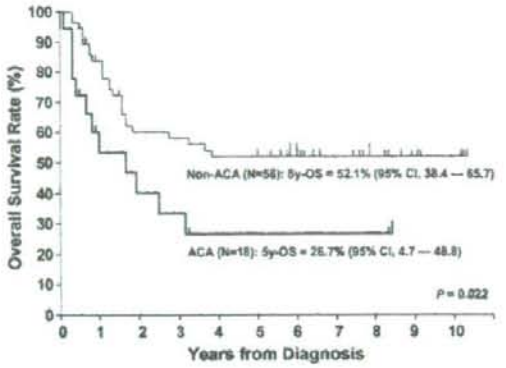


Fig. 2. Overall survival estimates for 74 infants with ALL and *MLL* gene rearrangements in the MLL96 and MLL98 studies; a comparison between patients with additional chromosomal abnormalities and patients with sole 11q23 abnormality excluding normal karyotype with *MLL* gene rearrangements. Median follow-up period: 78 months (range, 8–124 months).

Table 3
Multivariate analysis of prognostic factors in *MLL* rearranged ALL infants

| | Parameter estimates | Risk ratio (95% CI) | *P*-value |
|---|---|---|---|
| Age, less than 6 months | 0.724 | 2.063 (1.026–4.146) | 0.041 |
| Additional chromosomal abnormalities | 0.418 | 1.519 (0.771–2.993) | 0.226 |
| t(4;11)(q21;q23) | 0.345 | 1.413 (0.744–2.683) | 0.290 |
| WBC ≥ 300,000/uL | 0.387 | 1.473 (0.771–2.812) | 0.239 |
| CNS leukemia | 1.166 | 3.209 (1.497–6.881) | 0.002 |
| Registered in the MLL98 study | 0.387 | 1.472 (0.756–2.865) | 0.254 |

CI, confidence intervals; WBC, white blood cell; CNS, central nervous system.

(95% CI, 38.4–65.7%) ($P = 0.022$) (Fig. 2). In a multivariate analysis, only age at diagnosis (younger than 6 months) and positive central nervous system leukemia were significant prognostic factors for poor outcome in this study (Table 3).

## 4. Discussion

This study demonstrated that complex chromosomal abnormalities were associated with poor outcome in infant ALL with *MLL* gene rearrangements. The previous study described by Moorman et al. showed different findings, in that no prognostic effect of additional chromosomal abnormalities was observed in infants and children with ALL and 11q23 abnormalities [17]. However, it is difficult to simply compare between the study by Moorman et al. and ours as follows. First, Moorman et al. collected data from several cooperative study groups, which comprise different treatment cohorts. Secondly, accurate analyses of karyotypes and *MLL* gene rearrangements were not performed in all patients. Thirdly, the EFS rate in this previous study was too low to evaluate the effect of the additional chromosomal abnormalities in infants with *MLL*-rearranged ALL.

Moorman et al. stated that the frequency of additional chromosomal abnormalities depends on the different 11q23 translocations: high frequency of +X in t(4;11) and t(11;19), involvements in chromosomes 6, 9, and 12 in del(11)(q23) and other 11q23 [17]. In our study, several novel translocations were observed: t(2;4)(q31;q32), t(9;11)(p22;q13), t(2;9)(p10;q10), and t(6;11)(p10;q10). Other frequent chromosomal changes were +X and involvements of chromosomes 4, 7, and 11. In our study, a three-way 11q23 translocation was observed in four patients: t(4;11;15), t(4;11;5), t(4;11;9), and t(4; 11; 21). Different three-way translocations have been also detected in several other reports [15,17,19]. Complex structural chromosomal changes were observed in four patients, including insertion of a 4q21 fragment to the 11q23 locus or insertion of 10p12 to the 11q23 locus in our study. Kowarz et al. described ten patients with three-way translocation or complex structural chromosomal changes in MLL-AF4$^+$/AF4-MLL$^-$ ALL [16]. These findings indicate that complex chromosomal changes in leukemic cells disrupt several genes owing to the "cut and paste" recombination mechanism [16].

Recently, the functions of the partner genes fused to *MLL* gene located in 11q23 locus have been clarified: *AF4* at 4q21, *AF9* at 9p22, *ENL* at 19p133, *ELL* at 19p13.1, *AFX* at Xq13, and *AF6q21* at 6q21 are all transcription factors; *CBP* at 16p13 is a transcriptional coactivator; *AF1q* at 1q21 is a growth factor; and *AF17* at 17q21 is a dimerization protein [7]. In addition, several known genetic changes, such as *p53*, *p16*, and *RAS* mutations, are present in some cases in addition to *MLL* gene rearrangement, which might indicate the essential role of additional genetic changes in combination with *MLL* gene translocation in leukemogenesis [20]. Disruption of the Ikaros gene is also detected as an additional alteration in infant ALL [21]. Table 4 summarizes the genes at the breakpoint region of complex chromosomal abnormalities observed in our study, which have been reported only in hematologic malignancies, such as leukemia or lymphoma [22–29]. The function of each gene varies: *PMS1* at 2q31 and *FANCG* at 9p13 are a mismatch or DNA repair gene [23,27]; *Pax5* also located at 9p13, a differentiation factor of B-cells; and *HOXD13* also located at 2q13, a homeobox gene [24,28]. *PML* at 15q22, usually observed as *PMR-RARα* in acute promyelocytic leukemia with t(15;17), and *E2A* at 19p13, usually observed as *E2A-PBX1* in pre-B ALL with t(1;19), are both transcription factors [34,37]. Other genes such as *CHIC2* at 4q11 is associated with exocytosis, *SYK* and *NR4A3* at 9q22 are a tyrosine kinase and membrane receptor, respectively [29], and *CCND1* (*BCL1*) at 11q13 is associated with cell cycle [31]. Thus, if these genes are functionally disrupted after chromosomal changes, this could promote leukemogenesis.

In our study, the overall survival was significantly worse in the ACA group than that in the non-ACA group, but ACA was

Table 4
Breakpoint of chromosomes and possible located genes

| Breakpoint | Located genes | Function | Associated translocation | Associated disease | Reference |
|---|---|---|---|---|---|
| 1q32 | | | t(1;13)(q32;q14) | Diffuse large B-cell lymphoma | [22] |
| 2q31 | *PMS1* | Mismatch repair gene | t(2;12)(q31;p13) | Non-Hodgkin lymphoma, MDS | [23] |
| | *HOXD13* | Homeobox gene | t(2;11)(q31;p15) | Therapy-related AML | [24] |
| 4q11 | *CHIC2* | Exocytosis | t(4;12)(411;p13) | AML | [25] |
| 7p11 | | | dic(7;9)(p11-13;p11) | Pre-B ALL | [26] |
| 9p13 | *FANCG* | DNA repair | t(2;9)(p11;p13) | Pre-B ALL | [27] |
| | *Pax5* | B-cell differentiation | t(7;9)(q11;p13) | B-ALL | [28] |
| 9q22 | *SYK* | Tyrosine kinase | t(5;9)(q33;q22) | Peripheral T-cell lymphoma | [29] |
| | *NR4A3* | Membrane receptor | t(9;12)(q22;p12) | MDS | [29] |
| 11p11 | | | t(11;14)(p11;q32) | Splenic marginal-zone B-cell lymphoma | [30] |
| 11q13 | *CCND1 (BCL1)* | Cell cycle control | t(11;14)(q13;q32) | Mantle cell lymphoma, others | [31] |
| | *MYBOV (Cyclin D)* | | | Multiple myeloma | [32] |
| 15q22 | *PML* | Transcription factor | t(5;15)(q33;q22) | CML | [33] |
| | | | t(15;17)(q22;q21) | APL | [34] |
| 16p11 | | | t(3;16)(q27;p11) | Diffuse large B-cell lymphoma | [35] |
| | | | t(16;21)(p11;q22) | AML | [36] |
| 19p13 | *E2A* | Transcription factor | t(1;19)(q23;p13) | Pre-B ALL | [37] |
| | | | t(2;19)(p11;p13) | AML | [38] |
| | *LYL1* | Transcription factor | t(7;19)(q34;p13) | T-ALL | [39] |
| | | | t(17;19)(q22;p13) | ALL | [37] |

MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia.

not a significant factor in the multivariate analysis. However, given that young age and central nervous system leukemia are significant prognostic factors by multivariate analysis, it is likely that the poor survival outcome seen in the ACA group is associated with the combination of young age, positive central nervous system leukemia and ACA. Since another report showed no effect of additional chromosomal changes in *MLL* positive infant ALL [17], an analysis of the data from a greater number of patients treated with identical treatment protocols is underway to address this issue. In our study, the genes affected by the chromosomal changes varied among the patients, and the function of each gene was different. However, it can be postulated that some genetic alterations induced by additional chromosomal changes might be associated with leukemogenesis and disease progression in *MLL* positive infant ALL.

## 5. Conflict of interest

All the authors do not have any commercial or other associations that might pose a conflict of interest.

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

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***Background.*** *Mixed-lineage leukemia* (*MLL*)-partial tandem duplication (PTD) is associated with poor prognosis in adult acute myeloid leukemia (AML), but its relationship to pediatric AML is unknown. ***Procedure.*** One hundred fifty-eight newly diagnosed AML patients, including 13 FAB-M3 and 10 Down syndrome (DS) patients, who were treated on the Japanese Childhood AML Cooperative Treatment Protocol AML 99 were analyzed for *MLL*-PTD, as well as internal tandem duplication (ITD) and the kinase domain mutation (D835Mt) in the *FLT3* gene. ***Results.*** We found *MLL*-PTD in 21 (13.3%) of 158 AML patients, but not in FAB-M3 or DS patients. The differences between patients with and without *MLL*-PTD were significant for 3-year overall survival (OS) (56.3% vs. 83.2%, $P=0.018$), disease-free survival (DFS) (41.7% vs. 69.6%, $P=0.010$), and relapse rate (RR) (54.3% vs. 27.6%, $P=0.0085$) of 135 AML patients excluding the FAB-M3 and DS patients. Furthermore, ITD and D835Mt in the *FLT3* gene were found in 17 (12.6%) and 8 (5.9%) of these 135 patients, respectively. The differences between patients with *FLT3*-ITD and the wild-type allele were significant for 3-year OS (35.3% and 84.3%, $P<0.0000001$), DFS (40.0% and 66.9%, $P<0.003$), and RR (52.4% and 30.3%, $P<0.005$). Coduplication of both genes was found in only 3 (1.9%) patients. ***Conclusion.*** AML patients with *FLT3*-ITD, but not D835Mt, showed a poor prognosis. AML patients with *MLL*-PTD were also correlated with poor prognosis in this study. Pediatr Blood Cancer 2008;50:264–269. © 2007 Wiley-Liss, Inc.

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

## INTRODUCTION

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

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

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

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

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## PATIENTS AND METHODS

### Patients

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

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

### Detection of *MLL*-PTD

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

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

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

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

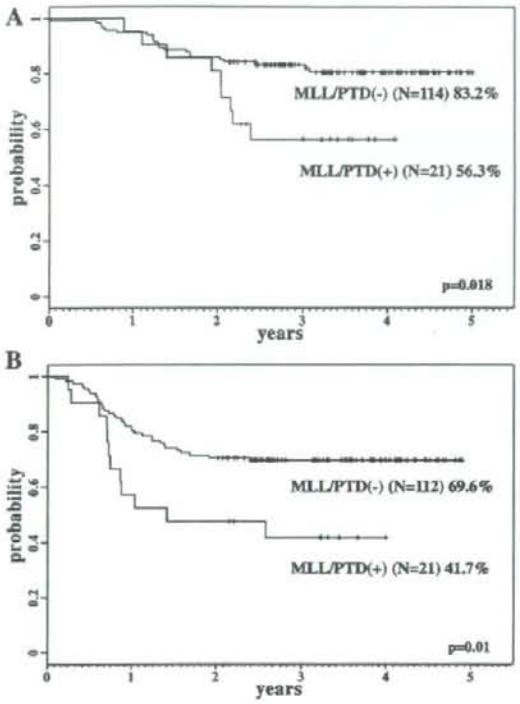


**Fig. 1.** Probabilities of 3-year OS (**A**) and 3-year DFS (**B**) in 135 AML patients excluding those with FAB-M3 and Down syndrome. Kaplan–Meier method estimates for patients with and without *MLL*-PTD are shown. The difference in patient numbers between OS and DFS resulted from the death of two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

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

### Detection of *FLT3*-ITD and D835Mt

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

### Statistical Analysis

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

## RESULTS

### *MLL*-PTD

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

### *FLT3*-ITD and D835Mt

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

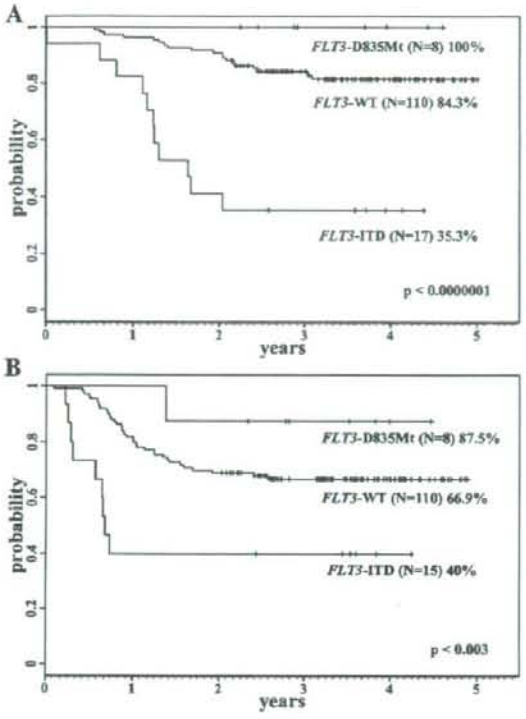


Fig. 2. Probabilities of 3-year OS (A) and 3-year DFS (B) in 135 AML patients, excluding those with FAB-M3 and Down syndrome. The Kaplan–Meier method for patients with *FLT3*-ITD, D835Mt, and WT is shown. The difference in patient numbers between OS and DFS resulted from the death of two patients during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

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

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

## Coduplication of the *MLL* and *FLT3* Genes

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

## Multivariate Analysis of Clinical Outcome

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

## DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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ONCOGENOMICS

# Identification of the novel *AML1* fusion partner gene, *LAF4*, a fusion partner of *MLL*, in childhood T-cell acute lymphoblastic leukemia with t(2;21)(q11;q22) by bubble PCR method for cDNA

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**The *AML1* gene is frequently rearranged by chromosomal translocations in acute leukemia. We identified that the *LAF4* gene on 2q11.2–12 was fused to the *AML1* gene on 21q22 in a pediatric patient having T-cell acute lymphoblastic leukemia (T-ALL) with t(2;21)(q11;q22) using the bubble PCR method for cDNA. The genomic break points were within intron 7 of *AML1* and of *LAF4*, resulting in the in-frame fusion of exon 7 of *AML1* and exon 8 of *LAF4*. The *LAF4* gene is a member of the *AF4/FMR2* family and was previously identified as a fusion partner of *MLL* in B-precursor ALL with t(2;11)(q11;q23), although *AML1-LAF4* was in T-ALL. *LAF4* is the first gene fused with both *AML1* and *MLL* in acute leukemia. Almost all *AML1* translocations except for *TEL-AML1* are associated with myeloid leukemia; however, *AML1-LAF4* was associated with T-ALL as well as *AML1-FGA7* in t(4;21)(q28;q22). These findings provide new insight into the common mechanism of *AML1* and *MLL* fusion proteins in the pathogenesis of ALL. Furthermore, we successfully applied bubble PCR to clone the novel *AML1-LAF4* fusion transcript. Bubble PCR is a powerful tool for detecting unknown fusion transcripts as well as genomic fusion points.**

