Rapid and inexpensive screening of chromosomal abnormalities in leukemia

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Rapid and inexpensive screening of chromosomal abnormalities in leukemia

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ABSTRACT

We made a proposal for combined use of multiplex polymerase chain reaction (M-PCR) and interphase fluorescence *in situ* hybridization (I-FISH) in screening of the clinically important leukemogenetic abnormalities in hematological disorders (acute myeloid leukemia; AML, acute lymphoblastic leukemia; ALL and chronic myelogenous leukemia; CML). We used M-PCR to detect 6 types of chimeric gene transcripts (AML1/ETO, PML/RARα, CBFβ/MYH11, BCR/ABL, TEL/AML1 and E2A/PBX1), M-PCR/restriction fragment length polymorphism (RFLP) for FMS-like tyrosine kinase 3 (FLT3) mutations (internal tandem duplication; ITD and D835 mutations) and I-FISH for screening of mixed-lineage leukemia gene (MLL) abnormalities (rearrangements, amplification and deletion). Our combination assay effectively and correctly enables to detect total 11 kinds of leukemogenetic abnormalities. And we also used microchip electrophoresis (ME) for sizing PCR products, which provides us accurate results easily and rapidly.

We demonstrate here the clinical value of our method in 27 AML, 11 ALL and 11 CML patients. The chimeric gene transcripts were detected in 6 (22.2%) of the 27 AML and in 5 (45.5%) of the 11 ALL cases. In the 11 CML cases, BCR/ABL were detected in all cases. FLT3 ITD was only detected in 6 (22.2%) of the 27 AML cases. And in 2 of these 6 FLT3 ITD positive cases, wild type transcripts were not detected, only ITD type transcripts were detected (hemizygote). FLT3 D835 mutations were not detected in our cases. MLL rearrangements were detected in 3 (11.1%) of the 27 AML and in 1 (9.1%) of the 11 ALL cases. MLL amplification was detected in only one of the AML cases (3.7%). Total 32 (65.3%) of 49 in our cases, we could detect some abnormalities by this combination assay in 1 to 2 days. Furthermore, in 9 (33.3%) of 27 leukemogenetically abnormal cases (exclude FLT3 mutated cases), abnormalities were detected only by our method not by karyotyping.

In conclusion, our combination assay of M-PCR followed by ME and I-FISH seems to be a very powerful tool for the rapid screening of leukemogenetic abnormalities in AML, ALL and CML.

KEY WORDS

multiplex PCR, interphase FISH, leukemia, chromosomal abnormality, FLT3 mutation

Introduction

The diagnosis of leukemia is multidisciplinary, morphology, immunology and cytogenetics as the most often used methodologies. Cytogenetics plays an important role in delineating patients with a defined prognosis. Conventional cytogenetic analysis (karyotyping) is time-consuming, needs considerable technical expertise and in some cases, especially in acute lymphoblastic leukemia (ALL) yields sufficient metaphases only in 60 to 80% of the bone marrow samples. Therefore some medical institutions use molecular biological methods, e.g., fluorescence *in*

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Table 1. PCR primers used in the multiplex PCR.

	Name	Sequence 5' to 3'		Name	Sequence 5' to 3'	Split-out PCR
M-PCR 1st round	AML1A:1863U21	GATGGCACTCTGGTCACTGTG	M-PCR 2nd round (A)	AML1A:1885U20	TGGCTGGCAATGATGAAAAC	A/E
	ETO:327L23	TCTCCTATCTCGGGTGAAATGTC		ETO:116L22	CGTTGTCGGTGTAAATGAACTG	A/E
	CBFB:267U22	TTTGAAGGCTCCCATGATTCTG		CBFB:344U21	TGGGCTGTCTGGAGTTTGATG	C/M
	MYH11:2198L22	AGGTCCCCTTCCAGCTTCTTCT		MYH11:2041L19	TGAGCGCCTGCATGTTGAC	C/M
	MYH11:1438L24	GAGCTGGATGTTGAGAGTGGAGAT		MYH11:1387L20	TCCTCGTCCAGCTGGTCTTG	C/M
	PBX1:459L18	GCCACGCCTTCCGCTAAC		PBX1:436L21	CATGTTGTCCAGCCGCATCAG	E/P
	E2A:1173U19	CTACGACGGGGGTCTCCAC		E2A:1173U19	CTACGACGGGGGTCTCCAC	A/E, C/M, E/P
	E2A:1883L22	TTTTCCTCTTCTCGCCGTTTCA		E2A:1884L19	AGGTTCCGCTCTCGCACTT	A/E, C/M, E/P
	BCR:1698U19	CGCTCTCCCTCGCAGAACT	M-PCR 2nd round (B)	BCR:1777U19	ACTGCCCGGTTGTCGTGTC	B/A
	BCR:3060U23	GAGTCACTGCTGCTGCTTATGTC		BCR:3128U22	CACGTTCCTGATCTCCTCTGAC	B/A
	ABL:661L20	TTTTGGTTTGGGCTTCACAC		ABL:642L23	ACACCATTCCCCATTGTGATTAT	B/A
	PML3:1211U19	CAAGAAAGCCAGCCCAGAG		PML3:1370U21	GCCAGTGTACGCCTTCTCCAT	P/R
	PML3:861U19	GTGCGCCAGGTGGTAGCTC		PML3:930U20	CAGCGCGACTACGAGGAGAT	P/R
	RARA:540L19	AAGCCCTTGCAGCCCTCAC		RARA:508L22	CCCATAGTGGTAGCCTGAGGAC	P/R
	TEL-1 EX	ACCAGGAGTCCTACCCTCTGTCAGTG		TEL-2 IN	CCGGCAGGAGAGCACACGCGTGATCCAG	T/A
	AML1-2 IN	AACGCCTCGCTCATCTTGCCTGGGCTCAG		AML1-2 IN	AACGCCTCGCTCATCTTGCCTGGGCTCAG	T/A
				E2A:1173U19	CTACGACGGGGGTCTCCAC	B/A, P/R, T/A
				E2A:1884L19	AGGTTCCGCTCTCGCACTT	B/A, P/R, T/A

