to have *E2A-PBX1* (22), *TEL-AML1*, *MLL-AF4* (19), *MLL-ENL* (19), and *MLL-AF5q31* (11) fusion genes, respectively. Infant *MLL*-Re-ALL patients were mainly treated according to the MLL-96 protocol (23).

RNA Extraction and High-Density Oligonucleotide Array Analysis. Total RNA and genomic DNA were isolated from frozen cells using the ISOGEN reagent (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The quality of total RNA was examined by gel electrophoresis to confirm that the ribosomal 28S and 18S RNA bands were intact. The experimental procedures for GeneChip (Affymetrix, Santa Clara, CA) were performed according to the Affymetrix GeneChip expression analysis technical manual as described previously (24, 25). Briefly, 3–5 μg of total RNA were used to synthesize biotin-labeled cRNA, which was then hybridized to a GeneChip Human U95 V2 oligonucleotide array (Affymetrix). After washing, the arrays were stained with streptavidin-phycoerythrin and analyzed on a Hewlett-Packard Scanner to collect the image data. GeneChip Analysis Suite software 4.0 was used to calculate the AD for each gene probe set on the array, which was shown as an intensity value of the gene expression. The AD values were normalized for each array so that the average of all AD values was 100. Raw data are available on the Internet.[4]

Statistical Analysis. For each expression data set, where the AD values lay outside the range (10–8000), the value was reset to a minimum of 10 and a maximum of 8000. Subsequently, all values were log transformed for further analysis. Hierarchical clustering analysis was performed using GeneSpring (Silicon Genetics, Inc., Redwood, CA), CLUSTER, and TREEVIEW software (Eisen Lab.; Ref. 26).

Genes that correlated with particular class distinctions were identified as described by Golub *et al.* (14). We used the signal-to-noise statistic $(\mu_0 - \mu_1)/(\sigma_0 + \sigma_1)$, where μ and σ represent the mean and SD of expression, respectively, for each class. We also carried out 100,000 permutations of the samples by Mann-Whitney *U* and Kruskal-Wallis *H* tests to determine whether the correlations were more significant than would be expected by chance alone. Applying PCA, the coordinates of the first three principal components for each sample were selected. An SVM algorithm (27) was also applied to classify the samples using a modified version of the SVM light.

RT-PCR and Sequence Analysis. cDNA was reverse transcribed from 5 μg of total RNA using a cDNA synthesis kit (Invitrogen, Carlsbad, CA). PCR amplification was performed with the Advantage 2 PCR kit (Clontech, Palo Alto, CA) by incubating at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s and 68°C for 2 min using *FLT3*-specific primers (F1; 5′-CCCAACTG-CACAGAAGAGATCACAG-3′ and F2; 5′-TACAGCCTGTTAGGGATAG-GTGGAGGG-3′). The PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and subjected to direct sequencing with primers (5′-CCAGCATGCCTGGTTCAAGAG-3′, 5′-GCCCTGAGA-TTTGATCCGAGTC-3′, 5′-GTGGGAAATCTTCTCACTTGG-3′, 5′-ATC-CTAGTACCTTCCCAAACTC-3′, 5′-AGAGAGGCACTCATGTCAGAAC-3′, F1 and F2) using DYEnamic ET Terminator Kits (Amersham Biosciences, Piscataway, NJ) on an Applied Biosystems DNA sequencer. Internal tandem duplications of the *FLT3* gene were investigated by RT-PCR using the primers (5′-TGTCGAGCAGTACTCTAAACA-3′ and 5′-ATCCTAGTACCTTCCC-AAACTC-3′) and electrophoresis as described previously (20).

Genomic PCR and Restriction Fragment-length Polymorphism Analysis. We amplified the exon 20 of the *FLT3* gene by genomic PCR using the primers (5′-GTTTGTTGCACATCATCATGGCCG-3′ and 5′-CCACAGT-GAGTGCAGTTGTTTACCATG-3′) incubating at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s and 68°C for 30 s. Amplified products were digested with *Eco*RV and subjected to electrophoresis on an agarose gel (Fig. 4).

RESULTS

Gene Expression Profiling Can Identify the Translocation Type in ALL Samples. We analyzed 32 pro-B or early pre-B ALL samples, including those with *MLL* rearrangements (n = 23), *TEL-AML1* (n = 6), and *E2A-PBX1* (n = 3), with Affymetrix oligonucleotide microarrays containing 12,600 probe sets. All samples showed high *CD19* expression signals. Relatively higher expression of *CD44* and lower of *CD10* (*MME*), *CD22*, *CD24*, and *CD79B* were found in patients with *MLL* rearrangements rather than in those with *TEL-AML1* and *E2A-PBX1* (supplementary information is available on the Internet).[4]

The results of the PCA were plotted with three-dimensional scaling to determine whether we could identify the ALL translocation types from their gene expression profiles. Samples carrying *MLL* rearrangements, those with *TEL-AML1* and *E2A-PBX1*, were resolved with this method. In contrast, no distinct subgroups were observed for defined *MLL* rearrangements, such as *MLL-ENL*, *MLL-AF4*, or *MLL-AF5q31* fusion genes (Fig. 1*A*). To classify the samples according to the similarity of their gene expression patterns and classify the genes according to the expression similarities over the samples, we applied a two-dimensional hierarchical clustering algorithm. In this analysis, samples with *TEL-AML1* and *E2A-PBX1* fusion were also subclassified into their respective clusters (Fig. 1*B*).

We next selected a list of genes whose expression patterns were

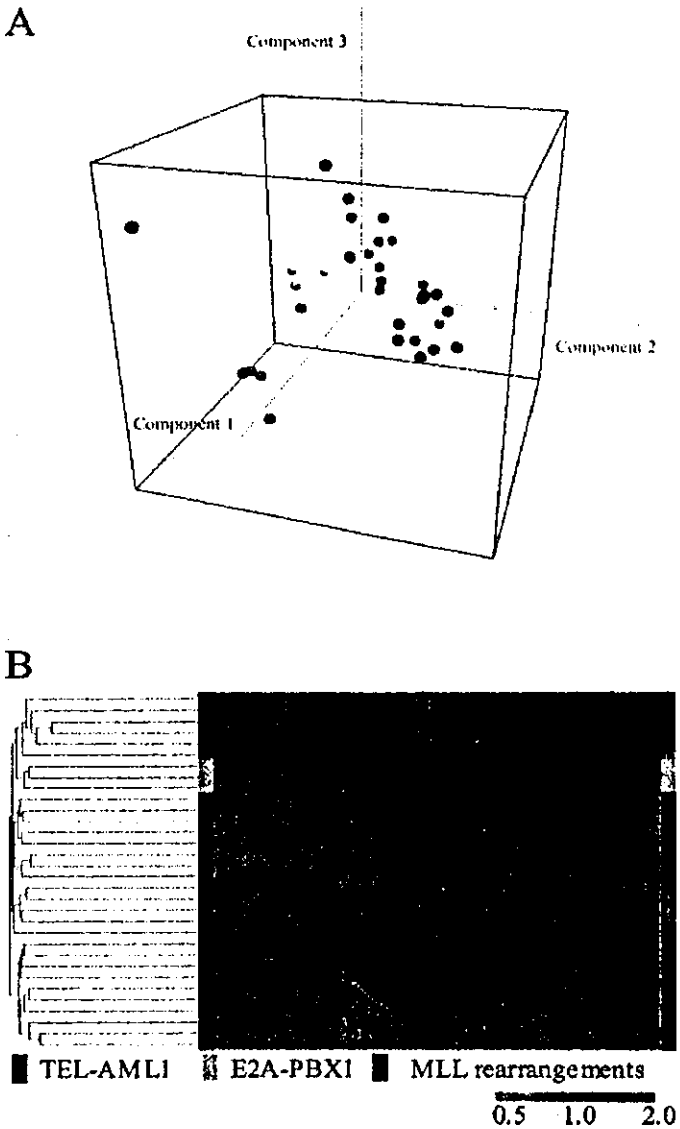

Fig. 1. *A*, comparison of gene expression in ALL associated with specific translocations. PCA plot of ALL with *TEL-AML1* fusion gene (*red*), *E2A-PBX2* (*yellow*), *MLL-AF4* (*blue*), *MLL-ENL* (*green*), and *MLL-AF5q31* (*purple*) carried out using 8322 genes that passed filtering. *B*, unsupervised two-dimensional hierarchical clustering analysis on ALL samples. This analysis was carried out using 3847 genes that passed filtering. Each *column* represents a gene and each *row* a sample. Relative expression levels are shown in *red* (relatively high) and *cyan* (relatively low).

[4] Internet address: http://www2.genome.rcast.u-tokyo.ac.jp/MLL.

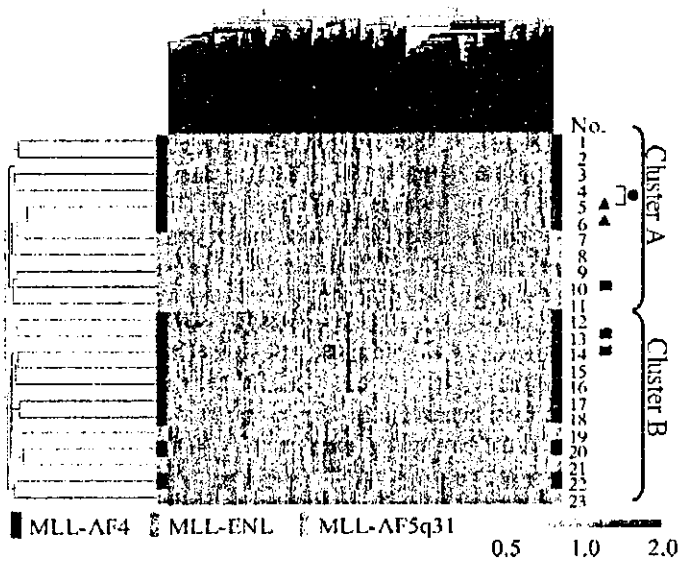

Fig. 2. Two-dimensional hierarchical clustering analysis on *MLL*-Re-ALL samples. Each *column* represents a gene and each *row* a sample. Relative expression levels are shown in *red* (relatively high) and *cyan* (relatively low). The *colored bar* on either side of the cluster identifies the fusion gene of each sample (*blue, MLL-AF4*; *green, MLL-ENL*; *purple, MLL-AF5q31*). ●, samples from the same patient. Two samples were taken at relapse (▲). Asp835 mutations were found in three samples (■).

correlated with particular translocations (*MLL* rearrangements, *E2A-PBX1* and *TEL-AML1*) using signal-to-noise analysis (supplementary Fig. 7; Ref. 14). Among 50 genes that were uniquely expressed in a subset of *MLL* rearrangements, 13 genes were also found in 50 unique genes for *MLL*-Re-ALL in a previous report (18). These included *LGALS1*, *CD44*, *CD45*, and *PMX1*. Furthermore, we found that *FLT3* and *MEIS1* were expressed highly, and *CD10* (*MME*) was less expressed in the samples with *MLL* rearrangements.