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**Keywords:** *AML1/RUNX1*; *LAF4*; T-cell acute lymphoblastic leukemia; *MLL*

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## Introduction

A large number of leukemias have been found to be associated with specific chromosomal aberrations. Recent studies have demonstrated that several chromosomal rearrangements and molecular abnormalities are strongly associated with distinct clinical subgroups and can predict clinical features and therapeutic responses (Rowley, 1999; Taki and Taniwaki, 2006). Some genes have been associated with recurrent rearrangements and have many fusion partner genes, such as *MLL* at 11q23, *TEL (ETV6)* at 12p13 and *NUP98* at 11p15; *AML1* (*RUNX1*, *CBFA2*) at 21q22 is one of the most frequent targets of these chromosomal rearrangements in both acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (Miyoshi *et al.*, 1991; Hayashi, 2000; Kurokawa and Hirai, 2003). To date, a number of in-frame fusion partners of *AML1* have been cloned: *YTHDF2* at 1p35 (Nguyen *et al.*, 2006), *ZNF687* at 1q21.2 (Nguyen *et al.*, 2006), *MDS1/EVI1* at 3q26 (Mitani *et al.*, 1994), *FGA7* at 4q28 (Mikhail *et al.*, 2004), *SH3D19* at 4q31.3 (Nguyen *et al.*, 2006), *USP42* at 7p22 (Paulsson *et al.*, 2006), *MTG8* (*ETO*, *CBFA2T1*) at 8q22 (Erickson *et al.*, 1992; Miyoshi *et al.*, 1993), *FOG2* at 8q23 (Chan *et al.*, 2005), *TRPS1* at 8q24 (Asou *et al.*, 2007), *TEL* (*ETV6*) at 12p13 (Golub *et al.*, 1995), *MTG16* at 16q24 (Gamou *et al.*, 1998) and *PRDX4* at Xp22 (Zhang *et al.*, 2004). Most *AML1* translocations, except for *TEL-AML1*, are associated with AML, involving the N-terminus Runt domain and lacking the C-terminus transactivation domain (Kurokawa and Hirai, 2003). AML1 fusion proteins are associated with leukemogenesis by dominantly interfering with normal *AML1*-mediated transcription and acting as a transcriptional repressor (Okuda *et al.*, 1998; Wang *et al.*, 1998). Clinically, patients with AML harboring t(8;21) in both children and adults show a high rate of complete remission, and its prognosis is considered better than that of patients with a normal karyotype or other chromosomal aberrations (Grimwade *et al.*, 1998).

In the present study, we analysed pediatric T-ALL with t(2;21)(q11;q22) and identified the *LAF4* gene,

which is one of the fusion partners of *MLL*, as a novel fusion partner of the *AML1* gene.

## Results

### *Case report*

A 6-year-old boy with a high leukocyte count (64 700 $\mu l^{-1}$), containing 84% blasts in peripheral blood and with a mediastinal mass, was diagnosed as having T-ALL. A bone marrow smear was hypercellular with 69% blasts and negative for myeloperoxidase. The leukemic cells, after gating of CD45-positive cells, were positive for CD5 (90.7%), CD7 (90.7%), CD58 (69.9%) and cytoplasmic CD3 (92.8%), and negative for HLA-DR, IgG, IgM, Igκ, Igλ, CD8, CD13, CD14, CD19, CD20 and CD33. He was treated on the Tokyo Children's Cancer Study Group (TCCSG) L04-16 extremely high-risk (HEX) protocol, including stem cell transplantation, because the response to initial 7-day prednisolone (60 mg $m^{-2}$) monotherapy was poor. He achieved complete remission after the induction phase. After the early consolidation phase and two courses of the consolidation phase, he received allogeneic bone marrow transplantation from an unrelated HLA-matched donor 4 months after diagnosis. He has been in complete remission for 17 months.

The patient's leukemic cells at diagnosis were analysed after written informed consent was obtained from his parents, and the ethics committee of Kyoto Prefectural University of Medicine approved this study.

### *Identification of the* AML1-LAF4 *fusion transcript*

Cytogenetic analysis of the leukemic cells of the patient using routine G-banding revealed 47, XY, add(1)(p36), +der(2)t(2;21)(q13;q22), t(2;21)(q13;q22), −9, −9, +mar1, +mar2, and spectral karyotyping (SKY) analysis revealed 47, XY, der(1)t(1;17)(p36.1;q23), der(2)t(2;21)(q11.2;q22), +der(2)t(2;21)(q11.2;q22), del(5)(p15.1), del(9)(q22), del(9)(p13), der(21)t(2;21)(q11.2;q22) (Supplementary Figure S1). Since *AML1* is located at 21q22, we inferred that *AML1* was rearranged in this case. Fluorescence *in situ* hybridization analysis using *AML1*-specific BAC (bacterial artificial chromosome) clones showed split signals of *AML1* on two der(2)t(2;21)(q11.2;q22) and der(21)t(2;21)(q11.2;q22) chromosomes (Figure 1a).

To isolate fusion transcripts of *AML1*, we performed the bubble PCR method for cDNA (Figure 2) and obtained various-sized products (Figure 3a). Four different-sized products were sequenced and two products contained *AML1* sequences fused to unknown sequences. Basic local alignment search tool (BLAST) search revealed that the unknown sequences were part of the *LAF4* gene and both products had the same in-frame junctions (Figure 3b). *LAF4* was located on chromosome 2q11.2–12, which was compatible with the result of spectral karyotyping analysis. We next performed reverse transcription-PCR to confirm *AML1-LAF4* fusion transcripts, and obtained three different-sized *AML1-LAF4* fusion products, including only one in-frame product (Figures 3c and d); however, reciprocal *LAF4-AML1* fusion transcripts were not generated (Figure 3c). Type 2 transcript is an out-of-frame fusion and generated premature termination in exon 9 of *LAF4* (Figure 3d). On the other hand, type 3 transcript is an in-frame fusion of exon 7 of *AML1* and exon 8 of *LAF4*, the same as the type 1 transcript; however, the type 3 transcript contained an 85-bp intronic sequence between exons 9 and 10 of *LAF4*, which might be due to splicing error, and appeared as a premature termination codon within the intronic sequences (Figure 3d). *AML1-LAF4* fusions were also confirmed by fluorescence *in situ* hybridization analysis (Figure 1b).

