BCR/ABL Rearrangement in Philadelphia Chromosome Negative CML Patients

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Abstract

Reverse transcription-polymerase chain reaction (RT-PCR) was used to study 34 patients with chronic myelogenous leukemia (CML) associated with negative Philadelphia (Ph) chromosome. This report showed evidence of a chimeric BCR/ABL transcript in 18 (52.9%) and 28 (82.4%) cases by first PCR and seminested PCR, respectively. In these BCR/ABL transcript positive cases, the incidence of BCR exon3/ABL exon2 (B3A2) and BCR exon 2/ABL exon2 rearrangement was 25 (89.3%) and 3 (10.7%) cases, respectively. The other 6 Ph negative patients showed no evidence of reciprocal translocation of BCR to chromosome 9. This data demonstrates that seminested PCR is sufficiently sensitive to detect BCR/ABL fusion transcript in Ph chromosome negative CML patients.

Key word : BCR/ABL, Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Chronic Myelogenous Leukemia (CML)

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Chronic myelogenous leukemia (CML) is a clonal disease resulting in an expansion of a subset of myeloid progenitor cells genetically characterized by fusion of the BCR and ABL genes, which involves the reciprocal translocation t(9;22) (q 34; q11)[1-3]. This translocation results in the cytogenetically distinct Philadelphia (Ph) chromosome, observed by cytogenetic analysis in 90 per cent to 95 per cent of CML patients[1,4,5]. As a consequence of the translocation, part of the ABL proto-oncogene moves from chromosome 9 to the breakpoint cluster region (BCR) on chromosome 22, leading to the formation of two types of hybrid mRNAs, which codify for abnormal proteins bear-

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ing a tyrosine kinase activity[6]. There are 2 types of hybrid transcripts, BCR exon 2 fused to ABL exon2 (B2A2 type) and in the other, BCR exon 3 fused to ABL exon2 (B3A2 type). Ph positive CML is a progressive multistep disease that usually begins with a chronic phase of variable duration which can be easily controlled with therapy[7]. The disease terminates in an acute phase called blastic crisis with decreased responsiveness to chemotherapy. However, it is difficult to monitor the BCR/ABL rearrangement in Ph negative CML.

Although the presence of Ph chromosome has traditionally been documented by cytogenetics other techniques such as Southern blotting and reverse transcription-polymerase chain reaction (RT-PCR) are also widely used to identify the translocation at the molecular level[8]. In this report, we present the detection of the chimeric BCR/ABL rearrangement unique to Ph chromosome by RT-PCR technique in CML patients with negative Ph chromosome.

MATERIAL AND METHOD

Specimen

Heparinized bone marrow or peripheral blood were obtained from 34 CML patients whose samples were sent for chromosomal analysis at the Human Genetics Unit, Department of Pathology, Ramathibodi Hospital from December 1992 to June 1997. K562 Ph chromosome positive cell line was used as a positive control for BCR/ABL amplified products.

Chromosome preparation

Heparinized blood or bone marrow samples were washed twice with Hank's balanced salt solution. One to two million cells were cultured in 20 per cent FCS/RPMI-1640 in CO_2-incubator overnight. The cells were harvested by adding colchicine solution. Finally, metaphase cells were dropped onto microscopic glass slides. GTG-banded chromosomes were prepared and analyzed.

RNA preparation

RNA was isolated from peripheral blood and bone marrow cells as decribed by Groffen et al[6].

Reverse transcription and PCR (RT-PCR)

RT-PCR was followed from a previously published protocol[9]. Briefly, cDNA was synthesized from 250 ng of RNA in a 20 µl reaction volume using 200 U RNAsin (Promega, Madison, WI), 1 mM of each dNTP (Promega), and 100 pmol of random hexamer (Promega). After incubation for 1 hour at 37°C, reaction mixture was denatured by heating at 95°C for 5 minutes and plunged immediately on ice. Reaction mixture was diluted with 30 µl of RT-PCR reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH9.0), 0.1 per cent Triton X-100, 1 mM $MgCl_2$] followed by the addition of the 5' and 3' specific amplification primers and 0.25 U Taq DNA Polymerase (Perkin Elmer/Cetus)[5]. Cycles of PCR consisted of denaturing by heating at 94°C for 30 seconds, annealing of primers at 55°C for 30 seconds and primer extension at 72°C for 30 seconds. The cycle was repeated 25 times by using a programmable heat block (Perkin Elmer/Cetus). Numerous precautions were taken to avoid false positive results due to contamination of reagents with genomic DNA, mRNA or PCR products.

