Comprehensive analysis of BCR-ABL transcript types in Korean CML patients using a newly developed multiplex RT-PCR

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Diagnosis of chronic myeloid leukemia (CML) is based on the detection of BCR-ABL gene or Philadelphia chromosome (Ph chromosome), and fusion proteins with different sizes are encoded depending on the breakpoint in the BCR gene. In general, 3 breakpoint cluster regions in the BCR gene have been described: major (M-bcr), minor (m-bcr), and micro (μ-bcr). This study was designed to determine the frequency of BCR-ABL transcripts using one-step multiplex reverse transcription polymerase chain reaction (RT-PCR). Bone marrow (BM) or peripheral blood (PB) samples at diagnosis from 548 patients were obtained with a referring diagnosis of Ph-positive (Ph+) CML, and multistep RT-PCR and newly developed one-step multiplex RT-PCR were applied on each sample. Compared with the previous multistep RT-PCR, one-step multiplex RT-PCR with the primers is the more rapid and accurate method to identify the BCR-ABL breakpoints. Most patients (538/548, 98.18%) were found to have b3a2 or b2a2, and total frequency of occurrence of c3a2, e1a2, b2a3, b1a1, and e1a3 or coexpression of b2a2 and b3a2 was less than 2.00%. No differences were observed between women and men. As the multiplex RT-PCR technique distinguishes BCR-ABL transcripts in all samples with high sensitivity and specificity, it easily could be applied at early stages of diagnosis. The incidence of one or the other rearrangement in CML patients varies in different reported series, and the frequency in each type of BCR-ABL transcript in Korean CML patients seems to be different from those of Western countries. (Translational Research 2006;148:249–256)

Abbreviations: AML = acute myeloid leukemia; ALL = acute lymphoid leukemia; BM = bone marrow; cDNA = complementary DNA; CML = chronic myeloid leukemia; CP = chronic phase; DEPC = diethylpyrocarbonate; DNA = deoxyribonucleic acid; FISH = fluorescence *in situ* hybridization; M-bcr = major breakpoint cluster region; m-bcr = minor breakpoint cluster region; μ-bcr = micro breakpoint cluster region; MNC = mononuclear cell; mRNA = messenger RNA; PB = peripheral blood; Ph chromosome = Philadelphia chromosome; Ph+ = Philadelphia chromosome-positive; RNA = ribonucleic acid; RQ-PCR = real-time quantitative polymerase chain reaction; RT-PCR = reverse transcription polymerase chain reaction; WBC = white blood cell

CML is a malignant clonal disorder of pluripotent hematopoietic stem cell.[1] The diagnosis of CML is based on detection of the BCR-ABL gene or Ph chromosome that is derived from the reciprocal translocation between chromosomes 9 and 22. The BCR-ABL gene encodes different fusion proteins that vary in size depending on the breakpoint in the BCR gene, but these proteins share a high ty-