	Name	Sequence 5' to 3'
FLT3 mutations	FLT3:R5	TGTCGAGCAGTACTCTAAACA
	FLT3:R6	ATCCTAGTACCTTCCCAAACTC
	FLT3:17F	CCGCCAGGAACGTGCTTG
	FLT3:17R	GCAGCCTCACATTGCCCC

situ hybridization (FISH) and/or reverse transcriptase polymerase chain reaction (RT-PCR) to detect chimeric gene or transcripts that result from chromosomal translocations. However, standard (uniplex) PCR and FISH are unsuitable for screening of leukemogenetic abnormalities, because they require different parameters (primer or probe sets, annealing temperature, etc) for each abnormality. This problem could be overcome by using multiplex PCR (M-PCR). M-PCR methods for leukemia have been reported[1-8]. But a variety of combinations of detectable chimeric gene transcripts were differing in each report. In the new World Health Organization (WHO) classification of acute leukemia, 7 kinds of chromosomal (leukemogenetic) abnormalities are introduced[9-11]. We picked up only these clinically important abnormalities (exclude 11q23 (MLL) abnormalities) from previously reported method[1] and modify a part of it to simplify and to be economical for routine work. We used interphase FISH (I-FISH) for detecting MLL abnormalities, because a number of MLL fusion partners have been identified[12-17]. This method can detect not only rearrangement but also amplification[18, 19] and deletion[20].

Furthermore, we made it possible to detect simultaneously 2 kinds of FMS-like tyrosine kinase 3 (FLT3) mutations (internal tandem duplication; ITD and D835 point mutation), which were known as poor prognostic factor in AML[21-33], with use of the identical template (cDNA synthesized from total RNA) and the same PCR condition of M-PCR for chimeric gene transcripts.

Finally, we used microchip electrophoresis (ME) to detect PCR products. It provided us accurate assessment of M-PCR results easily and rapidly to compare with agarose gel electrophoresis.

Materials and Methods

1. Patients' samples

Bone marrow (BM) or peripheral blood (PB) samples from 49 patients with hematological disorder (27 AML, 11 ALL and 11 CML), diagnosed in Kanazawa University Hospital or associated hospitals between August 2003 and July 2004, were analyzed. The diagnosis of leukemia was made according to French-American-British (FAB) classification with standard

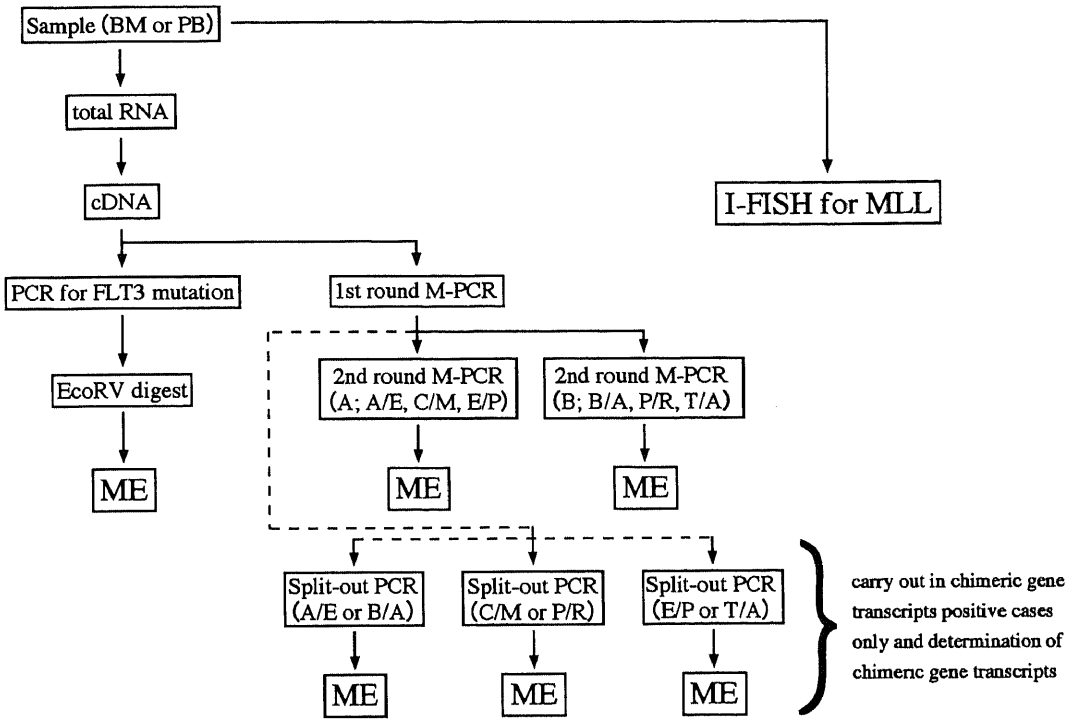

Figure 1. Protocol for combination assay of M-PCR and I-FISH.
Abbreviation: ME, microchip electrophoresis; A/E, AML1/ETO; C/M, CBFβ/MYH11; E/P, E2A/PBX1; B/A, BCR/ABL; P/R, PML/RARα; T/A, TEL/AML1

morpho-cytochemical criteria[34-38] and immunophenotypic analysis[39,40]. It was also evaluated according to new WHO classification[9-11]. Informed consent was obtained from every patient before obtaining samples.

2. RNA preparation

Total RNA was extracted from BM or PB samples (about 5 x 10^6 cells) using the QIAamp RNA Blood Mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions and dissolved in 60μl of distilled water (dH_2O).

3. cDNA synthesis

Ten microliter of RNA (about 8-30 μg) was heated at 65℃ for 10 minutes to denature and then cooled at 4℃ for 5 minutes. Following denaturation, RNA was preincubated at 37℃ for 10 minutes in a total volume of 20 μl with 10 U of ReverTra Ace™ transcriptase (Toyobo, Osaka, Japan) and random hexamer primers. And then RNA was reverse transcribed by incubation at 45℃ for 20 minutes. Subsequently, the cDNA was heated to 99℃ for 5 minutes to inactivate the reverse transcriptase.

