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* ≥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].

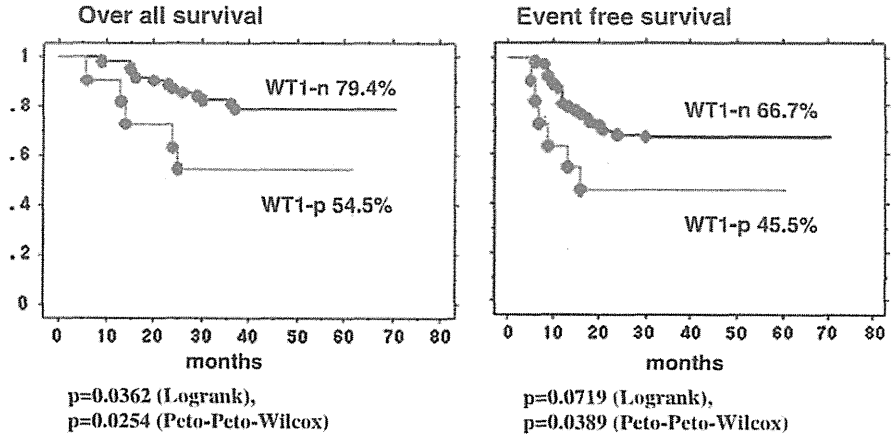

Fig. 1 The 5-year overall survival and event free survival rates for 74 AML patients according to *WT1* expression after 1st induction chemotherapy. *WT1-p WT1* mRNA expression had more than 2,500copies/μRNA, *WT1*-n; *WT1* mRNA expression had less than 2,500copies/μRNA. Overall survival is quite different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

Outcome in 146 patients with paediatric acute myeloid leukaemia treated according to the AML99 protocol in the period 2003–06 from the Japan Association of Childhood Leukaemia Study

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Summary

The acute myeloid leukaemia (AML) 99 trial conducted previously in Japan for the treatment of *de novo* paediatric AML showed excellent results, with a 5-year overall survival (OS) and event-free survival (EFS) of 75·6% and 61·6%, respectively. To examine reproducibility of these results in another cohort, the outcome of 146 newly diagnosed AML paediatric patients prospectively registered in the Japan Association of Childhood Leukaemia Study (JACLS) from 2003 to 2006 was compared to that of 240 patients in the original AML 99 clinical trial. The 5-year EFS and OS achieved in the new cohort was 66·7 ± 4·0% and 77·7 ± 8·0% respectively, which were comparable to those obtained in the original AML 99 clinical trial, although less frequent core-binding factor (CBF) AML (29·5% vs. 37%) and an almost equal frequency of allogeneic haematopoietic stem cell transplantation (allo-HSCT) during first complete remission (16·5% vs. 19%) were observed. The 5-year EFS in patients with a normal karyotype (NK) (n = 35, 54·9 ± 15·1%) was inferior in the present cohort when compared to the original AML99 trial. This study confirmed the excellent outcome of the original AML99 protocol.

Keywords: paediatric acute myeloid leukaemia, AML 99, Japan Association of Childhood Leukaemia Study, core-binding factor leukaemia, allo-HSCT.

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The acute myeloid leukaemia (AML) 99 trial was conducted in Japan between January 2000 and December 2002. The study recruited 240 children with newly diagnosed *de novo* AML. The 5-year overall survival (OS) and event-free survival (EFS) was 75·6% [95% confidence interval (CI), 70·3–81·4%] and 61·6% (95% CI, 55·8–68·1%), respectively. These results were superior to those of other clinical trials taking place at the time (Tsukimoto *et al*, 2009). However, it took approximately 4 years from the end of the AML99 trial to begin the nation-wide clinical trial, AML-05, for newly diagnosed *de novo* AML. We were interested in determining whether the positive outcome achieved in the original AML99 trial was reproducible in another cohort. To answer this question, we prospectively analysed the outcome of 146 paediatric patients newly diagnosed with AML and treated according to the AML 99 protocol in 57 institutes participating in the Japan Association of Childhood Leukaemia Study (JACLS) from 2003 to 2006. The results were compared to those of 240 patients in the original AML 99 clinical trial. We also analysed the data to determine prognostic factors for *de novo* paediatric AML.

Design and methods

Patients

Between 1 April, 2003 and 31 October, 2006, a total of 146 children with newly diagnosed AML were registered and treated according to the AML 99 protocol (Tsukimoto *et al*, 2009). Informed consent was obtained from the patients' guardians according to the Declaration of Helsinki and the protocol of treatment was approved by the institutional review boards of the participating institutes. The diagnosis of AML was based on morphological findings of bone marrow aspirates and immuno-phenotype analyses of leukaemic cells by flow cytometry or immune histochemical analysis. Children with acute promyelocytic leukaemia (APL), acute megakaryocytic leukaemia (AMKL) associated with Down syndrome, and therapy-related AML (t-AML) were excluded from this study.

Karyotypic analysis

Determination of karyotype was based on G-banding or fluorescence *in situ* hybridization analysis. Karyotypic analysis was successfully performed in 145 out of 146 patients.

Therapy

The details of the AML99 protocol were previously reported (Tsukimoto *et al*, 2009). Briefly, children younger than 2 years or those with a white blood cell (WBC) count $<100 \times 10^9$/l at diagnosis were treated with induction A. Children older than 2 years and with WBC count of $\geq 100 \times 10^9$/l were treated with induction B. Induction C was a rescue regimen for children who showed M3 marrow after induction A. Consolidation therapy consisted of five (for low- and intermediate-risk group) or six (for high-risk group) courses and triple intrathecal therapy was given as a part of each courses. Details of induction chemotherapeutic regimens are summarized in Table SI. Complete remission (CR) was defined by the absence of blast cells in the peripheral blood, fewer than 5% blast cells in the bone marrow (BM) aspirate, normal cellularity and trilineage haematopoiesis, and absence of blast cells in the cerebrospinal fluid and elsewhere. After the first consolidation course, patients in remission were stratified into three risk groups: low-risk (LR) patients were defined as those with t(8;21), inv (16), or aged younger than 2 years without high risk factors: high risk patients (HR) were those with abnormalities of monosomy 7, 5q-, t(16;21) or t(9;22); and intermediate risk (IR) patients were those who were not assigned to the LR or HR group. Allogeneic haematopoietic stem cell transplantation (allo-HSCT) was performed on 23 patients in first CR (CR1); three in the LR group, nine in the IR group, and 10 in the HR group. The preparative regimens and stem cell sources differed between the participating institutes. Although allo-HSCT was recommended for all HR patients in first remission and for IR patients with matched sibling donors as soon as possible (recommended after two courses of consolidation therapy), each individual institution decided allocation of allo-HSCT in CR1. The cumulative dose of cytarabine, anthracyclines and VP-16 (etoposide) was 59·4–78·4 g/m^2, 300–375 mg/m^2 and 3150–3200 mg/m^2, respectively.

Statistics

Based on the data at diagnosis and the annual follow-up survey, the 5-year EFS and OS were analysed. The Kaplan–Meier method was used to estimate survival rates and comparisons were based on the two-sided log-rank test. Failure to achieve remission, relapse, death during continuous complete remission (CCR), and secondary malignancy were considered as events. Death from any cause was the sole event used in determining OS. In both analyses, the observation time was censored at the last follow-up date if no event was noted.

Results

Patients' characteristics

The characteristics of the 146 patients are summarized in Table I. The median age at diagnosis was 7·6 years (range 0·3–16·2 years). Nine patients were infants. Four out of nine infants showed 11q23 abnormalities. The median leucocyte count at diagnosis was $14·7 \times 10^9$/l (range 0·6–243·6). Ninety patients (62%) showed WBC counts $<20 \times 10^9$/l, which was higher than those in the cohort of original

Table I. Patients' characteristics.