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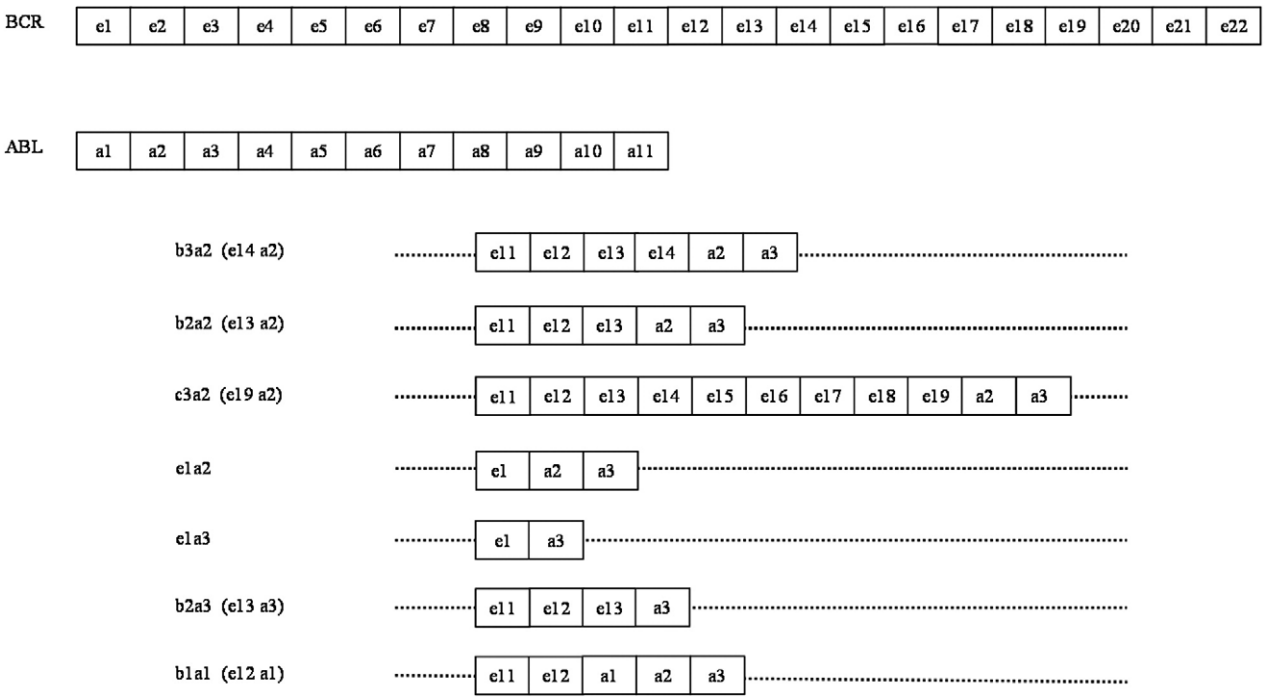

Fig 1. Schematic representation of BCR-ABL transcripts.

rosine kinase activity. Figure 1 represents the BCR-ABL transcript types schematically. In general, three breakpoint cluster regions in the BCR gene have been described: M-bcr, m-bcr, and μ-bcr.[2] More than 95% of Ph+ CML patients have a breakpoint in the M-bcr region. Two major breakpoints are b2a2 (e13a2) and b3a2 (e14a2), and both fusion mRNAs are translated into the p210 BCR-ABL protein.[3] The breakpoint in the m-bcr region results in an e1a2 junction, which is translated into a smaller p190 BCR-ABL protein, and it is involved in two thirds of ALL cases and in rare cases of CML and AML. In some cases, the breakpoint between BCR exon 19 and 20 in the μ-bcr region induces a larger p230 BCR-ABL protein.[4–7] In addition to these breakpoints, BCR-ABL transcripts with other junctions have been described in several rare cases, and they involve splicing between whole exons, insertion of small sequences, or genomic breakpoints within exons.[8–15] Sometimes, unusual breakpoints in ABL lead to rare transcripts, but analysis of BCR-ABL mutants has shown that the ABL SH2 domain, which is encoded by exons a3 and a4, is essential for transformation.[16] Therefore, it is extremely unlikely that any BCR-ABL fusion lacking either of these exons would cause CML.

The type of the fusion gene in CML is thought to be related to the clinical course and outcome of each patient, and several molecular methods such as Southern blotting, FISH, and RT-PCR are currently used for detection of BCR-ABL gene. RT-PCR is the most sensitive of the various techniques available, and RQ-PCR overcoming the quantitative limitation of RT-PCR has been developed and broadly used recently. However, before RQ-PCR can be applied, complications exist as RT-PCR steps have to be repeated using several primer sets to distinguish various transcript types. Therefore, one-step multiplex RT-PCR has to be performed before RQ-PCR to determine the type of fusion gene in each patient.

CML incidence relatively varies depending on the ethnic background, and the different frequency of BCR-ABL transcripts was observed among ethnic backgrounds.[17] Although several papers have reported on the frequency of BCR-ABL transcripts in CML patients in other countries, data regarding the frequency of BCR-ABL fusion genes in Korean CML patients are limited. Therefore, this study was designed to determine the frequency of BCR-ABL transcripts and to know the feasibility of the one-step multiplex RT-PCR in 548 Korean patients with Ph+ CML.

