* expression was also quite different between M1 marrow (median, 200 copies/μgRNA, $n = 67$) versus the M2/M3 marrow (median, 14,000 copies/μgRNA, $n = 7$) ($p < 0.0001$). There was a moderate to strong correlation between *WT1* positivity and M1/M2/M3 marrow. Finally, three out of seven M2/M3 marrow patients did not achieve CR after two consecutive induction chemotherapy cycles.

FLT3-ITD was found in 8 patients, *FLT3*-D835Mt in 5, *KIT*-Mt in 7, *MLL*-PTD in 8, *RAS*-Mt in 6 and *KRAS*-Mt in 8 patients out of the 74 diagnostic samples. Eight patients with *FLT3*-ITD showed higher *WT1* expression at the time of diagnosis (median, 60,000 copies/μgRNA) and after the initiation of chemotherapy (median, 1,850 copies/μgRNA) compared with those without *FLT3*-ITD (at diagnosis 15,500 copies/μgRNA, after induction chemotherapy 200 copies/μgRNA, $n = 66$). Three out of 4 patients with *WT1* positive after induction chemotherapy relapsed and died.

Interestingly, a total of 11 *WT1* positive patients after induction chemotherapy included 5 *FLT3*-ITD, 2 *MLL*-PTD, 1 *FLT3*-D835Mt, and 1 *KIT*-Mt. In particular, three out of four patients with *WT1* expression with more than 10,000 copies/μgRNA had the *FLT3*-ITD.

Multivariate analyses in 74 AML patients revealed that *FLT3*-ITD, *MLL*-PTD and *KIT* mutations were associated with poor prognosis; however, *NRAS* Mt, *KRAS* Mt and high *WT1* expression (>10,000 copies/μgRNA) did not show poor prognosis in patients with 5-year OS and EFS (see Supplemental Tables 1 and 2).

WT1 mRNA expression at the subsequent time point

Only 2 of the 56 (3.6 %) patients presented with *WT1* positive after three consecutive courses of chemotherapy. Unfortunately, both patients relapsed and died. Four of the 47 (8.5 %) patients at the finalization of therapy showed an expression of *WT1* positive and 3 patients relapsed (at 8, 9 and 16 months after the initiation of induction chemotherapy) and died. We found *MLL*-PTD in one patient.

Discussion

The present study aimed to investigate *WT1* mRNA expression and examined association between *WT1* expression and five gene alterations including *FLT3*, *KIT*, *MLL*, *NRAS* and *KRAS* in de novo 158 pediatric AML bone marrow samples. High *WT1* expression at diagnosis was reported to be associated with a poor prognosis in adult AML studies [6–9]; however, others have suggested the inverse results [10, 11, 15]. Consistent with previous works, our results also suggested that *WT1* expression was significantly different in subgroups according to FAB classification and karyotypic abnormalities [10, 11, 13–15].

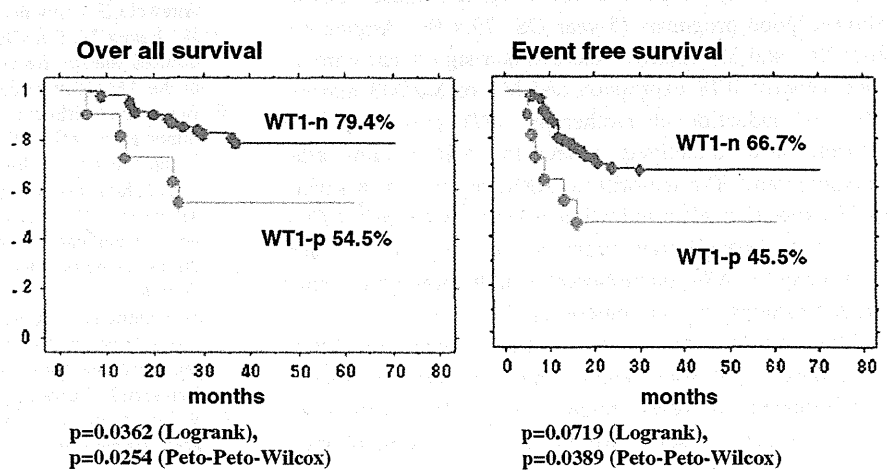

Fig. 1 The 5-year overall survival and event free survival rates for 74 AML patients according to *WT1* expression after 1st induction chemotherapy. *WT1-p WT1* mRNA expression had more than 2,500copies/μRNA, *WT1*-n; *WT1* mRNA expression had less than 2,500copies/μRNA. Overall survival is quite different according to *WT1*-p/n status

WT1 expression was higher in FAB-M0, M3, M7 and lower in M4 or M5. This may be partially explained by the fact that *WT1* expression was down-regulated with differentiation [35]. *WT1* expression in AML patients with 11q23 abnormalities was significantly lower, and thus, resulted in low *WT1* expression of FAB-M4 or M5, as previously reported [10, 13]. Conversely, t(8;21), inv(16) and t(15;17) displayed higher *WT1* expression and good prognosis as in previous studies [10, 11, 13]. Our results demonstrated that *WT1* expression of t(8;21) with *KIT* mutations (3,300 copies/μgRNA) was statistically lower than that without *KIT* mutations (30,000 copies/μgRNA) ($p = 0.020$). The reason for this finding remains unclear; however, a recent study suggested that *RUNX1-RUNX1T1* (AML1-ETO) rapidly induces AML in conjunction with *WT1* expression using a mouse model [36]. Furthermore, *FLT3*-ITD and D835Mt were strongly associated with higher expression of *WT1* mRNA in a total of 158 AML patients, which is consistent with previous adult AML [9, 10]. Patients with *FLT3*-ITD or D835Mt showed higher *WT1* expression, however, the prognosis was quite different between patients with *FLT3*-ITD and D835Mt in our study. Moreover, *WT1* expression in about 20 % of AML patients was lower than the specified cut-off value. The majority of AML patients with 11q23 abnormalities except for t(6;11)(q27;q23) showed lower *WT1* expression. We concluded that patients presenting with high *WT1* expression were comprised of several subgroups with heterogeneous clinical outcomes. Recently, a study suggested that *WT1* played a significant role in both tumor suppression and oncogenic potential [16, 17, 37]. There still remains an unexplained difference for higher *WT1* mRNA expression in clinically diverse prognostic subgroups (including those with good and poor outcomes). We could not discriminate *WT1* isoform in both status in this method. Further studies are needed to resolve this issue [38, 39].

From the analysis of paired 85 AML samples, *WT1* mRNA expression decreased to be below the cut-off value for the majority of patients (83.8 %), and these patients showed good prognosis (5-year OS: 79.4 %). Among the M1, M2, and M3 marrow, there was a significant correlation between *WT1* expression and M1 or M2/M3 marrow after 1st induction chemotherapy. *WT1* expression was considered to effectively reflect the MRD status after chemotherapy. The majority of AML patients with higher *WT1* expression after induction chemotherapy had *FLT3*-ITD and showed poor prognosis in the study. Thus, examining the *WT1* expression after induction therapy may predict patients' clinical outcomes.

The majority of the patients continuously presented with an expression of *WT1*-negative after the three course of chemotherapy; however, re-elevation of *WT1* expression was observed in some patients before haematological relapse was found and thus, showed poor prognoses. However, we also observed re-elevation of *WT1* expression after stem cell transplantation preceded the haematological relapse. We must await furtherer study to investigate which *WT1* expression in bone marrow or peripheral blood is more sensitive for haematological relapse.

We concluded that *WT1* mRNA expression was different in each karyotypic subgroup. *WT1* expression at diagnostic sample does not have any prognostic value; however, *WT1* expression after 1st induction chemotherapy would be associated with minimal residual disease or *FLT3*-ITD. Thus, it could be a good prognostic marker for assessing pediatric AML patients.