### *Detection of* AML1-LAF4 *genomic junctions*

Southern blot analysis using a cDNA probe within exon 7 of *AML1* detected a rearranged band derived from an

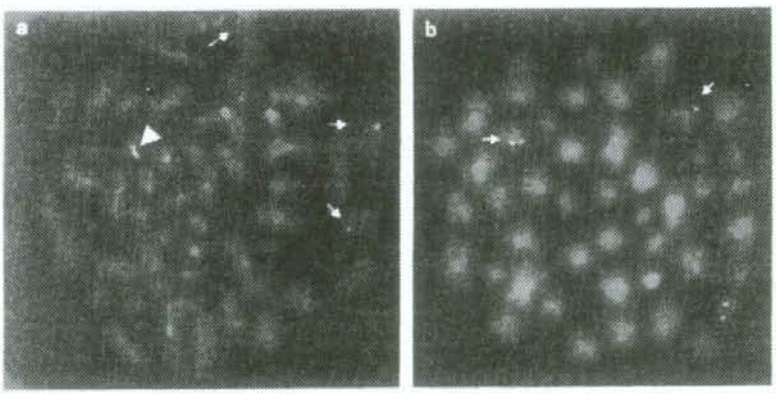


**Figure 1** Fluorescence *in situ* hybridization analysis of the leukemic metaphase. (**a**) Both RP11-272A3 (green, 3′ side of *AML1*) and RP11-994N6 (red, 5′ side of *AML1*) were hybridized to normal chromosome 21 (arrowhead), RP11-272A3 to der(21)t(2;21)(q11.2;q22) (arrow, green signal) and RP11-994N6 to two der(2)t(2;21)(q11.2;q22) chromosomes (arrows, red signal). (**b**) Two fusion signals of RP11-994N6 (5′ of *AML1*, red signals) and RP11-527J8 (3′ of *LAF4*, green signals) were detected on two der(2)t(2;21)(q11.2;q22) chromosomes (arrows).

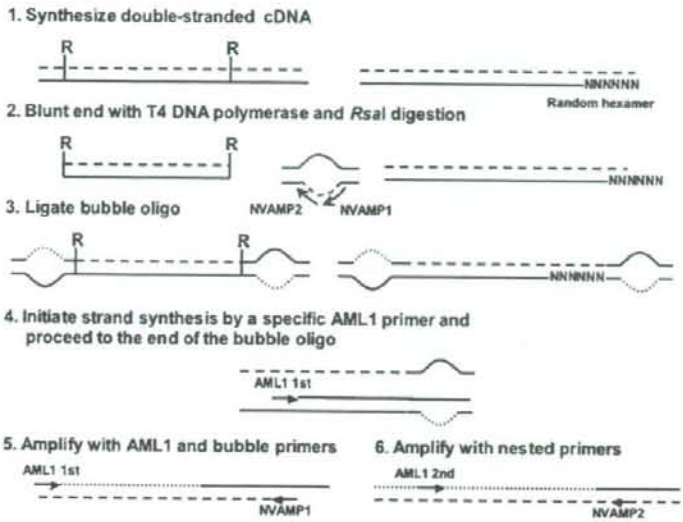


**Figure 2** Outline of bubble PCR for cDNA. Bubble PCR primers (NVAMP-1 and NVAMP-2) can only anneal with one complementary sequence for bubble oligo synthesized with *AML1* primer, but not bubble oligo itself; therefore, this single-stranded bubble provides the specificity of the reaction.

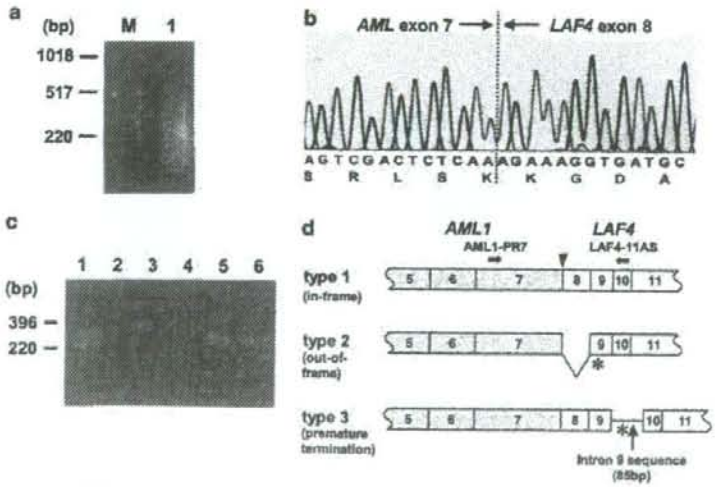


**Figure 3** Identification of *AML1-LAF4* fusion transcript. (**a**) Bubble PCR products by nested PCR using AML1-5S and NVAMP1 for first PCR, and AML1-E6S and NVAMP2 for second PCR (lane 1). M, size marker. (**b**) Sequence analysis of *AML1-LAF4* fusion transcript. The single letter amino-acid sequences surrounding the fusion point are shown at the bottom of the figure. (**c**) Detection of *AML1-LAF4* fusion transcripts by reverse transcription-PCR. Primers were AML1-PR7 and LAF4-11AS (lanes 1 and 3), AML1-PR8 and LAF4-PR5 (lanes 2 and 4), and β-actin, respectively. Lanes 1, 3 and 5, patient's leukemic cells; lanes 2, 4 and 6, normal peripheral lymphocytes. (**d**) Three fusion transcripts of *AML1-LAF4* are schematically depicted. Gray/dotted boxes denote predicted *AML1* exons and white boxes represent predicted *LAF4* exons. Type 3 contains the *LAF4* intron 9 splicing donor site. AML1-PR7 and LAF4-11AS indicate the primers used for reverse transcription-PCR. Asterisk shows the termination codon.

approximately 11 kb *Bgl*II germline fragment on chromosome 21 (data not shown). To isolate the fusion point of chromosomes 2 and 21, we next performed bubble PCR on genomic DNA and detected nested PCR products using primers AML1-GNM8-2S and NVAMP2 (Figure 4a). Sequence analysis of the subcloned PCR product revealed the genomic junction of 5′-*AML1-LAF4*-3′ (Figures 4c and d), and the result

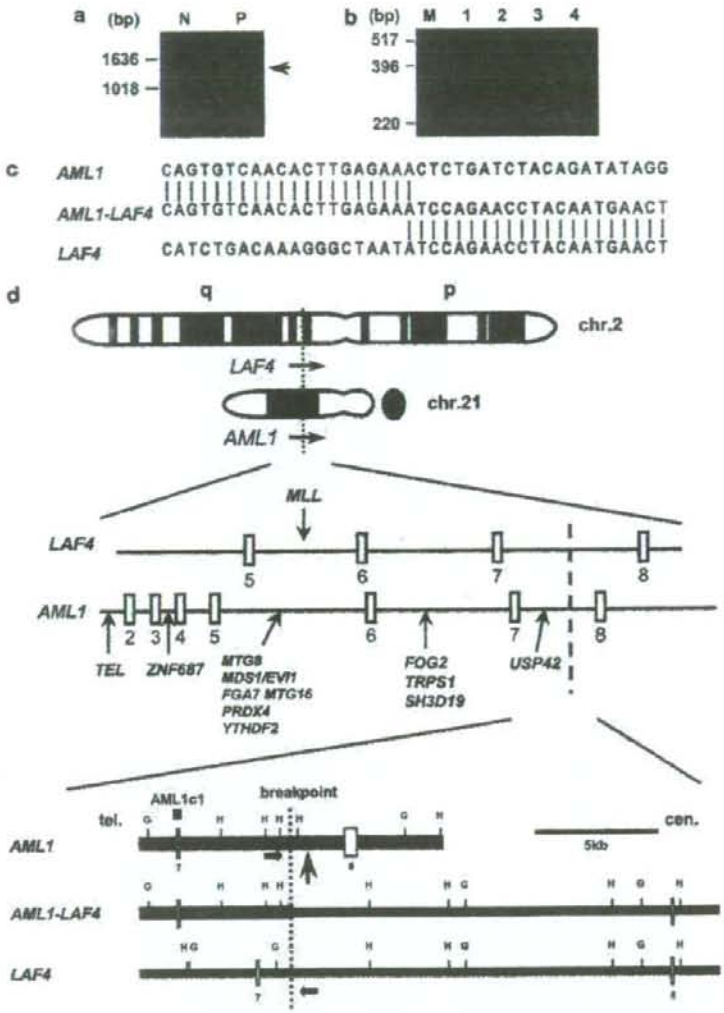


**Figure 4** Cloning of the genomic junction of *AML1* and *LAF4*. (**a**) Bubble PCR for genomic DNA. N, normal human lymphocytes; P, patient's leukemic cells. (**b**) Detection of the genomic fusion point of *AML1-LAF4* by PCR. Primers were AML1-GNM8-4S and LAF4-GNM11-2AS (lanes 1 and 3), and LAF4-GNM11-2S and AML1-GNM8-2AS (lanes 2 and 4). Lanes 1 and 2, patient's leukemic cells; lanes 3 and 4, normal peripheral lymphocytes. M, size marker. (**c**) Sequences of breakpoints in the patient's leukemic cells. (**d**) Physical map of the breakpoint regions. Open vertical boxes represent defined exons in each gene. Horizontal arrows show the primers used. Restriction sites are indicated by capital letters: G, *Bgl*II; H, *Hin*dIII. AML1c1 indicates the position of the cDNA probes for Southern blot analysis. A vertical arrow shows *AML1-USP42* breakpoint.

