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Fluorescence *in situ* Hybridization Analysis of 12;21 Translocation in Japanese Childhood Acute Lymphoblastic Leukemia

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Fluorescence *in situ* hybridization (FISH) analysis was applied to detect t(12;21) using two yeast artificial chromosome probes and cosmid probes covering the *TEL(ETV6)* and the *AML1* gene to clarify the incidence of abnormality of t(12;21) in Japanese childhood acute lymphoblastic leukemia (ALL). We detected seven *TEL/AML1* fusion positive patients (9.5%), all of whom were diagnosed as B-lineage ALL, among 74 childhood ALL. On the other hand, no *TEL/AML1* fusion positive patients were found among 37 adult ALL. The incidence among Japanese seemed to be lower than that among other nations. Of the seven patients with the *TEL/AML1* fusion, five exhibited normal karyotype, one was t(8;12)(q11;p13), i(21q) and the remaining one exhibited a near-triploid karyotype in conventional G-banding. The FISH method clearly demonstrated that all patients with the *TEL/AML1* fusion had subpopulations of leukemic cells with deletion of the normal *TEL* allele, which is significant for understanding the progression of leukemia with t(12;21).

Key words: 12;21 translocation — FISH — *TEL* gene

T(12;21)(p13;q22), first reported by Romana *et al.*,[1] is a nonrandom chromosomal translocation observed among patients with acute lymphoblastic leukemia (ALL) of childhood. The translocation results in a fusion of the *TEL* gene on 12p13 and the *AML1* gene on 21q22, comprising the helix-loop-helix domain of the *TEL* gene fused to almost all of the sequence of the *AML1* gene on the der(21) chromosome.[2, 3] According to previous reports,[4, 5] the frequency of t(12;21) was estimated as 15 to 30% in childhood ALL and the translocation has been recognized as the most common chromosomal aberration in childhood ALL. As the translocation is often difficult to detect by conventional G-banding analysis, molecular biological methods such as reverse transcriptase-polymerase chain reaction (RT-PCR) or fluorescence *in situ* hybridization (FISH) are useful for the diagnosis. In particular, FISH analysis has been applied to hematopoietic malignancies with subtle or complex chromosomal aberrations which are difficult or impossible to detect by standard cytogenetic analysis.[6, 7] We describe here the frequency, evaluated by the FISH method, and clinical features of t(12;21)-positive Japanese patients with childhood ALL.

MATERIALS AND METHODS

Patients ALL patients who were karyotyped in our laboratory between 1986 and 1996 were included in this study. Diagnosis of ALL was defined on the basis of morphology and immunophenotypic findings of the leukemic cells.

Cytogenetic analysis Bone marrow samples were separated by Ficoll sedimentation to obtain the mononuclear cells. After 24 h of unstimulated culture, samples were fixed in Carnoy's fixative solution (3:1 methanol and acetic acid). Slides for cytogenetic analysis were prepared by using the trypsin G-banding technique. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (ISCN 1995).[8]

Fluorescence *in situ* hybridization Bone marrow samples fixed in Carnoy's solution for cytogenetic analysis were subjected to FISH analysis. Sample preparation, FISH hybridization and detection were performed as described previously.[9] For the first screening of t(12;21), yeast artificial chromosome (YAC) 936e2 covering the *TEL* gene on 12p13[10] and YAC 812f11 covering the *AML1* gene on 21q22[11] were used as probes for dual-color FISH analysis. For those patients with a fusion signal of the two YACs, *TEL/AML1* gene fusion was confirmed by dual-color FISH on interphase nuclei with two cosmid probes, 184C4 which covers exons 4, 5, and intron 5 of the *TEL* gene[12] and Y107 which covers intron 2 to exon 3 of the *AML1* gene.[13] The YAC 936e2 and cosmid 184C4 probes were labeled with biotin-11-dATP by nick translation (GIBCO BRL, Gaithersburg, MD) and with avidin-fluorescein (FITC) (Boehringer Mannheim,

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Mannheim, Germany) to allow them to be detected as green signals. The YAC 812f11 and cosmid Y107 probes were labeled with digoxigenin-11-dUTP using a digoxigenin labeling system (Boehringer Mannheim) and with anti-digoxigenin-rhodamine (Boehringer Mannheim) to allow their detection as red signals. Interphase nuclei which showed adequate fluorescent signals were scored in at least 100 nuclei from each sample under a fluorescence microscope (Olympus Optical Co., Tokyo) fitted with appropriate absorption and excitation filters.