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References

1. Raimondi SC, Chang MN, Ravindranath Y, et al. Chromosomal abnormalities in 478 children with acute myeloid leukemia: clinical characteristics and treatment outcome in a Cooperative Pediatric Oncology Group Study—POG8821. Blood. 1999;94: 3707–16.
2. Webb DK, Harrison G, Stevens RF, et al. MRC Childhood Leukemia Working Party. Relationships between age at diagnosis, clinical features, and outcome of therapy in children treated in the Medical Research Council AML 10 and 12 trials for acute myeloid leukemia. Blood. 2001;98:1714–20.
3. Creutzig U, Ritter J, Zimmermann M, et al. Improved treatment results in high-risk pediatric acute myeloid leukemia patients after intensification with high-dose cytarabine and mitoxantrone: results of study acute myeloid leukemia-Berlin-Frankfurt-Munster 93. J Clin Oncol. 2001;19:2705–13.
4. Byrd JC, Mrozek K, Dodge RK, et al. Pretreatment cytogenetic abnormalities are predictive of induction success, cumulative incidence of relapse, and overall survival in adult patients with de novo acute myeloid leukemia: results from Cancer and Leukemia Group B (CALGB 8461). Blood. 2002;100:4325–36.
5. Rubnitz JE, Inaba H, Dahl G, Ribeiro RC, Bowman WP, Taub J, Pounds S, Razzouk BI, Lacayo NJ, Cao X, Meshinchi S, Degar B, Airewele G, Raimondi SC, Onciu M, Coustan-Smith E, Downing JR, Leung W, Pui CH, Campana D. Minimal residual disease-directed therapy for childhood acute myeloid leukaemia: results of the AML02 multicentre trial. Lancet Oncol. 2010;11:543–52.
6. Bergmann L, Miething C, Maurer U, et al. High levels of Wilms' tumor gene (wt1) mRNA in acute myeloid leukemias are associated with a worse long-term outcome. Blood. 1997;90:1217–25.
7. Trka J, Kalinova M, Hrusak O, et al. Real-time quantitative PCR detection of WT1 gene expression in children with AML: prognostic significance, correlation with disease status and residual disease detection by flow cytometry. Leukemia. 2002;16:1381–9.
8. Garg M, Moore H, Tobal K, Liu Yin JA. Prognostic significance of quantitative analysis of WT1 gene transcripts by competitive reverse transcription polymerase chain reaction in acute leukaemia. Br J Haematol. 2003;123:49–59.
9. Barragan E, Cervera J, Bolufer P, et al. Prognostic implications of Wilms' tumor gene (WT1) expression in patients with de novo acute myeloid leukemia. Haematologica. 2004;89:926–33.

10. Gaiger A, Schmid D, Heinze G, et al. Detection of the WT1 transcript by RT-PCR in complete remission has no prognostic relevance in de novo acute myeloid leukemia. Leukemia. 1998; 12:1886–94.
11. Weisser M, Kern W, Rauhut S, et al. Prognostic impact of RT-PCR-based quantification of WT1 gene expression during MRD monitoring of acute myeloid leukemia. Leukemia. 2005;19:1416–23.
12. Miyawaki S, Hatsumi N, Tamaki T, Naoe T, Ozawa K, Kitamura K, Karasuno T, Mitani K, Kodera Y, Yamagami T, Koga D. Prognostic potential of detection of WT1mRNA level in peripheral blood in adult acute myeloid leukemia. Leuk Lymphoma. 2010;51:1855–61.
13. Lapillonne H, Renneville A, Auvrignon A, et al. High WT1 expression after induction therapy predicts high risk of relapse and death in pediatric acute myeloid leukemia. J Clin Oncol. 2006;24:1507–15.
14. Cilloni D, Renneville A, Hermitte F, Hills RK, Daly S, Jovanovic JV, Gottardi E, Fava M, Schnittger S, Weiss T, Izzo B, Nomdedeu J, van der Heijden A, van der Reijden BA, Jansen JH, van der Velden VH, Ommen H, Preudhomme C, Saglio G, Grimwade D. Real-time quantitative polymerase chain reaction detection of minimal residual disease by standardized WT1 assay to enhance risk stratification in acute myeloid leukemia: a European LeukemiaNet study. J Clin Oncol. 2009;27:5195–201.
15. Noronha SA, Farrar JE, Alonzo TA, Gerbing RB, Lacayo NJ, Dahl GV, Ravindranath Y, Arceci RJ, Loeb DM. WT1 expression at diagnosis does not predict survival in pediatric AML: a report from the Children's Oncology Group. Pediatr Blood Cancer. 2009;53:1136–9.
16. Keilholz U, Menssen HD, Gaiger A, et al. Wilms' tumour gene 1 (WT1) in human neoplasia. Leukemia. 2005;19:1318–23.
17. Yang L, Han Y, Saurez Saiz F, Minden MD. A tumor suppressor and oncogene: the WT1 story. Leukemia. 2007;21:868–76.
18. Meshinchi S, Stirewalt DL, Alonzo TA, et al. Activating mutations of RTK/ras signal transduction pathway in pediatric acute myeloid leukemia. Blood. 2003;102:1474–9.
19. Zwaan CM, Meshinchi S, Radich JP, et al. FLT3 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic significance and relation to cellular drug resistance. Blood. 2003;102:2387–94.
20. Dohner K, Tobis K, Ulrich R, et al. Prognostic significance of partial tandem duplications of the MLL gene in adult patients 16 to 60 years old with acute myeloid leukemia and normal cytogenetics: a study of the Acute Myeloid Leukemia Study Group Ulm. J Clin Oncol. 2002;20:3254–61.
21. Shimada A, Taki T, Tabuchi K, Taketani T, Hanada R, Tawa A, Tsuchida M, Horibe K, Tsukimoto I, Hayashi Y. 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. Pediatr Blood Cancer. 2008;50:264–9.
22. Care RS, Valk PJ, Goodeve AC, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. Br J Haematol. 2003;121:775–7.
23. Shimada A, Taki T, Tabuchi K, et al. KIT mutations, and not FLT3 internal tandem duplication, are strongly associated with a poor prognosis in pediatric acute myeloid leukemia with t(8;21): a study of the Japanese Childhood AML Cooperative Study Group. Blood. 2006;107:1806–9.
24. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. Blood. 2006;107:3847–53.
25. Spassov BV, Stoimenov AS, Balatzenko GN, Genova ML, Peichev DB, Konstantinov SM. Wilms' tumor protein and FLT3-internal tandem duplication expression in patients with de novo acute myeloid leukemia. Hematology. 2011;16:37–42.
26. Becker H, Marcucci G, Maharry K, Radmacher MD, Mrózek K, Margeson D, Whitman SP, Paschka P, Holland KB, Schwind S, Wu YZ, Powell BL, Carter TH, Kolitz JE, Wetzler M, Carroll AJ, Baer MR, Moore JO, Caligiuri MA, Larson RA, Bloomfield CD. Mutations of the Wilms tumor 1 gene (WT1) in older patients with primary cytogenetically normal acute myeloid leukemia: a Cancer and Leukemia Group B study. Blood. 2010;116:788–92.
27. Ho PA, Zeng R, Alonzo TA, Gerbing RB, Miller KL, Pollard JA, Stirewalt DL, Heerema NA, Raimondi SC, Hirsch B, Franklin JL, Lange B, Meshinchi S. Prevalence and prognostic implications of WT1 mutations in pediatric acute myeloid leukemia (AML): a report from the Children's Oncology Group. Blood. 2010;116: 702–10.
28. Tsukimoto I, Tawa A, Hanada R, et al. Excellent outcome of risk stratified treatment for childhood acute myeloid leukemia-AML99 trial: for the Japanese Childhood AML Cooperative Study Group. Blood 2005:106:261a. Abstract 889.
29. Kobayashi R, Tawa A, Hanada R, Horibe K, Tsuchida M. Japanese childhood AML cooperative study group. Extramedullary infiltration at diagnosis and prognosis in children with acute myelogenous leukemia. Pediatr Blood Cancer. 2007;48:393–8.
30. Taketani T, Taki T, Sugita K, et al. FLT3 mutations in the activation loop of tyrosine kinase domain are frequently found in infant ALL with MLL rearrangements and pediatric ALL with hyperdiploidy. Blood. 2004;103:1085–8.
31. Yamamoto Y, Kiyoi H, Nakano Y, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. Blood. 2001;97:2434–9.
32. Jamal R, Taketani T, Taki T, et al. Coduplication of the MLL and FLT3 genes in patients with acute myeloid leukemia. Genes Chromosomes Cancer. 2001;31:187–90.
33. Schnittger S, Wormann B, Hiddemann W, et al. Partial tandem duplications of the MLL gene are detectable in peripheral blood and bone marrow of nearly all healthy donors. Blood. 1998;92: 1728–34.
34. Sano H, Shimada A, Taki T, Murata C, Park MJ, Sotomatsu M, Tabuchi K, Tawa A, Kobayashi R, Horibe K, Tsuchida M, Hanada R, Tsukimoto I, Hayashi Y. RAS mutations are frequent in FAB type M4 and M5 of acute myeloid leukemia, and related to late relapse: a study of the Japanese Childhood AML Cooperative Study Group. Int J Hematol. 2012;95:509–15.
35. Sekiya M, Adachi M, Hinoda Y, Imai K, Yachi A. Downregulation of Wilms' tumor gene (wt1) during myelomonocytic differentiation in HL60 cells. Blood. 1994;83:1876–82.
36. Nishida S, Hosen N, Shirakata T, et al. AML1-ETO rapidly induces acute myeloblastic leukemia in cooperation with the Wilms tumor gene, WT1. Blood. 2006;107:3303–12.
37. Hossain A, Nixon M, Kuo MT, Saunders GF. N-terminally truncated WT1 protein with oncogenic properties overexpressed in leukemia. J Biol Chem. 2006;281:28122–30.
38. Siehl JM, Reinwald M, Heufelder K, Menssen HD, Keilholz U, Thiel E. Expression of Wilms' tumor gene 1 at different stages of acute myeloid leukemia and analysis of its major splice variants. Ann Hematol. 2004;83:745–50.
39. Ito K, Oji Y, Tatsumi N, et al. Antiapoptotic function of 17AA(+)WT1 (Wilms' tumor gene) isoforms on the intrinsic apoptosis pathway. Oncogene. 2006;25:4217–29.