was confirmed by PCR analysis using primers AML1-GNM8-4S and LAF4-GNM11-2AS (Figure 4b); however, no 5′-*LAF4-AML1*-3′ product was generated, suggesting interstitial deletion near genomic break points (Figure 4b). These sequences near the break points did not contain any lymphoid heptamer/nonamer sequences, *Alu* sequences or consensus topoisomerase II cleavage sites.

## Discussion

In this study, we identified that *LAF4* was fused to *AML1* in pediatric T-ALL with t(2;21)(q11;q22). Other regions with chromosomal aberrations in this patient were not considered to be associated with recurrent cytogenetic changes involving T-ALL, except for the deletion of the short arm of chromosome 9. Spectral

karyotyping analysis detected del(9)(p13), and additional analysis of genome array (Human Mapping 50 K Hind Array, Affymetrix, Tokyo, Japan) revealed homozygous deletion of 4.5 Mb within the 9p21 region, including the *CDKN2A/p16/p14* locus (data not shown), which is frequently deleted in T-ALL (Ohnishi *et al.*, 1995).

Although the patient showed a complex chromosomal abnormality, t(2;21)(q11;q22) can form regular head-to-tail fusion transcripts of both *AML1* and *LAF4*, because the transcription direction of *AML1* and *LAF4* is telomere to centromere. Furthermore, fluorescence *in situ* hybridization analysis revealed two der(2)t(2;21)(q11.2;q22) creating *5′-AML1-LAF4-3′*, suggesting that *5′-AML1-LAF4-3′* is critical for leukemogenesis.

*LAF4* was previously reported to be a fusion partner of *MLL* in pediatric B-precursor ALL with t(2;21)(q11;q23) (von Bergh *et al.*, 2002; Bruch *et al.*, 2003; Hiwatari *et al.*, 2003). *LAF4* is the first gene fused to both *AML1* and *MLL*, and both *AML1-LAF4* and *MLL-LAF4* contained the same domains of *LAF4* (Figure 5). During the preparation of this manuscript, we found another pediatric T-ALL patient with *AML1-LAF4* reported in the Meeting Abstract (Abe *et al.*, *Blood* (ASH Annual Meeting Abstracts) 2006; **108**: 4276), suggesting that t(2;21)(q11;q23) is a recurrent cytogenetic abnormality and that the *AML1-LAF4* fusion gene is associated with the T-ALL phenotype. Both putative fusion proteins of AML1-LAF4 observed in two patients contained the Runt domain of AML1, and the transactivation domain, nuclear localization sequence and C-terminal homology domain of LAF4, although the fused exon of *LAF4* differed in the two cases. Several studies have reported that the fusion partners of *MLL* fused with different genes such as *MLL-AF10* and *CALM-AF10*, *MLL-CBP* and *MOZ-CBP* or *MLL-p300* and *MOZ-p300* (Ida *et al.*, 1997; Taki *et al.*, 1997; Chaffanet *et al.*, 2000). Comparison of the structure and function between *AML1-LAF4* and *MLL-LAF4* will facilitate our understanding of the molecular mechanisms underlying *AML1*- and *MLL*-related leukemia.

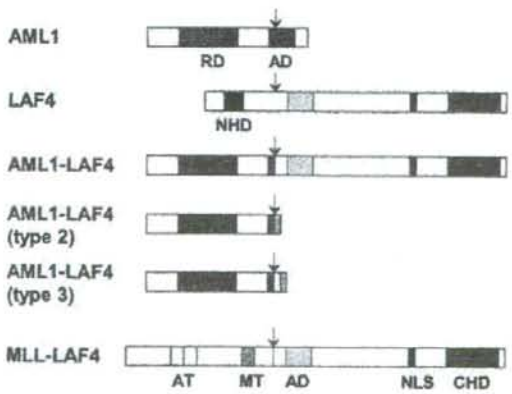


Figure 5 Schematic representation of putative AML1, LAF4 and AML1-LAF4 fusion proteins. Putative MLL-LAF4 fusion protein is also indicated for comparison. Arrows, break points or fusion points; AD, transactivation domain; AT, AT hooks; CHD, C-terminal homology domain; DNA, methyltransferase homology region; RD, RUNT domain; MT, DNA methyltransferase homology region; NLS, nuclear localization sequence.

The only *AML1* fusion partners in T-ALL are *LAF4* and *FGA7*. It is not known how *FGA7* is associated with T-ALL leukemogenesis, because *FGA7* does not show any significant sequence homology to any known protein motifs and/or domains (Mikhail *et al.*, 2004). Both patients with *AML1-LAF4* and *MLL-LAF4* fusions were diagnosed as having ALL, but they have different lymphoid lineages. *MLL-LAF4* is associated with B-lineage ALL; however, *AML1-LAF4* generates T-ALL. Our previous study showed that *LAF4* was expressed not only in B-lineage ALL but also in T-lineage ALL cell lines (Hiwatari *et al.*, 2003). *LAF4* showed strong sequence similarity to *AF4* (Ma and Staudt, 1996), which has a role in the differentiation of both B and T cells in mice (Isnard *et al.*, 2000). Furthermore, it was reported that *AML1* also plays an important role in T- and B-cell differentiation, because *AML1*-deficient bone marrow increased defective T- and B-lymphocyte development (Ichikawa *et al.*, 2004). These findings support that both *AML1* and *LAF4* are associated with T-ALL, respectively. Further functional analysis of the *AML1-LAF4* fusion gene will provide new insights into the leukemogenesis of *AML1*-related T-ALL. Recently, it has been reported that C-terminal truncated AML1-related fusion proteins play critical roles in leukemogenesis (Yan *et al.*, 2004, Agerstam *et al.*, 2007), suggesting that the two additional types of fusion transcripts observed in our patient (types 2 and 3 in Figures 3d and 5) have an additional function in leukemogenesis other than that of the entire AML1-LAF4 fusion protein.