Characteristics	Patients, *n*	%
Patients analysed	146	100
Age, years		
<1	9	6
1–9	86	59
≥10	51	35
Sex		
Male	67	46
Female	76	52
Unknown	3	2
WBC, $\times 10^9$/l		
<20	90	62
20–<50	25	17
50–<100	17	12
≥100	12	8
Unknown	2	1
FAB type		
M0	7	5
M1	20	14
M2	54	37
M4	24	16
M5	22	15
M6	5	3
M7	10	7
Unclassifiable/unknown	4	3
Cytogenetics		
t(8;21)	34	23
inv 16	9	6
11q23 abnormality	20	14
High risk	10	7
Normal	35	24
Others	37	25
Unknown	1	1

FAB, French-American-British classification.

AML99 clinical trial (n = 115, 48%). The median follow-up period of the surviving patients was 52 months.

Overall results

The CR rate after the first consolidation courses or induction C was 95·9% (140 out of 146 patients). None of the patients demonstrated induction death. Six patients with induction failure received allo-HSCT treatment. Five of these patients died and one patient, with refractory central nervous system (CNS) leukaemia, is still alive in CCR. Of the 146 patients, 135 patients were treated with induction A and 11 were treated with induction B. Nine of 135 patients were treated with induction C, resulting in four CRs and five induction failures. The 5-year EFS and OS for all 146 patients were 66·7 ± 4·0% and 77·7 ± 8·0%, respectively (Fig 1). These figures were comparable to those in the original AML99 protocol study. Although the incidence of death in CR1 was 3·5% in original AML99 protocol study, only two patients (1·4%)

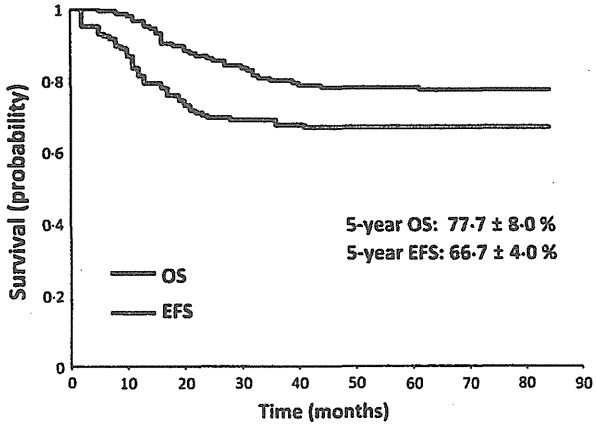

Fig 1. Probability of overall survival (OS) and event-free survival (EFS) in 146 paediatric patients with acute myeloid leukaemia.

died in CR1, one died of sepsis during the consolidation phase and the other one died of transplantation-related complication.

The cumulative risk of relapse was 30·2% (42 out of 139 patients). Four patients experienced relapse after treatment with allo-HSCT during CR1. Thereafter, all four patients died. Thirty-eight patients relapsed in CR during/after chemotherapy. The relapse site was predominantly BM (83·3%; 35 of 42). Seven patients experienced extramedullary relapse: two patients relapsed in the skin; two patients relapsed in the CNS; one patient relapsed in the mediastinum; one patient experienced a combined relapse in the CNS and BM; and one patient experienced a combined relapse in the testis and BM.

Results according to risk stratification

Among those who achieved first remission, 64 patients were stratified into the LR group, 63 into the IR group, and 13 into the HR group. The 5-year EFS and OS in each of the risk groups were 79·3 ± 6·8% and 85·7 ± 4·9% in the LR group; 64·9 ± 10·1% and 72·5 ± 8·8% in the IR group; and 69·2 ± 20·4% and 69·2 ± 20·4% in the HR group (Table II). The 5-year EFS and OS in the LR and IR groups were comparable to those in the AML 99 trial (Table II). However, in the IR group, the frequency of allo-HSCT in CR1 was much lower than in the original AML 99 trial (8/63 vs. 21/92). The 5-year EFS and OS rates in the HR group were better than those in the original AML 99 trial (Table II), although the frequency of allo-HSCT in CR1 was slightly higher (10/13 vs. 16/23). In the AML99 trial, the 5-year OS of 16 patients who received allo-HSCT in CR1 was 68·8%, which is comparable to the 5-year OS (70·1%) of 10 patients who received allo-HSCT in CR1 in the current study. Conversely, the relapse rate of patients who did not receive allo-HSCT in the HR group was lower than in the original AML 99 trial (1/3 vs. 5/7).

Table II. Comparison of 5-year EFS and OS between the AML99 trial and the current study (JACLS AML99) according to risk stratification.

	AML99			JACLS AML99		
	LR $n = 112$	IR $n = 92$	HR $n = 23$	LR $n = 64$	IR $n = 63$	HR $n = 13$
5-year EFS	71·3	59·8	56·5	79·3 ± 6·8	64·9 ± 10·1	64·3 ± 20·4
5-year OS	86·2	72·3	56·5	85·7 ± 4·9	72·5 ± 8·8	64·3 ± 20·4
SCT in 1st CR (n)	1	21	16	3	9	10

JACLS, Japan Association of Childhood Leukaemia Study; LR, low risk; IR, intermediate risk; HR, high risk; EFS, event-free survival; OS, overall survival; SCT, stem cell transplantation; CR, complete remission.

Results according to genetic abnormality or FAB classification

Based on a karyotypic analysis, 43 (29·7%) patients were categorized as having core-binding factor (CBF) leukaemia, consisting of karyotypic abnormalities such as t(8;21) in 35 patients or inv (16) in eight patients; 20 (13·8%) patients had 11q23 abnormalities; 35 (24·1%) patients had a normal karyotype (NK); and 10 (6·8%) patients had a high-risk karyotype including t(9;22), t(16;21), 5q- and -7 (Table I). The 5-year OS and EFS rates in each karyotype group are summarized in Table III. The 5-year EFS in patients with CBF leukaemia was 81·3 ± 6·1%. This was significantly higher than for patients with non-CBF leukaemia (60 ± 5·3%, $n = 103$, log rank $P = 0·015$) (Table III and Figure S1). On the other hand, the 5-year EFS in patients with a NK ($n = 35$) or a karyotype other than CBF, NK, 11q23 abnormality and high risk (others, $n = 37$), was inferior to that of patients with CBF leukaemia or 11q23 rearranged leukaemia (Table III). The 5-year EFS in patients with a high-risk karyotype was 70 ± 20·7% ($n = 10$), which was better than the 5-year EFS for patients with a NK or others, probably due to treatment with allo-HSCT during CR1 (Table III). However, of ten patients with a high-risk karyotype, three relapsed after allo-HSCT treatment and eventually died. The 5-year EFS in 11q23 rearranged leukaemia was 69·1 ± 15·2% ($n = 20$), which was relatively better in this study compared to the original AML99 trial. However, five out of six patients who relapsed subsequently died (Table SII). The 5-year EFS and OS rates, based on the FAB classification are summarized in Table III. The 5-year EFS in FAB types M2, M4 and M5 were relatively better, because CBF and 11q23 rearranged leukaemia were mainly categorized in one of the three classifications (Table III). On the other hand, the 5-year EFS in M0 patients was 28·6 ± 25·6% ($n = 7$), which was inferior even to that of non-CBF leukaemia (62·4 ± 8·1%, $n = 96$, log rank $P = 0·015$) (Figure S2). The characteristics of M0 patients are summarized in Table IV. Three out of seven patients had a high-risk karyotype. Four out of seven patients without a high-risk karyotype experienced induction failure, suggesting that leukaemia in M0 patients in this cohort possessed extremely poor biological properties.

Table III. 5-year EFS and OS according to the karyotypes and FAB classification.

	n	CR rate	5-year EFS	5-year OS
Cytogenetics				
CBF	43	100	81·3 ± 6·1	93 ± 4·2
NK	35	100	54·9 ± 15·1	68·3 ± 11·6
High*	10	90	70 ± 20·7	70 ± 20·7
11q23	20	95	69·1 ± 15·2	73·3 ± 14·0
Others†	37	89	58·6 ± 14·0	72·7 ± 10·2
FAB classification				
M0	7	57·1	28·6 ± 25·6	28·6 ± 25·6
M1	20	90	60 ± 17·9	75 ± 12·9
M2	54	100	74·1 ± 8·0	86·8 ± 5·3
M4	24	100	73·7 ± 12·6	82·2 ± 9·9
M5	22	100	71·8 ± 13·6	80·7 ± 10·8
M6	5	100	60 ± 36·5	60 ± 36·5
M7	10	90	45 ± 36·9	60 ± 25·8

CR, complete remission; EFS, event-free survival; OS, overall survival; CBF, core-binding factor; NK, normal karyotype; FAB, French-American-British classification.