METHODS

Patients. To compare the feasibility of the one-step multiplex RT-PCR with the conventional multistep RT-PCR technique, BM or PB samples at diagnosis from 548 patients were obtained with a referring diagnosis of Ph+ CML. The median age was 40 years (range 16–76), and 234 patients

Table I. RT-PCR: primer sequences

Primer sequences in first-round RT-PCR	
Major BCR	gctacggagaggctgaagaa (5′–3′) on bcr exon 11
	cgtgatgtagttgcttggga (5′–3′) on abl exon 3
Minor BCR	gcagctccaatgagaacctc (5′–3′) on bcr exon 1
	acaccattccccattgtgat (5′–3′) on abl exon 3
Micro BCR	tgctgtggtcaccaagagag (5′–3′) on bcr exon 18
	ctaagacccggagcttttca (5′–3′) on abl exon 3
Primer sequences in nested RT-PCR	
Major BCR	gtgcagagtggagggagaac (5′–3′) on bcr exon 12
	acaccattccccattgtgat (5′–3′) on abl exon 3
Minor BCR	caacagtccttcgacagcag (5′–3′) on bcr exon 1
	tgttatctccactggccaca (5′–3′) on abl exon 2
Micro BCR	cttcgacgtcaaagcccttc (5′–3′) on bcr exon 19
	ctaagacccggagcttttca (5′–3′) on abl exon 3

Table II. Multiplex RT-PCR: Primer sequences

Primer sequences in multiplex RT-PCR	
Sense primers	A1: caacagtccttcgacagcag (5′–3′) on bcr exon 1
	B1: gctacggagaggctgaagaa (5′–3′) on bcr exon 11
Anti-sense primer	C1: cgtgatgtagttgcttggga (5′–3′) on abl exon 3
Primer sequences in nested RT-PCR	
Sense primers	A2: caacagtccttcgacagcag (5′–3′) on bcr exon 1
	B2: gtgcagagtggagggagaac (5′–3′) on bcr exon 12
Anti-sense primer	C2: acaccattccccattgtgat (5′–3′) on abl exon 3

(42.70%) were women and 314 (57.30%) were men. These patients were examined and treated at the Catholic University of Korea between November 1999 and May 2005. This study was carried out according to the principles of the Declaration of Helsinki, informed consent was obtained, and the hospital review board approved the study.

RNA extraction & cDNA synthesis. MNC separated from BM or PB was used for RNA extraction using an RNAqueous Kit (Ambion, Austin, Tex). After RNA was extracted, RNA quantity and quality was assessed using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Del), and if 260/280 is less than 1.8, RNA extraction had to be repeated. Overall, 1 μg of RNA was used for reverse transcription using an AMV-RT Kit (Roche, Basel, Switzerland), and the reaction medium was made up to 20 μL with DEPC-treated water. The cDNA synthesis was carried out at 25°C for 10 min and then at 42°C for 60 min. AMV RT was denatured by incubating the reaction at 99°C for 5 min and then cooling at 4°C for 5 min.

Conventional RT-PCR. Primer 3 software, as provided by the Whitehead Institute for Biomedical Research/MIT Center, was used to design primers. For the detection of b3a2 or b2a2, primers specific for e11 of the BCR gene and a3 of the ABL gene were chosen. Primers specific for e1 of the BCR gene and a3 of the ABL gene, and primers specific for e18 of the BCR gene and a3 of the ABL gene, were chosen to be able to amplify e1a2 and c3a2, respectively (Table I). The RT-PCR reaction medium consisted of 1-μL cDNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2.5U Taq DNA polymerase (Roche), and primers in a final volume of 25μL. RT-PCR was performed in duplicate under the following cycling conditions: predenaturation at 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 5 min. For all RT-PCR reactions, the K562 cell line for positive control and water for negative control were amplified simultaneously to exclude any false-positive and false-negative results. Products of first-round RT-PCR were visualized using ethidium bromide-stained agarose gel electrophoresis, and the band should be as follows: 616bp for b3a2; 552bp for b2a2; 506bp for e1a2; and 350bp for c3a2. The first-round RT-PCR for the detection of b3a2 or b2a2 was performed first as they are the most common types found in the Korean population. If no band was detected, first-round RT-PCR for e1a2, and then for c3a2, were preformed sequentially.