Brief report

Somatic mosaicism for oncogenic *NRAS* mutations in juvenile myelomonocytic leukemia

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Juvenile myelomonocytic leukemia (JMML) is a rare pediatric myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML. In the present study, we report 2 patients with somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S) associated with the development of JMML. The mutated allele frequencies quantified by pyrosequencing were various and ranged from 3%-50% in BM and other somatic cells (ie, buccal smear cells, hair bulbs, or nails). Both patients experienced spontaneous improvement of clinical symptoms and leukocytosis due to JMML without hematopoietic stem cell transplantation. These patients are the first reported to have somatic mosaicism for oncogenic *NRAS* mutations. The clinical course of these patients suggests that *NRAS* mosaicism may be associated with a mild disease phenotype in JMML. (*Blood*. 2012;120(7):1485-1488)

Introduction

Juvenile myelomonocytic leukemia (JMML) is a rare myeloid neoplasm characterized by excessive proliferation of myelomonocytic cells. Somatic mutations in genes involved in GM-CSF signal transduction, such as *NRAS*, *KRAS*, *PTPN11*, *NF1*, and *CBL*, have been identified in more than 70% of children with JMML.[1-3] The term "somatic mosaicism" is defined as the presence of multiple populations of cells with distinct genotypes in one person whose developmental lineages trace back to a single fertilized egg.[4] Somatic mosaicism of various genes, including some oncogenes, has been implicated in many diseases. For example, somatic mosaicism for *HRAS* mutations is found in patients with Costello syndrome.[5-7] Whereas germline mutations in causative genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in JMML patients,[3,8-11] the presence of somatic mosaicism for these genes has never been reported. In the present study, we describe 2 cases of JMML in which the patients display somatic mosaicism for oncogenic *NRAS* mutations (G12D and G12S).

Study design

Written informed consent for sample collection was obtained from the patients' parents in accordance with the Declaration of Helsinki, and molecular analysis of the mutational status was approved by the ethics committee of the Nagoya University Graduate School of Medicine (Nagoya, Japan).

Patient 1. A 10-month-old boy had hepatosplenomegaly and leukocytosis (72.1 × 10^9/L) with monocytosis (13.3 × 10^9/L; Table 1). The patient's BM contained 7% blasts with myeloid hyperplasia. Cytogenetic analysis revealed a normal karyotype and colony assay of BM mononuclear cells (BM-MNCs) showed spontaneous colony formation but GM-CSF hypersensitivity assay was not tested. The diagnostic criteria for JMML, as developed by the European Working Group on Myelodysplastic Syndrome in Childhood, was fulfilled,[12] and the patient was treated with IFN-α and 6-mercaptopurine. His clinical and laboratory findings gradually resolved without hematopoietic stem cell transplantation. However, 11 years after the diagnosis of JMML, the patient developed thrombocytopenia (7.6 × 10^9/L) and BM findings showed trilineage dysplasia with low blast count compatible with refractory anemia. The patient did not have any physiologic abnormalities, such as facial deformity, and there was no family history of malignancy or congenital abnormalities.

Patient 2. A 10-month-old boy had anemia, hepatosplenomegaly, and leukocytosis (31.8 × 10^9/L) with monocytosis (6.4 × 10^9/L; Table 1). The patient's BM exhibited myeloid hyperplasia and granulocytic dysplasia with 5% blasts. Cytogenetic

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Table 1. Patient characteristics

	Patient 1	Patient 2
Age, mo	10	10
Sex	Male	Male
Liver, cm	12	5
Spleen, cm	8	10
WBCs, $\times 10^9$/L	72.1	31.8
Monocytes, %	18.5	20
Blasts, %	4	2
Hb, g/dL	8.9	5.4
Platelets, $\times 10^9$/L	59	100
HbF, %	2.1	1.7
BM blasts, %	7	5
Karyotype	46,XY [20/20]	46,XY [20/20]
Monosomy 7 (FISH)	Negative	Negative
Spontaneous colony formation	Positive	Positive
Gene mutation	*NRAS*, G12D 35G > A	*NRAS*, G12S 34G > A
Treatment	IFN-α-2b, 6-MP	None
Observation period, mo	231	103
Outcome	Alive	Alive
Fraction of mutant alleles, % (pyrosequencing)		
Nail (whole)	24	12.5 (average)
Nail (left hand)	ND	26
Nail (right hand)	ND	13
Nail (left foot)	ND	8
Nail (right foot)	ND	3
Buccal smear cells	43	21
Hair bulbs	5	ND
Family studies		
Father	Wild-type	Wild-type
Mother	Wild-type	Wild-type
Sibling	ND	Wild-type

Hb indicates hemoglobin; 6-MP, 6-mercaptopurine; and ND, not done.

analysis revealed a normal karyotype. Colony assay of BM-MNCs showed spontaneous colony formation and GM-CSF hypersensitivity. Although the diagnostic criteria for JMML were fulfilled,[12] the patient's clinical symptoms and leukocytosis improved spontaneously within a few months without cytotoxic therapy or hematopoietic stem cell transplantation. The patient has remained healthy and has experienced no hematologic or physiologic abnormalities. The most recent follow-up examination was conducted when the patient was 8 years of age.

Detailed methods for experiments are described in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

Results and discussion

DNA sequencing for JMML-associated genes (ie, *NRAS*, *KRAS*, *PTPN11*, and *CBL*) was performed (Figure 1 and Table 1). In Patient 1, the *NRAS* G12D mutation was identified in BM-MNCs at the time of diagnosis of both JMML and MDS. We identified the same G12D mutation in DNA derived from buccal smear cells and nails of both hands; however, the sequence profile of the nails showed a low signal for the mutant allele compared with signal of blood cells. In Patient 2, the *NRAS* G12S mutation was identified in DNA from BM-MNCs, buccal smear cells, and nails of the left hand. However, the sequence profiles of buccal smear cells and nails of the left hand showed a low signal for the mutant variant. No mutation was detected in DNA from the PB-MNCs of the patient's parents or sibling.

We used pyrosequencing to quantify the fraction of mutated alleles in DNA samples from different somatic tissues (Figure 1 and Table 1). The frequency of mutated alleles varied by tissue type as follows. For Patient 1: BM-MNCs, 50%; nails, 24%; buccal smear cells, 43%; and hair bulbs, 5%. For Patient 2: buccal smear cells, 21%; nails of left hand, 26%; nails of right hand, 13%; nails of left foot, 8%; and nails of right foot, 3%. We cloned the PCR product of *NRAS* exon 2 from the nails of Patient 1 and picked up 15 clones. The clones were sequenced. Four of the 15 clones (27%) contained the mutant allele, which is consistent with the results of pyrosequencing analysis (24% mutant allele). Because the confirmed detection level by pyrosequencing technique was above 5%, results with a low percentage (< 5%) of mutant allele (ie, hair bulbs in Patient 1) should be interpreted with caution.[13,14]

We diagnosed 2 JMML patients as having somatic mosaicism of *NRAS* mutations: G12D for Patient 1 and G12S for Patient 2. The diagnoses were based on negative familial studies and mutational allele quantification analyses that showed diversity in the chimeric mutational status of different somatic tissues. Although DNA from buccal smear cells might be contaminated with WBCs, we also identified mutations in DNA from the nail tissue, which is known to be a good biologic material without contamination from hematopoietic cells, in both patients. These data suggest that a portion of the *NRAS*-mutated somatic cells were derived from one cell that acquired the mutation at a very early developmental stage. Although both somatic and germline mutations of RAS pathway genes (ie, *PTPN11*, *NRAS*, *NF1*, and *CBL*) are found in some JMML patients,[3,8-11] somatic mosaicism for these genes has never been reported. To the best of our knowledge, the present study is