*High-risk karyotype includes 5q-, -7, Ph, and t(16;21).

†Others include karyotypes other than CBF, NK, 11q23 abnormality, and high-risk karyotype.

Results of allo-HSCT

Allo-HSCT in CR1 was performed on 22 patients; sibling BM ($n = 9$), one matched sibling peripheral blood stem cell (PBSC, $n = 1$), unrelated cord blood ($n = 7$) and unrelated BM ($n = 5$). Only one patient died of transplantation-related complication. The 4 year-OS in these patients was 78·3 ± 8·9%. Three patients in the LR group received allo-HSCT in CR1, based on institutional decision. Two of whom were M6 patients. One of theses three patients had AML with t(8;21)(q22;q22) and showed positive minimal residual disease (MRD). All of them are alive in CCR. As a salvage therapy, allo-HSCT was performed on 32 patients experiencing relapse after attaining CR1. Twenty patients received allo-HSCT in second remission, and 12 of these patients survived (4-year OS; 57·6 ± 22·2%). On the other hand, 12 patients received allo-HSCT without second CR (CR2) but only two patients survived (4-year OS; 16·7 ± 64·5%, Figure S3). Survival rates of patients receiving allo-HSCT during

Table IV. Clinical and biological characteristics of M0 patients.

Patient	Gender	Age (years)	Karyotype	Response to induction	HSCT in CR1	Prognosis
1	M	11	-7	CR	+	CCR
2	F	8	5q-	CR	+	CCR
3	M	13	47,XY,+6,t(11;19)(q23;p13·3)	IF	–	Dead
4	F	14	47,XX,+4	IF	–	Dead
5	F	0·6	-7	CR	+	Dead*
6	F	1	47,XX + 3	CR	–	Dead*
7	F	11	46XX,del(7)(q?)	IF	–	Dead

HSCT, haematopoietic stem cell transplantation; CR1, first complete remission; CR, complete remission; CCR, continuous complete remission; IF, induction failure: M, male; F, female. +, HSCT in CR1 was performed; -, HSCT in CR1 was not performed.
*Both patients died of disease after relapse.

CR1 were significantly better than those of patients receiving allo-HSCT without CR1 (12/20 vs. 2/12, $P = 0·027$).

Discussion

The 5-year EFS of 66·7 ± 4·0% and 5-year OS of 77·7 ± 8·0% achieved in the present cohort are comparable to those in the original AML 99 clinical trial. On the other hand, the incidence of CBF leukaemia was lower than in the original AML99 trial (29·5% vs. 37%) and a very similar frequency of CR1 with allo-HSCT treatment (16·5% vs. 19%) was observed. Compared to the UK Medical Research Council (MRC)-AML12 study (Gibson *et al*, 2005, 2011), the current study showed better overall EFS and OS rates (Table V). Strikingly, the overall results of the current study were comparable to those of the AML02 study from St. Jude Children's Research Hospital (SJCRH), which was based on a risk stratification determined by measuring MRD and performed allo-HSCT in 27% of the patients (Rubnitz *et al*, 2010). In addition, the low incidence of death in CR1 (1·4%), which is much better than that in the MRC-AML12 or AML02 studies, might contribute to the excellent outcome in the present study (Table V). These findings suggest that the chemotherapeutic regimen and the risk stratification system in the AML99 protocol were quite effective in the treatment of paediatric *de novo* AML.

According to the risk groups, 5-year EFS and OS rates in the LR and IR groups in this cohort were comparable to those in the original AML99 clinical trial (Table II). Interestingly, the frequency of allo-HSCT in CR1 in the IR group was much lower than in the original AML99 clinical trial (9/63 vs. 21/92), suggesting that allo-HSCT during CR1 did not contribute to an improvement of the prognosis in this risk group. This finding is also consistent with the results that no improvements in 5-year OS in the IR group were achieved by allo-HSCT during CR1 in the original AML99 clinical trial (Tsukimoto *et al*, 2009). The 5-year EFS and OS in the HR group in the current cohort were better than in the original AML99 clinical trial (Table II). Because the 5-year OS of HR patients receiving allo-HSCT during CR1 was similar to that in the AML99 original trial (66·7% vs. 68·8%), the low relapse rate in HR patients who did not receive allo-HSCT (1/4 vs. 5/7) contributed to a better prognosis in these patients. These findings suggest that some patients in the HR group did not need to receive allo-HSCT during CR1. Therefore, the more precise evaluation system for initial treatment response might be useful to identify patients in a HR group who can be cured by chemotherapy only.

It is well known that the frequency of CBF leukaemia has a good prognostic impact on the overall results of paediatric AML clinical trials (Gibson *et al*, 2005; Tomizawa *et al*, 2007; Tsukimoto *et al*, 2009; Rubnitz *et al*, 2010). The present study included 43 (29·5%) patients with CBF leukaemia, which is relatively higher than the 17·5% in the MRC-AML12 study and the 24·8% in the SJCRH AML02 study. Furthermore, the overall 5-year EFS and OS of patients with CBF leukaemia were 81·3 ± 6·1% and 93 ± 4·2%, respectively. These figures were superior to those in the MRC-AML12 study (10-year OS: 84%) and were similar to those

Table V. Summary of overall results of clinical trials for paediatric AML from major research groups.

	n	CR rate	CBF	HSCT in CR1	Death in CR1	EFS	OS
AML99 (Tsukimoto *et al*, 2009)	240	94	37	19	3·5	61·6 (5-year)	75·6 (5-year)
SJCRH AML02 (Rubnitz *et al*, 2010)	230	94	24·8	27	8·7	63 (3-year)	71 (3-year)
MRC-AML12 (Gibson *et al*, 2005, 2011)	455	92	20	7	6	56 (5-year)	66 (5-year)
AML99 (JACLS) (present study)	146	95	29·5	16·5	1·4	66·7 (5-year)	77·7 (5-year)

CR, complete remission; CBF, core binding factor; HSCT, haematopoietic stem cell transplantation; EFS, event-free survival; OS, overall survival; SJCRH, St. Jude Children's Research Hospital; MRC, Medical Research Council; JACLS, Japan Association of Childhood Leukaemia Study.

in the AML02 study (3-year EFS: 85·8%, 3-year OS: 90·6%). Therefore, high frequency and a favourable outcome of CBF leukaemia contributed to the better overall results in this study. It is well known that *NPM1* mutation defines another subgroup with favourable prognosis (Hollink *et al*, 2009). However, a previous study determined this genetic alteration was not prevalent in paediatric AML in Japan (Shimada *et al*, 2007). Thus, screening for *NPM1* mutation was not performed in the current study. On the other hand, the 5-year EFS of the non-CBF leukaemia patients was 60 ± 5·3%, which was also better than that in AML02 study.

The 5-year EFS and OS of 11q23 rearranged AML in this cohort was 69·1% and 73·3%, respectively. Although several reports showed that t(9;11) positive AML shows a better prognosis than AML with other 11q23 abnormalities (Rubnitz *et al*, 2010), only four out of 20 patients had t(9;11). Thus, the 5-year EFS and OS of 11q23 rearranged AML in this cohort were similar to those of patients with t(9;11) (5-year EFS: 66·7%, 5-year OS: 80%) and superior to those of patients with other 11q23 abnormalities (5-year EFS: 50%, 5-year OS: 55·9%) observed in the AML02 study. We believe that the improved prognosis of patients with 11q23 abnormalities may also contribute to improved results in this study.