4. Multiplex PCR (M-PCR) for chimeric gene transcripts

To obtain certain sensitivity and specificity while simply and economically, we used non-parallel nested or semi nested PCR protocol (one 1st round PCR tube followed by two 2nd round PCR tubes) with a GeneAmp PCR system 2400 (Perkin-Elmer Corp., Emeryville, Calif.). And to verify the integrity of the isolated RNA and the correct synthesis of the cDNA, we included an internal positive control in which a 690 bp segment of the ubiquitously expressed transcription factor E2A mRNA was co-amplified in the same PCR tubes. The primer sequences are listed in Table 1. We used commercially available multiplex PCR kit (Qiagen) which already optimized for M-PCR. In the 1st round M-PCR, 1 μl of cDNA was added to the PCR solution, which consisted of 25 μl of 2x QIAGEN Multiplex PCR master mix, 5 μl of 10x primer mix (2 μM of each primer, including 16 kinds of primers), 5 μl of 5x Q-solution (Qiagen) and

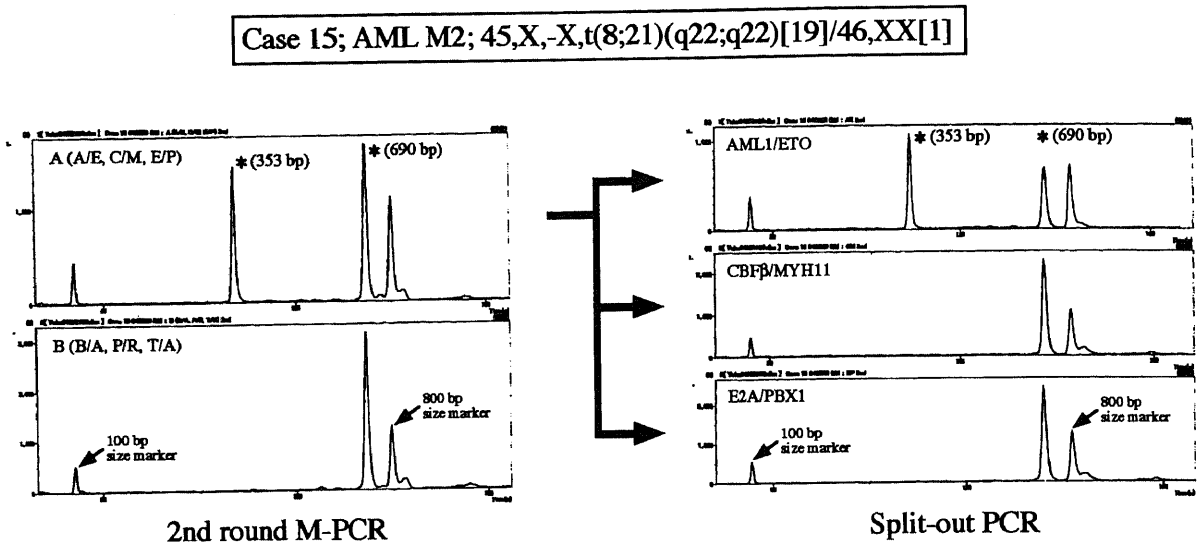

Figure 2. Example of chimeric gene transcripts detected by M-PCR with ME. Amplification products of two 2nd round PCR (left panels A and B) and the corresponding split-out PCR (right panels) from AML1/ETO positive patient (case 15). Arrows indicate DNA size markers of 100 bp (left) and 800 bp (right), and asterisks indicate PCR products (690 bp size PCR products indicate internal positive control).

14 μl of dH_2O. Thermocycling conditions were initial activation of the polymerase step at 95℃ for 15 minutes, followed by 35 amplification cycles of 30 seconds at 94℃, 90 seconds at 58℃ and 90 seconds at 72℃, with a final extension step at 72℃ for 10 minutes. One microliter of thousand-fold diluted 1st round M-PCR product with dH_2O was subjected to the two 2nd round PCR tubes (tube A for screening of AML1/ETO, CBFβ/MYH11 and E2A/PBX1, tube B for BCR/ABL, PML/RARα and TEL/AML1). The 2nd round M-PCR conditions were different in the number of amplification cycles of 20. After the 2nd round M-PCR, two PCR products were analyzed with microchip electrophoresis (ME) instrument (see below). Because both of the 2nd round M-PCR solution contained a number of primers (A; 8 and B; 10 kinds) for 3 kinds of chimeric genes with several breakpoint or splice variants, it is very risky to determine a chimeric gene transcript by sizing. Therefore, if specific peak was detected in one of them, we performed split-out PCR separately using individual primer sets, as outlined in Fig. 1 and 2. The split-out PCR was performed using the 1 μl of thousand-fold diluted 1st round M-PCR product with dH_2O as template, 2nd round individual PCR primer sets and the same reaction conditions as for the 2nd round M-PCR. After the split-out PCR, three PCR products were analyzed with ME and determined a chimeric gene transcript.

5. Cytogenetic analysis

Cytogenetic analyses were performed on BM samples after 24 hours of unstimulated culture. GTG bands with trypsin were obtained. Karyotypes were reviewed and defined according to the International System for Human Cytogenetic Nomenclature (ISCN) 1995 criteria[41].

6. Interphase FISH (I-FISH) for MLL gene

I-FISH was performed on uncultured BM or PB sample using commercially available probes for flanking the breakpoints within the MLL according to the protocol of the manufacturer (Vysis, Stuttgart, Germany). The signals were viewed with a Zeiss Axiosplan 2 (Zeiss, Jena, Germany) and captured to the analyzing system ISIS (MetaSystems, Altlussheim, Germany). For each case 100 to 200 interphase nucleuses were evaluated.