The sequences of primers for nested RT-PCR are listed in Table I, and the band should be as follows: 443bp for b3a2; 368bp for b2a2; 313bp for e1a2; and 206bp for c3a2. The nested RT-PCR was performed in the same condition as the first-round RT-PCR, and 2.5 μL of the first-round RT-PCR product was used.

Multiplex RT-PCR. A primer specific for e11 of the BCR gene was chosen to be able to amplify all variants in M-bcr and μ-bcr regions, and the specific primer for e1 was chosen for detection of breakpoints in the m-bcr region. The ABL primer is specific for a3 and can thus detect junctions in a2 and a3 (Table II). The PCR reaction medium consisted of 2.5-μL cDNA, 1X PCR buffer, 0.25 mM dNTP, 2.5U i-StarTaq DNA polymerase (Intron, Kyungki-do, Korea), and primers in a final volume of 25 μL. Concentrations of primers on bcr exon 11 and abl exon 3 were 0.5 μM each, and the concentration of the primer on bcr exon 1 was 0.125 μM. PCR was performed in duplicate under the following cycling conditions: predenaturation at 94°C for 3 min, 30 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 5 min. The band should be as follows: 627bp for b3a2; 552bp for b2a2; 378bp for b2a3; 580bp for b1a1; 429bp for e1a2; 1167bp for c3a2; and 255bp for e1a3.

To determine the sensitivity of the assay, PCR amplifications of each transcript type were performed using cDNA from patient samples or K562 as a template, and the amplified PCR products for each transcript type were purified using

Wizard SV Gel and PCR Clean-Up System (Promega, Madison, Wisc). The purified PCR products were cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, Calif), and this recombinant vector was transformed into competent *E. coli* (Invitrogen) according to the manufacturer's instructions. After overnight incubation on LB plates containing 50-μg/mL ampicillin, a single colony was inoculated into 2 mL of LB containing 50-μg/mL ampicillin overnight, and then plasmid DNA was extracted using a DNA-spin Plasmid DNA Purification Kit (Intron). It was serially diluted in 10-fold series in water, and the sensitivity was determined by gel electrophoresis after PCR amplification.

Nested RT-PCR. The sequences of primers for nested RT-PCR are listed in Table II, and the band should be as follows: 443bp for b3a2; 368bp for b2a2; 194bp for b2a3; 396bp for b1a1; 378bp for e1a2; 983bp for c3a2; and 204bp for e1a3. The nested RT-PCR was performed in the same condition as the multiplex RT-PCR, but the annealing temperature increased to 65°C. Concentrations of primers on bcr exon 12 and abl exon 3 were 1 μM each, and the concentration of the primer on bcr exon 1 was 0.25 μM. Overall, 0.2 μL of the multiplex RT-PCR product was used.

RQ-PCR. To evaluate the response to treatment, RQ-PCR was performed in duplicate using Real-Q BCR-ABL Quantification Kit (BioSewoom, Seoul, Korea) and LightCycler software 4.0. PCR was performed in duplicate under the following cycling condition: denaturation at 95°C for 10 min, 45 cycles at 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s, followed by a cooling step at 40°C for 30 s. A total of 20μL of reaction medium contained a 10-μL PCR reaction mixture (2X), probe and primer mixture, sterile water, and 2-μL cDNA. ABL gene was used as a control gene in RQ-PCR. If the value of ABL gene in RQ-PCR amplification was less than 10^4 copy numbers, this sample was not qualified for multiplex RT-PCR analysis and RNA extraction was repeated for those samples. This kit also contains the plasmid DNAs with defined copy number for BCR-ABL and ABL to be used as standards. The quantity of BCR-ABL transcript was normalized to the ABL expression level, and the result was expressed as the ratio of BCR-ABL copy number to ABL copy number.