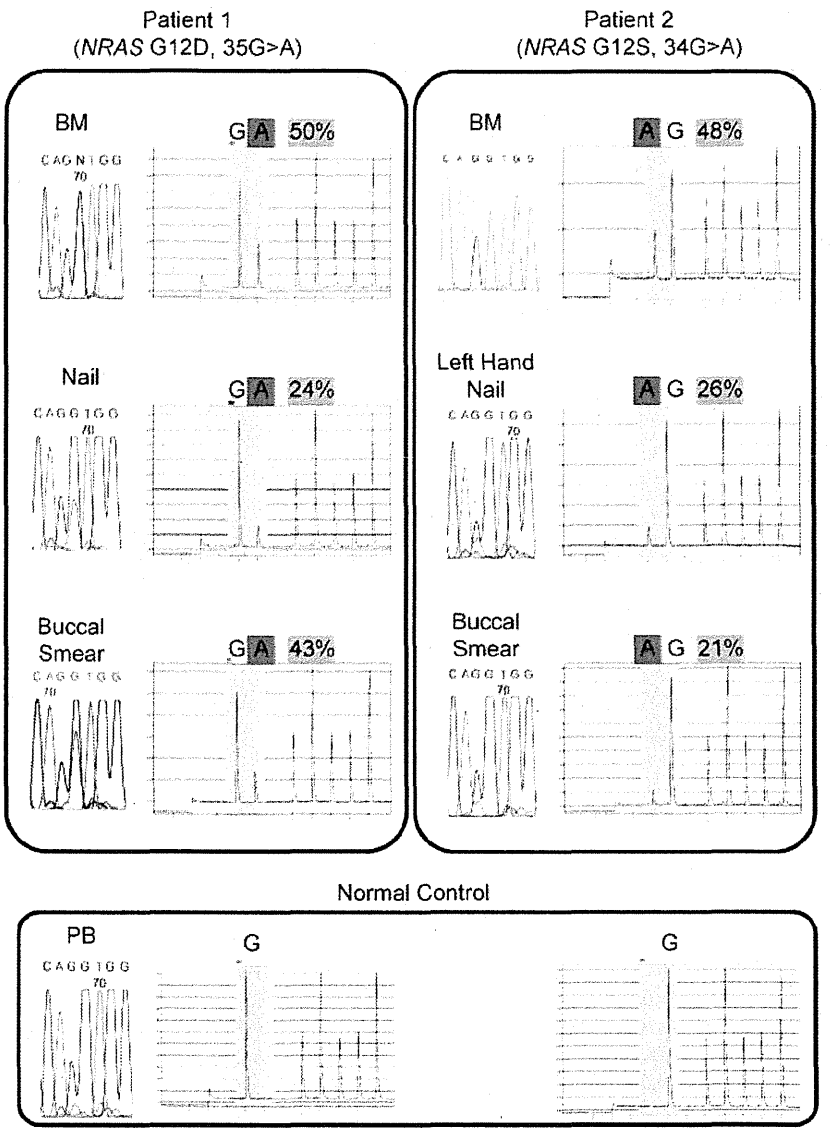

Figure 1. Direct sequencing and quantitative mutational analysis of *NRAS* in JMML patients. *NRAS* mutations are detected by direct sequencing and quantified by pyrosequencing. Direct sequencing identified oncogenic *NRAS* mutations: for Patient 1, G12D, 35G > A; for Patient 2, G12S, 34G > A) in BM-MNCs at diagnosis of JMML and in the nails and buccal smear cells. Quantification by pyrosequencing revealed that the fractions of mutated allele varied among different tissue types. For Patient 1: BM, 50%; nail, 24%; and buccal smear, 43%. For Patient 2: BM, 48%; left-hand nail, 26%; and buccal smear, 21%.

the first report of JMML patients with somatic mosaicism of mutations in RAS pathway genes.

Germline RAS pathway mutations are often associated with dysmorphic features similar to Noonan syndrome or its associated diseases. Correspondingly, JMML patients with germline *NRAS* or *CBL* mutations exhibit characteristic dysmorphic features.[3,10] Although our patients did not show any dysmorphic or developmental abnormalities, they should receive careful medical follow-up, especially for the occurrence of other cancers, because of the oncogenic nature of the mutations.

In general, JMML is a rapidly fatal disorder if left untreated.[8] However, recent clinical genotype-phenotype analyses have revealed heterogeneity in their clinical course. We and other researchers have reported that patients with *PTPN11* mutations have a worse prognosis than patients with other gene mutations, including *NRAS* and *KRAS*.[15,16] Both of the JMML patients in the present study with somatic mosaicism of oncogenic *NRAS* mutations have had a mild and self-limiting clinical course. We analyzed nails of other 3 JMML patients with RAS mutations who experienced aggressive clinical course and none showed somatic mosaicism (data not shown). In analogy to the mild phenotype of JMML patients with germline mutations in *PTPN11*, we speculate that JMML patients with somatic mosaicism of RAS genes might have a mild clinical course. We are planning to confirm these observations in larger cohort.

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Authorship

Contribution: S.D. and H.M. designed and conducted the research, analyzed the data, and wrote the manuscript; A.S., M.M.-E., M. Sato, H.K., A.K., M. Sotomatsu, and Y.H. treated the patients; Y.T., Y.F.-H., K.Y., H.H., H.K., N.Y., H.S., A.N., X.W., O.I., Y.X.,

N.N., M.T., A.H., and K.K. conducted the research; and S.K. designed the research, analyzed the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Flotho C, Kratz CP, Niemeyer CM. How a rare pediatric neoplasia can give important insights into biological concepts: a perspective on juvenile myelomonocytic leukemia. *Haematologica.* 2007; 92(11):1441-1446.
2. Muramatsu H, Makishima H, Jankowska AM, et al. Mutations of an E3 ubiquitin ligase c-Cbl but not TET2 mutations are pathogenic in juvenile myelomonocytic leukemia. *Blood.* 2010;115(10): 1969-1975.
3. Niemeyer CM, Kang MW, Shin DH, et al. Germline CBL mutations cause developmental abnormalities and predispose to juvenile myelomonocytic leukemia. *Nat Genet.* 2010;42(9):794-800.
4. Cotterman CW. Somatic mosaicism for antigen A2. *Acta Genet Stat Med.* 1956;6(4):520-521.
5. Gripp KW, Stabley DL, Nicholson L, Hoffman JD, Sol-Church K. Somatic mosaicism for an HRAS mutation causes Costello syndrome. *Am J Med Genet A.* 2006;140(20):2163-2169.
6. Sol-Church K, Stabley DL, Demmer LA, et al. Male-to-male transmission of Costello syndrome: G12S HRAS germline mutation inherited from a father with somatic mosaicism. *Am J Med Genet A.* 2009;149A(3):315-321.
7. Girisha KM, Lewis LE, Phadke SR, Kutsche K. Costello syndrome with severe cutis laxa and mosaic HRAS G12S mutation. *Am J Med Genet A.* 2010;152A(11):2861-2864.
8. Niemeyer CM, Arico M, Basso G, et al. Chronic myelomonocytic leukemia in childhood: a retrospective analysis of 110 cases. European Working Group on Myelodysplastic Syndromes in Childhood (EWOG-MDS). *Blood.* 1997;89(10): 3534-3543.
9. Tartaglia M, Niemeyer CM, Fragale A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat Genet.* 2003; 34(2):148-150.
10. De Filippi P, Zecca M, Lisini D, et al. Germ-line mutation of the NRAS gene may be responsible for the development of juvenile myelomonocytic leukaemia. *Br J Haematol.* 2009;147(5):706-709.
11. Side LE, Emanuel PD, Taylor B, et al. Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. *Blood.* 1998;92(1): 267-272.
12. Pinkel D. Differentiating juvenile myelomonocytic leukemia from infectious disease [letter]. *Blood.* 1998;91(1):365-367.
13. Fakhrai-Rad H, Pourmand N, Ronaghi M. Pyrosequencing: an accurate detection platform for single nucleotide polymorphisms. *Hum Mutat.* 2002;19(5):479-485.
14. Ogino S, Kawasaki T, Brahmandam M, et al. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. *J Mol Diagn.* 2005; 7(3):413-421.
15. Bresolin S, Zecca M, Flotho C, et al. Gene expression-based classification as an independent predictor of clinical outcome in juvenile myelomonocytic leukemia. *J Clin Oncol.* 2010; 28(11):1919-1927.
16. Yoshida N, Yagasaki H, Xu Y, et al. Correlation of clinical features with the mutational status of GM-CSF signaling pathway-related genes in juvenile myelomonocytic leukemia. *Pediatr Res.* 2009;65(3):334-340.