RT-PCR Total RNA was isolated with an RNA extraction kit (RNeasy Mini Kit, QIAGEN, Santa Clarita, CA). RT-PCR to detect the *TEL/AML1* fusion gene was performed with an RT-PCR Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA by using Moloney murine leukemia virus-reverse transcriptase and one-tenth of the reaction product was submitted to nested PCR amplification using the following specific primers: TEL940f (5′-CTCTCTCATCGGGAAGACCTGGC-3′) and AML1-R41 (5′-ATTGCCAGCCATCACAGTGAC-3′) for the first-step PCR and TEL981f (5′-GGTCTCT-GTCTCCCCGCCTGAAG-3′) and AML1-R42 (5′-CAGAGTGCCATCTGGAACAT-3′) for the second-step PCR. The sequences of AML1-R41 and AML1-R42 were designed as described previously[14] and were located on exon 4 of the *AML1* gene. The sequences of TEL940f and TEL981f were located on exon 5 of the *TEL* gene. Thirty cycles of PCR reaction (94°C×1 min, 65°C×1 min and 72°C×1 min) were performed in the first- and the second-step PCR on a DNA thermal cycler (Perkin Elmer, Norwalk, CT). PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide.

Southern blot analysis of the *TEL* rearrangement High-molecular-weight DNA was extracted from a bone marrow sample. Southern blot analysis was performed according to standard methods. Extracted DNA was digested with *Bam*HI, electrophoresed and transferred to a nylon membrane (Hybond N+, Amersham, Buckinghamshire, UK). The transferred membrane was hybridized to the TEL probe covering exon 5 of the *TEL* gene. The probe was made by PCR amplification from a cDNA library of normal human lymphocytes using the following primers: TEL540f (5′-CCCTCCCACCATTGAACTGT-TGC-3′) and TEL939r (5′-CCAGGTCTTCCCGATGA-GAGAGG-3′).

RESULTS

Chromosome aberrations in childhood and adult ALL patients Seventy-four childhood (less than 16 years old) and 37 adult (16 years or older) ALL patients were enrolled in this study. The childhood patients included 72 with B-lineage ALL and two with T-lineage ALL, and the adult patients included 35 with B-lineage ALL and two with T-lineage ALL. Numbers of patients with each type of chromosome aberration are listed in Table I. Forty-seven out of 74 childhood patients had a normal karyotype. The others showed chromosomal aberrations such as hyperdiploid karyotypes, t(9;22), t(1;19) and 11q23 translocations. Three patients with abnormalities of the short arm of chromosome 12 were also included. The adult patients included in this study were restricted to those exhibiting normal karyotype or 12p11-13 abnormality, including deletions or translocations.

Detection of t(12;21) by FISH analysis in ALL patients In dual-color FISH analysis using the YAC 936e2 and the 812f11 probes, the YAC 936e2 green signal fused to the 812f11 red signal on the der(21) chromosome in the metaphase leukemic cells from t(12;21)-positive control samples. The fusion yellow signal in the interphase nuclei was sufficiently intense to facilitate the diagnosis of t(12;21). From the control study using three t(12;21)-negative ALL cell lines and five normal bone marrow (non-leukemic patients) samples, the level of false-positive results of this method was estimated as 2.1±1.1%. Therefore, 4.3% (mean+2SD) was used as the cut-off level in this study.