7. FLT3 mutation analysis

To make it possible to detect simultaneously 2 kinds of FLT3 mutations (ITD and D835 mutation)

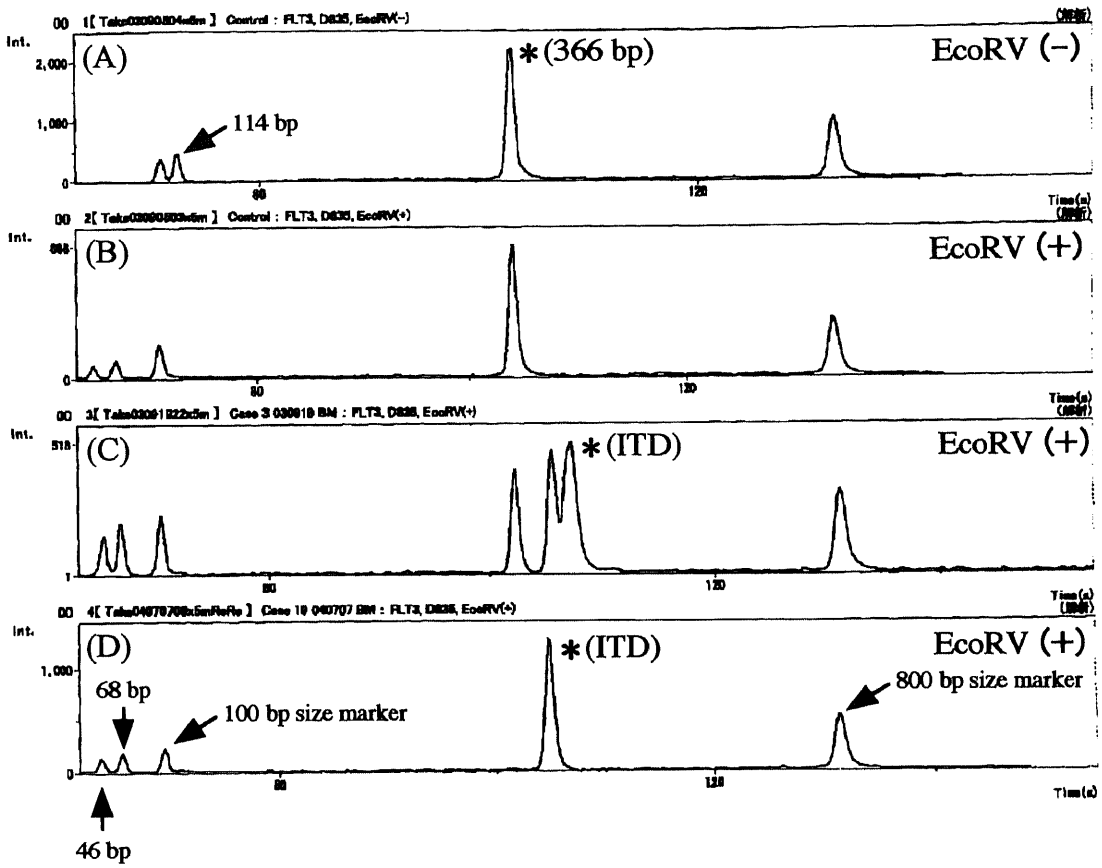

Figure 3. Examples of analysis for FLT3 mutations by M-PCR/RFLP with ME.
(A) ITD negative/D835 normal case (before *Eco*RV digestion), (B) ITD negative/D835 normal case (after *Eco*RV digestion), (C) ITD positive (heterozygote)/D835 normal case (after *Eco*RV digestion) and (D) ITD positive (hemizygote)/D835 normal case (after *Eco*RV digestion).

with chimeric gene transcripts, we used cDNA as template and the same PCR condition (exclude template volumes). The primer sequences are listed in Table 1. Five microliter of cDNA was added to the PCR solution, which consisted of 25 μl of 2x QIAGEN Multiplex PCR master mix, 5 μl of 10x primer mix (2 μM of each primer), 5 μl of 5x Q-solution (Qiagen) and 10 μl of dH_2O. The PCR condition was same as 1st round M-PCR for chimeric gene transcripts. After PCR amplification, 13.4 μl of PCR product was digested with 1.2 U of *Eco*RV restriction endonuclease (Toyobo) in 15 μl at 37°C for 1 hour. After digestion, the product was analyzed with ME (see below). The ITD positive cases showed 366 bp peak (wild type transcripts) and/or additional longer peak. D835 mutation led to the detection of the undigested products of 114 bp, in addition to the two 68 and 46 bp fragments corresponding to the digestion of the wild type transcripts (Fig. 3).

8. Microchip electrophoresis (ME) analysis

PCR products were analyzed with ME instrument (model SV1100; Hitachi Electronics Engineering Co. Ltd., Tokyo, Japan). The microchip had three sample wells, and wells were connected with a cross-channel 0.1 mm wide, 0.03 mm deep and 45 mm long. After the channel was filled with 0.6% hydroxypropyl methylcellulose containing ethidium bromide, a mixture of 2 μl of PCR products, 7 μl of Tris-EDTA (TE) buffer (pH 8.0) and 1 μl of loading buffer containing the internal standards (100 and 800 bp) was loaded into one of the sample wells of the microchip; the program was run at 300 V for 1 minutes (injection time) and at 750 V for 3 minutes (separation time). Accurate fragment size analysis based on the electrophoretic mobility of the sample relative to the

Table 2. Characteristics, results of the analysis (M-PCR for chimeric gene transcripts, 2 kinds of FLT3 mutations and I-FISH for MLL abnormalities) and cytogenetic data from the 27 AML patients.