Conversely, the 5-year EFS of patients with a normal karyotype was 54·9 ± 15·1%, which was less than that observed in patients with a high-risk karyotype (70·0 ± 20·7%). It is well known that various genetic alterations are related to a poor prognosis in the NK group, such as *FLT3* internal tandem duplication (ITD) (Zwaan *et al*, 2003; Shimada *et al*, 2008; Mizushima *et al*, 2010; Balgobind *et al*, 2011). These genetic alterations were not tested in this cohort, and this may have resulted in a lower 5-year EFS in the normal karyotype group. However, the 5-year OS was 68·3 ± 11·6% (Table III), which was better than that in AML12 study, suggesting allo-HSCT might salvage this group. As the patients with *FLT3*-ITD received allo-HSCT treatment in CR1 in the subsequent AML05 trial, it should be possible to draw conclusions on whether allo-HSCT during CR1 improves the prognosis of *FLT3*-ITD positive patients. In addition, the 5-year EFS was only 28·6 ± 25·6% in the FAB M0 patients. Four out of seven patients experienced induction failure. It is likely that these patients had adverse genetic alterations that were not identified. However, it is not possible to screen all genetic alterations related to a poor prognosis, suggesting that universal prognostic markers are essential for improving risk stratification. Rubnitz *et al* (2010) identified that a MRD of 1% or higher, measured by multi-colour flow cytometric analysis after induction therapy, was an adverse prognostic factor in the paediatric AML cohort. Thus, the risk stratification, along with a combined measurement of MRD and detailed genetic analysis, are expected to improve the outcome of paediatric AML.

AML-M0 was also associated with poor outcome in the present study. Barbaric *et al* (2007) described that AML-M0 patients have an inferior outcome compared with those with non-M0 AML in CCG-2891 and 2961 studies. However, the small number of patients with paediatric AML-M0 did not allow the prognosis of this group to be determined. Interestingly, AML-M0 is subdivided into two groups based on the mutation status of *RUNX1* (Silva *et al*, 2009). Further studies are required to determine whether *RUNX1* mutation is associated with the prognosis of AML-M0.

Finally, the treatment of relapsed paediatric AML patients remains a challenge. The current analysis revealed that 4-year OS was 57·6 ± 22·2% in patients transplanted in CR2. On the other hand, 4-year OS was only 16·7 ± 64·5% in patients transplanted without CR2. These findings suggest that achieving CR2 is critical for the success of HSCT, which is consistent with previous studies (Isoyama *et al*, 2010; Sander *et al*, 2010). Because of this, the establishment of an effective re-induction regimen is mandatory. Recent studies also identified new agents, including small molecular compounds targeting key pathways in AML blast cells, which show promising effects (Ravandi *et al*, 2010; Inaba *et al*, 2011).

In conclusion, the overall outcome in this cohort was comparable to that in the original AML99 protocol, suggesting that the AML99 regimen was highly effective in the treatment of paediatric AML, especially for patients with CBF leukaemia or 11q23 abnormalities. The establishment of more effective regimens, especially for relapsed patients, is necessary to further improve the outcome of paediatric AML.

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Authorship and disclosures

TI took primary responsibility for the paper, analysed the data and wrote the manuscript. KH collected the clinical data. AT and SA supervised this research. All authors followed the patients, participated in editing the manuscript and approved the final version. The authors reported no potential conflicts of interest.

Supporting Information

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

Fig S1. Comparison of the probability of event-free survival (EFS) in patients with core binding factor (CBF) leukaemia and non-CBF leukaemia.

Fig S2. Comparison of the probability of event-free survival (EFS) in patients with a FAB M0 phenotype and a non-CBF and non-FAB M0 phenotype.

Fig S3. Comparison of the probability of overall survival (OS) in patients receiving allogeneic haematopoietic stem cell transplantation (HSCT) according to the disease status at the time of HSCT.

Table SI. Summary of induction therapy of AML99 protocol.

Table SII. Summary of relapsed cases according to karyotype.

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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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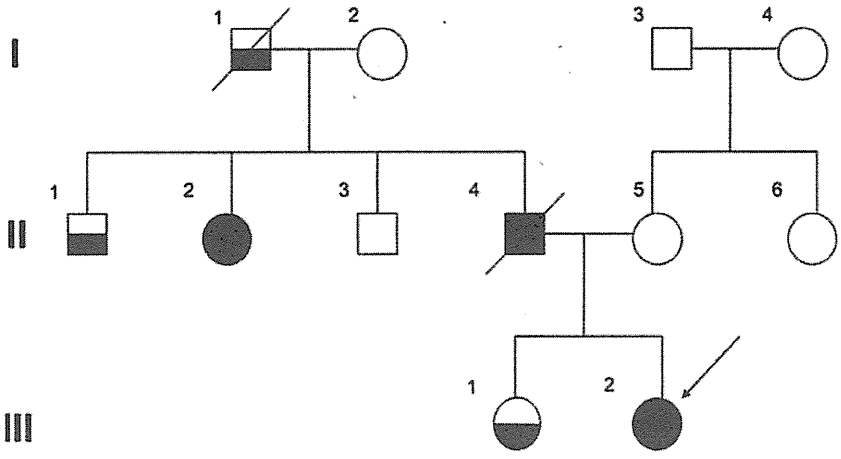

Figure 1. The family pedigree. Squares indicate males and circles indicate females. Open symbols represent unaffected persons, half-filled symbols represent persons affected by thrombocytopenia, and closed symbols represent persons affected by FPD who developed MDS/AML. The proband (III-2) is indicated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

thrombocytopenia (50-80 $\times$ $10^3/\mu L$), 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).

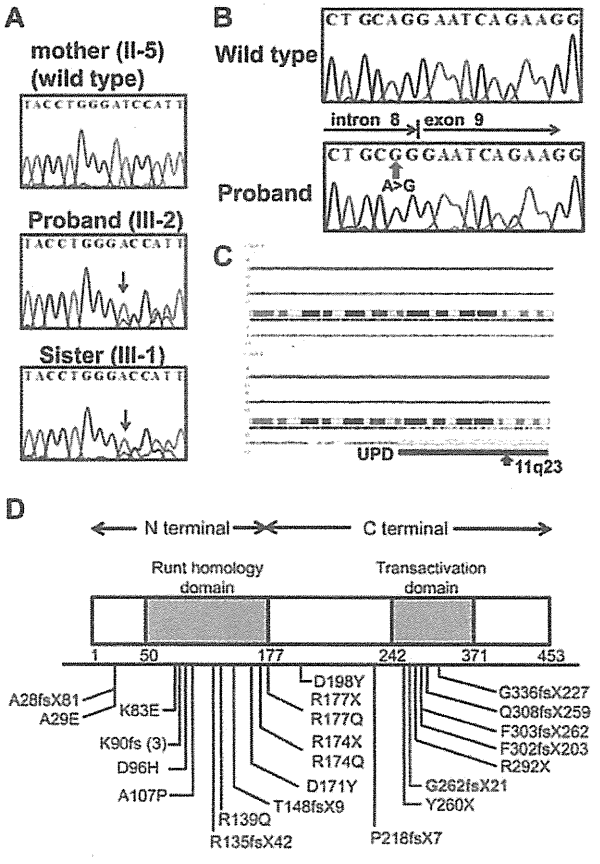

Figure 2. Mutation analysis of *RUNX1* and *CBL* genes in the pedigree. (A) Direct sequencing analysis of affected patients (III-1, III-2) and an unaffected family member (II-5) is shown. Arrow indicates a one-base deletion of adenine. (B) Mutated *CBL* is shown in the proband. (C) Identification of 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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bjh research paper

De novo childhood myelodysplastic/myeloproliferative disease with unique molecular characteristics

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Summary

Myelodysplastic/myeloproliferative uclassifiable (MDS/MPN-U) is a rare myeloid neoplasm characterized by myelodysplasia and myeloproliferation at the time of initial presentation, which is usually a diagnosis of exclusion. The molecular pathogenesis of MDS/MPN-U patients remains to be elucidated. Among five patients diagnosed with MDS/MPN-U, three patients harboured *RUNX1* (*AML1*) mutations; one carried somatic mosaicism of *RUNX1* mutation with *JAK2*^{V617F} mutation and one had dual *RUNX1* and *FLT3*-internal tandem duplication mutations with progression to acute myeloid leukaemia (AML). Germline mutation of *TP53* was detected as a sole genetic lesion in one patient. *JAK2*^{V617F} and somatic mosaicism of *KRAS* and *TET2* mutations co-existed in one patient. Otherwise, no alterations were detected in *PTPN11*, *NRAS*, *CBL* and *ASXL1* genes. *ETV6-PDGFRB* fusion transcript was not detected in all patients. Four patients recieved haematopoietic stem cell transplantation (HSCT); three patients relapsed and one achieved complete remission after three donor lymphocyte infusions. Our findings suggest that the mutational spectrum observed in childhood MDS/MPN-U is quite different from that seen in juvenile myelomonocytic leukaemia and, to some extent, resemble chronic myelomonocytic leukaemia. Moreover, two patients had constitutional alterations of genes frequently found in AML. Further investigations are required to define the roles of these genetic alterations in the pathogenesis of childhood MDS/MPN-U.