Of the 74 childhood patients with ALL, seven (9.5%) were found to have the fusion signal of the two YACs on leukemic cells (Table I). Among the 37 adult patients with ALL, none showed fusion signal-positive cells, even among the patients with 12p abnormalities. Because the YAC 936e2 and 812f11 probes span more than 1 megabase and might contain genes other than *TEL* and *AML1*, dual-color FISH analysis was applied using cosmid probes 184C4 and Y107, located within the *TEL* and *AML1* genes, respectively, to confirm the *TEL/AML1* gene fusion on the interphase leukemic cells with the fusion signal of

Table I. Numbers of Patients with *TEL/AML1* Fusion Signal

Chromosome aberration	No. of patients	No. of patients with *TEL/AML1* fusion signal
Childhood ALL (age<16)		
normal karyotype	47	5
12p translocation	3	1
t(9;22)(q34;q11)	6	0
t(1;19)(q23;p13)	6	0
11q23 translocation	3	0
hyperdiploid	3	0
other abnormalities	6	1
total	74	7
Adult ALL (age≥16)		
normal karyotype	33	0
12p translocation	4	0
total	37	0

Table II. Karyotypes and FISH Results of Seven Patients with *TEL/AML1* Fusion Signal

Patient	Karyotype	% of *TEL/AML1*-positive cells on interphase nuclei	% of cells with deletion of normal *TEL* gene
1	46,XY[10]	89.0	91.7
2	46,XX,t(8;12)(q11;p13),i(21q)[1]/46,XX[7]	85.6	10.0
3	46,XX[2]	94.5	60.5
4	46,XX[14]	96.0	18.5
5	46,XX[1]	96.5	85.4
6	46,XX[1]	96.5	64.5
7	near-triploid (modal number 67–69)[4]	95.5	n.d.[a)]

a) Patient 7 was excluded from the analysis of the deletion because those cells showed more than two copies of chromosome 12.

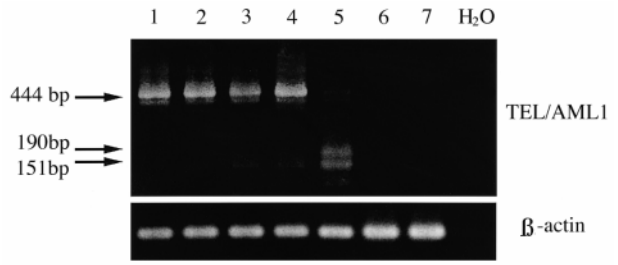

Fig. 1. RT-PCR analysis of t(12;21)-positive patients. The results of the first-step PCR are shown. Lanes 1 to 5 are from patients 1, 4, 5, 6 and 7 in Table II, respectively. Childhood ALL without t(12;21) was used for the negative control (lanes 6 and 7). Lanes 1 to 4: main PCR product showing a long type of *TEL*/*AML1* fusion gene; lane 5: two short types mainly detected. β-Actin was used as the control for expression.

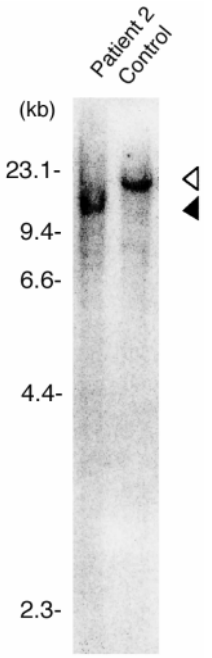

Fig. 2. Southern blot analysis of the genomic DNA from patient 2 with the *TEL* gene probe. The rearranged band in the patient (10 kbp) is indicated by a black arrowhead. The normal germline band (18 kbp) in the control sample is indicated by a white arrowhead, but the germline band was not found in the patient.

YACs. With these cosmids, the positive samples with *TEL/AML1* gene fusion showed a fused yellow signal on the der(21) chromosome in the metaphase and on the interphase nuclei of the leukemic cells. Therefore it was possible to confirm *TEL/AML1* gene fusion by using these cosmid probes. The cut-off level in this method was estimated to be 3.5%. All seven childhood ALL patients with a fusion signal of YACs showed also a fusion signal of the two cosmids on the interphase nuclei, confirming that they have *TEL/AML1* fusion gene. The karyotypes and the interphase FISH results of the seven patients with the *TEL/AML1* fusion signal are listed in Table II. In patients 3, 5 and 6 only one or two metaphases were available for cytogenetic analysis. A precise karyotype was not obtained from patient 7 because of poor morphology of the metaphases.