In this study, we first applied the panhandle PCR method, which is usually used for cloning the fusion partners of *MLL* or *NUP98* (Megonigal *et al.*, 2000; Taketani *et al.*, 2002); however, no fusion transcripts could be obtained. Therefore, we searched for another method to clone the fusion transcripts and adapted the bubble PCR method for cDNA cloning. To date, bubble PCR has been performed for cloning unknown genomic fusion points but not fusion cDNAs (Zhang *et al.*, 1995). Using double-stranded cDNA, we could apply the bubble PCR method for cloning fusion cDNA with fewer nonspecific products. The bubble PCR primer can only prime DNA synthesis after a first-strand cDNA has been generated by an *AML1*-specific primer because of the bubble-tag with an internal non-complementary region (Zhang *et al.*, 1995). Although bubble PCR for genomic DNA generated one or two amplification products (Smith, 1992), bubble PCR for cDNA generated a complex set of amplification products that appeared as a smear by SYBR green staining, suggesting that a random hexamer generated various double-stranded cDNA containing the *AML1* sequence. This means that various fusion points can be estimated, even if after bubble oligo ligation was generated. Furthermore, bubble PCR for cDNA could amplify in both 5′–3′ and 3′–5′ directions of the gene or transcript, and easily

**Table 1** Comparison between bubble PCR and panhandle PCR

| *Characteristics* | *Bubble PCR* | *Panhandle PCR* |
|---|---|---|
| Available orientation of fusion transcript | 5′–3′, 3′–5′ | Only 5′–3′ |
| *AML1*-specific random hexamer[a] | Not necessary | Necessary |
| Self-annealing | Not necessary | Necessary |
| Number of required polymerase reaction | 2 | 4 |
| Number of final products | Many (smear) | A few |
| Nonspecific product | Few | Few |
| Number of extra sequences other than targeted sequences in cloned product | 50–60 bp | > 100 bp |
| Search for other targeted exons | Easy | Hard[b] |

[a]30-mers *AML1*-specific oligonucleotide with random hexamer (AML1-N). [b]Necessary to use another *AML1*-specific random hexamer if the target exons are 5′ region of the initial target.

handle any exons fused to unknown partners for amplification. Once-ligated cDNAs are also available for cloning any genes, other than *AML1*, as the target. We demonstrated the efficiency and specificity of bubble PCR for cDNA (Table 1 and Supplementary Figure S2).

To date, a great number of fusion genes associated with chromosomal translocations have been cloned, although these fusion genes are found as a minor part of various malignancies. Recently, high frequencies of mutations in *NOTCH1* in T-ALL (James *et al*., 2005), *NPM* in AML with normal karyotype (Weng *et al*., 2004) and *JAK2* in myeloproliferative disorders (polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis) (James *et al*., 2005) have been reported, and these mutations are considered to be a good target for therapy. These genes were first identified as associated with chromosomal translocations in a small subset of specific phenotypes of hematologic malignancies (Ellisen *et al*., 1991; Morris *et al*., 1994; Lacronique *et al*., 1997). These findings suggest that continuing attempts to identify genes associated with chromosomal translocations can be expected to provide further insights into the significance of various gene alterations in cancer and the development of novel-targeted therapies (Taki and Taniwaki, 2006). The bubble PCR method for cDNA will contribute to identifying numerous novel translocation partners more easily and further functional analysis of chimeric transcripts.

## Materials and methods

*Spectral karyotyping analysis*
Spectral karyotyping analysis was performed with a SkyPainting kit (Applied Spectral Imaging, Migdal Ha'Emek, Israel). Signal detection was performed according to the manufacturer's instructions.

*Fluorescence* in situ *hybridization analysis*
Fluorescence *in situ* hybridization analysis of the patient's leukemic cells using *AML1*-specific BAC clones (RP11-272A3, 3′ of *AML1* and RP11-994N6, 5′ of *AML1*) was carried out as described previously (Taniwaki *et al*., 1994). Fusion of *AML1* and *LAF4* was analysed with the patient's leukemic cells using RP11-994N6 (5′ of *AML1*) and RP11-527J8 (3′ of *LAF4*).

*Bubble PCR for cDNA*
We modified the original bubble PCR method to apply for cDNA cloning (Figure 2; Supplementary Figure S2) (Smith, 1992; Zhang *et al*., 1995).

Poly(A)$^+$ RNA was extracted from the patient's leukemic cells using a QuickPrep Micro mRNA Purification Kit (GE Healthcare, Buckinghamshire, UK). Two hundred nanograms of poly(A)$^+$ RNA was reverse transcribed to cDNA in a total volume of 33 μl with random hexanucleotide using the Ready-To-Go You-Prime First-Strand Beads (GE Healthcare). Double-stranded cDNAs were synthesized from 10 μl of single-stranded cDNA with a phosphorylated random hexanucleotide, blunt ended with T4 DNA polymerase, digested with *Rsa*I endonuclease and ligated with bubble oligo. *Rsa*I, a 4-bp blunt-ended cutter, was chosen to shorten the bubble oligo-ligated fragments, so that almost all bubble oligo-ligated fragments would be easy to clone by standard PCR reaction. This suggests that poor-quality samples are also suited to this method, although it is unsuitable for cloning long products.

The sequences of the primers used are listed in Supplementary Table S1 and their positions in the *AML1* gene are shown in Supplementary Figure S2. Nested PCR was performed using primers NVAMP-1 (bubble oligo) and AML1-5S (exon 5) for first round PCR, and NVAMP-2 (bubble oligo) and AML1-E6S (exon 6) for nested PCR. NVAMP1 and NVAMP2 can only anneal to the newly synthesized unique sequence of the bubble oligo by AML1-5S.

We used poly(A)$^+$ RNA in bubble PCR for cDNA with the expectation that this approach could amplify fewer transcripts; however, total RNA is also suitable for this method.

*Bubble PCR for genomic DNA*
Bubble PCR for genomic DNA was performed as described previously (Smith, 1992; Zhang *et al*., 1995). Primers were as follows: NVAMP-1 and AML1-GNM8S for first round PCR, and NVAMP-2 and AML1-GNM8-2S for second round PCR (Supplementary Table S1).

*Reverse transcription–PCR and genomic PCR analyses*
Reverse transcription–PCR and genomic PCR analyses were performed as described previously. After 35 rounds of PCR (30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C), 5μl of PCR product were electrophoresed in a 3% agarose gel. Primers were as follows: AML1-PR7 and LAF4-11AS, and AML1-PR8 and LAF4-PR5 for reverse transcription-PCR; and AML1-GNM8-4S and LAF4-GNM11-2AS, and LAF4-GNM11-2S and AML1-GNM8-2AS for genomic PCR (Supplementary Table S1).

*Nucleotide sequencing*
Nucleotide sequences of PCR products and, if necessary, subcloned PCR products were analysed as described previously (Hiwatari *et al*., 2003).

*Southern blot analysis*
High-molecular-weight DNA was extracted from the patient's leukemic cells by proteinase K digestion and phenol/chloroform extraction. DNA (10 μg) was digested with *Bgl*II, subjected to electrophoresis on 0.7% agarose gel and transferred to a nylon membrane. Blots were hybridized to probes that were labeled by the Dig-labeled PCR method according to the manufacturer's instructions (Roche Applied Science, Tokyo, Japan). Probes

were 112 bp *AML1* cDNA fragments (AML1c1, nucleotides 1233–1344; GenBank accession no. NM_001754).

### Abbreviations

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.

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# Whole-genome profiling of chromosomal aberrations in hepatoblastoma using high-density single-nucleotide polymorphism genotyping microarrays

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**To identify the genomic profile and elucidate the pathogenesis of hepatoblastoma (HBL), the most common pediatric hepatic tumor, we performed high-density genome-wide single-nucleotide polymorphism (SNP) microarray analyses of 17 HBL samples. The copy number analyzer for GeneChip® (CNAG) and allele-specific copy number analysis using anonymous references (AsCNAR) algorithms enabled simple but sensitive inference of allelic composition without using paired normal DNA. Chromosomal aberrations were observed in 15 cases (88%). Gains in chromosomes 1q, 2 (or 2q), 8, 17q, and 20 and losses in chromosomes 4q and 11q were frequently identified. High-grade amplifications were detected at 7q34, 14q11.2, and 11q22.2. Several types of deletions, except homozygous deletion, were identified. Most importantly, copy-neutral loss of heterozygosity (uniparental disomy [UPD]) at 11p15 was detected in four of the 17 HBL samples. Insulin-like growth factor II (*IGF2*) and *H19* genes were located within this region. The methylated status of this region indicated the paternal origin of the UPD. The expression patterns of *IGF2* and *H19* were opposite between genes with and without the UPD. This difference in the expression patterns might influence the clinical features of HBL. (*Cancer Sci* 2008; 99: 564–570)**