Keywords: *RUNX1*, *TET2*, *JAK2*^{V617F}, *TP53*, MDS/MPN-U

Myelodysplastic/myeloproliferative neoplasms (MDS/MPNs) are clonal myeloid neoplasms that, at the time of initial presentation, are characterized by overlapped myelodysplasia and myeloproliferation (Vardiman *et al*, 2009). These disorders include chronic myelomonocytic leukaemia (CMML), juvenile myelomonocytic leukaemia (JMML), atypical chronic myeloid leukaemia (a-CML; *BCR-ABL1* negative) and myelodysplastic/MPN unclassifiable (MDS/MPN-U)(Cazzola *et al*, 2011). The MDS/MPN-U category comprises a provisional entity, defined as refractory anaemia with ringed sideroblasts (RARS) associated with marked thrombocytosis (RARS-T) (Vardiman *et al*, 2009). CMML and JMML are characterized by persistent peripheral blood (PB) monocytosis >1 × 10^9/l, no Philadelphia chromosome, fewer than 20% blasts in PB and bone marrow (BM), no eosinophilia, and dysplasia in one or more myeloid lineage (Vardiman *et al*, 2009), while a-CML is characterized by involvement of the neutrophil lineage with leucocytosis. Patients who present with MDS/MPN features and do not meet the criteria for CMML, JMML or a-CML are diagnosed as MDS/MPN-U (Vardiman *et al*, 2009).

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JMML is an aggressive disease of early childhood (<5 years), while CMML and a-CML are observed mainly in the elderly (median age at diagnosis: 65–75 years) (Emanuel, 2008); the rarity of MDS/MPN-U has contributed to the paucity of reports on this category. In paediatric fields, the term CMML is reserved for the secondary occurrences seen in patients previously treated with chemotherapy and radiotherapy (Hasle *et al*, 2003), and JMML is considered to be the paediatric equivalent of CMML.

Characterization of the molecular events associated with MDS/MPNs has provided great insights into the pathophysiology of these diseases and has guided more specific therapeutic approaches. It was recently reported that the genetic lesions found in JMML differ from those found in CMML (Kohlmann *et al*, 2010; Perez *et al*, 2010; Bacher *et al*, 2011). Specifically, genetic profiling of patients with JMML revealed that alterations in *PTPN11* and *NF1* genes are specific to JMML (Side *et al*, 1998; Tartaglia *et al*, 2003; Kratz *et al*, 2005). Conversely, *TET2*, *RUNX1* and *JAK2* mutations are unique genetic events for CMML (Kohlmann *et al*, 2010; Bacher *et al*, 2011). In addition, *RAS*, *CBL* and *ASXL1* alterations are encountered in both diseases (Perez *et al*, 2010; Sugimoto *et al*, 2010). On the other hand, the activating *JAK2*^{V617F}, *NRAS*, *KRAS*, *TET2*, *CBL* and *EZH2* mutations have been reported in some cases with a-CML (Grand *et al*, 2009; Reiter *et al*, 2009; Ernst *et al*, 2010). Recently, our understanding of the molecular basis of RARS and RARS-T has improved considerably. Somatic mutations of *SF3B1*, *JAK2* and *MPL* genes have been reported to play a pivotal role in RARS and RARS-T molecular pathogenesis (Cazzola *et al*, 2011). At present, the molecular genetic abnormalities involved in the pathogenesis of other MDS/MPN-U patients (rather than RARS and RARS-T) have not been fully clarified.

We aimed to define the molecular characteristics of a group of paediatric patients who could not be diagnosed as having JMML and were provisionally given a diagnosis of MDS/MPN-U based on clinical, laboratory and morphological findings.

Methods

Patients and samples

Five patients (median age at diagnosis, 8 years, range; 6–15 years) not satisfying the diagnostic criteria for JMML, CMML or a-CML and diagnosed with MDS/MPN-U (according to the World Health Organization classification; Vardiman *et al*, 2009) were referred to our department between 1991 and 2011. One of these patients (Patient 4) was reported previously (Shimada *et al*, 2007); the data of this patient are summarized, included in the cohort analysis and complemented by new molecular data. Cytogenetic analysis was performed for all patients. There was no history of radiation exposure or chemotherapy prior to diagnosis. Peripheral blood (PB) and/or bone marrow (BM) samples were collected from all patients and mononuclear cell fractions were isolated using a Ficoll gradient. Genomic DNA was isolated using the QIAmp DNA blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was also extracted from nails (Patients 2, 3 and 5), hair follicles and buccal epithelial cells (Patients 2 and 5) and sarcomatous skin lesion (Patient 5) using lysis buffer, followed by purification with a QIAmp DNA investigator kit (Qiagen). Parental blood samples (Patients 3 and 5) were analysed in order to confirm whether the change was *de novo* or inherited. Progression to acute myeloid leukaemia (AML) during the course of the disease occurred in two out of five patients, and DNA was available at the time of AML progression. Informed consent was obtained from the guardians of all patients, in accordance with institutional guidelines, and study protocols were approved by the Institutional Review Boards of Nagoya University Graduate School of Medicine.

Mutational analysis

We investigated the following candidate genes at known mutational hot spots: *PTPN11* (exons 3, 8 and 13), *NRAS* (exons 2 and 3), *KRAS* (exons 2 and 3), *TET2* (exons 3–11), *RUNX1* (exons 3–8), *ASXL1* (exon 12) and *CBL* (exons 7, 8, 9) (Perez *et al*, 2010). Moreover, the complete coding region for *TP53* (Guinn *et al*, 1995) and the juxter membrane domain of the *FLT3* gene using primer pairs R5 and R6 (Xu *et al*, 1999) were investigated by sequencing analysis after polymerase chain reaction (PCR) amplification of genomic DNA. PCR amplification and purification were performed in a 25-μl PCR mix containing at least 50 ng of template DNA using quick Taq PCR™ HS Dye mix (Qiagen) under the following conditions: 94°C for 2 min (first denaturing step); 94°C for 30 s; 65°C for 30 s; 35 cycles of 68°C for 30 s to 1 min, depending on PCR product length; and 68°C for 7 min (final extension step). PCR products were purified from the reaction mixture using the QIA quick PCR purification kit (Qiagen), and were directly sequenced on a DNA sequencer (ABI PRISM 3100 Genetic Analyser; Applied Biosystems, [Life Technologies, Carlsbad, CA, USA]) using a Big Dye terminator cycle sequencing kit (Applied Biosystems). All DNA sequence abnormalities were confirmed by three independent experiments and were validated by two investigators.

Allele-specific PCR

In order to efficiently screen *JAK2*^{V617F} mutations, we carried out allele-specific PCR analysis, as described previously (Baxter *et al*, 2005).

RT-PCR for the ETV6-PDGFRB fusion transcript

RNA was extracted, reverse transcribed with random hexamer primers and tested for the presence of *ETV6-PDGFRB* fusion by single-step reverse transcriptase polymerase chain

reaction (RT-PCR) according to procedures described elsewhere (David *et al*, 2007).

Results

Clinical and haematological characteristics of MDS/MPN-U patients

Five patients fulfilling the diagnostic criteria of MDS/MPN-U with a median age at onset of 8 years (range 6–15 years) were included in this study. The main clinical features of all patients are described in Table I. The median white blood cell (WBC) count, percentage of peripheral monocytes, PB and BM blast counts were 23·6 × 10^9/l (range; 9·2–70·6 × 10^9/l), 17% (range: 11–36%), 1% (range; 1–2%) and 2% (range; 1·0–4·0%), respectively. No clinical or laboratory evidence of chronic infections was detected in this group of patients. Clinical manifestations of neurofibromatosis type 1 were absent in all patients. BM examination showed hypercellularity with marked increase of myeloid cells and variable degrees of dysplastic changes in the blood lineages (Fig 1). Cytogenetic analysis revealed that two patients had normal karyotypes and aberrant changes were identified in three patients, including trisomy 8, 19, 21 and mixed changes of t (1;11)(p32;p15), add (10)(q11·2),add (11)(p15) add(16)(q13) (Table I).