Sequencing analysis. For the product of unexpected size, the nested RT-PCR product was purified, and the single-strand reaction was carried out with the primer sets and BigDye Terminator v 3.1 Cycle Sequencing Kit [Applied Biosystems (API), Foster City, Calif]. The cDNA sequence was obtained with an ABI PRISM 310 Genetic Analyzer (ABI) and analyzed with the Sequencing Analysis 3.7 software.

RESULTS

Multiplex RT-PCR was used to detect several kinds of rearrangements in BCR-ABL transcripts in a single reaction (Fig 2). Then RQ-PCR was performed using appropriate primer sets for each type of rearrangement, and nested RT-PCR was performed in case of a negative result in RQ-PCR. The sensitivity of multiplex RT-PCR and nested RT-PCR was measured for each transcript type, and the results are shown in Fig 3. The sensitivity of multiplex RT-PCR was similar for all transcripts.

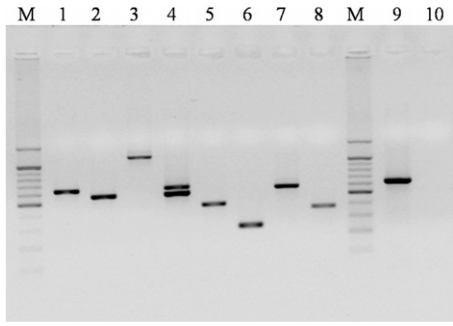

Fig 2. Multiplex RT-PCR (M, 100bp marker; *lane 1,* b3a2; *lane 2,* b2a2; *lane 3,* c3a2; *lane 4,* coexpression of b2a2 and b3a2; *lane 5,* e1a2; *lane 6,* e1a3; *lane 7,* b1a1; *lane 8,* b2a3; *lane 9*, K562 positive control; *lane 10,* H_20 negative control).

Most patients (538/548, 98.18%) expressed one p210 BCR-ABL rearrangement, and of the cases positive for p210, 364 patients (67.66%) corresponded to b3a2, and 174 (32.34%) corresponded to b2a2. The b3a2 was expressed in 64.82% of female patients and 53.75% of male patients, and b2a2 was expressed in 33.22% of female patients and 29.88% of male patients. No differences were observed between women and men. Currently, survival rates of b3a2 and b2a2 patients are 84.62% (308/364) and 93.10% (162/174), respectively. The rest had c3a2 (4), e1a2 (1), coexpression of b2a2 and b3a2 (2), b2a3 (1), e1a3 (1), or b1a1 (1) (Table III).

In the multiplex RT-PCR, the product of unexpected size not detectable in conventional RT-PCR was detected in 1 patient, and e1a3 was confirmed by the direct sequencing of this product (Fig 4).

DISCUSSION

Conventional RT-PCR without cytogenetics analysis could miss the detection of rare cases that are not covered by the primers used in conventional RT-PCR, unless the proper primers are used. Therefore, analysis by cytogenetics in the initial evaluation of a patient with suspected CML has been the standard for diagnosis.[18] However, this technique suffers from serious limitations as BM metaphases are required and aspiration and cultivation of proliferating cells are not always sufficient. Furthermore, this technique is relatively insensitive because typically a maximum number of only 20–50 metaphases are analyzed.[19] If the primers used in the multiplex RT-PCR can cover all BCR-ABL variants, cytogenetics analysis might not be required at the time of diagnosis.

Before the introduction of multiplex RT-PCR, the previous RT-PCR protocols for BCR-ABL analysis in