Brief report

CBL mutation in chronic myelomonocytic leukemia secondary to familial platelet disorder with propensity to develop acute myeloid leukemia (FPD/AML)

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Familial platelet disorder with a propensity to develop acute myeloid leukemia (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia, abnormal platelet function, and a propensity to develop myelodysplastic syndrome (MDS) and AML. So far, > 20 affected families have been reported. Recently, a second *RUNX1* alteration has been reported; however, no additional molecular abnormalities have been found so far. We identified an acquired *CBL* mutation and 11q-acquired uniparental disomy (11q-aUPD) in a patient with chronic myelomonocytic leukemia (CMML) secondary to FPD with *RUNX1* mutation but not in the same patient during refractory cytopenia. This finding suggests that alterations of the *CBL* gene and *RUNX1* gene may cooperate in the pathogenesis of CMML in patients with FPD/AML. The presence of *CBL* mutations and 11q-aUPD was an important "second hit" that could be an indicator of leukemic transformation of MDS or AML in patients with FPD/AML. (*Blood.* 2012; 119(11):2612-2614)

Introduction

Familial platelet disorder with a propensity to develop acute myeloid leukemia (FPD/AML) is a rare autosomal dominant disease characterized by thrombocytopenia, abnormal platelet function, and a propensity to develop myelodysplastic syndrome (MDS) and AML.[1,2] Since Song et al reported haploinsufficiency of the *RUNX1/CBFA2* gene,[3] more than 20 affected families have been reported.[4-8] Notably, various types of mono-allelic mutations of the *RUNX1* gene have been found in patients with AML secondary to FPD.[3,7-9] *RUNX1*, which is a key regulator of definitive hematopoiesis and myeloid differentiation, is also commonly involved in sporadic cases of MDS and AML, by translocations in AML[10] and by point mutations in AML[11,12] and MDS.[13] Recently, a second *RUNX1* alteration has been reported[8]; however, no additional molecular abnormalities have been found so far.

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing because these *CBL* mutations have been shown to result in aberrant tyrosine kinase signaling, which would also lead to the activation of RAS signaling pathways. So far, we and others have reported that *CBL* mutations occurred in a variety of myeloid neoplasms, including de novo AML,[14,15] MDS,[16,17] and myeloproliferative neoplasm,[16,17] especially in chronic myelomonocytic leukemia (CMML)[16,17] and juvenile myelomonocytic leukemia.[18] The importance of *CBL* mutations for leukemogenesis has substantially increased, which prompted us to search for possible *CBL* mutations in this pedigree.

Here, we reported that *CBL* mutation developed at the time of diagnosis of CMML, but not during refractory cytopenia, in a Japanese patient with FPD/AML harboring a *RUNX1* mutation.

Methods

RUNX1 mutation analysis

DNA and RNA were extracted from peripheral blood (PB) of the proband, her sister, and their mother after obtaining informed consent. We performed mutation analysis of the *RUNX1* gene by PCR followed by direct sequencing with the use of an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). For further confirmation of deletion mutations, the PCR products were subcloned with the use of a TOPO TA Cloning Kit (Invitrogen) and then sequenced. Mutations were screened from exons 1-8 of the *RUNX1* gene.

CBL mutation analysis

Because *CBL* mutations thus far reported almost exclusively involved exons 8-9 that encode Linker/RING finger domains, we confined our mutation analysis to these exons, which were subjected to direct sequencing. Because the frequency of 11q-acquired uniparental disomy (11q-aUPD) was reported as ~ 85%-90% in *CBL* mutations, we also analyzed the sample with Affymetrix GeneChip 250K *Nsp*I.[17-19] Genome-wide detection of copy number abnormalities or allelic imbalances was performed with CNAG/AsCNAR Version 3.0 software (http://www.genome.umin.jp), which enabled sensitive detection of copy number neutral loss of heterozygosity (or aUPD).[19] In addition, we examined mutations of the following genes in the proband as previously reported: *FLT3*, *KIT*, *RAS*, *JAK2*, *PTPN11*, *ASXL1*, *IDH1/2*, and *MPL*.[20-22] The study adhered to the principles of the Helsinki Declaration and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Center.

Results and discussion

The proband (III-2), who was the second child of nonconsanguineous parents, underwent an 8-year follow-up of mild to moderate

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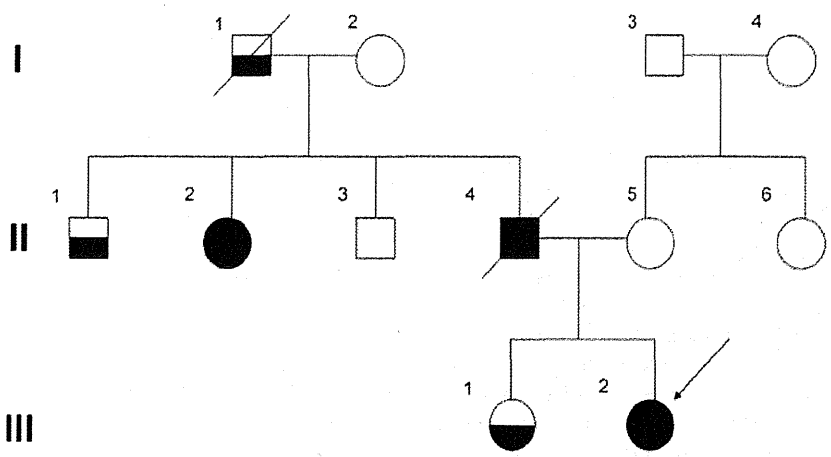

Figure 1. The family pedigree. Squares indicate males and circles indicate females. Open symbols represent unaffected persons, half-filled symbols represent persons affected by thrombocytopenia, and closed symbols represent persons affected by FPD who developed MDS/AML. The proband (III-2) is indicated by an arrow.

thrombocytopenia (50-80 × $10^3/\mu L$), and at that age of 10 years, her condition was diagnosed as refractory cytopenia. Cytogenetic analysis found a normal karyotype, and FISH showed neither monosomy 7 nor trisomy 8. The proband had been closely observed without any therapy for 2 years and 9 months because she did not require transfusion and her disease remained stable; however, at the age of 12 years, leukocytosis and monocytosis developed and she became dependent on platelet transfusions. Finally, the disease evolved to CMML, and allogeneic bone marrow (BM) transplantation from an unrelated donor was performed. During the entire course, the number of blast cells in PB was constantly < 2%, and no additional symptoms were observed, such as hepatosplenomegaly. Her elder sister (III-1) was also followed for 10 years with mild thrombocytopenia; however, the morphologic findings of PB or BM were not compatible with myeloproliferative neoplasms.[17] Because her platelet count has been gradually decreasing, allogeneic BM transplantation is being considered. Although her father (II-4) developed MDS at the age of 41 and died 2 years later, her paternal aunt (II-2) developed MDS at the age of 49 and has remained in complete remission for 11 years after successful allogeneic cord blood transplantation. Her paternal grandfather (I-1) and uncle (II-1) also had a history of thrombocytopenia (Figure 1). Direct sequencing analysis of *RUNX1* found a one-base deletion of adenine at position 2364 within exon 7, resulting in a frameshift mutation that corresponded to AML1b transcript in the proband and her sister (Figure 2A). This resulted in a frameshift after amino acid change G262GfsX21. This mutation was not detected in their mother. All these data suggested that her paternal grandfather (I-1), uncle (II-1), aunt (II-2), and her father (II-4) were considered to have FPD/AML, carrying the same *RUNX1* mutation.

Although no *CBL* mutations were found in the proband sample of refractory cytopenia before development of CMML, homozygous mutation of the *CBL*, which was located in the splice acceptor site of intron 8 (Figure 2B), was identified in the proband sample in the CMML. We also found 11q-aUPD (Figure 2C) in the proband sample, confirming a strong association of *CBL* mutations with 11q-aUPD, as previously described[16-18]; however, no mutations of any other genes, including *FLT3*, *KIT*, *RAS*, *JAK2*, *PTPN11*, *ASXL1*, *IDH1/2*, and *MPL*, were found and no additional somatic *RUNX1* alterations. No *CBL* mutations were found in her sister's sample at this time.