Two Distinct Patterns of Gene Expression among *MLL*-Re-ALL Samples. Gene expression patterns for leukemias with *MLL* rearrangements have been reported to be unique compared with those in other ALL without *MLL* rearrangements or AML (18). To investigate the variations in gene expression patterns among *MLL*-Re-ALL samples, we used a two-dimensional hierarchical clustering analysis on gene expression profiles for our 23 *MLL*-Re-ALL samples. This analysis produced two major sample clusters (Fig. 2). Two expression profiles generated from samples at onset and relapse from the same patient were classified in the same cluster. Excluding the two relapse samples, a random permutation test showed that the expression profiles of these two groups were statistically different ($P < 0.01$ by Mann-Whitney U test, see supplementary Fig. 9). Fourteen hundred of the 4200 genes expressed in this analysis showed distinct ($P < 0.05$) expression patterns between the two major clusters of *MLL*-Re-ALL samples. These probabilities were more significant than those between the two groups based on translocations (6 *MLL-ENL* samples *versus* 13 *MLL-AF4* samples). In fact, samples No. 20 and No. 21 carrying different *MLL*-partner genes had a correlation value of 0.49, which was much higher than the value of −0.07 found between samples No. 1 and No. 20 that have the same *MLL* partner gene. The two groups of patients were not significant in age or WBC counts at diagnosis (by Mann-Whitney U test), gender, or treatment (by χ^2 test; data not shown).

Gene Expression Signature Has Prognostic Relevance for *MLL*. To elucidate the possible clinical significance associated with the two main expression profile groups, Kaplan-Meier analysis was performed for relapse and survival (Fig. 3). Excluding the two samples obtained at relapse, Kaplan-Meier analysis showed that Cluster B in Fig. 2 was associated with a distinctly favorable prognosis. As shown in Fig. 3*B*, the overall probability of survival at 3 years was 92 ± 8% SE for Cluster B and 0% for Cluster A ($P = 0.0005$ by Log-rank analysis). The probability of event-free survival at 3 years was 73 ± 14% for Cluster B and 0% for Cluster A ($P = 0.01$). *MLL*-Re-ALL patients < 1 year old are reportedly associated with a poor prognosis, whereas those with t (4;11) and >1 year old have relatively good prognosis (2, 4, 6). In our study, only 1 patient (No. 22 on Fig. 2) was older than 1 year and subclassified in the favorable cluster B.

As reported previously (18), we found that *MLL*-Re-ALL is characterized by elevated levels of *FLT3* expression (shown in supplementary Fig. 8*A*). The AD values of *FLT3* showed no significant difference between the two clusters. Internal tandem duplication of the *FLT3* gene has been reported in 20–30% of adult AML (28, 29) and 15% of childhood AML (30). In addition, mutations of the *FLT3* gene have been reported in 5% of adult AML (31). We investigated Asp835 mutations of the *FLT3* gene by direct sequence and restriction fragment-length polymorphism analysis of genomic PCR products. Three (14%) of 21 *MLL*-Re-ALL patients, 1 from Cluster A and 2 from Cluster B, were found to have Asp835 mutations (Fig. 4). This was confirmed by sequence analysis of *FLT3* cDNA. No other mutations were found in the intracellular region of *FLT3* by sequence analysis of

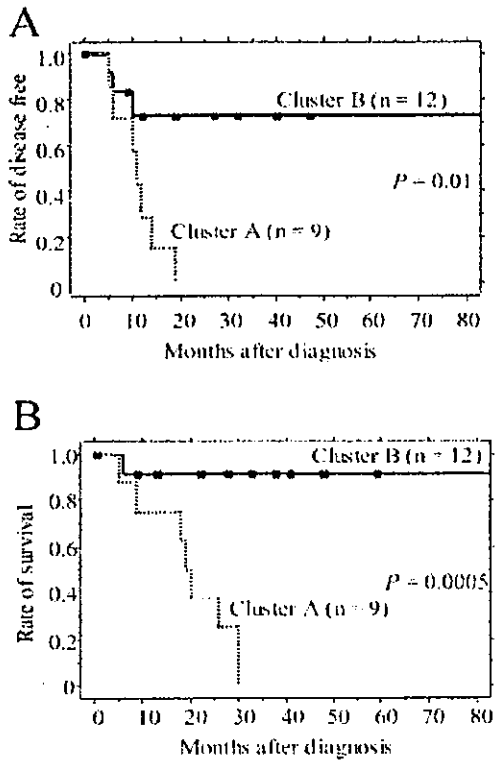

Fig. 3. Survival analysis of *MLL*-Re-ALL. Kaplan-Meier curves for Cluster A ($n = 9$) *versus* Cluster B ($n = 12$). *A*, disease-free survival; *B*, overall survival.

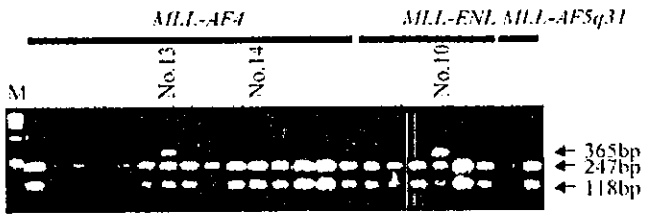

Fig. 4. Detection of Asp835 mutation of the *FLT3* gene. The *arrow* (*356 bp*) indicates Asp835 mutation of the *FLT3* gene. The *lane numbers* correspond to the sample numbers in Fig. 2.

cDNA. RT-PCR analysis failed to reveal internal tandem duplications of the *FLT3* in any of the samples (data not shown).

Biological Aspects between the Two Differential Prognostic Clusters. To investigate the biological features of the two clusters shown in Fig. 2, signal:noise expression ratios were calculated for the two groups as described previously (14), and the top and bottom 30 ranked genes that differentiated between the two groups were selected (Fig. 5). Transcription factors/coactivators were found among the top 30 discriminating genes for both clusters. In Cluster A (Gene list U), *TRIP3* and *CBF2*, and in Cluster B (gene list F), *CDP*, *NCOR1*, *USF2*, *ZFP36L2*, and *SMARCC2* were found among the top discriminating genes. We compared the promoter targets for these transcription factors with the upstream promoter sequences of other genes in our lists. CDP was reported to bind to at least two homeotic CCAAT motifs located upstream of the TATA element and to suppress histone gene expression (32). Four of the genes in gene list U (*TRIP3*, *H2BFL*, *C14orf2*, and *MDH1*), with expression suppressed in samples with elevated *CDP* expression, have two 5′-CCAAT-3′ motifs (5′-CCAAT-3′-53–72 bp-5′-CCAAT-3′), located between 500 bp upstream and 100 bp downstream of the transcription start site. This indicates that the expression patterns of these genes may be functionally related.

The precise subclassification of unknown samples into the two clusters by gene expression profiling is especially important in *MLL*-Re-ALL because there are few conventional methods for predicting the prognosis of those types of ALL. A supervised SVM was used against these higher and lower signal-to-noise genes to classify the samples. The test sample was classified using a leave-one-out model for the remaining 20 samples. Through all 21 cycles, 100% accuracy in predicting prognosis was achieved with between 21 and 1000 genes selected for higher or lower signal-to-noise values (data not shown). This result suggested that the prognosis of *MLL*-Re-ALL could be predicted reliably by using the expression profiles of selected genes.

DISCUSSION

To make an accurate diagnosis of pediatric ALL, many clinical diagnostic examinations are required. It is necessary to consider the interrelationship of various prognostic factors, including chromosomal translocations (1, 2, 4, 12). ALL patients with t (12;21; *TEL-AML1*) are associated with a good clinical outcome (2, 4), whereas *MLL*-Re-ALL patients are associated with a poor outcome (1, 2, 4, 10, 12). Our data suggest that *MLL*-Re-ALL can be diagnosed from gene expression profiles. *FLT3*, *MEIS1*, and the 13 genes reported previously (18) were also found in the top 50 genes expressed highly in our *MLL*-Re-ALL samples. *HOXA9*, a heterodimer partner of *MEIS1*, was significantly expressed in *MLL*-Re-ALL samples ($P < 0.05$ by Mann-Whitney U test).

One of the most interesting findings in this study was the remarkable variance in the gene expression signatures of *MLL*-Re-ALL. This difference was more significant than that between 13 samples with t t(4;11) and 6 with t(11;19). This result strongly suggests that at least two subgroups exist in *MLL*-Re-ALL independent of the *MLL* partner genes, with patients in one subgroup (Cluster A) having a remarkably poor prognosis.

It was reported that an internal tandem duplication of *FLT3* in AML predicted poor prognosis, and recently, mutations of *FLT3* have been reported to be rare in AML (31). Our result showed Asp835 mutations of *FLT3* in 3 (14%) of 21 *MLL*-Re-ALL patients, but we found no tandem duplications of *FLT3* similar to our previous report (20). Elevated expression of the *FLT3* gene was not associated with either cluster, and Asp835 mutations were not associated with prognosis. Except for *SPN*, expression of these leukocyte markers, *CD10*, *CD19*, CD22, *CD24*, *CD44*, *CD79B*, and *TdT*, were not significantly correlated with the two clusters (supplementary Fig. 6). It was reported that, with intensive treatment, including hematopoietic stem cell transplantation, 30–40% of *MLL*-Re-ALL infants remained free of relapse

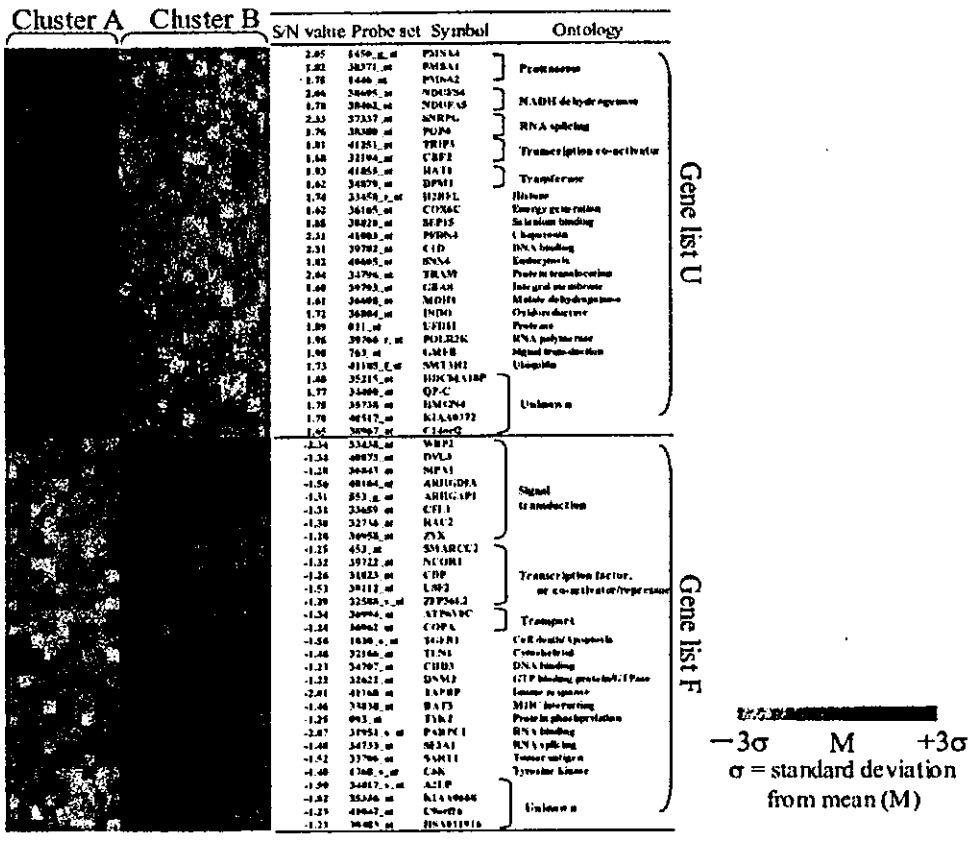

Fig. 5. Genes specifically expressed in Cluster A or Cluster B. The top 30 genes (Gene list U) and bottom 30 genes (Gene list F) by signal-to-noise values are shown. Each *column* represents a leukemia sample and each *row* a gene. The signal-to-noise value (S/N value) for the probe set of U95A array, gene symbol, and gene ontology is shown on the *right*. In each gene list, the genes are arranged according to their ontologies. Relative expression levels are shown in *red* (high) and *cyan* (low).