Mutation profiling of candidate genes in paediatric patients with MDS/MPN-U

To reach a precise definition of the genetic background of the five children with MDS/MPN-U diagnosis, we analysed the sequences of several candidate genes recently shown to be involved in the pathogenesis of MDS/MPNs. Genetic profiling revealed several lesions involving the *RUNX1* (*AML1*), *TET2, KRAS, JAK2, TP53* and *FLT3*-internal tandem duplication (ITD) genes. Specifically, Patient 1 had an 84-bp deletion in the runt domain of *RUNX1* that led to V90-K117del (causing deletion of 28 amino acids) at the onset of diagnosis (Fig 2). Patient 2 had concomitant missense point mutations in *KRAS* (G13D) and *TET2* (L346P) in a diagnostic BM sample obtained at age 6 years. Furthermore, *JAK2*^{V617F} mutation was concurrently detected (by allele-specific PCR combined with sequence analysis) with the *KRAS* and *TET2* genetic alterations in a PB sample obtained at the time of splenectomy at age 15 years. Interestingly, we also found *KRAS* and *TET2* mutations in the patient's nail and PB samples. In addition, the *TET2* mutation was identified in the buccal mucosal epithelial cells (Fig 3), but neither the *KRAS* mutation nor the *TET2* mutation was detected in hair samples obtained from the same patient. Samples from the patient's family were not available. Patient 3 was found to have a missense mutation in exon 2 c.31G > C (E11Q) of *TP53* in PB and nail samples obtained at the initial time of diagnosis (Fig 4). Screening of *TP53* missense mutations in fa-

Table I. Clinical and molecular characteristics of paediatric MDS/MPN-U patients.

Patient No.	Sex/ age (years)	WBC (× 10^9/l)	Mo (%)	Hb (g/dl)	Blasts (%)	Plt (× 10^9/l)	BM Blast (%)	Karyotype	Molecular alterations					Therapy	Survival (years)	AML transformation
									JAK2 V617F	*RUNX1*	*TET2*	Others				
1	F/14	23·6	17	3·9	1	9·000	4	47, XX + 21	–	V90–K117del	–	–		RBMT	Dead (4)	–
2	F/6	9·2	21	4·4	1	23·000	3	46, XX	+	–	L346P	*KRAS* G13D		No	Alive (>20)	–
3	F/8	36·8	36	3·9	1	54·000	1	48, XX, +8 + 19	–	–	–	*TP53* E11Q		URBMT + DLI	Alive (>2)	–
4	F/6	70·6	11	12·1	2	560·000	1	46, XX	–	Val117fsX124	–	*FLT3*-ITD		URBMT	Dead (3)	+
5	M/15	20·6	11	15·4	2	67·000	2	46, XY t(1;11)(p32;p15), add (10)(q11·2), add (11)(p15) add(16)(q13)	+	T300fsX311	–	–		RBMT	Alive (>0·5)	+

F, female; M, male; WBC, White blood cell count; Mo, Monocytes; Hb, Haemoglobin; Plt, Platelet count; RBMT, Related Bone Marrow Transplantation; URBMT, Unrelated Bone Marrow Transplantation; *FLT3*-ITD, *FLT3*-Internal tandem duplication; DLI, Donor Lymphocyte infusion.

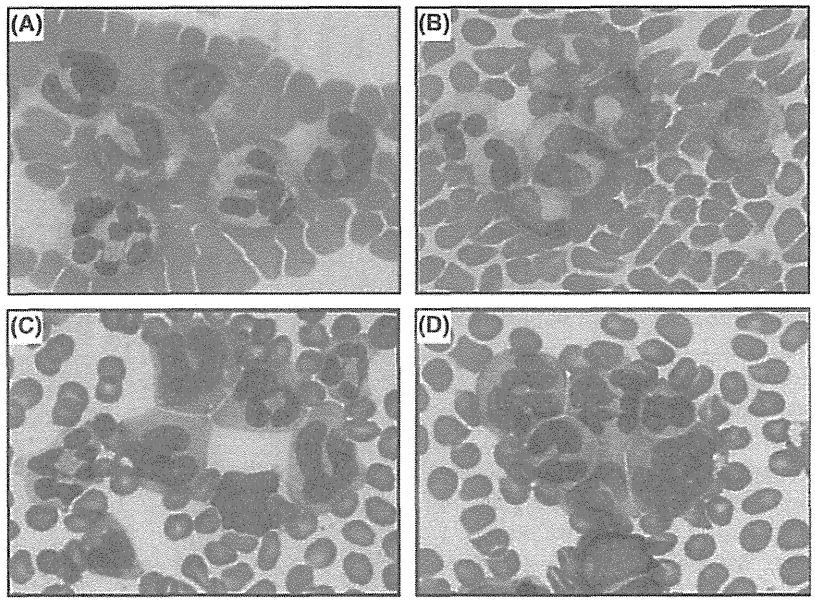

Fig 1. Representative peripheral blood (PB) and bone marrow (BM) smears from paediatric patients with MDS/MPN-U. The upper panels are representative of PB changes while the lower panels are representative of BM changes. PB smear (A and B) showing a variety of dysplastic changes involving granulocytic lineage, in addition to the presence of myeloblast. May-GrÜwald-Giemsa (MGG) staining; magnification, ×1000. BM smear (c and d) showing hyper-cellularity and increased myeloid cells with <5% blasts.

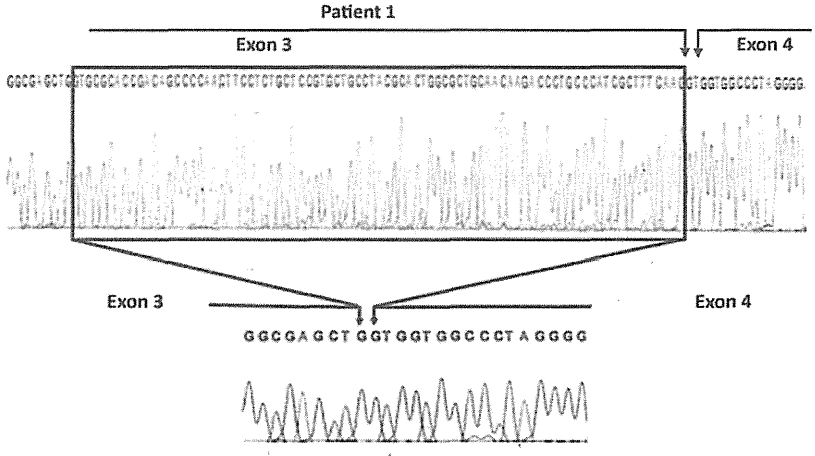

Fig 2. Sequence data for *RUNX1* gene in Patient 1. Nucleotide sequences showing wild-type exon 3 and 4 boundary of *RUNX1* in cDNA obtained from normal control and 84-bp deletion from exon 3 resulting in a frameshift mutation in Patient1.

mily members confirmed that three individuals had the same mutation (the patient's mother and two sisters). *RUNX1* mutations in the chronic phase could not be examined in Patient 4, as samples were not available, but an 8-bp insertion was seen in the exon 3–4 boundary in *RUNX1*, which caused a frameshift mutation and a stop codon in exon 4; this genetic alteration was detected at the time of AML transformation (Shimada *et al*, 2007). Simultaneously, the patient showed a cooperating *FLT3*-ITD mutation after AML progression. Patient 5 had a 2-bp insertion (898_899insAA) in exon 7 *RUNX1*, which resulted in a frameshift mutation in the C-terminal region and a stop at codon 310 (Fig 5). This patient also carried a $JAK2^{V617F}$ mutation in the diagnostic PB sample (Fig 3C). Importantly, the patient had the same *RUNX1* mutation in the buccal epithelial cells, hair and nails, while other family members carried the wild-type *RUNX1* gene. All patients were negative for *PTPN11, NRAS, CBL* and *ASXL1* genetic alterations. The *ETV6-PDGFRB* fusion transcript could not be detected in all patients.