Seminested PCR

Five microliters of RT-PCR products were used as templates for seminested PCR. Primers for the steps were upstream primer (5'GGAGCTGCA GATGCTGACCAAC 3') the sequence locations of which were BCR 587-608 and ABL 251-228, respectively. The amplified products were fractionated by Nusieve : agarose gel electrophoresis, stained with ethidium bromide and visualized by a UV - transilluminator.

RESULT

Karyotype of 27 samples, from bone marrow and peripheral blood samples of 34 CML patients were normal (46, XX for female, and 46, XY for male), whereas, one case of bone marrow analysis showed abnormal karyotype of 46, XX, t(1;9). Six samples of bone marrow and peripheral blood samples had no cytogenetic data. Then, the RNA from bone marrow and peripheral blood was isolated and *in vitro* amplification was performed to detect BCR/ABL rearrangement. The PCR based assay for the detection of BCR/ABL rearrangement at the genomic level could define two mRNA species which differed based upon the BCR exons included by the translocation (Fig. 1). The PCR results of 34 patients are shown in Table 1. In 27 patients with normal karyotype, BCR/ABL rearrangement were detected in 13 and 21 cases by

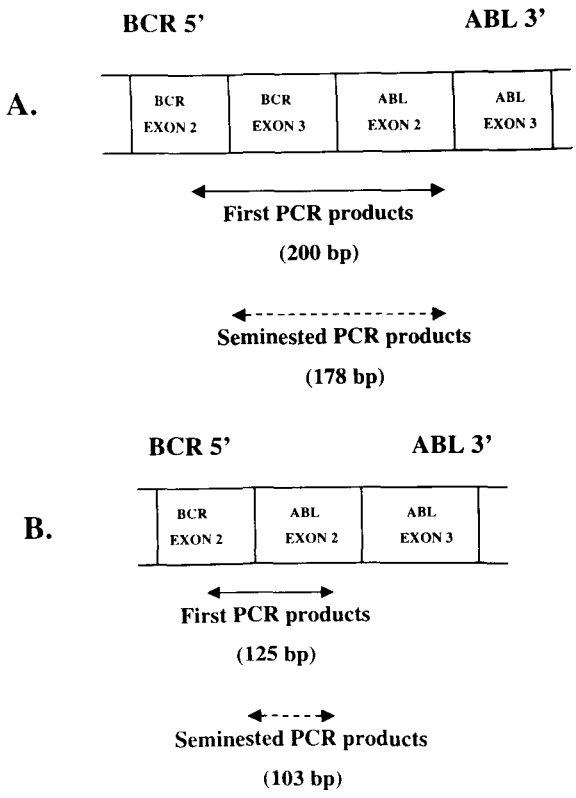

Fig. 1. Schematic diagram illustrating the chimeric BCR/ABL transcripts unique to Ph chromosome. The BCR/ABL rearrangement and amplified products generated by first and seminested PCR products from (A) BCR exon3/ABL exon2 or B3A2, and (B) BCR exon2/ABL exon2 or B2A2.

first and seminested PCR, respectively. No rearrangement was detected in the remaining 6 cases. A patient with abnormal karyotype of 46, XX,t (1;9) did not show BCR/ABL rearrangement by first PCR but by seminested PCR the BCR/ABL rearrangement was present. The other 6 patients who had no cytogenetic information were found to have BCR/ABL rearrangemetnt for 5 and all 6 patients by first and seminested PCR, respectively. Therefore, from a total of 34 Ph negative CML patients, first PCR assay was sufficiently sensitive to detect the BCR/ABL rearrangement in 18 patients (52.9%) and in addition, the seminested PCR technique could detect such rearrangement in 28 patients (82.4%), whereas, 6 patients (17.6%) had no evidence of the rearrangement detected by both first PCR and seminested PCR assay.