(4, 23, 33, 34), suggesting the existence of two patient groups with differential prognosis. Our results demonstrate that gene expression profiling is able to predict the prognosis of these distinct groups of *MLL*-Re-ALL more accurately than the conventional methods, such as karyotype analysis.

The top 30 and bottom 30 genes that were differently expressed between the two clusters (Clusters A and B) provided us with an insight into the biological behavior of ALL. In Gene list U, we found the transcriptional co-activators *TRIP3* and *CBF2*. *CBF2* was reported as a co-activator of NF-Y, which also bound the CCAAT motif (35–38). On the other hand, *CDP* (in Gene list F) also recognizes the CCAAT motif (32). CDP plays an essential role in the differentiation of hematopoietic cells (39). Loss of heterozygosity and reduced *CDP* expression has been observed in human uterine leiomyoma and breast cancer, providing the first evidence that CDP can act as a potential a tumor suppressor (40, 41). Our analysis of the promoter sites of the listed 60 genes suggested that CDP might suppress the expression of four genes in Cluster B samples. The transcription factors, CBF2 and CDP, may regulate the expression of, and be correlated quantitatively with, many genes that were differently expressed between the two clusters. For example, CBF2 was reported to induce *CDC2*, which has tandem CCAAT motifs in its promoter site (37, 42). Actually, *CDC2* showed a higher expression in Cluster A samples ($P < 0.05$ by unpaired t-test).

In the 30 genes expressed highly in Cluster B, we found 8 signal transduction genes, including four Rho family genes (*RAC2*, *ARHGDIA*, *ARHGAP1/GRAF*, and *CFL1*) and two GTP-associated genes (*DNM2* and *SIPA1*). This result suggests that different pathways are activated in Cluster B. *ARHGAP1/GRAF* is one of the partner genes of *MLL* (46). Biallelelic mutations of the *ARHGAP1/GRAF* gene have been identified in samples of myelodysplastic syndrome and AML. Recent studies have confirmed the oncogenic potential of Rho proteins (47, 48), and several studies suggest that Rho GTPases might be overfunctional in human cancers (49). It seems probable that the Rho pathway plays some roles in the leukemogenesis of patients with Cluster B.

In conclusion, these different gene expressions between the subgroups provided us with valuable information for clarifying the mechanism of leukemogenesis in *MLL*-Re-ALL. Further analysis of *MLL*-Re-ALL should lead to more accurate characterization of the key molecules of leukemogenesis and help in the search for new drug targets and diagnostic markers.

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RESEARCH ARTICLE

AML1/RUNX1 Mutations Are Infrequent, but Related to AML-M0, Acquired Trisomy 21, and Leukemic Transformation in Pediatric Hematologic Malignancies

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AML1/RUNX1, located on chromosome band 21q22, is one of the most important hematopoietic transcription factors. *AML1* is frequently affected in leukemia and myelodysplastic syndrome with 21q22 translocations. Recently, *AML1* mutations were found in adult hematologic malignancies, especially acute myeloid leukemia (AML)–M0 or leukemia with acquired trisomy 21, and familial platelet disorder with a predisposition toward AML. Through the use of polymerase chain reaction–single-strand conformation polymorphism analysis, we examined the *AML1* gene for mutations in 241 patients with pediatric hematologic malignancies, and we detected *AML1* mutations in seven patients (2.9%). Deletion was found in one patient, and point mutations in four patients, including three missense mutations, two silent mutations, and one mutation within an intron resulting in an abnormal splice acceptor site. All of the mutations except for one were heterozygous. Mutations within the runt domain were found in six of seven patients. Six of seven patients with *AML1* mutations were diagnosed with AML, and one had acute lymphoblastic leukemia. In three of these seven patients, AML evolved from other hematologic disorders. *AML1* mutations were found in two of four AML-M0 and two of three patients with acquired trisomy 21. Patients with *AML1* mutations tended to be older children. Three of four patients with *AML1* mutations who received stem cell transplantation (SCT) are alive, whereas the remaining three patients with mutations without SCT died. These results suggest that *AML1* mutations in pediatric hematologic malignancies are infrequent, but are possibly related to AML-M0, acquired trisomy 21, and leukemic transformation. These patients may have a poor clinical outcome.

INTRODUCTION

The *AML1/RUNX1* gene is located on chromosome band 21q22 and is recognized as an essential transcription factor for normal hematopoiesis, composed of AML1 and core-binding factor (CBF) β–DNA complex (Okuda et al., 2001). *AML1* is frequently affected in leukemia and myelodysplastic syndrome (MDS) with 21q22 translocations, including t(8;21)(q22;q22) (*AML1-MTG8/ETO*) (Miyoshi et al., 1991), t(3;21)(q26;q22) (*AML1-EVI1/EAP/MDS1*) (Mitani et al., 1994; Nucifora et al., 1994), t(16;21)(q24;q22) (*AML1-MTG16*) (Gamou et al., 1998), and t(12;21)(p13;q22) (*TEL-AML1*) (Golub et al.,1995; Romana et al., 1995). In all of these fusion transcripts, the whole runt domain encoding the DNA binding of AML1 is retained; however, in TEL-AML1, only the whole transactivation domain is retained. Therefore, the runt domain of AML1 is considered to play an important role in leukemogenesis. Recently, *AML1* mutations were found in leukemia (Osato et al., 1999; Preudhomme et al., 2000), MDS (Imai et al., 2000), and familial platelet disorder with a propensity to develop acute myeloid leukemia (FPD/AML) (Song et al., 1999; Buijs et al., 2001). Most of the mutations were clustered within the runt do-

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TABLE I. Frequency of *AML1* Mutations in Pediatric Hematological Malignancies

Diagnosis	No. of patients	No. of *AML1* mutations (%)
AML	100	6 (6.0)
M0	4	2[a] (50.0)
M1	7	1[b] (14.3)
M2	21	0 (0)
M3	3	0 (0)
M4	18	1 (5.6)
M5	11	0 (0)
M6	0	
M7	30[c]	0 (0)
MDS-r-AML[d]	3	2[e] (66.7)
unclassified	3	0 (0)
MDS	30	0 (0)
CML	5	0 (0)
ALL	106	1 (0.9)
B precursor	87	1[a] (1.1)
T	19	0 (0)

[a]Silent mutation.
[b]Developed from Kostmann syndrome.
[c]Including 25 patients with Down syndrome.
[d]MDS-r-AML, MDS-related AML.
[e]One patient's AML developed from RAEB-T, the other from JMML.

main of *AML1*, and the function of these mutants was decreased or lost (Osato et al., 1999; Imai et al., 2000; Michaud et al., 2002). *AML1* mutations are frequently found in AML-M0 and myeloid malignancies with acquired trisomy 21 in adults (Osato et al., 1999; Preudhomme et al., 2000; Langabeer et al., 2002). There have been no reports of *AML1* mutations in pediatric hematologic malignancies. We therefore examined *AML1* mutations in pediatric hematologic malignancies and evaluated the relationship between *AML1* mutations and the clinical features.

MATERIALS AND METHODS

Patient Samples

We analyzed 241 pediatric patients (age, 0–15 years; median, 6 years) with hematologic malignancies, including 100 patients with AML (including 25 with acute megakaryoblastic leukemia [AMKL] and Down syndrome), 30 with MDS, five with CML (three chronic phase and two blastic crisis), 106 with acute lymphoblastic leukemia (ALL), in addition to 75 normal healthy donors (Table 1). AML, MDS, and CML patients were treated between 1991 and 1999, and ALL patients were treated between 1995 and 1999 in several institutes in Japan. These patients were diagnosed according to the morphological and immunophenotypic criteria of the French–American–British (FAB) classification (Bennett et al., 1991). AML patients were treated with the Japanese childhood AML protocol of the Ministry of Health and Welfare of Japan, and ALL patients were mainly treated with the Tokyo Children's Cancer Study Group (TCCSG) L95-14 protocol (Tsuchida et al., 2000). Some patients were treated with the modified TCCSG L95-14 protocol. MDS and CML patients were treated according to the protocol of each institute. Informed consent was obtained from all of these patients or their parents and from the normal healthy donors.

Polymerase Chain Reaction–Single-Strand Conformation Polymorphism (PCR-SSCP) Analysis

DNA or total RNA was extracted from bone marrow or peripheral blood samples from these patients by standard methods (Jamal et al., 2001; Taketani et al., 2002b). We analyzed mutations in the runt domain of *AML1* by PCR or reverse transcriptase (RT)-PCR single-strand conformation polymorphism (SSCP) (Takita et al., 2001). The primers used for these analyses were the same as those used in previous studies (Song et al., 1999; Imai et al., 2000). DNA (50 ng) or 1 μl of the cDNA solution was amplified by PCR in a total volume of 10 μl with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, 5% DMSO, 250 μM of each dNTP, 1.14 μCi of [α-^{32}P]dCTP, 2.5 units of *Taq* polymerase (Ampli*Taq* Gold; Applied Biosystems, Foster City, CA), and 10 pmol of each primer. PCR amplification was performed with this mixture by use of a DNA thermal cycler (Applied Biosystems) under the following conditions: after initial denaturation at 95°C for 9 min, 35 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min, followed by a final elongation at 72°C for 7 min. The PCR products were denatured and loaded on a nondenaturing polyacrylamide gel containing 5% polyacrylamide (99:1 acrylamide to bisacrylamide) and TME (30 mM Tris; 35 mM MES {2-[*N*-morpholine] ethanesulfonic acid}, Dojin Chemicals; 1 mM Na_2EDTA, pH 6.8), and electrophoresed in TME buffer at room temperature (Takita et al., 2001). The gels were dried and exposed to Kodak XAR films (Kodak, Rochester, NY) for 12 hr at room temperature.