Therapy and survival

Patient 1 underwent allogeneic bone marrow transplantation (BMT) from her sister. However, she relapsed and received

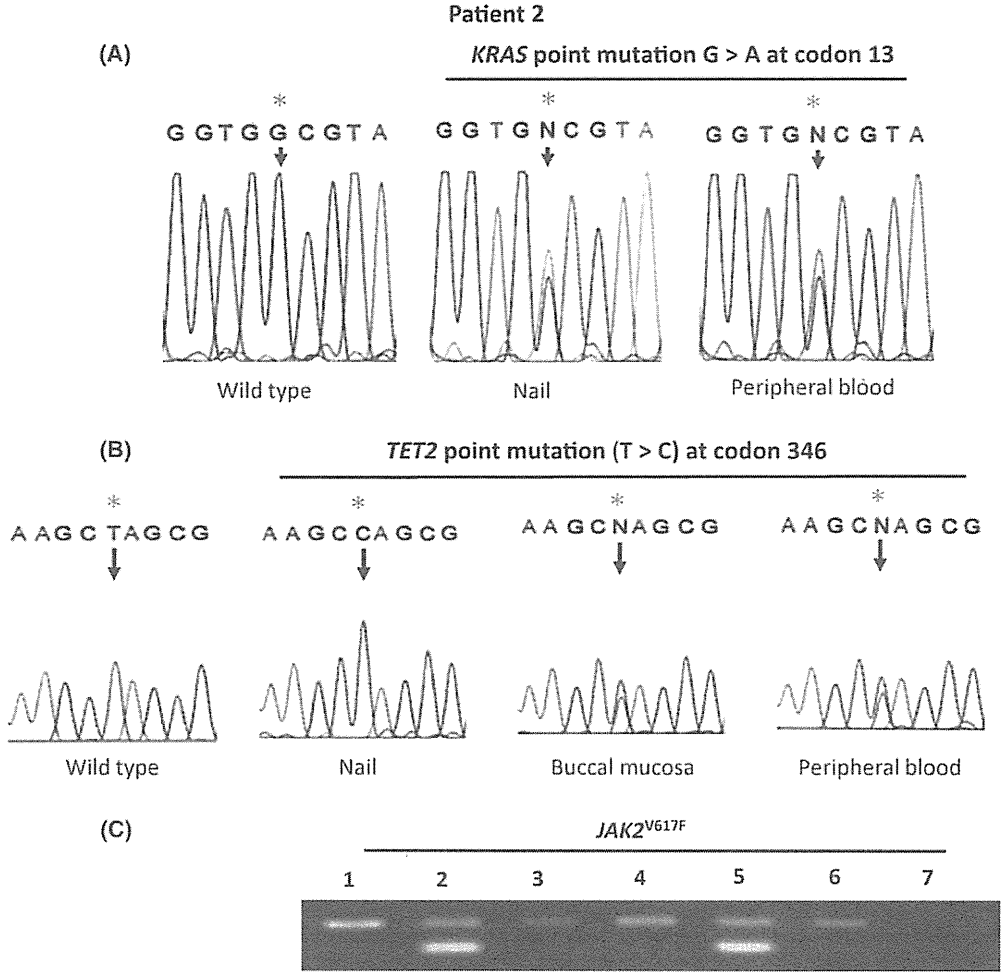

Fig 3. (A) Somatic mosicism of *KRAS* mutation. Nucleotide sequences of *KRAS* showing A to G point mutation at codon 13 in peripheral blood and nails from Patient 2. (B) Somatic mosicism of *TET2* mutation. Sequence traces showing *TET2* exon 3, C to T mutation at codon 346 in peripheral blood, nails and buccal mucosa cells from the same patient. (C) Allele-specific PCR analyses of *JAK2* displayed results of CMML cases. The lower band (203 bp) indicates mutant DNA with $JAK2^{V617F}$ mutation while the upper band (364 bp) indicates wild-type DNA, which was used as an internal control. Lanes 1–5 represent CMML patients; Lane 6: healthy volunteer (negative control); Lane 7: no template; Lanes 2 and 5: $JAK2^{V617F}$ positive cases.

a second BMT from the same donor, but died in 2nd relapse. In Patient 2, persistent thrombocytopenia and splenomegaly were noted; she became transfusion-dependent and underwent splenectomy at the age of 15 years. After splenectomy, her platelet count recovered, and she showed persistent leucocytosis. Subsequently, she became transfusion independent and survived for more than 20 years after the onset of diagnosis. Patient 3 underwent Human Leucocyte Antigen (HLA)-matched unrelated allogeneic BMT, but she relapsed 5 months later. She achieved complete remission after three donor lymphocyte infusions, and remains alive. Interestingly, *TP53* mutation (the only genetic abnormality observed in this patient) was not detected at the time of complete remission. Patients 4 and 5 developed acute mixed-lineage leukaemia at 4 years and 3 months from the onset of diagnosis, respectively. They received allogenic stem cell transplantation (SCT) after AML-oriented chemotherapy; Patient 4 died as a result of relapse, while Patient 5 is still alive (Table I).

Discussion

The molecular hallmarks of MDS/MPNs reported by many investigators have markedly improved our understanding of the pathogenesis of these diseases. Here, we provide insight into some genetic alterations observed in paediatric patients with MDS/MPN-U diagnosis that could be useful for identification of this disease. Among five cases diagnosed with MDS/MPN-U, we found that three patients had *RUNX1* mutations, two patients carried $JAK2^{V617F}$ mutation, one patient had *KRAS* and *TET2* mutations, while genetic alteration of *TP53* was confirmed as the sole genetic lesion in one

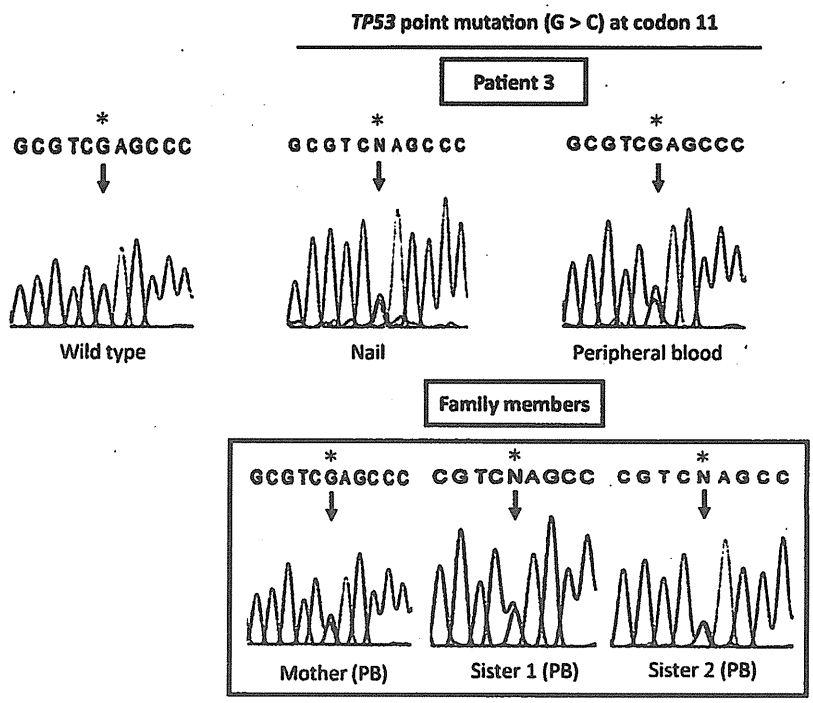

Fig 4. Germline mutation of *TP53* in Patient 3. Nucleotide sequences of *TP53* showing G to C point mutation at codon 11 in nails and peripheral blood from Patient 3. This mutation was also identified in DNA extracted from the peripheral blood (PB) obtained from the patient's mother and sisters.