The subspecies of BCR/ABL rearrangement could be distinguished by a different size of amplified products (Fig. 1). In our 34 Ph negative CML patients, the incidence of B3A2 and B2A2 rearrangement was 25 (89.3%) and 3 (10.7%) cases, respectively (Table 2). The predominant hybrid transcripts were observed in patients No. 1-6 gene-

Table 2 BCR/ABL rearrangement in Ph negative CML patients.

BCR/ABL rearrangement	No. of patients
B3A2	25
B2A2	3

B3A2 = BCR exon3/ABL exon2 rearrangement
B2A2 = BCR exon2/ABL exon2 rearrangement

Table 1. The *in vitro* amplification of BCR/ABL rearrangement in 34 Ph negative CML patients.

Karyotype analysis		PCR detection		
Karyotype	No. of patient	No. of positive patient by first PCR	No. of positive patient by seminested PCR	No. of negative patient
normal	27	13	21	6
abnormal*	1	-	1	-
no result	6	5	6	-
total	34	18	28	6
(% of total)	(100%)	(52.9%)	(82.4%)	(17.6%)

* 46, XX, t (1;9)

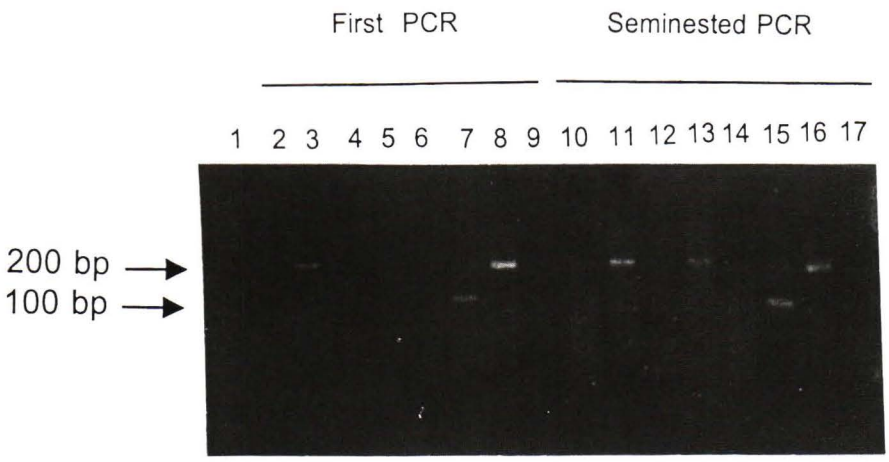

Fig. 2. Agarose gel electrophoresis of amplified products generated by first and seminested PCR from 6 Ph negative CML patients. K562, human Ph positive cell line carrying B3A2 transcripts, was positive control, and H_2O was negative control. The amplified products of B3A2 generated by first and seminested PCR were 200 and 178 bp, respectively, while those of B2A2 were 125 and 103 bp by first and seminested PCR, respectively. Lane 1, molecular weight marker. Lane 2-9, first PCR products from patients No. 1-6, K562 and H_2O, respectively. Lane 10-17, seminested PCR products from patients No. 1-6, K562 and H_2O, respectively.

rating either B3A2 or B2A2 chimera (Fig. 2). The first PCR products of B3A2 were observed in patient No. 2 and positive control K562 cell line, while seminested PCR products of those were seen in patients No. 2, 4 and K562 cells. The first and seminested PCR products of B2A2 were generated in patient No. 6. The amplified products of patients No. 1, 3, 5 and H_2O negative control are underobservation.

DISCUSSION

The Ph chromosome is a cytogenetic hallmark of CML, although 5-10 per cent of cases do not have it[10]. At cytogenetic level, variant Ph translocation, involving chromosome 22 and some chromosomes other than 9 (simple variant Ph translocations) or one or more chromosomes in addition to chromosomes 9 and 22 (complex variant translocations), have been described in a small proportion (3-8%) of CML patients[11]. In this report, we investigated whether RT-PCR was a viable alternative for karyotype analysis in the diagnosis and therapeutic monitoring of the condition of CML patients.