Sequence Analysis

The PCR products showing an abnormal band-shift in SSCP were subcloned into a pCR2.1-TOPO vector (Invitogen, Carlsbad, CA) (Taketani

TABLE 2. Clinical Features of Acute Leukemia Patients With *AML1* Mutations*

Pt. no.	Diagnosis	Age	Sex	Karyotype	SCT	Prognosis	*AML1* mutation
1	AML-M0	2	F	47,XX,+7	−	dead	del55(exon3)→ter
2	AML-M0	10	M	46,XY	+	alive	Ile87Ile
3	AML-M1	11	F	47,XX,+21	+	alive	Leu117Pro
4	AML-M4	14	F	46,XX,3q+,7q−	−	dead	Asp171Asn
5	AML-M4	13	M	46,XY,t(9;22)(q34;q11)	−	dead	Arg49His*
6	AML-lym	10	F	47,XX,+21	+	dead	IVS 3−10;t>a→ter
7	ALL**	3	M	46,XY	+	alive	Ile87Ile
	normal						Gly42Arg*

*Pt. no., patient number; SCT, stem cell transplantation; AML-lym, AML with lymphoid characteristics; normal, normal healthy donor; del, deletion; ter, induced to stop codon; IVS, intervening sequence; *, outside runt domain; **, B-precursor ALL.

et al., 2002a) and were sequenced in both directions to confirm the mutations.

Statistical Analysis

We used the nonparametric Mann–Whitney *t* test or Fisher's exact test to compare the clinical characteristics between the patients with and those without *AML1* mutations.

RESULTS

In 241 pediatric hematologic malignancies, *AML1* mutations were found in seven patients (2.9%) (Tables 1 and 2), including two patients with AML-M0 (patients 1 and 2), one each with AML-M1 (patient 3), AML-M4 (patients 4 and 5), AML with lymphoid characteristics (patient 6), and one with B-precursor ALL (patient 7). No mutations were detected in de novo MDS and CML. Among 75 normal healthy donors, only one *AML1* polymorphism was detected (Table 2). Chromosomal analysis of these patients showed acquired trisomy 21 in two patients (patients 3 and 6), −7/7q− in two (patients 1 and 4), a normal karyotype in two (patients 2 and 7), and t(9;22)(q34;q11) in one (patient 5). No mutations were found in 25 AMKL patients with constitutional trisomy 21 (Down syndrome) and in acute leukemias with 21q22 translocations, including eight AMLs with t(8;21)(q22;q22) and 11 ALLs with t(12;21)(p13;q22).

In patient 1, a 55-bp deletion (*AML1b* mRNA nucleotides [nt] 1616 to 1670; GenBank accession no. D43968) in exon 3 was detected, resulting in a stop codon (Fig. 1A). Patients 3, 4, and 5 had missense mutations: Leu117Pro (T1928C substitution), Asp171Asn (G2089A), and Arg49His (G1724A), respectively (Fig. 1B, C). Patients 2 and 7 had the same silent mutation: Ile87Ile (C1839A). In patient 6, a point mutation occurred in intron 3 (intervening sequence 3–10;t→a). Because this alteration produced AG, a splicing acceptor site, mutant *AML1* mRNA was added to the 8-bp insertion in intron 3, which led to a stop codon. One normal healthy donor in 75 had a missense mutation of Gly42Arg (G1702C). All of the mutations, except for that in patient 3, were heterozygous because analysis of the direct sequencing confirmed that all patients with *AML1* mutations had both one mutated allele and one normal allele (data not shown). SSCP analysis showed that the remaining normal band was absent in patient 3 (Fig. 1B). Mutations within the runt domain (codons 50 to 177) were found in six patients, and outside in one patient, and in one normal healthy donor (Fig. 2).

A comparison of the clinical characteristics between patients with or without *AML1* mutations showed that the white blood cell and platelet counts at onset, sex, immunophenotype, hepatosplenomegaly, and involvement of the central nervous system had no relation to *AML1* mutations (data not shown). The age of patients with *AML1* mutation was 2–14 years (median, 10 years), whereas that of patients without *AML1* mutation was 0–15 years (median, 6 years). Because of the small sample size, the difference between the two groups was not significant. With regard to the prognosis of seven patients with *AML1* mutations, three of four patients treated with stem cell transplantation (SCT) are alive, whereas the remaining three patients without SCT died. In three (patients 3, 4, and 6) of seven patients with *AML1* mutations, acute leukemia evolved from other hematologic disorders. Patient 3 was initially diagnosed with severe congenital neutropenia (Kostmann syndrome) and was treated with granulocyte–colony stimulating factor (G-CSF). She had mutations of both the *elastase2* and *G-CSF receptor* genes. When she developed AML-M1, both trisomy 21 and an *AML1* mutation appeared in her bone marrow cells. Patient 4, who was initially diagnosed with refrac-

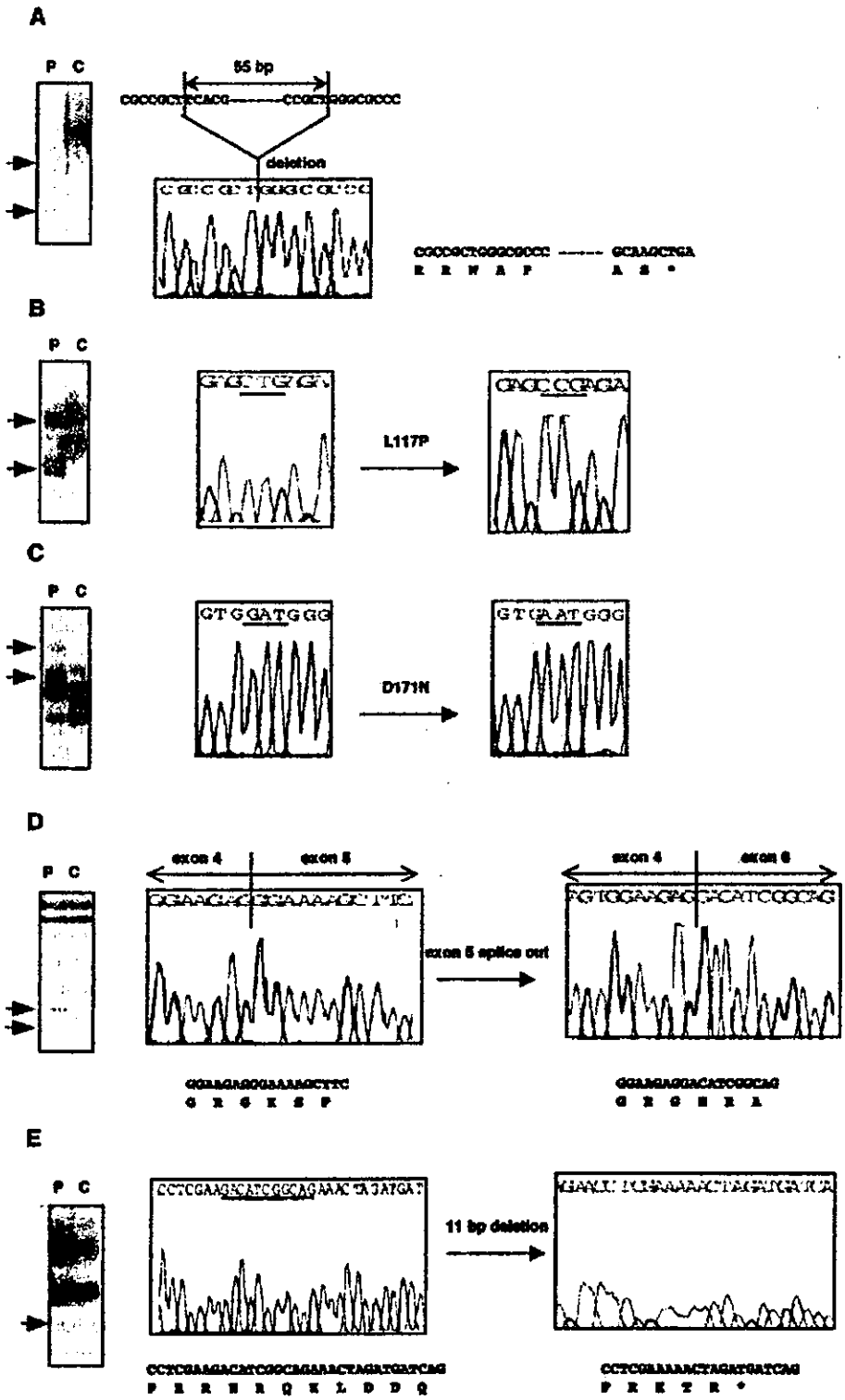

Figure 1. Mutation analysis and alternative splicing forms of the *AML1* gene. SSCP (left) and sequence (right) analysis; arrows in SSCP analysis indicate shifted bands. P, patient; C, normal healthy donor; underlines in sequence analysis indicate an altered nucleotide leading to protein change. A: Patient 1. B: Patient 3. C: Patient 4. D: Patient 8. E: Patient 9.

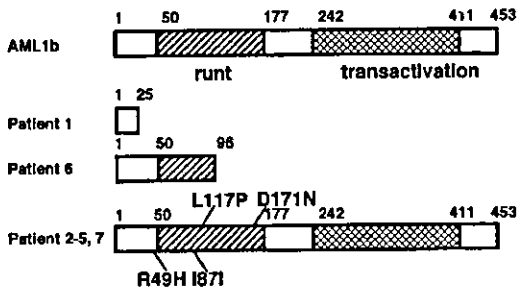

Figure 2. Schematic diagram of *AML1* mutations in pediatric hematologic malignancies.

tory anemia with excess blasts in transformation (RAEB-T), was treated with VP16, AraC, and anthracyclines. She developed AML-M4 9 months after the diagnosis. Patient 6 had been diagnosed with juvenile myelomonocytic leukemia with a normal karyotype 4 years previously. She developed AML with a lymphoid marker and rearrangements of the *IG* and *TCR* genes accompanied by the presence of both trisomy 21 and an *AML1* mutation. Interestingly, her leukemic cells were negative for myeloperoxidase before she was treated with SCT, suggesting the lineage switch to ALL.

RT-PCR–SSCP and sequence analyses also revealed two novel splicing forms of *AML1* in three ALL patients, including two with B-precursor ALL and one with T-ALL. In patient 8, although exon 5 was spliced out, this altered *AML1* gene was transcribed in frame (Fig. 1D). In patients 9 and 10, an 11-bp deletion in exon 6 (nts 1848 to 1859), which was induced by recognition of AG (nts 1858–1859) as a splicing acceptor site, led to a stop codon (Fig. 1E). Age, the white blood cell and platelet counts at onset, sex, immunophenotype, and chromosomal findings had no relation to *AML1* mutations (data not shown). All of these patients are still alive.