Hepatoblastoma (HBL) is the most common pediatric hepatic tumor predominantly observed in infants and children aged less than 3 years.[1–3] The dramatic increase in the survival of patients that has been observed during the last three decades is mainly due to advances in the use of chemotherapy and surgical techniques.[1–3] Currently, approximately 75% of children with HBL can be cured completely, although a large tumor, a multifocal tumor, and metastatic spread are all associated with a fatal outcome.[3] The etiology of HBL remains unknown. Most HBL are sporadic; however, an association with prematurity or low birth weight,[4] and genetic disorders such as familial adenomatous polyposis (FAP),[5] or Beckwith–Wiedemann syndrome (BWS) has been documented.[6] These findings imply that an alteration at 11p15, which is the critical region in BWS and critical to the wingless signaling pathway involving the adenomatosis polyposis coli (*APC*) gene that is constitutionally mutated in FAP patients,[7,8] could also play a role in the genesis of sporadic HBL. Indeed, the loss of heterozygosity (LOH) at 11p15 and mutations in the *APC* and β-catenin genes have also observed in some sporadic HBL.[9,10]

LOH and deletion of tumor suppressor genes are observed frequently in malignant cells and can be associated with the deregulation of cell fate and apoptosis.[11] Similarly, amplification of the chromosomal regions can increase the expression of oncogenes during tumor progression. Conventional cytogenetic analyses of chromosomal aberrations in HBL performed using standard karyotyping,[12–16] fluorescence *in situ* hybridization (FISH),[17–20] and comparative genomic hybridization (CGH),[21,22] have been reported. Although these analyses have identified several chromosomal aberrations in HBL, predominantly the gains in chromosomes 1q, 2, 8q, 17q, and 20 and the loss in chromosome 4q, the tumor-associated genes of HBL involved in these genomic copy number (CN) alterations are yet to be identified.

In recent years, a high-resolution genomic approach has been used for the systematic screening of chromosomal CN alterations. The availability of microarray-based high-density single-nucleotide polymorphism (SNP) analysis allows a reproducible and rapid determination of genome-wide alterations.[23–25] The Affymetrix® GeneChip® platform, originally developed for large-scale SNP typing, has a unique feature compared with array-based CGH: it enables the genome-wide detection of LOH in addition to extremely high-resolution CN analysis of cancer genomes by using large numbers of SNP-specific probes. The density, distribution, and allele specificity of SNP render them an excellent candidate for the high-resolution analyses of LOH and CN alterations in cancer genomes.[26,27] Conventionally, LOH analyses require the comparison of the genotypes of the tumor and its normal germline counterpart. However, for the analysis of cell line, xenograft, leukemia, and archival samples, paired normal DNA is often unavailable. In the absence of a paired normal DNA sample, LOH is inferred only based on the lower-than-expected frequencies of heterozygous SNP calls in the tumor samples. However, the low tumor content within the samples greatly hampers the sensitive detection of LOH due to increased heterozygous SNP calls. To overcome these difficulties with the current algorithms, we have recently developed novel algorithms (copy number analyzer for GeneChip® [CNAG] and allele-specific copy number analysis using anonymous references [AsCNAR]) to analyze the allelic composition of cancer genomes based on the microarray data obtained from the GeneChip® platform.[27,28] These algorithms calculate the allele-specific CN independent of the availability of a paired control DNA, enabling the sensitive detection of both LOH and CN alterations in a wide spectrum of primary tumor specimens. The performance of the new algorithm was demonstrated by detecting the neutral CN LOH or uniparental disomy (UPD) in a large number of acute leukemia samples.[28]

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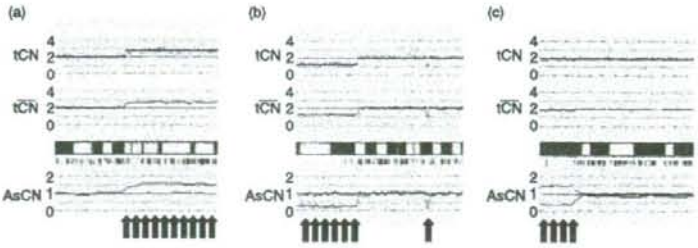


Fig. 1. Representative results of the allele-specific copy number analysis using anonymous references (AsCNAR) program with regard to copy number (CN) alterations detected in our series at particular loci, such as (a) gain (b) chromosomal loss, and (c) uniparental disomy (UPD), which have not been detected using conventional algorithms. The red dots indicate the raw CN plot for each single-nucleotide polymorphism (SNP), and the blue lines indicate the local mean CN of five SNP. The vertical green bar indicates the heterozygous SNP calls.

In the present study, to identify the novel genomic alterations in sporadic HBL cases, we performed high-resolution analyses of genome-wide CN alterations such as gains, losses, allelic imbalances, and amplifications of small chromosomal regions. Due to the high resolution of the SNP arrays and the new algorithm AsCNAR, we could systematically identify several amplifications, deletions, and allelic imbalances, including the UPD.

## Materials and Methods

**Patients and samples.** We obtained 17 primary HBL samples at the time of diagnosis from five patients treated at the Gunma Children's Medical Center and 12 patients treated at different institutes in Japan, including Saitama Children's Medical Center. No patient had received chemo- and/or radiotherapy before the biopsy of the primary tumors. After obtaining informed consent from the parents and approval for the study from the institutional review board of each institute, all the HBL samples were subjected to genomic DNA extraction using the QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Total RNA was extracted from the frozen stocked tumors using the Isogen reagent (Nippon Gene, Osaka, Japan), according to the manufacturer's instructions. The total RNA was reverse transcribed to synthesize cDNA using the Ready-To-Go T-Primed First-Strand Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

**SNP array analysis.** The array experiments were performed according to the standard protocol of Affymetrix® GeneChip® Mapping 50K *Xba*I Array (Affymetrix, Inc., Santa Clara, CA, USA). In brief, the total genomic DNA (250 ng from each sample) was first digested with a restriction enzyme (*Xba*I). The digested DNA was then ligated to an appropriate adapter that recognized the four cohesive base pair (bp) overhangs, and polymerase chain reaction (PCR) amplification was performed using a single primer that recognized the adapter sequence. After fragmentation with DNase I, the PCR products were labeled with a biotinylated nucleotide analog using terminal deoxynucleotidyl transferase, and the labeled products were hybridized to the GeneChip® Human Mapping 50K Array for 17 h. Subsequently, the arrays were washed, stained, and scanned.

The genotype calls and the intensity of the SNP probes were determined using GeneChip Operation software (GCOS; Affymetrix, Inc.). The SNP CN and chromosomal regions with gains or losses were individually evaluated using the CNAG[27] and AsCNAR algorithms,[28] which enabled an accurate determination of allele-specific CN as well as the sensitive detection of LOH even in the presence of normal cell contamination of up to 70–80% without requiring constitutive DNA (Fig. 1; http://www.genome.umin.jp).