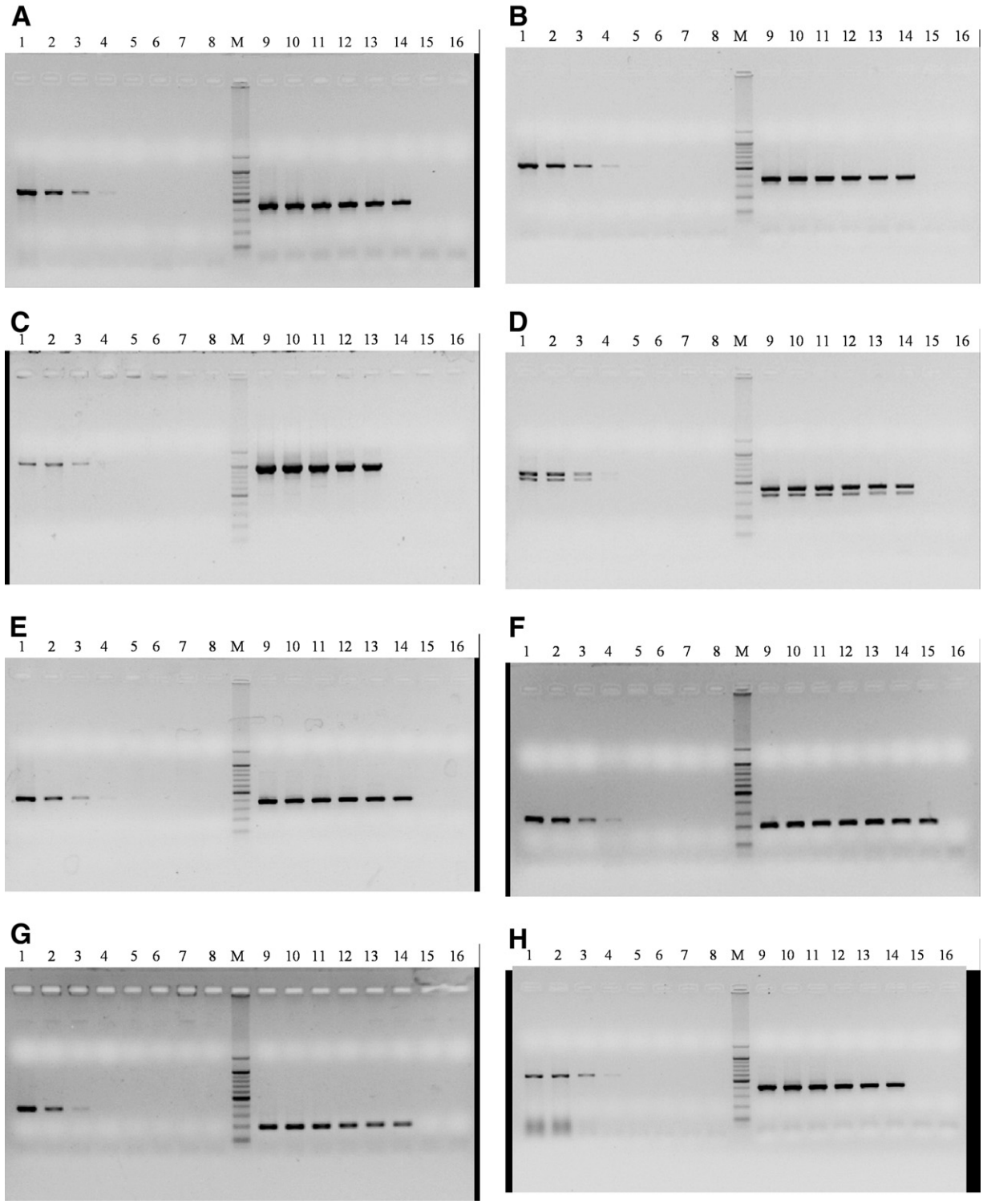

Fig 3. Sensitivity of RT-PCR for eight BCR-ABL transcript types: (**A**) b3a2, (**B**) b2a2, (**C**) c3a2, (**D**) b2a2 +b3a2, (**E**) e1a2, (**F**) e1a3, (**G**) b2a3, and (**H**) b1a1. Multiplex RT-PCR (*lane* 1 ~ 8), nested RT-PCR (*lane* 9 ~ 16) 2×10^6 copy no. (*lane* 1, 9); 2×10^5 copy no. (*lane* 2, 10); 2×10^4copy no. (*lane* 3, 11); 2×10^3 copy no. (*lane* 4, 12); 2×10^2 copy no. (*lane* 5, 13); 2×10^1 copy no. (*lane* 6, 14); 2×10^0 copy no. (*lane* 7, 15); 2×10^{-1} copy no. (*lane* 8, 16). M, 100bp marker. Multiplex RT-PCR could detect down to 2×10^4copy no. of c3a2 and b2a3, whereas down to 2×10^3copy numbers of b3a2, b2a2, b2a2+b3a2, e1a2, e1a3, and b1a1 could be detected. In nested RT-PCR, down to 2×10^2copy no. of c3a2 could be detected. Nested RT-PCR could detect down to 2×10^1 copy no of b3a2, b2a2, b2a2+b3a2, e1a2, b2a3, and b1a1, whereas down to 2×10^0 copy no. of e1a3 could be detected.