DISCUSSION

In this study, we investigated *AML1* mutations in pediatric hematologic malignancies. We found *AML1* mutations in seven (2.9%) of 241 patients with pediatric hematologic disorders. Previous studies showed that *AML1* mutations, including insertion, deletion, or duplication, were detected in AML, MDS, CML, and ALL, but not in any leukemia cell lines (Osato et al., 1999; Imai et al., 2000; Preudhomme et al., 2000; Vegesna et al., 2002). *AML1* germline mutations were also found in FPD/AML (Song et al., 1999; Buijs et al., 2001). The frequency of *AML1* mutations in pediatric AML in this study was six (6.0%) of 100, which was almost similar to that of adult AML (six of 109 [5.5%] [Osato et al., 1999] and 11 of 131 [8.4%] [Preudhomme et al., 2000]), whereas that of *AML1* mutations in pediatric ALL was one of 116 (0.9%) compared with one of 37 (2.7%) in adult ALL (Osato et al., 1999). The incidence of *AML1* mutations was 50% (two of four) in AML-M0 and 4.1% (three of 74) in other FAB subtypes of AML, respectively. *AML1* mutations in adult patients were more frequently found in AML-M0 and in myeloid malignancies with acquired trisomy 21, less frequently in CML and ALL (Osato et al., 1999; Preudhomme et al., 2000; Steer et al., 2001; Langabeer et al., 2002). In pediatric AML-M0, the frequency of *AML1* mutations was as high as that in adults. *AML1* mutations in AML-M0 might lead to prematurely terminating mutations because AML1 protein regulates the *myeloperoxidase* gene, and AML-M0 was negative for myeloperoxidase (Osato et al., 1999; Preudhomme et al., 2000). Most mutations in adult AML-M0 are biallelic mutations. However, a monoallelic mutation was found in two AML-M0 pediatric patients in this study. The frequency of *AML1* mutations in this study may not be accurate because the patients enrolled in this study were not sequential and our selection of patients was biased. A large sequential prospective study is needed to elucidate the exact incidence of *AML1* mutations in pediatric hematologic malignancies.

Most of the mutations were clustered within the runt domain of *AML1*, whereas the other *AML1* allele was wild-type, and showed loss of heterozygosity, or another mutation. Mutant analysis in vitro showed that the functions of AML1, DNA-binding and transactivation activities, were almost all decreased or lost (Osato et al., 1999; Imai et al., 2000; Michaud et al., 2002). The 55-bp deletion in patient 1 and the point mutation of intron 3 in patient 6 caused a frameshift, leading to a stop codon. These alterations resulted in a truncated AML1 protein, lacking a runt domain and transactivation domain. These truncated proteins would decrease or lose the activities of DNA binding, and of CBFβ interaction and transactivation. Asp171 makes a hydrogen bond with Arg174 and fixes the orientation for interaction with the DNA (Tahirov et al., 2001); therefore, Asp171 is one of the essential residues for DNA binding. An in vitro assay also revealed that the Asp171Ala mutant decreased both DNA binding and heterodimerization with CBFβ (Tahirov et al., 2001). Asp171Asn in patient

4 might have a function similar to that of the Asp171Ala mutant. Leu117 consists of runt domain loop βD (Warren et al., 2000). This loop, which is not implicated in DNA binding, makes a number of contacts with CBFβ through the side chain of Tyr113. Leu117Pro substitution in patient 3 leads to a decline in heterodimerization with CBFβ. Arg49 exists outside the runt domain, but is highly conserved in three human AML1 family proteins (AML1, AML2, and AML3) as well as in AML1 homologs of several species. This implies that this residue is an important residue of AML1 and that this mutant may compromise AML1 function or conformation. A silent mutation, Ile87Ile, in patients 2 and 7 was previously reported in Japanese leukemia patients (Osato et al., 1999). Although no mutations or polymorphisms of the runt domain of *AML1* were found in normal healthy donors in this and previous studies (Osato et al., 1999; Preudhomme et al., 2000), this mutation might be a polymorphism peculiar to the Japanese because it was detected only in Japanese patients. However, it is unknown whether the silent mutation affects the function of AML1.

Chromosomal analysis revealed that *AML1* mutations were found in two of three patients with acquired trisomy 21: two AML patients, and one with ALL. A high incidence of *AML1* mutations in myeloid malignancies with acquired trisomy 21 (Preudhomme et al., 2000) and a low incidence in hematologic disorders with constitutional trisomy 21 (Down syndrome) were previously reported (Taketani et al., 2002c). It was reported that acquired trisomy 21 appeared when some patients with Kostmann syndrome or 8p11 myeloproliferative disorder developed AML (Kalra et al., 1995; Macdonald et al., 2002). Patient 4 also developed AML from RAEB-T, although the chromosomal abnormality did not show trisomy 21, but 7q−. We could not examine *AML1* mutations at the time of initial diagnosis of these patients because the samples were not available. However, these results suggest that the occurrence of the *AML1* mutation might be associated with leukemic transformation of MDS or other hematological diseases.

Clinically, *AML1* mutations were found in older pediatric patients (median, 10 years) compared with pediatric patients without *AML1* mutation (median, 6 years). The clinical outcome of patients with *AML1* mutations has not been reported previously. In this study, all of the patients treated with SCT are alive except for patient 6, who died of a treatment-related disorder. The remaining three patients who did not receive SCT died. Further investigation of a larger number of patients is needed to clarify whether pediatric patients with *AML1* mutations have a dismal prognosis.

AML1 has three main types of alternative splicing forms with a runt domain (Miyoshi et al., 1995). Furthermore, there were a large variety of spliced *AML1* transcripts, including splicing out of exon 6, exon 2–3, and exon 3 (Levanon et al., 1996; Chimienti et al., 2000). We detected two new spliced forms of *AML1*. No mutations were detected at an exon–intron boundary by direct sequence analysis (data not shown). These spliced forms were quantitatively smaller than normal *AML1* because the aberrant band was weaker than the normal band. It was reported that AML1a overexpression, which lacked a transactivation domain, suppressed granulocytic differentiation and stimulated cell proliferation in 32Dcl3 murine myeloid cells, but these AML1a effects were canceled by the concomitant overexpression of AML1b (Tanaka et al., 1995). The spliced form of the 11-bp deletion in exon 6 was structurally similar to AML1a. This suggested that a small number of spliced transcripts might not influence the function of the normal AML1 transcript.

In conclusion, our results showed that *AML1* mutations in pediatric hematologic malignancies are infrequent, but are related to AML-M0, acquired trisomy 21, and leukemic transformation. Patients with this mutation may have a poor clinical outcome.

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Risk-directed treatment of infant acute lymphoblastic leukaemia based on early assessment of *MLL* gene status: results of the Japan Infant Leukaemia Study (MLL96)

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Summary. We studied the effectiveness of risk-directed therapy for infants younger than 13 months of age with acute lymphoblastic leukaemia (ALL). Fifty-five infants were assigned to different treatment programs (from December 1995 to December 1998) on the basis of their *MLL* gene status at diagnosis. Forty-two cases (76·3%) had a rearranged *MLL* gene (*MLL*$^+$) and were treated with remission induction therapy followed by sequential intensive chemotherapy, including multiple genotoxic agents (MLL9601 protocol). Haematopoietic stem cell transplantation (HSCT) was attempted if suitable donors were available. Thirteen infants (23·7%) were classified as *MLL*$^-$ and treated for 2·5 years with intensive chemotherapy for high-risk B-ALL (MLL9602 protocol). Complete remission was induced in 38 of the 42 infants (90·5%) with *MLL*$^+$ ALL and in all 13 patients (100%) with *MLL*$^-$ disease. In the *MLL*$^+$ subgroup, the estimated event-free survival (EFS) rate at 3 years post diagnosis was 34·0% ± 7·5%, compared with 92·3% ± 7·4% in the *MLL*$^-$ subgroup (overall comparison, $P = 0·001$ by log-rank analysis). Both age less than 6 months (hazard ratio = 6·87, 95% CI = 0·91–52·3; $P = 0·013$) and central nervous system (CNS) involvement at diagnosis (hazard ratio = 2·92 95% CI = 1·29–6·63; $P = 0·015$) were significant independent predictors of an inferior outcome. These findings indicate a strategic advantage in classifying infant ALL as either *MLL*$^+$ or *MLL*$^-$ early in the clinical course and selecting therapy accordingly. Standard chemotherapy for high-risk B-lineage ALL appeared adequate for *MLL*$^-$ cases. Novel therapeutic initiatives are warranted for infants with *MLL*$^+$ disease, particularly those with initial CNS leukaemic involvement or age less than 6 months, or both.

Keywords: infants, childhood leukaemia, *MLL* gene rearrangement, CNS leukaemia, haematopoietic stem cell transplantation, intensive chemotherapy.

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Although the treatment outcome in children with acute lymphoblastic leukaemia (ALL) has improved markedly over the past 10 years (Pui & Evans, 1998), the prognosis for

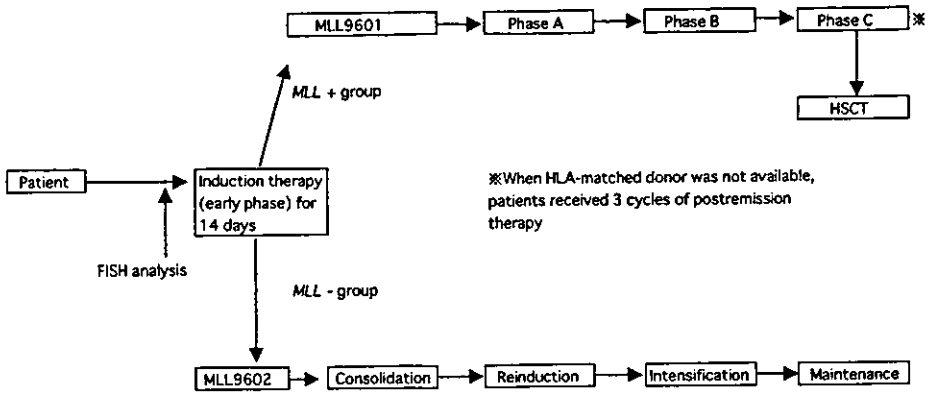

Fig 1. Flow diagram illustrating the design of protocols MLL9601 and MLL9602. Drug doses and schedules are given in Tables I and II. HSCT, haematopoietic stem cell transplantation.

those diagnosed in the first 12 months of life remains disappointing, despite the wide use of intensive chemotherapy (Reaman *et al*, 1985; Reaman *et al*, 1987; Ishii *et al*, 1991; Chessells *et al*, 1994; Ferster *et al*, 1994). ALL in infants is often accompanied by hyperleucocytosis, hepatosplenomegaly and leukaemic infiltration of the central nervous system (CNS) at the initial diagnosis. In most cases of infant ALL, the lymphoblasts express a $CD10^{-}CD19^{+}HLA\text{-}DR^{+}$ phenotype and one or more myeloid antigens, in contrast to the predominantly $CD10^{+}$ phenotype of childhood B-cell precursor ALL, indicating that the disease in infancy reflects an earlier stage of B-cell development with characteristics of both lymphoid and myeloid leukaemia (Basso *et al*, 1994; Pieters *et al*, 1998). In addition, many cases of infant ALL possess cytogenetic abnormalities, including chromosomal translocations affecting the 11q23 chromosome region (Ishii *et al*, 1991; Greaves, 1996). The gene disrupted by such rearrangements has been cloned and designated *MLL* (also *ALL-1* or *HRX*; Cimino *et al*, 1991; Ziemin-vanderpoel *et al*, 1991). Very recently, investigations at the Dana-Farber Institute in Boston showed that infant ALL with a rearranged *MLL* gene has a unique gene expression profile that clearly distinguishes it from other types of ALL as well as acute myeloid leukaemias (Armstrong *et al*, 2002).