**Validation of CN alterations using the interphase FISH.** We performed FISH to validate the CN status obtained using the SNP array analysis. FISH probes were prepared using the BAC clones RP11-185M22, RP11-80P10, and RP11-86M15. Each BAC DNA was purified, and 100 ng of the clone was labeled with digoxigenin-dUTP using random primers; these labeled clones were used as probes for FISH analysis by following the established protocols.[29,30]

**Quantitative real-time PCR and reverse transcription (RT)-PCR.** Real-time quantitative PCR (RQ-PCR) and real-time quantitative RT-PCR (RQ-RT-PCR) analyses were carried out to quantify the relative CN of several amplifications in the HBL samples and the expression levels of the defender against cell death 1 (*DAD1*), EPH receptor B6 (*EphB6*), *ErbB4*, insulin-like growth factor II (*IGF2*), and *H19* genes using a Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with an ABI prism 7700 real-time PCR detection system (Applied Biosystems). The primer pairs were designed using PrimerExpress software (Applied Biosystems) and synthesized by Invitrogen (Carlsbad, CA, USA).The primer sets used for the RQ-PCR experiments are listed in Table 1. Data were captured using Sequence Detection

**Table 1. Primers used for polymerase chain reaction (PCR) analyses**

| Gene | Primer forward | Primer reverse |
|---|---|---|
| (Genomic RQ-PCR) | | |
| EphB6 | GGACTGCAACTGAACGTCAA | TCTGGAAAGGAAGCAAAGGA |
| DAD1 | GTTATGTCGGCGTCGGTAGT | GTCCCCACGAGGAGACAGTA |
| (RQ-RT-PCR) | | |
| ERBB4 | AACAGCAGTACCGAGCCTTG | CCAGAGGCAGGTAACGAAAC |
| DAD1 | CGAGCCTTTGCTGATTTTCT | TCCAATAAGCTGCCATCTCC |
| IGF2 | CTCTCCGTGCTGTTCTCTCC | TATCGGGAAATGAGGTCAGC |
| H19 | GAAGGAGGTTTAGGGGATCG | TTGCTCTTTCTGCCTGGAAC |
| (Bisulfite PCR/RQ-PCR) | | |
| H19DMR (Methylated) | GGTACGGTTTTTTTAGGTTTATGTC | ACCCCTACAACCTCCTTACTACG |
| H19DMR (Unmethylated) | TATGGTTTTTTTAGGTTTATGTTGG | ACCCCTACAACCTCCTTACTACAC |

Primers and probes were designed using Primer Express software and MethPrimer software. RQ-PCR, real-time quantitative PCR; RQ-RT-PCR, real-time quantitative reverse transcription-PCR.

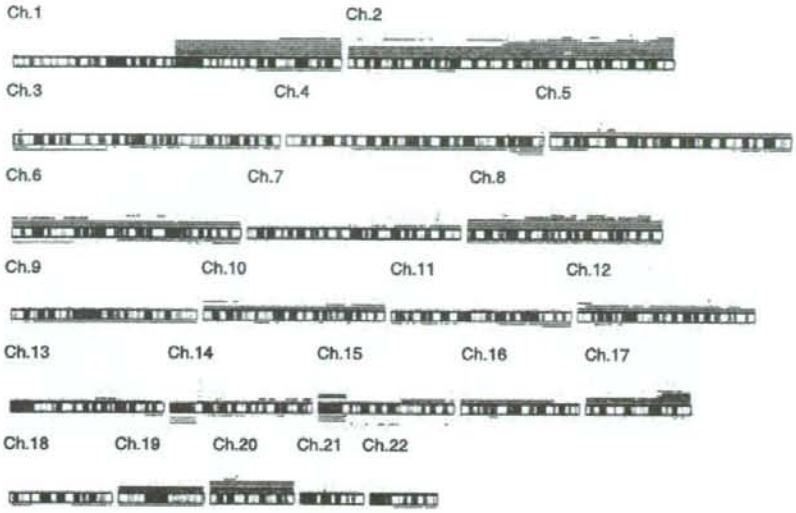


Fig. 2. Overview of the DNA copy number (CN) gains and losses detected in 17 hepatoblastoma (HBL) samples. A gain is indicated by the red bar above the chromosome ideogram, and a loss is indicated by the green bar under the chromosome ideogram. Each horizontal line represents an aberration detected in a single tumor.

software (version 1.7a; Applied Biosystems). For each primer pair, a standard curve was generated from five-fold serial dilution from approximately 50–80 pg of control DNA from a healthy individual. The amounts of genomic DNA and cDNA used in each test and the reference marker for all HBL samples were calculated using the appropriate standard curve. Normalization was performed using the β-actin gene as the internal control.

**Sodium bisulfite modification and methylation-specific PCR.** The genomic DNA from the tumor samples was treated with sodium bisulfite as described previously.[31] Briefly, 1 μg of DNA was denatured with sodium hydroxide and modified with sodium bisulfite. The modified DNA was then purified with the Wizard® DNA Clean-Up System (Promega, Madison, WI, USA), precipitated with ethanol, resuspended in Tris-EDTA (TE) buffer (pH 8.0), and either used immediately or stored at –20°C until use. The bisulfite-modified DNA was amplified with primer pairs for the methylated and unmethylated complete sequences upstream of the *H19* promoter CpG islands in the HBL samples with UPD in 11p15. The primer pairs were designed using MethPrimer software,[32] and synthesized by Invitrogen. The primers for methylation-specific DNA and unmethylation-specific primers are listed in Table 1. Normal lymphocyte DNA was used as the control. PCR was carried out in a 25 μL reaction volume using Ex Taq Hot Start Version (TaKaRa Bio Inc., Kyoto, Japan). The PCR conditions were as follows: 1 cycle at 95°C for 10 min; followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min; and a final extension at 72°C for 5 min. The PCR products were separated on 3% agarose gels and visualized under UV illumination after ethidium bromide staining. To quantify the ratio of the methylation status, we also carried out the methylation-specific RQ-PCR analysis.

## Results

**Detection of CN alterations in HBL samples.** We investigated 17 HBL samples obtained from the sporadic cases of HBL by using the Affymetrix® GeneChip® 50K *Xba*I Mapping Array. Although these specimens did not contain paired control DNA and had varying degrees of normal tissue contamination, the genomic alterations were accurately determined in most specimens by our CNAG/AsCNAR program (Fig. 1). The real CN and LOH status was inferred from the observed signal ratios of the tumor to the reference, based on the hidden Markov models implemented in the CNAG/AsCNAR program; these are summarized in Fig. 2. The CN data were validated at a number of SNP sites using FISH analysis of the cell nuclei extracted from the HBL samples (Fig. 3). The CN data obtained using the FISH analyses were consistent with those obtained using SNP mapping.

Numerical chromosomal aberrations were observed in 15 HBL samples (88%), excluding two HBL samples (HBL_22 and HBL_250). These 15 cases had variable degrees of CN gains and losses; however, the gains including the amplifications were more frequent than the losses (Table 2 and Fig. 2). Total or partial gains in chromosomes 1q and 2 were the most frequent aberrations detected in eight of the 17 patients (47%). The gain in chromosome 8 was the second most frequent aberration detected in five of the 17 samples (29%). The gains in chromosomes 17q and 20 were observed in 24% of the cases (four of 17 cases). The LOH in chromosomes 4q and 11q was observed in three (18%) and two (12%) of the 17 samples, respectively. However, these regions were usually large, and we could not determine the presence or absence of alterations in specific genes within these regions.

**High-grade amplification and common deletion.** High-grade amplifications are of particular interest because they may indicate the loci of oncogenes. The regions with high-grade amplification were defined as segments with at least five SNP loci with an inferred CN of >5. High-grade amplifications of 7q34 and 14q11.2 were observed in five (29%) and nine (53%) HBL samples, respectively. For the validation of the amplifications observed using the SNP array, FISH analysis and genomic RQ-PCR were performed. To determine the genes that are potentially affected at 14q11.2, several genes localized at the 14q11.2 chromosomal region with overlap or proximity to the BAC-RP11-85M16 were examined using the UCSC browser (www.genome.ucsc.edu). Genes that map to these regions include *EphB6* and *DAD1*, which are identified as the negative regulators of apoptosis. These two genes were subjected to RQ-PCR. FISH analysis with RP11-85M16 BAC clone probe showed multiple signals, confirming



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