Case	Age (y)	Sex	FAB	Sample	M-PCR for chimeric gene	FLT3 ITD	FLT3 D835	MLL	Karyotype
1	65	M	M0	BM	neg	neg	neg	neg	Failed
2	55	M	M0	PB	neg	neg	neg	neg	Failed
3	49	F	M1	BM	neg	WT+/ITD+	neg	R	46,XX,t(6;11)(q27;q23)[5]/46,XX,der(4)t(1;4)(q25;q33),t(6;11)(q27;q23)[15]
4	70	F	M1	BM	neg	neg	neg	neg	46,XX[20]
5	46	F	M1	BM	neg	WT+/ITD+	neg	neg	46,XX[20]
6	78	F	M1	PB	neg	WT+/ITD+	neg	neg	46,XX[20]
7	86	F	M1	PB	neg	WT+/ITD+	neg	neg	46,XX[20]
8	88	M	M1	BM	neg	neg	neg	neg	47,XY,+8[11]/46,XY,del(20)(q11)[4]/46,XY[5]
9	69	M	M2	BM	neg	neg	neg	neg	46,XY,+8,add(16)(q13),-17[11]
10	59	M	M2	BM	neg	neg	neg	neg	46,XY[20]
11	73	F	M2	BM	neg	neg	neg	neg	46,XX [20]
12	91	F	M2	PB	neg	neg	neg	neg	46,XX,add(6)(p11),-16,+mar[4]/45,XX,-5,add(6)(p11),-16,+mar[1]/46,XX,-5,add(6)(p11),+15,-16,+mar[2]/44,XX,-5,add(6)(p11),add(8)(p11),del(11)(q21),-13,+15,-16,add(22)(p11)[3]/45,XX,-5,add(6)(p11),add(8)(p11),del(11)(q21),-13,+15,-16,add(22)(p11),+mar[5]/45,XX,-5,add(6)(p11),add(8)(p11), del(11)(q21),del(11)(q21),-13,+15,-16,del(17)(p11),add(22)(p11),+mar[1]
13	80	F	M2	BM	neg	WT-/IDT+	neg	neg	46,XX[15]
14	44	M	M2	PB	neg	neg	neg	neg	45,XY,-7,-11,+mar, inc[20]
15	48	F	M2	BM	AML/ETO1	neg	neg	neg	45,X,-X,t(8;21)(q22;q22)[19]/46,XX[1]
16	62	M	M2	PB	AML/ETO1	neg	neg	neg	45,X,-Y,t(8;21)(22;q22),inc[7]/46,XY[2]
17	19	M	M2	PB	neg	neg	neg	neg	47,XY,+X[6]/47,idem,t(7;11)(p15;p15)[14]
18	57	M	M2	BM	AML/ETO1	neg	neg	neg	45,X,-Y,t(8;21)(q22;q22)[2]/44,X,-Y,t(8;21)(q22;q22),-13[1]/46,XY[17]
19	38	F	M2	BM	neg	WT-/IDT+	neg	neg	46,XX [20]
20	83	M	M3	PB	PML/RARα	neg	neg	neg	46,XY[20]
21	56	M	M3	PB	PML/RARα	neg	neg	neg	46,XY,add(7)(q22),t(15;17)(q22;q?21),inc[17]/46,XY[3]
22	65	M	M4	BM	neg	neg	neg	A	47,XY,del(5)(q23q32),+14,add(17)(q25),add(19)(q13),del(20)(q11)[11]/47,XY,del(5)(q23q32),+14,-17,add(17)(p11),add(19)(q13),del(20)(q11),+mar[7]/46,XY,del(5)(q23q32),+14,-17,add(17)(p11),add(19)(q13),-20,+mar[1]/46, XY [1]
23	54	M	M4Eo	BM	CBFβ/MYH11	neg	neg	neg	46,XY,inv(16)(p13q22)[20]
24	20	M	M5a	PB	neg	neg	neg	R	47,XY,+i(8)(q10),t(11;19)(q23;p13)[5]/53,XY,+X,+5,+6,t(11;19)(q23;p13),+der(11)t(11;19),+12,+16,+add(19)(p13)[4]/54,XY,+X,+5,+6,t(11;19)(q23;p13),+der(11)t(11;19),+12,+16,+add(19)(p13),+20[1]/46,XY[10]
25	25	M	M5a	BM	neg	neg	neg	R	46,XY,+8,t(9;11)(p22;q23) [20]
26	45	F	M5b	BM	neg	neg	neg	neg	46,XX [20]
27	80	M	M6	PB	neg	neg	neg	neg	46XY,+4,der(1;7)(q10;p10) [1]/46XY,+4,der(1;7)(q10;p10),del(20)(q11)[19]

Abbreviation: M, male; F, female; BM, bone marrow; PB, peripheral blood; neg, negative; WT, wild type; R, rearrangement; A, amplification.

Table 3. Characteristics, results of the analysis (M-PCR for chimeric gene transcripts, 2 kinds of FLT3 mutations and I-FISH for MLL abnormalities) and cytogenetic data from the 11 ALL patients.

Case	Age (y)	Sex	Immunophenotype	Sample	M-PCR for chimeric gene	FLT3 ITD	FLT3 D835	MLL	Karyotype
28	7	F	Pre-B	BM	E2A/PBX1	neg	neg	neg	Failed
29	24	M	Pre-B	PB	BCR/ABL	neg	neg	neg	45,XY,-7,del(14)(q22),add(15)(p11),add(16)(q22),del(19)(q11),inc [2]/46,XY [10]
30	14	M	Common or Pre-B	BM	neg	neg	neg	neg	46,XY[20]
31	2	F	Common	BM	neg	neg	neg	neg	Failed
32	6	M	Common	BM	E2A/PBX1	neg	neg	neg	46,XY[5]
33	7	M	Common	BM	neg	neg	neg	neg	46,XY,del(9)(q22q34),+10,-20,-21,add(22)(q13),+mar[12]/46,XY[8]
34	1	M	Common	BM	neg	neg	neg	neg	Dry Tap
35	15	M	Common	PB	TEL/AML1	neg	neg	neg	Dry Tap
36	14	M	Common	BM	neg	neg	neg	neg	46,XY[20]
37	2	F	Pro-B	PB	neg	neg	neg	R	46,XX[11]
38	32	F	mixed lineage	BM	BCR/ABL	neg	neg	neg	46,XX,i(8)(q10),t(9;22)(q34,q11.2),-16,-20,+der(22)t(9;22),+mar[18]/ 46,XX,t(9;22)[1]

Abbreviation: M, male; F, female; BM, bone marrow; PB, peripheral blood; neg, negative; R, rearrangement.

internal standards was achieved by using DNA size analysis software (SV1100b, ver.1.1.0.0; Hitachi Electronics Engineering Co. Ltd.).