Inherited *RUNX1* mutations were clustered in the N-terminal region in exons 3-5, which affect the runt homology domain. Mutations in the C-terminal region, detected in the present pedigree, have been reported less frequently so far and are considered to affect the transactivation domain (Figure 2D).

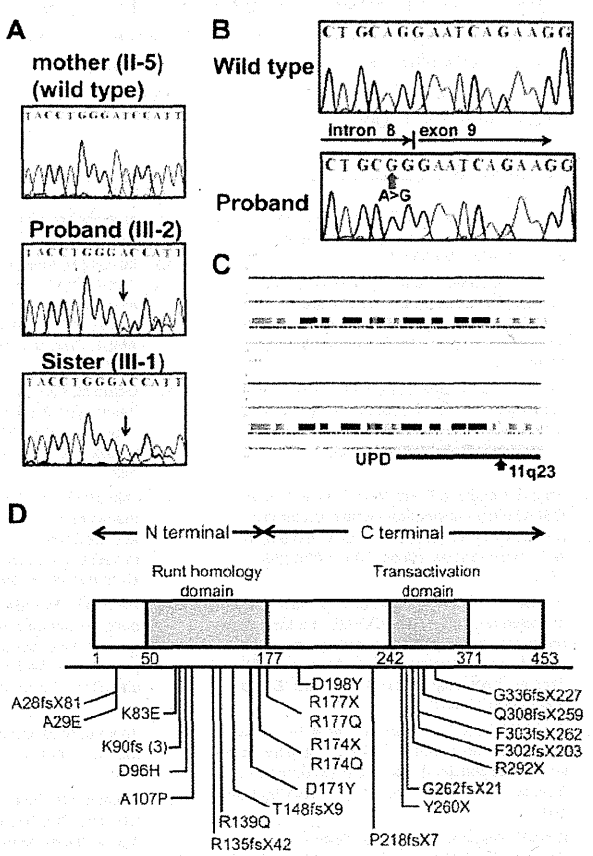

Figure 2. Mutation analysis of *RUNX1* and *CBL* genes in the pedigree. (A) Direct sequencing analysis of affected patients (III-1, III-2) and an unaffected family member (II-5) is shown. Arrow indicates a one-base deletion of adenine. (B) Mutated *CBL* is shown in the proband. (C) Identification of acquired uniparental disomy of 11q in the proband. Total copy number (tCN; red plot) is shown above the cytoband, and the results of allele-specific copy number analysis with anonymous references (AsCNAR) plots are shown below the cytoband. Larger allele is presented by a red line, and the smaller allele is presented by a blue line. Allele-specific analysis showed 11q-aUPD (blue line), which contained the *CBL* region (arrow). (D) Schematic representation of wild-type and mutated *RUNX1*. The affected *RUNX1* is truncated at the C terminus of the transactivation domain (TAD). Part of TAD is lacking in this proband (red line).

It has been postulated that disruption of the *RUNX1* gene is not sufficient to cause AML, as previously reported with monoallelic and biallelic inactivation of *Runx1* in mice[23,24] and in mice carrying the knocked-in *Runx1-Eto* chimeric gene. These data indicate that a second-hit mutation in addition to the dysfunction of *RUNX1* is required for the development of AML. Minelli et al postulated that the mutations seen in FPD cases have a mutation effect that induces additional genetic abnormalities and promotes progression to hematologic malignancies.[25]

Marked associations between chromosome translocation and gene mutations have been reported: *KIT* mutation in core binding leukemia, t(8;21)/*AML1-ETO* and inv(16)(p13q22)/*CBFβ-MYH11*, *FLT3*-ITD in leukemia with t(15;17)/*PML-RARα*, or with t(6;9)/*DEK-CAN*. We consider that it is important to find an association to administer clinically relevant treatment. In addition to the germline *RUNX1* mutation, we identified an acquired *CBL* mutation in the proband and assumed it to be a second hit mutation by which FPD evolved into CMML. To our knowledge, this is the first patient with FPD/AML in whom *CBL* mutation has developed. This finding suggests that alterations of the *CBL* gene and *RUNX1* could cooperate in the pathogenesis of CMML or AML in patients with FPD/AML. The presence of 11q-aUPD provided evidence that loss of the wild-type copy of *CBL* with duplication of the mutant copy was an important second hit that could be an indicator of leukemic transformation in patients with FPD/AML.

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Authorship

Contribution: Y.H. and C.O. designed the study; A.M., C.O., and D.H. provided critical reagents and samples; N.S., M.P., A.S.-O., and C.M. performed the experiments; H.A. and S.O. supervised the work; N.S. and M.P. analyzed the results; N.S. and D.H. constructed the figures; N.S. and Y.H. wrote the paper; and all the authors critically reviewed and revised the manuscript.

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References

1. Dowton SB, Beardsley D, Jamison D, Blattner S, Li FP. Studies of a familial platelet disorder. *Blood.* 1985;65(3):557-563.
2. Ho CY, Otterud B, Legare RD, et al. Linkage of a familial platelet disorder with a propensity to develop myeloid malignancies to human chromosome 21q22.1-22.2. *Blood.* 1996;87(12):5218-5224.
3. Song WJ, Sullivan MG, Legare RD, et al. Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. *Nat Genet.* 1999;23(2):166-175.
4. Buijs A, Poddighe P, van Wijk R, et al. A novel CBFA2 single-nucleotide mutation in familial platelet disorder with propensity to develop myeloid malignancies. *Blood.* 2001;98(9):2856-2858.
5. Michaud J, Wu F, Osato M, et al. In vitro analyses of known and novel RUNX1/AML1 mutations in dominant familial platelet disorder with predisposition to acute myelogenous leukemia: implications for mechanisms of pathogenesis. *Blood.* 2002;99(4):1364-1372.
6. Owen CJ, Toze CL, Koochin A, et al. Five new pedigrees with inherited RUNX1 mutations causing familial platelet disorder with propensity to myeloid malignancy. *Blood.* 2008;112(12):4639-4645.
7. Kirito K, Sakoe K, Shinoda D, Takiyama Y, Kaushansky K, Komatsu N. A novel RUNX1 mutation in familial platelet disorder with propensity to develop myeloid malignancies. *Haematologica.* 2008;93(1):155-156.
8. Preudhomme C, Renneville A, Bourdon V, et al. High frequency of RUNX1 biallelic alteration in acute myeloid leukemia secondary to familial platelet disorder. *Blood.* 2009;113(22):5583-5587.
9. Heller PG, Glembotsky AC, Gandhi MJ, et al. Low Mpl receptor expression in a pedigree with familial platelet disorder with predisposition to acute myelogenous leukemia and a novel AML1 mutation. *Blood.* 2005;105(12):4664-4670.
10. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y, Ohki M. t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. *Proc Natl Acad Sci U S A.* 1991;88(23):10431-10434.
11. Osato M, Asou N, Abdalla E, et al. Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2alphaB gene associated with myeloblastic leukemias. *Blood.* 1999;93(6):1817-1824.
12. Taketani T, Taki T, Takita J, et al. AML1/RUNX1 mutations are infrequent, but related to AML-M0, acquired trisomy 21, and leukemic transformation in pediatric hematologic malignancies. *Genes Chromosomes Cancer.* 2003;38(1):1-7.
13. Harada H, Harada Y, Niimi H, et al. High incidence of somatic mutations in the AML1/RUNX1 gene in myelodysplastic syndrome and low blast percentage myeloid leukemia with myelodysplasia. *Blood.* 2004;103(6):2316-2324.
14. Sargin B, Choudhary C, Crosetto N, et al. Flt3-dependent transformation by inactivating c-Cbl mutations in AML. *Blood.* 2007;110(3):1004-1012.
15. Caligiuri MA, Briesewitz R, Yu J, et al. Novel c-CBL and CBL-b ubiquitin ligase mutations in human acute myeloid leukemia. *Blood.* 2007;110(3):1022-1024.
16. Dunbar AJ, Gondek LP, O'Keefe CL, et al. 250K single nucleotide polymorphism array karyotyping identifies acquired uniparental disomy and homozygous mutations, including novel missense substitutions of c-Cbl, in myeloid malignancies. *Cancer Res.* 2008;68(24):10349-10357.
17. Sanada M, Suzuki T, Shih LY, et al. Gain-of-function of mutated C-CBL tumour suppressor in myeloid neoplasms. *Nature.* 2009;460(7257):904-908.
18. Shiba N, Kato M, Park MJ, et al. CBL mutations in juvenile myelomonocytic leukemia and pediatric myelodysplastic syndrome. *Leukemia.* 2010;24(5):1090-1092.
19. Nannya Y, Sanada M, Nakazaki K, et al. A robust algorithm for copy number detection using high-density oligonucleotide single nucleotide polymorphism genotyping arrays. *Cancer Res.* 2005;65(14):6071-6079.
20. Pardanani AD, Levine RL, Lasho T, et al. MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. *Blood.* 2006;108(10):3472-3476.
21. Rocquain J, Carbuccia N, Trouplin V, et al. Combined mutations of ASXL1, CBL, FLT3, IDH1, IDH2, JAK2, KRAS, NPM1, NRAS, RUNX1, TET2 and WT1 genes in myelodysplastic syndromes and acute myeloid leukemias. *BMC Cancer.* 2010;10:401.
22. Tartaglia M, Niemeyer CM, Fragale A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. *Nat Genet.* 2003;34(2):148-150.
23. Sun W, Downing JR. Haploinsufficiency of AML1 results in a decrease in the number of LTR-HSCs while simultaneously inducing an increase in more mature progenitors. *Blood.* 2004;104(12):3565-3572.
24. Ichikawa M, Asai T, Saito T, et al. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. *Nat Med.* 2004;10(3):299-304.
25. Minelli A, Maserati E, Rossi G, et al. Familial platelet disorder with propensity to acute myelogenous leukemia: genetic heterogeneity and progression to leukemia via acquisition of clonal chromosome anomalies. *Genes Chromosomes Cancer.* 2004;40(3):165-171.