Table III. Frequency of expression of the BCR-ABL rearrangements in 548 Korean patients with CML

Rearrangement BCR-ABL	One-step multiplex RT-PCR (n = 548)	Multistep RT-PCR (n = 548)
Major	538	538
Minor	1	1
Micro	4	4
Others		
b1a1	1	1
b2a3	1	1
b2a2/b3a2	2	2
e1a3	1	0

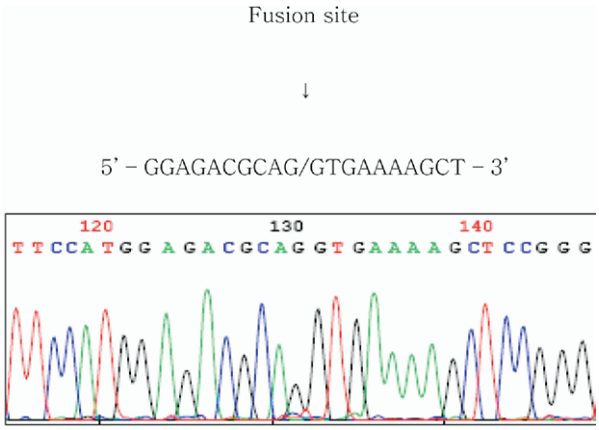

Fig 4. cDNA sequence results demonstrating the e1a3 fusion site between bases 130 and 131, corresponding to BCR e1 and ABL a3.

the laboratory used 3 separate reactions: one for b3a2 and b2a2, a second for e1a2, and a third for c3a2. Compared with the previous RT-PCR, multiplex RT-PCR with the primers is a more rapid and accurate method to identify the various BCR-ABL breakpoints based on the size of the amplified bands, and it is expected to distinguish the theoretical in-frame fusion mRNAs because all patients demonstrated at least 1 transcript of BCR-ABL mRNAs, which were reported previously. In addition to BCR-ABL transcript types detected in the patients, atypical BCR-ABL transcript types involving various parts of BCR exons have been reported in other ethnic groups, and some of them are expected to be covered by primers sets used in the multiplex RT-PCR. Rare transcripts such as b3a3, e2a2, e2a1, e6a1, and e8a2 have been reported, and band size would be 453, 488, 600, 1071, 1167, and 1320, respectively, if the multiplex RT-PCR is applied.[8–15]

In one case of this study, the product a bit smaller than c3a2 was detected in multiplex RT-PCR, and sequencing revealed a rare in-frame e1a3 transcript. A 42-year-old man presented in September 2002, and his white blood cell count was 3700/mm^3 and the platelet count was 282K/mm^3. Although the morphology of the BM biopsy was diagnostic for CML in CP and all metaphases analyzed were Ph+, his BCR-ABL transcript type could not be identified by the previous RT-PCR method. He was started on imatinib 400mg/day in March 2003, and by classic cytogenetics, the Ph+ metaphases decreased from 100% before imatinib to 0% by 6 months. He has shown no other chromosomal abnormalities and still remains in CP. In the previous RT-PCR method, his BCR-ABL transcript types could not be determined because exon 1 of the BCR gene is not covered by M-bcr-specific primers, and exon 3 of the ABL gene is not covered by m-bcr-specific primers in the nested RT-PCR. Therefore, a false-negative result was obtained during the follow-up of this patient. Application of multiplex RT-PCR enabled identification of e1a3 as shown in Fig 4. A newly designed multiplex RT-PCR method was routinely used to exclude rare variants, and the PCR product with unexpected band size is sequenced to determine the transcript type.