Results

1. Chimeric gene transcripts

Eleven (28.9%) of the 38 acute leukemia cases studied were positive for chimeric gene transcripts by our M-PCR (Table 2 and 3). In particular, chimeric gene transcripts were detected in 6 (22.2%) of the 27 AML cases and in 5 (45.5%) of the 11 ALL cases. In the 27 of AML cases, AML1/ETO chimeric gene transcripts was detected in 3 cases (11.1%), PML/RARα in 2 cases (7.4%) and CBFβ/MYH11 in 1 cases (3.7%). In the 11 of ALL cases, BCR/ABL chimeric gene transcripts (minor type; e1a2) was

Table 4. Characteristics, results of M-PCR for chimeric gene transcripts and cytogenetic data from the 11 CML patients.

Case	Age (y)	Sex	Stage	Sample	M-PCR for chimeric gene		Karyotype
39	38	F	CP	BM	BCR/ABL	b2a2	46,XX,t(9;22)(q34;q11)[20]
40	76	F	CP	BM	BCR/ABL	b2a2	46,XX,t(9;22)(q34;q11)[20]
41	72	M	CP	BM	BCR/ABL	b2a2	46,XY,t(9;22)(q34;q11)[15]/46,XY[5]
42	53	M	CP	BM	BCR/ABL	b2a2	46,XY[20]
43	85	M	CP	PB	BCR/ABL	b2a2	46,XY,t(9;22)(q34;q11)[19]/46,XY[1]
44	61	M	CP	PB	BCR/ABL	b3a2	46,XY[19]/46,X,-Y[1]
45	30	M	CP	PB	BCR/ABL	b3a2	46,XY,t(9;22)(q34;q11)[19]/46,XY[1]
46	60	F	CP	PB	BCR/ABL	b2a2	46,XX,t(9;22)(q34;q11)[20]
47	78	M	CP	PB	BCR/ABL	b3a2	46,XY,t(9;22)(q34;q11)[20]
48	75	F	CP	BM	BCR/ABL	b2a2	46,XX,t(9;22)(q34;q11)[20]
49	48	F	AP	BM	BCR/ABL	b3a2	46,XX,t(9;22)(q34;q11)[20]

Abbreviation: M, male; F, female; CP, chronic phase; AP, accelerated phase; BM, bone marrow; PB, peripheral blood.

detected in 2 cases (18.2%), TEL/AML1 in 1 cases (9.1%) and E2A/PBX1 in 2 cases (18.2%). In these 11 chimeric gene transcripts detected cases, only 6 cases (54.5%) showed associated translocations by karyotyping. And in remaining 5 cases (45.5%), karyotyping failed to detect these clinically important translocations.

To test the ability of our M-PCR for major type BCR/ABL chimeric gene transcripts (b2a2 and b3a2), the 11 chronic myelogenous leukemia (CML) cases (10 chronic phase and 1 accelerated phase) were examined. In all these cases, major type BCR/ABL were detected (7 b2a2 and 4 b3a2). Furthermore, in the Philadelphia (Ph) chromosome negative 2 CML cases, BCR/ABL chimeric gene transcript was detected by our M-PCR perfectly (Table 4).

Except one case of myelofibrosis, no chimeric gene transcripts were detected in 2 healthy adult volunteers and 29 hematological disorder's (exclude AML, ALL and CML) BM or PB samples. In that myelofibrosis case, minor type (e1a2) BCR/ABL chimeric gene was detected, and involved t(9;22)(q34;q11) abnormality was also detected by karyotyping (data not shown).

2. MLL gene abnormalities

MLL rearrangements were detected in 3 (11.1%) of the 27 AML and in 1 (9.1%) of the 11 ALL cases (Table 2 and 3). Furthermore, in only one of the AML cases (case 22), MLL amplification was observed (Fig. 4). This case showed complex abnormalities by karyotyping but not detected MLL rearrangement by I-FISH.

3. FLT3 mutations

FLT3 ITD of exon 14 (formerly called exon 11) were only detected in 6 (22.2%) of the 27 AML cases (Table 2). In these cases, five of them showed normal karyotype and remaining one case showed t(6;11)(q27;q23) by karyotyping and MLL rearrangement by I-FISH (case 3). Furthermore, in 2 of these 6 FLT3 ITD positive cases (case 13 and 19), wild type transcripts were not detected, only ITD type transcripts were detected (hemizygote, Fig. 3). In contrast, FLT3 ITD was not detected in any cases of ALL. Except of the 2 ITD hemizygote cases of AML, wild type transcripts of exon 14 were detected in all determined samples (derived from 25 AML, 11 ALL, 11 CML, 2 healthy adult volunteers and 29 other hematological disorders). FLT3 D835 mutations were not detected in our samples.

Discussion

Our main aim was to develop a rapid, technically simple and cost-effective method for screening of the clinically important leukemogenetic abnormalities incorporated in the new WHO classification for the