DNMT3A mutations are rare in childhood acute myeloid leukaemia, myelodysplastic syndromes and juvenile myelomonocytic leukaemia

Acute myeloid leukaemia (AML) is a complex disease caused by mutations and deregulated gene expression, leading to increased proliferation and decreased differentiation of haematopoietic progenitor cells. Contemporary treatments have resulted in 5-year event-free survival rates of almost 60% for paediatric AML (Pui *et al*, 2011).

Recently, a whole genome sequencing study of AML uncovered recurrent mutations of an epigenetic regulator, the *DNA methyltransferase 3A* (*DNMT3A*) gene, in approximately 20% of adult AML patients (Ley *et al*, 2010; Yamashita *et al*, 2010; Yan *et al*, 2011). In these studies, *DNMT3A* mutations were frequently associated with *FLT3*-internal tandem duplication (ITD), *nucleophosmin 1* (*NPM1*) and *isocitrate dehydrogenase 1* (*IDH1*) mutations (Ley *et al*, 2010; Yan *et al*, 2011). *DNMT3A* mutations were also found in adult myelodysplastic syndromes (MDS) (8%, 12/150) (Walter *et al*, 2011), AML secondary to myeloproliferative neoplasms (MPNs) (14%, 5/35), myelofibrosis (15%, 3/20) and polycythaemia vera (7%, 2/30) (Stegelmann *et al*, 2011).

DNMT3A is involved in epigenetic regulation of genes by enzymatic de novo addition of methyl groups to the cytosine residue of CpG dinucleotides. *DNMT3A* mutations were significantly enriched with a cytogenetic profile associated with intermediate risk, including a normal cytogenetic profile, as well as the M4 and M5 subtypes, according to the French-American-British (FAB) classification system (Ley *et al*, 2010; Yan *et al*, 2011). In AML patients with a normal karyotype and *FLT3*-ITD, patients with *DNMT3A* gene mutations showed a worse prognosis than those without *DNMT3A* gene mutations (Ley *et al*, 2010; Yan *et al*, 2011); however, the frequency and clinical impact of *DNMT3A* gene mutations in paediatric AML and myeloproliferative neoplasms (MPN) remain uncertain. We searched for *DNMT3A* gene mutations in 149 AMLs who were treated on the Japanese Childhood AML Cooperative protocol, AML 99 (range: 0–15 years old, M0: 5, M1: 23, M2: 44, M3: 13, M4: 22, M5: 21, M6: 1, M7: 17, unclassified: three patients), 40 juvenile myelomonocytic leukaemias (JMMLs; range: 2 months to 8 years), 24 myelodysplastic syndromes (MDSs) and 20 paediatric therapy-related leukaemia/MDSs (t-Leuk/MDSs, range: 1–17 years). *FLT3*-ITD and *NPM1* gene alterations have been reported in these 149 AML patients (Shimada *et al*, 2007, 2008).

Total RNA 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). *DNMT3A* mutations were thus far reported to be almost exclusively involved in exons 16-23 (especially codon R882 in exon 23) (Ley *et al*, 2010; Yamashita *et al*, 2010; Stegelmann *et al*, 2011; Walter *et al*, 2011; Yan *et al*, 2011); thus, we confined our analysis to these exons. cDNA was amplified using the following primers: *DNMT3A* cDNA 15F, 5′-CAGGTGCTTTTGCGTGGAGTGT-3′ and 19R, 5′-ATGC AGGAGGCGGTAGAACTCA-3′, 17F, 5′-AAGATCATGTAC-GTCGGGGA-3′ and 22R, 5′-CTTTGCCCTGCTTTA TG-GAG-3′ and 20F, 5′-CCCTGTGATGATTGATGCCA-3′ and 23R, 5′-GTATTTCCGCCTCTGTG-GTT-3′ for AML samples. For JMML, MDS and t-Leuk/MDS, we confined our analysis to exon 23, including the hotspot of codon R882, of the *DNMT3A* gene using the following primers: *DNMT3A* DNA 23F, 5′-AGAACTAAGCAGGGCC-TCAGAGGA-3′ and 23R, 5′-GTATTTCCGCCTCTGTGGTT-3′. Subsequently, direct sequencing was performed on a DNA sequencer (ABI 310; Applied Biosystems, Foster City, CA, USA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). The study adhered to the principles of the Helsinki Declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Centre.

No *DNMT3A* mutations were detected in any AML patients in our study. Recently, *DNMT3A* mutations have been reported in paediatric AML patients (Ho *et al*, 2011; Thol *et al*, 2011). Only two patients were identified (both 15 years old). Combined with these and our data, the frequency of *DNMT3A* mutations is extremely rare (2/524, 0·4%) in childhood AML. Furthermore, we did not identify *DNMT3A* mutations in MDS, JMML or paediatric t-Leuk/MDS. These findings were not compatible with those of adult MDS and MPN, suggesting that the frequency of *DNMT3A* gene mutations depends on age.

On the other hand, we found *FLT3*-ITD in 20 (13%) of 149 AML patients; however, no *NPM1* mutations were found (Shimada *et al*, 2007, 2008). Nine AML patients with *FLT3*-ITD were found to lack *DNMT3A* mutation. *DNMT3A* mutations have been correlated with *FLT3*-ITD and *NPM1* in adult AML, but not in paediatric AML. Although patients with *DNMT3A* mutations have been associated with FAB-M4, M5, especially *MLL*-negative M5, no mutations in these paediatric M4/M5 patients were found in this study. *DNMT3A* mutations have not been detected in any adult AML with favourable cytogenetics, including *t*(8;21) and inv(16) (Ley *et al*, 2010; Yan *et al*, 2011). Higher frequencies of *t*(8;21) and inv(16) in

paediatric than in adult AML patients may be associated with rare *DNMT3A* mutations in paediatric AML. These data suggest that the pathology of paediatric AML may be different from that of adult AML. We concluded that *DNMT3A* mutations, as well as *NPM1* mutations, may be infrequent in paediatric AML and MDS patients, especially those <15 years old.

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Authorship

TT and YH designed the study. AS, MS, SA, AT, KH and MT provided critical reagents and samples. NS and MP performed the experiments. RH, IT and HA supervised the work. NS and MP analysed the results. NS, TT, and YH wrote the paper and all the authors critically reviewed and revised it.

Conflict of interest

The authors declare no conflicts of interest.

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References

Ho, P.A., Kutny, M.A., Alonzo, T.A., Gerbing, R.B., Joaquin, J., Raimondi, S.C., Gamis, A.S. & Meshinchi, S. (2011) Leukemic mutations in the methylation-associated genes DNMT3A and IDH2 are rare events in pediatric AML: a report from the Children's Oncology Group. *Pediatric Blood & Cancer*, **57**, 204–209.