RT-PCR and Southern blot analysis RNA samples were available from five patients (patients 1, 4–7) out of the seven *TEL/AML1* fusion positive patients (patient 1 was in relapse, patients 4, 5 and 6 were at the onset and patient 7 was at 1 week after the onset). Results of RT-PCR are shown in Fig. 1. All the samples examined showed *TEL/AML1* fusion transcript of the expected size in the first-step PCR, and this was confirmed by nested PCR. According to Satake *et al*.,[14)] there are four types of *TEL/AML1* fusion transcripts; type A: *TEL* exon 5 fused to *AML1* exon 2, but exon 3 of *AML1* spliced out, type B: *TEL* exon 5 fused to *AML1* exon 2, type C: *TEL* exon 5 fused to *AML1* exon 4, type D: *TEL* exon 5 fused to

Table III. Clinical Findings of *TEL/AML1* Fusion Signal-positive Patients

Patient	Age	Sex	WBC (10^9/liter)	Surface marker[a)]	Organomegaly	CNS	EFS
1	7y4m	M	19	CD10, CD13, CD19, CD34	—	—	26m
2	2y10m	F	274	CD10, CD19, CD34, HLA-DR	—	—	60m+
3	7y6m	F	93	CD10, CD19, HLA-DR	—	—	36m+
4	3y11m	F	112	CD10, CD19, CD34, HLA-DR	—	—	34m+
5	2y7m	M	25	CD10, CD13, CD19, CD34, HLA-DR	liver, LN	—	17m+
6	9y3m	M	33	CD10, CD19, CD34, HLA-DR	—	—	12m+
7	8y0m	M	16	CD10, CD19, CD34, HLA-DR	—	—	12m+

CNS: involvement of the central nervous system, EFS: event-free survival, LN: lymph node, WBC: white blood cell, +: alive.
a) Surface markers observed in more than 50% of bone marrow cells are recorded.

AML1 exon 3. The expected sizes of the first-step PCR products were 190 bp, 444 bp, 151 bp and 405 bp, respectively. For patients 1 and 4 to 6, the main product of the first-step PCR showed 444 bp, the type B transcript, and for patient 7, the main products were 190 bp and 151 bp, namely, the type A and C transcripts. Eight childhood ALL patients without any *TEL/AML1* fusion signal by FISH analysis were also analyzed as negative controls and none had a *TEL/AML1* chimeric transcript.

Southern blot analysis was performed for patient 2, whose RNA sample was not available, and a 10 kbp rearranged band was detected with the *TEL* exon 5 cDNA probe (Fig. 2). Patient 2 lacked the normal germline band of the *TEL* gene, indicating deletion of the *TEL* gene on the normal chromosome 12.

Deletion of the normal *TEL* allele of leukemic cells with t(12;21) Three green signals of YAC 936e2 were observable in the leukemic cells harboring the *TEL/AML1* fusion gene without a deletion of the normal *TEL* allele. However, the leukemic cells with deletion of the normal *TEL* allele exhibited only two green signals of YAC 936e2, one of which was fused to a red signal of YAC 812f11. To estimate the percentage of deletion in each patient, the YAC 936e2 green signals were enumerated on the interphase leukemic cells. Before applying this method to *TEL/AML1* fusion-positive patients, a control study was performed using PHA-stimulated peripheral blood from three normal adults, three normal bone marrow samples and three ALL cell lines without 12p abnormality. The false-positive level of this method, namely, the percentage of the cells with one signal of YAC 936e2, was estimated as 3.2±2.1%. Therefore, a value of 7.4% (mean+2SD) was used as the cut-off level in this deletion study. The results in *TEL/AML1* fusion-positive patients are shown in Table II. All six patients studied (1–5, and 7) had subpopulations of leukemic cells with deletion of the normal *TEL* allele.