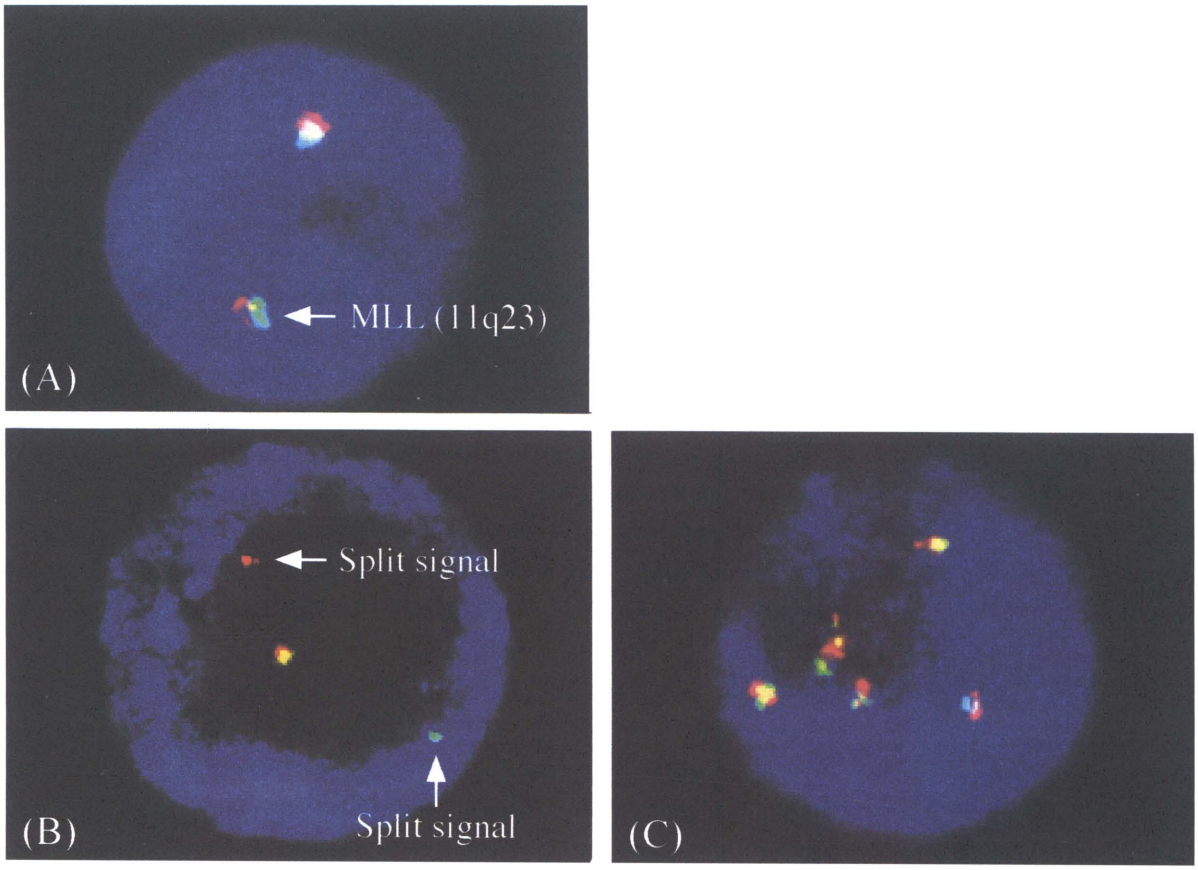

Figure 4. Various signal patterns observed in I-FISH for MLL.
(A) normal , (B) rearrangement and (C) amplification patterns.

acute leukemia (AL). Namely, our main targets for detection are 1) t(8;21)(q22;q22); (AML1/ETO), 2) inv(16)(p13;q22) or t(16;16)(p13;q22); (CBFβ/MYH11), 3) t(15;17)(q22;q12); (PML/RARα), 4) t(9;22)(q34;q11); (BCR/ABL), 5) t(1;19)(q23;p13); (E2A/PBX1), 6) t(12;21)(p12;q22); (TEL/AML1) and 7) 11q23 (MLL) abnormalities. Some groups have described M-PCR methods to detect various pair of chimeric gene[1-8]. One of them, Pallisgaard et al.[1] reported M-PCR method that enabled to detect 29 separate translocations and could detect chimeric gene transcripts in about 45% of the AL. Strehl et al.[4] also reported similar result using a commercially available M-PCR assay kit (HemaVision®). But these methods require 8 parallel nested M-PCRs even if in chimeric genes negative cases. In positive cases, 3 to 6 parallel nested M-PCRs were required to gain the final results. Therefore, it is too difficult to take it in daily routine works in hospital laboratory. Furthermore, although their method contains 11 kinds of MLL related abnormalities and occupies about 38% of their detectable abnormalities, it cannot detect a part of MLL rearrangements. MLL is well known as promiscuous gene. MLL fusion partners have been reported over 30 kinds[12, 13]. And new MLL fusion partners have been reported every year[14-17]. It is very difficult to detect all MLL rearrangement by PCR, even if M-PCR used. So, we switched strategy for detection of MLL abnormalities using from M-PCR to I-FISH. I-FISH can detect almost MLL rearrangement on diagnostic material[42-44]. In fact, we could detect MLL rearrangements in 3 (11.1%) of the 27 AML and in 1 (9.1%) of the 11 ALL cases by using I-FISH. Furthermore I-FISH can also detect amplification[18, 19] and deletion[20]. Actually, one of our AML cases (case 22) showed amplification pattern. The amplification of MLL has reported as poor prognosis in myeloid leukemia[18, 19]. Therefore, it is very important to detect this abnormality in AML. We picked up remaining 6 types of clinically important chimeric genes

incorporated in new WHO classification of AL from Pallisgaard's method[1]. And we added a few modifications to it to simplify and to be cost effective for routine works. First, we used non-parallel nested or semi nested M-PCR (one 1st round PCR tube followed by two 2nd round PCR tubes) to decrease use of reagents and contamination (Fig. 1). As a result, 1st round PCR tube contained 16 kinds of primers (Table 1). Some groups also reported modified Pallisgaard's method but all of them used parallel nested M-PCR[4,6-8]. In our methods, only three or six PCR tubes (reagents) were required to determine existence of six chimeric gene transcripts. In our cases, 11 (28.9%) of the 38 AL cases were positive for one of six chimeric gene transcripts by our M-PCR method. Therefore, in average just 3.9 tubes (reagents) were required per sample. In contrast, if we used Pallisgaard's method to our AL cases, we could detect chimeric gene transcripts in 14 (36.8%) of the 38 AL cases, but we required 19.4 tubes (reagents) per sample at least. Therefore, we could save 15.5 tubes (reagents) per sample using by our method. All of three cases, chimeric gene transcripts detected by only Pallisgaard's M-PCR method and not by our M-PCR methods, were MLL rearrangement types. Therefore, we could know that these cases had MLL rearrangement abnormality by I-FISH but could not know MLL fusion partner. In almost cases, information of MLL fusion partner is not clinically important from the viewpoint of prognosis[45]. Second modification was to use random hexamer primers at cDNA synthetic process, whereas in Pallisgaard's method, they used gene specific primers. It is very useful for increase sensitivity and specificity at cDNA synthetic process to use gene specific primers. But cDNA synthesized by gene specific primers can be used only for specific assays. Our additional aim was simultaneous detection of 2 kinds of FLT3 mutations (ITD and D835 mutations) together with chimeric gene. These mutations were detected in 15 to 30% and 0 to 7% of AML, respectively and mainly accompanied with normal karyotype and reported as poor prognostic factors[21-33]. Therefore, it is very important to detect these abnormalities at onset. Majority of these previously reports used genomic DNA as PCR template and reports using RT-PCR were very rare[21,32,46]. But we challenged to use RT-PCR to detect FLT3 mutations because screening of chimeric gene transcripts had to use cDNA as template and it was very troublesome to extract genomic DNA only for FLT3 mutations analysis. Therefore we used random hexamer primers at cDNA synthetic process and used the same cDNA for FLT3 mutation analysis. This strategy was very useful in ITD hemizygote cases that were known as poorer prognostic factor to compare with heterozygote cases[27]. In ITD hemizygote cases, the methods using genomic DNA are affected by contamination of normal cells because wild type FLT3 alleles derived from normal cells are co-amplified[33]. FLT3 is primarily expressed in hemaotopoietic stem cells[47,48]. Therefore, contaminations of mature cells do not affect to RT-PCR assay. In our AML cases, two cases had ITD peak only (case 13 and 19), and we could easily judge these cases as hemizygote type. By our method, 6 (22.2%) of the 27 AML cases were positive for FLT3 ITD. This was consistent with the rate reported by other groups[22,23,26,27,29,30]. On the other hand, FLT3 D835 mutation was not detected in any our cases. This mutation had been described in approximately ~7% of patients with AML[26,28]. Therefore this result might be because of insufficient case numbers examined.