Ley, T.J., Ding, L., Walter, M.J., McLellan, M.D., Lamprecht, T., Larson, D.E., Kandoth, C., Payton, J.E., Baty, J., Welch, J., Harris, C.C., Lichti, C.F., Townsend, R.R., Fulton, R.S., Dooling, D.J., Koboldt, D.C., Schmidt, H., Zhang, Q., Osborne, J.R., Lin, L., O'Laughlin, M., McMichael, J.F., Delehaunty, K.D., McGrath, S.D., Fulton, L.A., Magrini, V.J., Vickery, T.L., Hundal, J., Cook, L.L., Conyers, J.J., Swift, G.W., Reed, J.P., Alldredge, P.A., Wylie, T., Walker, J., Kalicki, J., Watson, M.A., Heath, S., Shannon, W.D., Varghese, N., Nagarajan, R., Westervelt, P., Tomasson, M.H., Link, D.C., Graubert, T.A., DiPersio, J.F., Mardis, E.R. & Wilson, R.K. (2010) DNMT3A mutations in acute myeloid leukemia. *New England Journal of Medicine*, **363**, 2424–2433.

Pui, C.H., Carroll, W.L., Meshinchi, S. & Arceci, R.J. (2011) Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *Journal of Clinical Oncology*, **29**, 551–565.

Shimada, A., Taki, T., Kubota, C., Tawa, A., Horibe, K., Tsuchida, M., Hanada, R., Tsukimoto, I. & Hayashi, Y. (2007) No nucleophosmin mutations in pediatric acute myeloid leukemia with normal karyotype: a study of the Japanese Childhood AML Cooperative Study Group. *Leukemia*, **21**, 1307.

Shimada, A., Taki, T., Tabuchi, K., Taketani, T., Hanada, R., Tawa, A., Tsuchida, M., Horibe, K., Tsukimoto, I. & Hayashi, Y. (2008) 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. *Pediatric Blood & Cancer*, **50**, 264–269.

Stegelmann, F., Bullinger, L., Schlenk, R.F., Paschka, P., Griesshammer, M., Blersch, C., Kuhn, S., Schauer, S., Döhner, H. & Döhner, K. (2011) DNMT3A mutations in myeloproliferative neoplasms. *Leukemia*, **25**, 1217–1219.

Thol, F., Heuser, M., Damm, F., Klusmann, J.H., Reinhardt, K. & Reinhardt, D. (2011) DNMT3A mutations are rare in childhood acute myeloid leukemia. *Haematologica*, **96**, 1238–1240.

Walter, M.J., Ding, L., Shen, D., Shao, J., Grillot, M., McLellan, M., Fulton, R., Schmidt, H., Kalicki-Veizer, J., O'Laughlin, M., Kandoth, C., Baty, J., Westervelt, P., Dipersio, J.F., Mardis, E.R., Wilson, R.K., Ley, T.J. & Graubert, T.A. (2011) Recurrent DNMT3A mutations in patients with myelodysplastic syndromes. *Leukemia*, **25**, 1153–1158.

Yamashita, Y., Yuan, J., Suetake, I., Suzuki, H., Ishikawa, Y., Choi, Y.L., Ueno, T., Soda, M., Hamada, T., Haruta, H., Takada, S., Miyazaki, Y., Kiyoi, H., Ito, E., Naoe, T., Tomonaga, M., Toyota, M., Tajima, S., Iwama, A. & Mano, H. (2010) Array-based genomic resequencing of human leukemia. *Oncogene*, **29**, 3723–3731.

Yan, X.J., Xu, J., Gu, Z.H., Pan, C.M., Lu, G., Shen, Y., Shi, J.Y., Zhu, Y.M., Tang, L., Zhang, X.W., Liang, W.X., Mi, J.Q., Song, H.D., Li, K.Q., Chen, Z. & Chen, S.J. (2011) Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nature Genetics*, **43**, 309–315.

CBL mutations in infant acute lymphoblastic leukaemia

Infant acute lymphoblastic leukaemia (ALL) is relatively rare, occurring in approximately 2·5–5% of cases of childhood ALL (Biondi *et al*, 2000). Infant ALLs are much more likely to present with high leucocyte counts, hepatosplenomegaly and overt central nervous system (CNS) diseases (Taki *et al*, 1996). T cell phenotype is much less common in infants, while myeloid antigen co-expression and the absence of CD10 expression are more frequent in infants than in older children with ALL. When molecular techniques [such as fluorescence *in situ* hybridization (FISH) or Southern blot analysis] are used in addition to karyotype, *MLL* gene rearrangements (*MLL*-R) are found in 70–80% of infant ALL compared with only 2–4% of older children with ALL (Taki *et al*, 1996; Biondi *et al*, 2000). Thus, infant ALL appears to be biologically distinct from the disease in older children (more than 1 year old).

In this regard, recent reports of somatic mutations of the *CBL* proto-oncogene in myeloid neoplasms are intriguing, because these *CBL* mutations were shown to result in aberrant tyrosine kinase signalling, which also leads to activation of RAS signalling pathways. So far, we and others have reported that *CBL* mutations occur in a variety of myeloid neoplasms, including *de novo* acute myeloid leukaemia (AML) (Caligiuri *et al*, 2007), myelodysplastic syndrome (MDS), and myeloproliferative neoplasm, especially in chronic myelomonocytic leukaemia (CMML) (Sanada *et al*, 2009), and juvenile myelomonocytic leukaemia (JMML) (Shiba *et al*, 2010). The importance of *CBL* mutations regarding leukaemogenesis is substantially increased. Recently, we found *CBL* mutation in therapy-related AML with *MLL*-R (Shiba *et al*, 2011). Interestingly, the *MLL-CBL* fusion gene has been reported in a *de novo* AML case (Fu *et al*, 2003), and this prompted us to search for possible *CBL* mutations in infant ALL with *MLL-R*.

Because *CBL* mutations thus far reported were almost all clustered within exons 8–9 that encode Linker/RING finger domains (Caligiuri *et al*, 2007; Sanada *et al*, 2009; Shiba *et al*, 2010), we confined our mutation analysis to these exons, in which polymerase chain reaction-amplified exons 8–9 were subjected to direct sequencing using an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Branchburg, NJ, USA). The study adhered to the principles of the Helsinki Declaration, and was conducted under the regulations enacted by the Ethics Board of Gunma Children's Medical Centre.

CBL gene analysis was performed in 41 infant ALL patients in which *MLL*-R was found in 33 patients (80·5%), including 15 patients with t(4;11)(q21;q23), 4 with t(9;11)(p22;q23) and 5 with t(11;19)(q23;p13.3). Median age at diagnosis was 4·7 months (range, 0–12 months). We also performed *CBL* gene mutation analysis in 28 B cell precursor (BCP)-ALL patients (age range, 1–14 years).

Heterozygous mutations of the *CBL* gene were identified in 2 (4·9%) of 41 infant ALL patients, but not in older children with BCP-ALL. These were located in exon 8 (Fig 1). One patient was a 3-month-old female with t(4;11)(q21;q23) and the other patient was a 6-month-old male with t(11;19)(q23;p13.3). They were registered and treated on two Japanese infant leukaemia protocols, MLL96 and MLL98 respectively (Isoyama *et al*, 2002; Kosaka *et al*, 2004). Although strong association between *CBL* mutations and 11q-acquired uniparental disomy (aUPD) has been reported (Sanada *et al*, 2009), we did not perform the single nucleotide polymorphism array analysis due to lack of DNA.

MLL-R are more frequent in younger infants; up to 90% of infant ALL less than 6 months old at diagnosis have detectable *MLL*-R compared with 30–50% of infant ALL aged 6–12 months (Taki *et al*, 1996). *MLL*-R ALL has a characteristic gene expression profile that significantly differs from that of non-*MLL*-R BCP-ALL and of AML, confirming that *MLL*-R ALL is a biologically unique leukaemia subtype.

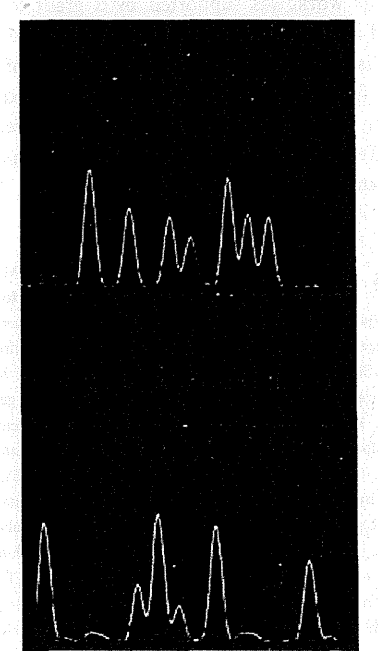

Fig 1. Identification of *CBL* mutations. Heterozygous mutations of the *CBL* gene were identified in Patients 7 and 21.