The most important prognostic factor in infant ALL is 11q23/*MLL* gene rearrangement (Chen *et al*, 1993; Heerema *et al*, 1994; Pui *et al*, 1994; Rubnitz *et al*, 1994; Cimino *et al*, 1995; Hilden *et al*, 1995; Taki *et al*, 1996; Reaman *et al*, 1999). The event-free survival rate among MLL^{-} rearranged cases generally ranges from 10% to 20%, compared with about 50% in cases with *MLL* in the germline configuration. Thus, improving the prognosis of infant ALL will require innovative strategies in which patients with or without an *MLL* gene rearrangement are regarded as discrete subgroups requiring different methods of clinical management. Molecular analyses, including Southern blotting, polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) techniques, are critical in determining the true incidence of *MLL* gene rearrangement, as some cases with an apparently normal karyotype have a rearranged *MLL* gene (Martinez-Climent *et al*, 1995; Greaves, 1996; Rubnitz *et al*, 1996; Uckun *et al*, 1998; Mathew *et al*, 1999). Hence, molecular assays applied early in the clinical course enable the characterization of the biological heterogeneity of infant ALL cases, leading in principle to a more rational selection of treatment protocols (Pinkel, 1996).

In the present study, we subdivided infants with ALL into two groups 14 d after the start of treatment, depending on whether or not an *MLL* gene rearrangement was detected at diagnosis by FISH analysis (Fig 1). Positive cases were treated with sequential intensive chemotherapy modelled on elements of both ALL and acute myeloid leukaemia regimens, followed when possible by haematopoietic stem cell transplantation (HSCT). Cases lacking an *MLL* gene rearrangement received standard intensive chemotherapy for high-risk B-lineage ALL (Chen *et al*, 1993; Rubnitz *et al*, 1994; Silverman *et al*, 1997).

PATIENTS AND METHODS

Patients. Fifty-five of 56 infants, 0–12 months of age, with newly diagnosed ALL were eligible for this study. They were registered consecutively at 34 Japanese medical centres and treated according to Japan Infant Leukaemia Protocol MLL9601 or 9602 between December 1995 and December 1998. According to institutional guidelines, informed consent was obtained from the patients' parents before the instigation of therapy. All research was approved by the respective institutional review boards. In addition to routine clinical and laboratory examinations, the patients were evaluated for the characteristics of their leukaemic cells, including morphological and cytochemical properties, immunophenotype, cytogenetics and molecular biological features. ALL was diagnosed when 25% or more lymphoblasts were present in the bone marrow. Smears obtained from bone marrow, peripheral blood and/or cerebrospinal fluid (CSF) were stained with standard Wright–Giemsa, periodic-acid Schiff, acid phosphatase, α-naphthylacetate esterase, and myeloperoxidase. The results were interpreted

by a single group of experienced investigators, who applied the French–American–British (FAB) classification system. CNS involvement at diagnosis was defined as more than five mononuclear cells per μl of CSF with obvious lymphoblastoid morphology.

Immunophenotypic analysis. Surface markers were analysed with an Epics Profileflow cytometer (Coulter Electronics, Hialeah, FL, USA) at the study centre (Department of Pediatrics, Tokyo Medical and Dental University, and Shakaihoken Chuo Hospital or Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto). Intracellular antigens were detected by treating mononuclear cells (Fix & Perm Kit; Caltag Laboratories, Burlingame, CA, USA) to render the cell membrane permeable before flow cytometric analysis. The monoclonal antibodies used in this study included: anti-CD3 (Leu4), anti-CD2 (pan-T), anti-CD4 (Leu3), anti-CD5 (Leu1), anti-CD8 (Leu2a), anti-CD13 (LeuM7), anti-CD15 (LeuM1), anti-CD33 (LeuM9), anti-CD34 (HPCA1), anti-CD56 (Leu19) and anti-HLA-DR (HLA-DR), all purchased from Becton Dickinson Chemistry System (Mountain View, CA, USA). Anti-CD1 (T6), anti-CD2 (T11), anti-CD7 (3A1), anti-CD14 (My4), anti-CD19 (B4) and anti-CD10 (J5) were obtained from Coulter Immunology, while anti-CD79a (MB1), anti-TdT (HT6) and anti-MPO (MPO7) were from Dako (Kyoto, Japan), anti-CD20 (IOB20a) and anti-GPA (glycophorin) from Immunotech (Marseille, France), and anti-CD41b (TP80) from Nichirei (Tokyo, Japan). Each surface antigen test was considered to be positive when > 25% of the mononuclear cells (> 80% of the lymphoblasts) reacted positively. In cases with a small proportion of lymphoblasts (25–79%), > 50% of the mononuclear cells had to show a positive reaction to the antibody.

Cytogenetic studies. Mononuclear cells were separated from bone marrow or peripheral blood. After 24 h of incubation without external stimulation, the samples were fixed in Carnoy's fixative (methanol and acetic acid, 3:1). Slides for cytogenetic analysis were prepared with use of the trypsin G-banding technique. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN) (Mitelman, 1995).

FISH analysis. The same metaphase samples prepared for cytogenetic studies were used for FISH analysis. The S1363 and LB140 cosmid probes, encompassing the 5′ and 3′ ends of the MLL gene, were kindly provided by Dr Misao Ohki (National Cancer Institute of Japan). They were labelled with biotin 11-dUTP or digoxigenin-11-dUTP (Boehringer-Mannheim, Mannheim, Germany) by PCR after sequence-independent amplification (Bohlander *et al*, 1994) and then were hybridized to obtain metaphase samples, as previously described (Arif *et al*, 1997). These probes were detected with avidin fluorescein (Vector Laboratories, Burlingame, CA, USA) or antidigoxigenin rhodamine (Boehringer-Mannheim), and then counterstained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI). Images of the hybridized signals were captured by fluorescence microscopy (Olympus Optical, Tokyo, Japan).

Southern blot analysis. The results of FISH analysis to detect *MLL* rearrangement were confirmed by Southern blot analysis (Yamamoto *et al*, 1993). High-molecular-weight DNA, extracted from bone marrow or peripheral blood mononuclear cells digested with *Bam*HI or *Hin*dIII, was subjected to electrophoresis and transferred to a nylon membrane (Hybond N+; Amersham, Bucks, UK). The transferred DNA on the membrane was hybridized to the 880 bp *MLL* cDNA probe (kindly provided by M. Greaves, Leukaemia Research Fund Centre, London, UK), which covers the breakpoint cluster region of the MLL gene. Cytogenetic and molecular studies were performed at the study centre (Department of Cancer Cytogenetics, Research Institute for Radiation Biology and Medicine, Hiroshima University, and Department of Paediatrics, Faculty of Medicine, University of Tokyo).

Treatment protocols. All infants received the same intensive 'early-phase' induction therapy. On the d 14 after the start of chemotherapy, patients were assigned to one of two protocols, depending on FISH detection of the presence or absence of *MLL* gene rearrangement at diagnosis. Patients with rearranged *MLL* (*MLL*$^+$) were treated according to the MLL9601 protocol for very high-risk patients (induction therapy and three different postremission regimens), while those with germline *MLL* (*MLL*$^-$) were placed on the MLL9602 protocol, which specifies repeated and intensive use of antimetabolites in addition to genotoxic agents. Before the initiation of chemotherapy, some patients with hyperleucocytosis (≥ 150 cells × 10^9/l) received 1 week of preinduction treatment with 60 mg/m^2 of intravenous prednisolone with or without exchange transfusion. The drugs and doses specified by the MLL9601 protocol are listed in Table I. The induction regimen consisted of six cytotoxic drugs administered sequentially. Only patients who achieved complete remission (CR, < 5% lymphoblasts in bone marrow) or partial remission (5–25% lymphoblasts in bone marrow) received three phases of postremission therapy (A, B and C). Therapy for subclinical CNS disease consisted of triple intrathecal therapy (TIT) with methotrexate (MTX), hydrocortisone and cytarabine, administered on the d 1 of each chemotherapy phase. The MLL9602 protocol for the *MLL*$^-$ group consisted of induction, consolidation, intensification, reinduction and maintenance therapies. The drugs and doses specified by the MLL9602 protocol are listed in Table II.

When human leucocyte antigen (HLA)-compatible donors were available, patients in the *MLL*$^+$ subgroup were scheduled for allogeneic HSCT during postremission therapy. Those lacking HLA-compatible donors received three courses of postremission therapy (phases A, B and C) without maintenance therapy. Treatment for overt CNS disease, regardless of MLL subgroup, consisted of additional cranial irradiation (15 Gy) or a regimen containing total body irradiation for HSCT when HLA-compatible donors were available.

Statistical analysis. Continuous variables were compared with the Mann–Whitney *U*-test. Differences in the distribution of categorical variables between subgroups were analysed with the chi-square test using the Yates' correction.

Table I. MLL9601 protocol for infant leukaemia with MLL gene rearrangement.

Induction therapy	
Early phase	
Vincristine	0·05 mg/kg i.v. push, weeks 1, 2
Doxorubicin	1·0 mg/kg i.v. drip, d 3, 5
Dexamethasone	0·3 mg/kg/d in 2 divided doses, i.v. push, d 1–14
Cyclophosphamide	40 mg/kg i.v. infusion within 2 h, d 2
Late phase	
Vincristine	0·05 mg/kg i.v. push, weeks 3, 4
Doxorubicin	1·0 mg/kg i.v. drip, d 17, 19
Dexamethasone	0·3 mg/kg/d in 2 divided doses, i.v. push, 14 d then tapered over 7 d
Cyclophosphamide	40 mg/kg i.v. infusion within 2 h, d 16
Etoposide	3·0 mg/kg i.v. infusion within 2 h, d 29–32
Cytarabine	20 mg/kg i.v. infusion for 4 h, then 4 h after the end of etoposide
Postremission treatment	
Phase A	
Etoposide	3·0 mg/kg i.v. infusion within 2 h, d 1–3
Cytarabine	10 mg/kg i.v. infusion for 12 h, d 4, 8
THP-doxorubicin	1·0 mg/kg i.v. drip, d 3, 5
Prednisolone	2 mg/kg/d in 3 divided doses, p.o., d 4–9
L-Asparaginase	600 U/kg i.v. infusion within 4 h, d 9
Phase B	
Vincristine:	0·05 mg/kg i.v. push, d 1
Dexamethasone	0·3 mg/kg/d in 2 divided dose, p.o., d 1–7
Methotrexate	100 mg/kg i.v. infusion for 24 h, d 1 15 mg/m^2 of folinic acid started at 36 h post methotrexate, then 6 h × 7
Cyclophosphamide	20 mg/kg/d in 2 divided doses, i.v. infusion within 2 h, d 2, 3
Phase C	
Mitoxanthrone	0·4 mg/kg i.v. push, d 1
Etoposide	3·0 mg/kg i.v. infusion within 2 h, d 1–5
Cytarabine	100 mg/kg i.v. infusion for 4 h, d 1–5, 4 h after the end of etoposide
TIT therapy	Age < 3 months: methotrexate, 3 mg; hydrocortisone, 10 mg; cytarabine, 6 mg Age ≥ 3 months: methotrexate, 6 mg; hydrocortisone, 10 mg; cytarabine, 12 mg

THP, tetrahydropyranyl; TIT, triple intrathecal therapy.