In this experiment, higher annealing temperature reduced unspecific bands, and modifications in the proportions of various primers were able to optimize the reaction. Initially, equimolar primer concentrations of 1 μM each were used in the multiplex RT-PCR. However, uneven amplification occurred, with some of the products barely visible even after the reaction was optimized for the cycling conditions. After repeating modifications, concentrations of primers on bcr exon 11, bcr exon 12, and abl exon 3 were determined to be 0.5 μM each, and the concentration of primer on bcr exon 1 was 0.125 μM.

In this study, the frequency and distribution of BCR-ABL transcript types in Korean patients with Ph+ CML was determined using multiplex RT-PCR and sequencing analysis, and to the authors' knowledge, this study included the largest number of patients with CML. Overall, 98.18% of patients were found to have fusion genes involving the M-bcr region, corresponding to p210 protein. Of the cases positive for p210, the number of patients with b3a2 were twice the number of patients with b2a2. Differently from b3a2 and b2a2 fusion transcripts, the total frequency of occurrence of c3a2, e1a2, b2a3, b1a1, and e1a3 or coexpression of b2a2 and b3a2 was less than 2.00% in Korean patients with CML. Male and female patients showed similar proportions in each type of fusion gene. Coexpression may be caused by alternative splicing or phenotypic variation, with clinical courses different from classic CML.[20]

The incidence of one or the other rearrangement in CML patients varies in different reported series, and the

frequency of each BCR-ABL transcript type in Korean patients with CML seems to be different from those of Western countries. Using the conventional RT-PCR technique, fusion transcript types in 250 Mexican patients with CML[20] and fusion transcript types in CML patients of the Mestizo ethnic group (a mixture of Spaniard and Amerindian)[21] were reported. In the Mexican population, 83.00% were positive for major BCR (48.00% b2a2 and 35.00% b3a2) and 4 patients (2.00%) had c3a2 transcript. Whereas only 2 patients in this study (0.36%) had coexpression of b2a2/b3a2, 15.00% of the Mexican population had various types of coexpressions such as b3a2/b2a2, e1a2/b3a2/b2a2, c3a2/b3a2, and e1a2/b2a2/c3a2. Among these 4 types of coexpressions, coexpression of b2a2/b3a2 had no significant differences from b3a2 or b2a2 with respect to the clinical findings in the study. In the Mestizo ethnic group, only 5.40% of CML patients presented the b3a2 rearrangement. Overall, 94.60% presented b2a2 rearrangement, and none of the patients had e1a2 fusion gene.

Using the same conventional RT-PCR technique in 2004, Ito et al reported the frequency and distribution of BCR-ABL transcript types in the Japanese population.[22] Overall, 67.50% (85/126) with b3a2, 30.20% (38/126) with b2a2, 0.80% (1/126) with b2a3, and 1.60% (2/126) with c3a2 were observed, and this result seems to be in accordance with the Korean population having similar ethnic backgrounds. Also, in Thailand, 92.00% of CML patients (91/99) were positive for major BCR (61.00% b3a2 and 31.00% b2a2).[23]

These differences could be caused by differences in the sensitivities of detection techniques used, but ethnic differences should be strongly taken into consideration. Also, HLA associations with CML have been investigated in several papers. HLA-B*37, DRB1*10, and DRB4 showed a risk association with CML, but HLA-B*35, DRB1*11, and DRB3 showed protective effects on CML.[24] Other papers also described the susceptibility association and other associations with clinical response to CML.[25–27] The mechanisms for how the HLA system modifies susceptibility to CML are unknown, and it is worthy of further studies. Therefore, experiments on the frequency of different BCR-ABL transcript types in different ethnic groups are recommended, and a comprehensive survey of these experiments might be able to solve what factors induce different BCR-ABL fusion transcripts. Finally, the authors suggest that the rational approach for detection of BCR-ABL transcripts in CML are one-step multiplex RT-PCR, specific RQ-PCR, and nested RT-PCR sequentially, which involve various molecular techniques to simplify the complicated process from diagnosis to monitoring.

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