At the beginning, we used another PCR conditions (annealing at 58℃, 30 cycles and using 1 μl cDNA) in 1st round M-PCR for chimeric gene. But using this condition for FLT3 analysis, exon 20 of FLT3 was not amplified enough. Therefore, we had to change PCR condition. We did not want to change the annealing temperature, because it would be expected to induce nonspecific reaction in chimeric gene analysis if we changed it to lower temperature. The thermal cycler used in this study had not thermogradient thermoblock. Therefore, we tried to change another parameters. Fortunately this problem was able to resolve by increasing template volumes from 1 to 5 μl and cycle numbers from 30 to 35. And we changed cycle numbers of 2nd round M-PCR from 25 to 20.

We used ME instrument for sizing of PCR products. Fujita et al.[49] reported that ME instrument could detect PCR products within 4 minutes per sample whereas agarose gel electrophoresis needed 2 hours to

determine the lengths of amplicons in their study. In fact, we could start sprit-out PCR within 30 minutes after the 2nd round PCR finished. Furthermore, the limit of resolution of the ME instrument was specified as 10 bp according to the document provided by the manufacturer. It is well known that the capillary electrophoresis (CE) systems are also able to be sizing of PCR products rapidly and correctly. Some groups reported M-PCR with CE systems to detect leukemogenetic abnormalities[2, 5, 50]. In these reports, split-out PCR were not necessary because their amplicons have characteristic fluorescent dye patterns, namely, the CE system always requires the PCR primers labeled by fluorescent dye. And in CE system, because of limited numbers of fluorescent dye enabled, the detectable numbers of chimeric genes are also limited. Furthermore, CE system requires denaturation of PCR products before electrophoresis. On the other hand, the ME instrument does not require the primers labeled by fluorescent dye and denaturation, but only needs addition of TE buffer and loading buffers to PCR products. Therefore we could know accurate PCR results rapidly, easily and inexpensively to compare with CE systems.

To collect all results, 21 (55.3%) of the 38 AL cases, we could detect some leukemogenetic abnormalities by our combined assay in one to two days. In contrast, in 7 (43.8%) of 16 cases detected these abnormalities (exclude FLT3 mutated cases), karyotype analyses failed to detect these clinically important abnormalities. Furthermore, in the cases detected these clinically important abnormalities using by karyotype analyses, it had to take for 10 to 14 days to obtain the results. These consequences showed that our combined assay had sufficient ability to screen of clinically important leukemogenetic abnormalities in AL at onset. Furthermore we could detect major type BCR/ABL chimeric gene transcripts in all of 11 CML cases (10 chronic phase and 1 accelerated phase). And 2 of these cases showed masked Ph chromosome in karyotype analyses. These results indicate that our M-PCR for chimeric gene is also applicable for CML cases and available for discrimination of ALL from CML in blast crisis.

In conclusion, we believe that our combined use of M-PCR with ME and I-FISH are not only useful but also inexpensive to detect clinically important leukemogenetic abnormalities in hematological malignancies at diagnosis.

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白血病由来染色体異常の、迅速かつ経済的な検出法に関する研究

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要　旨

我々はmultiplex PCR（M-PCR）と間期核FISH（I-FISH）を組み合わせ，急性骨髄性白血病（AML），急性リンパ性白血病（ALL），慢性骨髄性白血病（CML）に特徴的な遺伝子異常のスクリーニングを行った（以下，本法）。M-PCRによるキメラ遺伝子（AML1/ETO, PML/RARα, CBFβ/MYH11, BCR/ABL, TEL/AML1, E2A/PBX1）の検索，M-PCR/RFLPによるFLT3遺伝子異常（ITD，D835変異）の検索，そしてI-FISHによりMLL遺伝子の再構成，増幅ならびに欠失の検索を行った。また，PCR産物の確認にはマイクロチップ電気泳動装置を用い，簡便かつ迅速に，結果を得た。

実際に臨床検体（AML27例，ALL11例，CML11例）に本法の適用を試みた。その結果，全49例中，32例（65.3%）にて何らかの白血病関連遺伝子異常が検出可能であった。また，FLT3 ITD以外の異常が検出された27例中9例では，これらの染色体（遺伝子）異常は核型分析では検出されず，本法においてのみ検出された。

今回我々が用いた，M-PCRとI-FISHを組み合わせたコンビネーションアッセイ法はAML, ALL, CMLに特徴的な遺伝子異常を迅速かつ効率的に検出するのに非常に有用であった。