Event-free survival (EFS) was calculated from the date of remission induction to the last follow-up or to the first adverse event (failure to achieve remission, early death, resistant leukaemia, relapse or death from any cause). Patients who did not achieve a CR were assigned a failure time of zero. Results were updated on 28 February 2001 (median follow-up times for patients assigned to the MLL9601 and MLL9602 protocols were 936 and 1041 d respectively). EFS rates (mean ± standard errors) were estimated by the Kaplan–Meier method (Kaplan & Meier, 1958) and tested for significance by the log-rank test. All analyses were performed with the BMDP statistical software 3D, 4F, 1L and 2L (Dixon, 1992). A two-sided *P*-value of less than 0·05 was considered to be statistically significant. Selected variables (e.g. gender, leucocyte count, age, myeloid antigen expression and CNS involvement at diagnosis) were included as binary covariates in a stepwise multivariate EFS analysis based on the Cox proportional hazards model. Hazard ratios and 95% confidence intervals (CI) for the association between covariates and events were estimated from the Cox model (Cox, 1972). There were too few patients in the *MLL*$^-$ subgroup to attempt either univariate or multivariate analysis.

RESULTS

Clinical characteristics

Using FISH analysis with confirmation by Southern blotting, we identified *MLL* gene rearrangements in 42 patients (*MLL*$^+$) and germline *MLL* in 13 (*MLL*$^-$). Comparison of the presenting clinical and laboratory features of these subgroups (Table III) revealed that patients with a rearranged *MLL* gene were significantly more likely to girls, to be younger than 6 months, and to have bleeding and splenomegaly, higher leucocyte counts and lower platelet counts. Eleven patients in the *MLL*$^+$ subgroup (28%), compared with only one with germline *MLL* (9%), had cutaneous involvement. By French–American–British (FAB) morphological criteria, L2 leukaemic blast cells characterized 14 cases of *MLL*$^+$ ALL (26%) but none of the *MLL*$^-$ cases. Thus, the *MLL*$^+$ and *MLL*$^-$ cases in our series represented distinct clinical entities, with the latter possessing fewer unfavourable characteristics that might contribute to treatment failure.

Immunophenotyping and cytogenetic/molecular analysis

Surface antigen expression was analysed in 38 of the 42 *MLL*$^+$ patients and in 11 of the 13 *MLL*$^-$ patients. In the

Table II. MLL9602 protocol for infant leukaemia without MLL gene rearrangement.

Induction therapy/reinduction therapy	
Early phase	
Vincristine	0·05 mg/kg i.v. push, week 1, 2
Doxorubicin	1·0 mg/kg i.v. drip, d 3, 5
Dexamethasone	0·3 mg/kg/d in 2 divided doses, i.v. push, 14 d
Cyclophosphamide	40 mg/kg i.v. infusion within 2 h, d 2
Late phase	
Vincristine	0·05 mg/kg i.v. push, weeks 3, 4
Prednisolone	2 mg/kg/d in divided doses, i.v. push or p.o., d 15–28, then tapered over 7 d
Etoposide	3·0 mg/kg i.v. infusion within 2 h, d 29–32
Cytarabine	20 mg/kg i.v. infusion for 4 h, then 4 h after the end of etoposide
L-Asparaginase	300 U/kg i.v. infusion within 4 h, d 16–18 and d 23–25
Consolidation	
Methotrexate	100 mg/kg i.v. infusion for 24 h, d 1; 15 mg/m^2 of folinic acid started at 36 h post methotrexate, then 6 h, × 7
Cyclophosphamide	20 mg/kg in 2 divided doses i.v. infusion, within 2 h, d 3, 17 and 31
L-Asparaginase	300 U/kg i.v. infusion within 4 h, d 3, 17 and 31.
Prednisolone	2 mg/kg/d in 3 divided dose, i.v. push or p.o., d 1–3, 15–17 and 29–31.
Intensification	
6-Mercaptopurine	3 mg/kg p.o., d 1–15.
Vincristine:	0·05 mg/kg i.v. push week, d 1, 8 and 15.
Cytarabine	20 mg/kg i.v. infusion for 1 h, d 2–7 and 9–14.
Daunorubicin	1·0 mg/kg i.v. infusion, d 1, 8 and 15.
Maintenance	
6-Mercaptopurine	3 mg/kg p.o., d 1–15, 29–43 and 57–71.
Methotrexate	1 mg/kg i.v. push, d 1, 8, 29, 36, 57 and 64. 10 mg/kg i.v. infusion for 5 h, d 71.
Etoposide	5 mg/kg i.v. push, d 15 and 43.
Cytarabine	10 mg/kg i.v. infusion for 4 h, d 15 and 43 after the end of etoposide.
Vincristine:	0·05 mg/kg i.v. push, d 71.
Prednisolone	2 mg/kg/d in divided doses, i.v. push or p.o., d 71–85.
TIT therapy	Age < 3 months: methotrexate, 3 mg; hydrocortisone, 10 mg; Cytarabine, 6 mg Age ≥ 3 months: methotrexate, 6 mg; hydrocortisone, 10 mg; Cytarabine, 12 mg.

TIT, triple intrathecal therapy.

former subgroup, all patients had CD19$^+$ leukaemic cells, whereas only one (2·6%) was positive for CD10. T lineage-associated antigens (CD2, cytoplasmic CD3 or CD7) were positive in two cases (5·2%), while co-expression of a myeloid antigen (CD15) was noted in a third of the cases. HLA-DR and CD34 were expressed in 37 (95%) and 25 (64%) patients respectively. In the *MLL*$^-$ subgroup, all leukaemic cells expressed CD10, a common marker of B-cell precursor ALL, with or without co-expression of myeloid or T-lineage antigens.

As shown in Table III, 34 patients (81%) in the *MLL*$^+$ subgroup had 11q23 translocations, approximately half of which were t(4;11)(q21;q23). Infants with other 11q23 abnormalities included five patients with t(11;19)(q23;p13), four with t(9;11)(p22;q23), two with t(11;22)(q23;p13) and one with add(11)(q23). Importantly, seven patients (17%) had a normal karyotype despite the presence of *MLL* gene rearrangement by molecular analysis. Karyotypes or genotypes associated with *MLL* involvement were not observed in the *MLL*$^-$ subgroup, seven of whom (58%) had a normal karyotype. Taken together, these results underscore the more primitive cellular origin of *MLL*$^+$ *vs* *MLL*$^-$ ALL.

Treatment outcome

Figure 2 summarizes the results of induction and postremission therapies for the *MLL*$^+$ and *MLL*$^-$ subgroups. Twenty-six of the 42 infants with *MLL*$^+$ blast cells and hyperleucocytosis, and three of the 13 with *MLL*$^-$ blast cells, received 1 week of preinduction treatment with intravenous predonisolone (60 mg/m^2). Only three infants in the *MLL*$^+$ subgroup underwent exchange transfusion. There was no substantial difference in CR rates between the *MLL*$^+$ and *MLL*$^-$ cohorts (92·5% *vs* 100%). None of the patients with a rearranged *MLL* gene succumbed to complications during induction therapy. Of the 38 *MLL*$^+$ patients who achieved CR, 11 relapsed during postremission treatment (before HSCT), with four still alive on 28 February 2001. In contrast, only one infant treated on the MLL9602 protocol has relapsed.

Nineteen of the 27 *MLL*$^+$ infants in continuous CR underwent allogeneic HSCT; the remaining eight infants

Table III. Presenting characteristics of 55 infants with or without MLL gene rearrangement.

Feature	*MLL*$^+$ infants ($n = 42$)	*MLL*$^-$ infants ($n = 13$)	*P*-value
Sex			
Female	27 (64%)	1 (7%)	0·0012
Male	15 (36%)	12 (93%)	NS
Median (range) age (months)	4 (0·0–12·0)	9 (4·0–12·0)	0·001
Bleeding	21 (52%)	1 (9%)	0·026
Fever/infection	15 (36%)	6 (55%)	NS
Hepatomegaly	30 (71%)	5 (46%)	NS
Splenomegary	31 (74%)	2 (18%)	0·0024
Cutaneous involvement	11 (28%)	1 (9%)	NS
CNS involvement	11 (28%)	3 (25%)	NS
Median (range) WBC ($\times 10^9$/l)	234 (3·24–750)	21 (2·8–498)	0·0004
Median (range) platelet ($\times 10^9$/l)	33 (9–174)	45 (24–266)	0·0238
Median (range) haemoglobin (g/dl)	7·2 (3·1–15·9)	7·7 (5·7–10·9)	NS
FAB classification	($n = 39$)	($n = 10$)	
L1	25 (64%)	9 (90%)	
L2	14 (26%)	0 (0%)	
L1/2	0 (0%)	1 (10%)	
Chromosomal aberrations	($n = 42$)	($n = 12$)	
t(4;11)(q21;q23)	22 (52%)	0	
Other 11q23 abnormalities	12 (29%)	1 (8%)	
Other karyotypes	0	4 (34%)	
Normal karyotypes	7 (17%)	7 (58%)	
Undetermined	1 (2%)	0	
Detection of *MLL* gene rearrangement			
FISH analysis	42 (100%)	0	
Southern blot analysis	42 (100%)	0	

NS, not significant; FISH, fluorescence *in-situ* hybridization; WBC, white blood cell count.

lacked HLA-matched donors and therefore received only chemotherapy or autologous transplants. Table IV summarizes the characteristics, sources of donor cells, times to HSCT, conditioning regimens and outcomes of the 19 allogeneic transplant recipients. Similar proportions of patients < 6 months or 6–12 months of age were in this cohort: five of 27 (20·8%) *vs* four of 15 (28·5%). At a median post-transplant follow-up time of 615 d (range,