Clinical characteristics of t(12;21) ALL Clinical and immunophenotypic findings of the childhood ALL patients with the *TEL/AML1* fusion are listed in Table III. All seven patients were positive for CD10 and CD19, indicating a B-lineage phenotype of the leukemic cells. Furthermore, in six patients (patients 1, 2 and 4–7), CD34 was also positive. Six patients (patients 2–7) responded well to treatment with a standard regimen and remained in remission. Patient 1 relapsed and died 2 years after the onset.

DISCUSSION

Among 74 childhood ALL patients, seven were determined to have *TEL/AML1* fusion gene by FISH and RT-PCR analysis. On the other hand, no *TEL/AML1* fusion-positive patient was found among 37 adult patients. The frequency of the t(12;21) abnormality in childhood ALL was 9.5% (7 of 74 patients) in our study. Previously reported frequencies of t(12;21) ALL in Japanese children were 9.7% by FISH analysis[15)] and 13% by RT-PCR analysis.[16)] Because ALL patients included in this study were restricted to those for whom we had sufficient samples for FISH analysis, the distribution of the frequencies of each type of chromosome abnormality were slightly different from those in childhood ALL reported previously. For example, more than 30% of childhood ALL had hyperdiploid karyotype according to Heim and Mitelman,[17)] while only 4.1% was hyperdiploid karyotype in this study. Considering other reports on t(12;21) ALL in Japanese childhood ALL, the frequency among Japanese seems to be lower than that among other nations.[4, 5, 18)]

All of the t(12;21)-positive patients were positive for CD10 and CD19 and were considered to have B-lineage ALL. Six out of the seven patients (85.7%) with t(12;21) were also CD34-positive, indicating that the leukemic cells originated from primitive hematopoietic cells similar to those of ALL patients with t(9;22) or 11q23 abnormalities. These patients had relatively low white blood cell (WBC) counts at the time of diagnosis and a low incidence of invasion-indicating findings, such as

organomegaly and lymphadenopathy. Six out of the seven t(12;21)-positive patients are still in first remission, indicating a favorable outcome, as reported by others.[5)]

Deletion or loss of heterozygosity of the short arm of chromosome 12 is frequently observed in a variety of hematological malignancies.[19–21)] In t(12;21)-positive ALL patients, a deletion of the normal *TEL* gene in the non-translocated chromosome 12 is frequently observed by Southern blot analysis (loss of germline band) or by FISH analysis (loss of FISH signals on the normal chromosome 12). We investigated the percentage of leukemic cells with deletion of the normal *TEL* gene by counting the number of YAC 936e2 signals on the interphase leukemic cells. All seven patients with t(12;21) had subpopulations of leukemic cells with deletion of the normal *TEL* gene, revealing the presence of two groups of leukemic cells, one without the deletion of the normal *TEL* gene and the other with the deletion. The percentage of leukemic cells with the deletion varied among these patients. For example, almost all leukemic cells (91.7%) in patient 1 and only a small fraction of the leukemic cells in patients 2 and 4 (10.0% and 18.5%, respectively) had deletion of the normal *TEL* gene. The deletion of the normal *TEL* gene in t(12;21)-positive ALL was also reported by others.[22, 23)] Raynaud *et al*. reported that 11 out of 17 ALL patients with t(12;21) showed deletion of the normal TEL gene as determined by metaphase FISH of the leukemic cells.[22)] Two out of the 11 patients showed a subpopulation of leukemic cells with the deletion. In our study, FISH was applied to the interphase of the leukemic cells and revealed that all six patients with t(12;21) had a subpopulation of leukemic cells with deletion of the normal *TEL* gene. These results indicated that the deletion of the normal *TEL* gene occurred in essentially all t(12;21)-positive ALL, though the leukemic cells with the deletion comprised only a subpopulation as a result of a secondary event. In patient 2, only 10% of the leukemic cells appeared to have deletion of the normal *TEL* gene by FISH, but the patient showed complete loss of the germline band derived from the normal *TEL* gene in Southern blot analysis. These findings indicate that a small deletion involving the *TEL* gene, which could not be detected by FISH analysis, had occurred in patient 2. Such a small deletion of the normal *TEL* gene in t(12;21)-positive ALL may be more frequent than observed in this FISH study, and may be significant for the progression of leukemia with t(12;21).

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