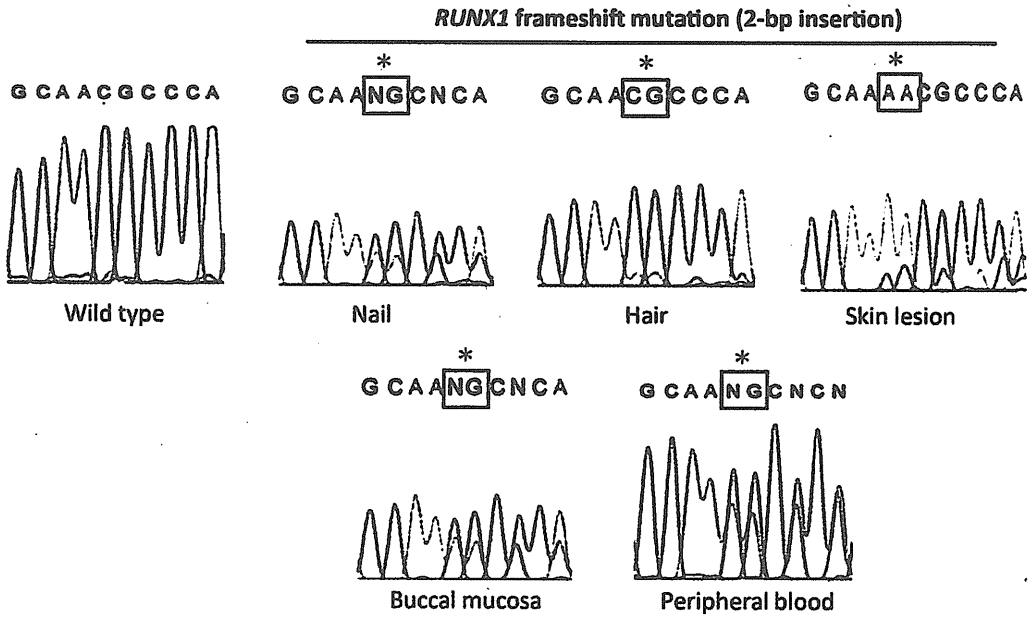

Fig 5. Mutation of *RUNX1* in Patient 5. Nucleotide sequences of *RUNX1* wild-type and 898_899 insAA show a frameshift mutation in exon 7 of the C-terminal region and a stop at codon 310 in peripheral blood, nail, hair, buccal mucosa and sarcomatous skin lesion from Patient 5. This mutation was not found in the patient's parents or siblings.

patient, and *FLT3* mutation was concurrent with *RUNX1* in one patient. Mutations in these genes are frequently detected in adult CMML, but not in JMML patients, and our provisional results suggest that the mutational spectrum observed in childhood MDS/MPN-U is quite different from that seen in JMML and, to some extent, resemble CMML. We detected

three *RUNX1* frameshift mutations, two at the N-terminus and one in the C-terminal region. The latter was found in DNA extracted from PB, buccal smear cells, hair bulbs and nails of the same patient (Patient 5), but we did not have the opportunity to investigate gonadal tissues in our patient and the parental blood samples were shown to carry the wild-type *RUNX1* allele. Therefore, we lack proof of the *de novo* *RUNX1* germline mutation and we have concluded that it might be somatic mosaicism of *RUNX1* gene mutation. Patients 1 and 4 had no family history of haematological malignancy; other somatic cells from these patients or their family members were not available for analysis, and therefore we could not exclude the possibility of *RUNX1* somatic mosaicism in those two patients. It has been reported that familial platelet disorder (FPD)/AML, a rare disease entity associated with germline mutation of *RUNX1*, is associated with a high predisposition to myeloid malignancy (Owen *et al*, 2008). In this study, progression to AML occurred in two patients; one had *RUNX1* mutation with a cooperating *FLT3*-ITD mutation and one had somatic mosaicism of *RUNX1* with the $JAK2^{V617F}$ mutation, suggesting an integrated role for the *RUNX1* mutation with *FLT3* and *JAK2* mutations in disease progression. Other investigators noted that *RUNX1* and *FLT3* mutations were concurrent in adult patients with CMML after AML transformation (Kuo *et al*, 2009). Moreover, a previous study reported that co-existence of class I and II mutations in adult CMML patients potentially cooperate in leukaemic transformation (Bacher *et al*, 2011). We noted a higher tendency of AML transformation in paediatric MDS/MPN-U patients harbouring *RUNX1* mutations, but a large cohort is required to better define the actual incidence of *RUNX1* mutations in childhood MDS/MPN-U and its role in disease progression. Previous reports have shown that leukaemic transformation is associated with *RUNX1* mutations in adult patients with CMML (Kuo *et al*, 2009; Bacher *et al*, 2011). In our cohort, trisomy 21 was detected 4 years after the initial diagnosis in one patient (Patient 4), who developed acute mixed-lineage leukaemia and carried dual *RUNX1* and *FLT3*-ITD mutations. In line with this result, Preudhomme *et al* (2000) reported that trisomy 21 was found with *RUNX1* mutations in the leukaemic phase. Mutations of *KRAS* and *TET2* genes were concomitantly detected in nail and PB samples (Patient 2), while hair samples were negative for both mutations. Moreover, *TET2* mutation was found in buccal mucosal epithelial cells from the same patient, thus suggesting somatic mosaicism of the *KRAS* and *TET2* mutations. We speculate that the occurrence of genetic defects in a mosaic pattern among children with MDS/MPN-U may explain the rarity of this disease and give this category unique molecular features that differ from other MDS/MPNs. This provisional speculation should be confirmed in subsequent studies in order to ascertain the actual incidence of MDS/MPN-U in children. Importantly, the patient with *KRAS* and *TET2* mutations developed $JAK2^{V617F}$ mutation 10 years later, and our findings support the notion that *TET2* mutations occur early in myeloid neoplasia (Abdel-Wahab *et al*, 2010) and are consistent with the results of Kosmider *et al* (2009a), who reported that the frequent *TET2* mutations among CMML patients may be responsible for the deregulated monocytic lineage. In our series, *TET2* mutation was detected in Patient 2, who has survived more than 20 years. Kosmider *et al*, 2009b) reported that *TET2* mutations could be used as a marker for good prognosis with long survival and lower incidence of leukaemic transformation in adult patients with MDS. With regard to *JAK2* alterations in relation to MDS/MPNs, it has been shown that $JAK2^{V617F}$ is present in a subset (7–10%) of adult CMML patients and 20–40% of adult patients with MDS/MPN-U (Levine *et al*, 2005; Yamada *et al*, 2007; Pich *et al*, 2009; Jekarl *et al*, 2010). Furthermore, it has been reported that $JAK2^{V617F}$ and *RAS* mutations are frequently seen in the proliferative phase rather than the dysplastic phase of CMML (Ricci *et al*, 2010). We have described a missense mutation of *TP53* that was detected in PB and nails of a CMML patient (Patient 3) and was confirmed in her family members, indicating the occurrence of germline mutations in *TP53*. Genetic alteration of *TP53* was recently shown to be involved in the transformation process in a proportion of CML patients (Guinn *et al*, 1995). Moreover, it has been noted that *TP53* mutations contribute to the development of MDS (Bartram, 1996). In our study, three out of four patients who received HSCT under a conventional conditioning regimen (busulfan, melphalan, and total body irradiation) relapsed and only one case entered remission after donor lymphocyte infusion. Therefore, additional therapeutic approaches with SCT should be considered to treat such patients in order to improve the therapeutic outcome. We believe that identification of molecular markers involved in the pathogenesis of MDS/MPN-U can contribute to an early diagnosis and proper planning for treatment of this category. In summary, this is the first report to shed the light on childhood MDS/MPN-U with special emphasis on its unique molecular features, including the presence of somatic mosaicism of *RUNX1*, *KRAS* and *TET2* mutations, in addition to $JAK2^{V617F}$ and *FLT3*-ITD mutations, and germline mutation of *TP53*. Molecular assessment can be useful as a diagnostic and prognostic tool for paediatric MDS/MPN-U and will open new therapeutic avenues. Additional studies are needed for the understanding of the pathogenetic mechanism of childhood MDS/MPN-U in order to find an appropriate management strategy for this category.

Conflicts of interest

The authors declare no conflict of interest.