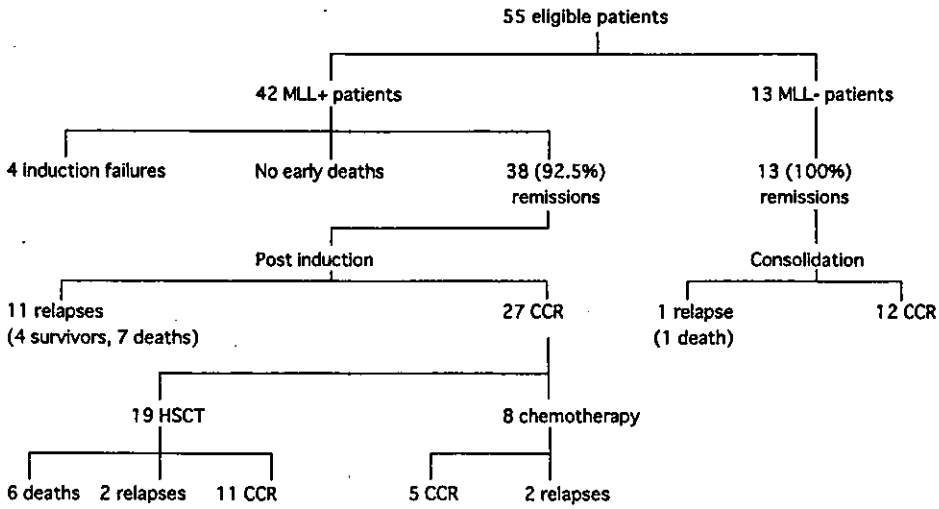

Fig 2. Distribution of events among patients treated on protocol MLL9601 ($n = 42$) or MLL9602 ($n = 13$). CCR, continuous complete remission; HSCT, haematopoietic stem cell transplantation.

Table IV. Haematopoietic stem cell transplantation for infant with *MLL*$^+$ ALL.

UPN	Age (months)	Type	Status at HSCT	Time to HSCT (d)	Conditioning	Outcome
81	3	allo BMT	1CR	142	TBI/BU/CY	CR
83	3	allo BMT	1CR	151	TBI/VP16/L-PAM	CR
85	4	allo BMT	1CR	267	TBI/BU/CY	CR
78	10	CBSCT	1CR	177	TBI/BU/CY	CR
89	7	CBSCT	1CR	228	BU/VP16/CY	CR
99	12	CBSCT	1CR	163	BU/VP16/CY	CR
119	3 week	CBSCT	1CR	177	TBI/VP16/CY	CR
121	3	CBSCT	1CR	219	TBI/VP16/CY	CR
10	12	UBMT	1CR	249	TBI/BU/CY	CR
42	8	UBMT	1CR	302	BU/VP16/CY	CR
111	4	UBMT	1CR	250	BU/VP16/CY	CR
90	7	allo BMT	1CR	238	BU/AraC//CY	Relapse
19	10	allo BMT	1CR	123	AraC/VP16/CY	Relapse
97	6	allo BMT	1CR	105	TBI/VP16/L-PAM	Death
12	4	allo BMT*	1CR	180	TBI/VP16/CY	Death
80	3	CBSCT	1CR	257	BU/CY	Death
13	3	UBMT	1CR	313	TBI/VP16/CY	Death
61	10	allo BMT	1CR	301	TBI/VP16/CY	Death
73	3 week	allo BMT	1CR	242	BU/Thio/CY	Death

*CD34-positive selection.

HSCT, haematopietic stem cell transplantation; UBMT, unrelated bone marrow transplantation; allo BMT, allogenic bone marrow transplantation; CBSCT, unrelated cord blood stem cell transplantation; TBI, total body irradiation; CY, cyclophosphamide; Ara C, cytarabine; BU, busulphan; Thio, thiotepa; L-PAM, melphalan; 1CR, first complete remission; VP16, etoposide.

28–1587 d), two patients had relapsed, six had died of complications and 11 remained in CR. Both of the infants who underwent autologous HSCT relapsed.

The mean (± SE) probability of EFS at 3 years was 34·0% ± 7·6% among patients with a rearranged *MLL* gene, compared with 92·3% ± 7·4% for patients with germline *MLL* (Fig 3A). The curves were significantly different by the log-rank test ($P = 0·001$). Although an estimated 55·5% ± 7·9% of the *MLL*$^+$ subgroup were alive at 3 years post diagnosis, their overall survival experience was still inferior to that of the *MLL*$^-$ subgroup ($P = 0·011$, data not shown). Multivariate analysis with a Cox proportional hazards model identified two factors as having an adverse impact on EFS at 3 years: age < 6 months (hazard ratio = 6·87, 95% CI = 0·91–52·3; $P = 0·013$) and CNS involvement at diagnosis (HR = 2·92, 95% CI = 1·29–6·63; $P = 0·015$). These relationships are illustrated by Kaplan–Meier curves in Fig 3B and C. Only one of the patients with overt CNS disease was an event-free survivor at 48 months of follow-up. Other factors (e.g. leucocyte count and co-expression of myeloid antigens by leukaemic cells) did not achieve significance in the multivariate model. The EFS rate for patients with the t(4;11)(q21;q23) did not differ significantly from the rates in subgroups with other 11q23 abnormalities or normal karyotypes (34·0% ± 10·5% *vs* 25·0% ± 12·5% *vs* 42·9% ± 18·7%, $P = 0·618$ by log-rank analysis; curves not shown). Importantly, comparison of clinical and biological factors between patients < 6 months or ≥ 6 months of age and between those with or without CNS involvement failed to reveal differences that might explain the dire outcomes in the higher-risk categories (data not shown).

Toxicity

The major toxic reactions of patients treated on the MLL9601 protocol are reported in Table V. There was a high frequency of infectious episodes associated with myelosuppression during the induction phase and after the A, B and C phases of chemotherapy. None of the septicaemic or neutropenic episodes associated with fever were fatal. To the contrary, nearly all infections resolved after supportive therapy with a combination of antibiotics and granulocyte colony-stimulating factor. One patient developed acute renal failure and renal tubular acidosis after high-dose methotrexate therapy during the first course of phase B. He recovered after peritoneal dialysis.

DISCUSSION

MLL gene rearrangement in infants with ALL is important because of the inadequacy of standard chemotherapy alone for patients with this molecular abnormality (Pui *et al*, 1994; Cimino *et al*, 1995; Hilden *et al*, 1995; Taki *et al*, 1996; Reaman *et al*, 1999). Once the *MLL* gene status is known, one can rationally decide between a highly toxic but potentially curative treatment, such as HSCT, and intensified chemotherapy. We used FISH to determine the *MLL* gene status at diagnosis because Southern blotting is

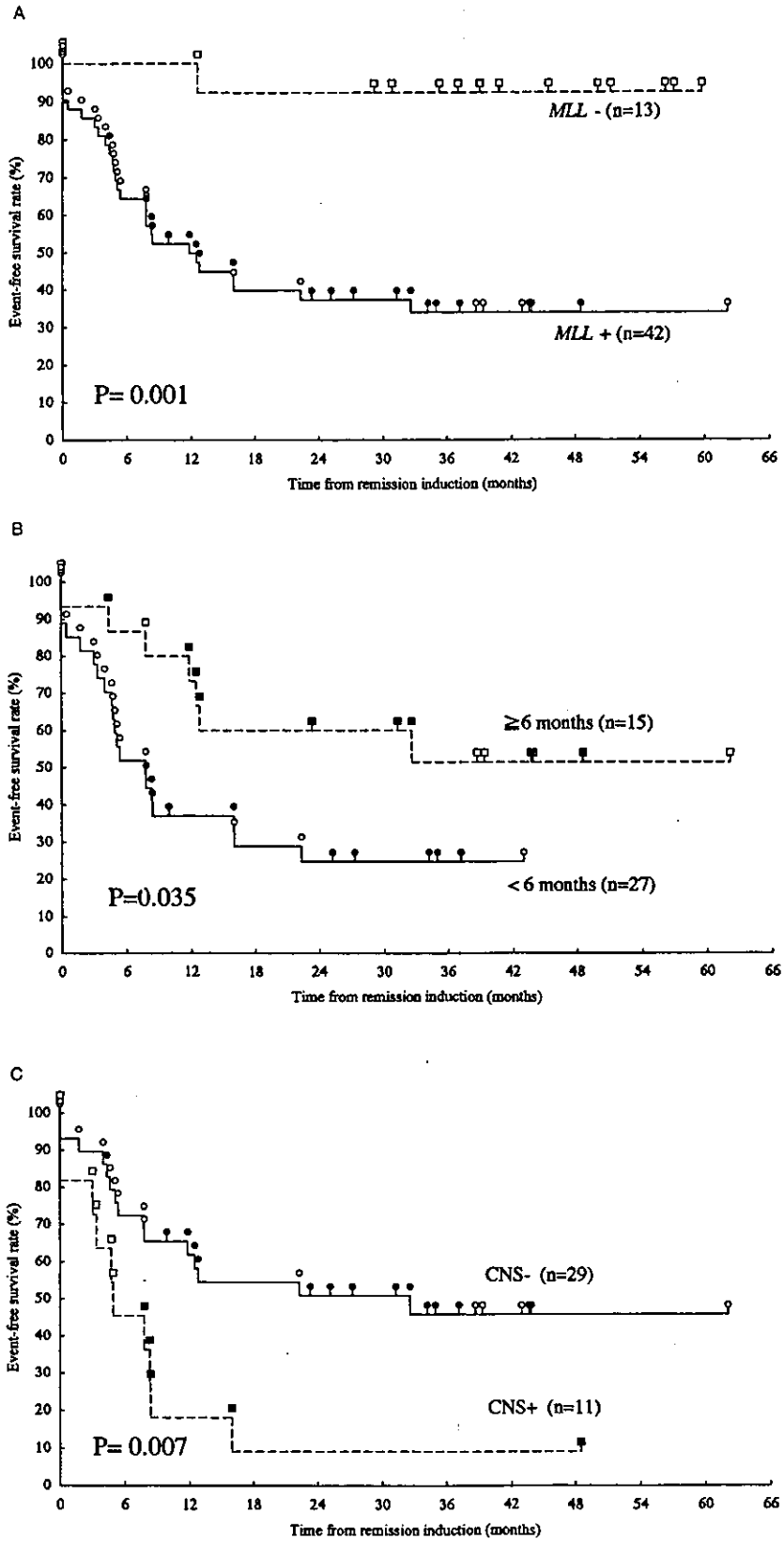

Fig 3. Kaplan–Meier analysis of event-free survival. (A) Overall comparison: MLL^+ ($n = 42$) *vs* MLL^- ($n = 13$). (B) MLL^+ subgroup: infants < 6 months old ($n = 27$) *vs* older infants ($n = 15$). (C) MLL^+ subgroup: infants with ($n = 11$) or without ($n = 29$) CNS involvement at diagnosis. In all panels, event-free survivors are indicated by marks on the curves. Open squares and circles, patients receiving chemotherapy only; closed squares and circles, haematopoietic stem cell transplantation. *P*-values are reported on the graphs.