The BCR/ABL chimeric gene was detected by first PCR analysis in 52.9 per cent of Ph negative CML patients, while by seminested PCR analysis, the chimeric gene was found in 82.4 per cent of the same patient group. Our study showed that the RT-PCR technique could detect BCR/ABL mRNA in 52.9-82.4 per cent of Ph negative CML cases with the incidence of B3A2 and B2A2 rearrangement of 89.3 per cent and 10.7 per cent, respectively. In addition, the detection by seminested PCR is more sensitive to detect BCR/ABL transcript than by first PCR. On the other hand, the true BCR/ABL fusion negative patients were found to be only 17.6 per cent of all Ph chromosome negative CML cases. The reasons for the latter group may be that (i) the presence of rare variants of BCR/ABL transcripts and of methodologic drawbacks linked to the PCR procedure might result in false diagnosis[12,13], (ii) these patients probably were suffering from a different hematologic disease, i.e. myelodysplastic syndrome[14], and (iii) the failure of RT-PCT procedure resulting from the highly degradable nature of the mRNA so that the samples must be carefully processed and stored, and the lack

of the specific band may imply failure to amplify the chimeric transcript (false-negative result)[15]. Another report of 2 Ph negative CML patients, one showing a normal karyotype and the other having inv (3) (q21q26) demonstrated the presence of BCR rearrangemant by Southern blot analysis[16]. In at least 14 CML cases having normal karyotype, molecular studies revealed rearrangement of the BCR/ABL fusion mRNA[17-20].

According to the widespread application of the PCR method for diagnostic purposes, we, therefore, suggest the following : (i) frequent cross-comparison of the PCR procedure among laboratories; (ii) great care to avoid contamination in all steps ; (iii) in case of doubt, performing a PCR directly on an RNA sample without reverse transcription so that cDNA contamination could be ruled out; (iv) harvesting and storing a back-up sample (at least for patients who will be given high-dose chemotherapy), a simple and useful method to reveal any contamination and to set up an internal quality control procedure[15].

We suggest that RT-PCR is one of the viable alternatives to karyotyping analysis in the detection of BCR/ABL fusion and its transcribed mRNA. Nevertheless, karyotyping is still important because it detects additional nonrandom chromosomal aberrations.

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การตรวจหายีนลูกผสม BCR/ABL ในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาว ชนิด CML ที่ไม่มีโครโมโซมฟิลาเดลเฟีย

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การศึกษาทางอณูพันธุศาสตร์ในผู้ป่วยโรคมะเร็งเม็ดโลหิตขาวชนิด CML ซึ่งไม่มีโครโมโซมฟิลาเดลเฟีย จำนวน 34 ราย โดยเทคนิค RT-PCR พบว่าผู้ป่วยจำนวน 18 ราย (52.9 เปอร์เซ็นต์) และ 28 ราย (82.4 เปอร์เซ็นต์) มียีนลูกผสม BCR/ABL โดยการตรวจวิธี PCR ชุดแรก และ PCR ชุด seminested ตามลำดับ การตรวจหายีนลูกผสม BCR/ABL โดยวิธี PCR ชุด seminested เป็นวิธีที่ไวกว่าวิธี PCR ชุดแรก ในผู้ป่วยกลุ่มนี้พบว่าผู้ป่วยจำนวน 25 ราย (89.3 เปอร์เซ็นต์) มียีน BCR exon3 เชื่อมติดกับยีน ABL exon2 และผู้ป่วยจำนวน 3 ราย (10.7 เปอร์เซ็นต์) มียีน BCR exon2 เชื่อมติดกับยีน ABL exon2 สำหรับผู้ป่วยกลุ่มที่สองจำนวน 6 ราย ตรวจไม่พบยีนลูกผสม BCR/ABL ฉะนั้นการตรวจยีนลูกผสม BCR/ABL ด้วยวิธี PCR ชุด seminested เป็นวิธีการหนึ่งที่ไวพอที่จะใช้ตรวจการแลกเปลี่ยนชิ้นเนื้อโครโมโซมระหว่างบริเวณที่มียีน BCR บนโครโมโซมแท่งที่ 22 และยีน ABL บนโครโมโซมแท่งที่ 9

คำสำคัญ : ยีนลูกผสม BCR/ABL, ปฏิกิริยา RT-PCR, มะเร็งเม็ดโลหิตขาว ชนิด CML

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