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Detection of the *BCR/abl* Gene Rearrangement by Reverse Transcriptase Based Polymerase Chain Reaction

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Abstract: The Philadelphia (Ph) chromosome is the single most intensively studied chromosome alteration characterizing a human malignancy. The specific genetic alteration of chronic myelogenous leukemia (CML) is the formation of the *BCR/abl* fusion gene in leukemic cells. The presence of the *BCR/abl* gene has important diagnostic and prognostic implications in CML. The detection of *BCR/abl* transcripts by reverse transcriptase based polymerase chain reaction (RT-PCR) was investigated in patients with CML in whom the Ph chromosome abnormality was documented by cytogenetic analysis. In a total of 68 CML patient cases, the Ph chromosome was found in 53 cases (77.9%) by cytogenetic analysis. On the other hand, sixty two cases (91.2%) were detected to have *BCR/abl* gene rearrangement. Of these, b3a2 was 44 cases (64.7%) and b2a2 was 17 cases (25.0%). There was one case with both b3a2 and b2a2 (1.5%). Of the fifteen cases of Ph chromosome negative by cytogenetic anlaysis, the *BCR/abl* gene was observed in nine cases. The results of *BCR/abl* fusion gene confirmed by the direct sequencing method correlated well with PCR analysis. The amplified PCR products were detected by 1×10^5 dilutions. In conclusion, PCR technique is sensitive, rapid and relatively simple for a laboratory test in detecting the *BCR/abl* fusion gene with CML regardless of the result of cytogenetic analysis.

Key words: *BCR/abl* fusion gene, chronic myelogenous leukemia, polymerase chain reaction, philadelphia chromosome.

Chronic myeloid leukemia (CML) was the first disease shown to be consistently associated with a cytogenetic abnormality, now known as the Philadelphia (Ph) chromosome (Kawasaki *et al.*, 1988). The Ph chromosome, which was first described by Nowell and Hyngerford in 1960 (Nowell and Hungerford, 1960), is a shortened chromosome 22 that results from a balanced translocation between chromosomes 9 and 22. This translocation relocates *ABL* from chromosome 9q34 to within the *BCR* (breakpoint cluster region) gene on chromosome 22q11 (Groffen *et al.*, 1984). Gene rearrangement of *BCR* and *ABL* is the molecular counterpart of the translocation t(9:22). By splicing of primary transcript, two types of chimeric mRNA are generated: b3a2 (*BCR* exon b3 fused to *ABL* exon 2) and b2a2 (*BCR* exon b2 fused to *ABL* exon 2) (Mills *et al.*, 1991; Shtivelman *et al.*, 1986). Present means of diagnosing CML include some combination of morphological, cytochemical (Yasukawa *et al.*, 1992), cytogenetic (Rowley, 1973; Sanchez *et al.*, 1973), immunological (Arthur *et al.*, 1988) and molecular analyses (Benn *et al.*, 1987; Kawasaki *et al.*, 1988; Tkachuk *et al.*, 1990). Each method has its merits but may be difficult to carry out if only limited clinical samples are available and/or only a small proportion of the cells are leukemic. A more sensitive method would be useful in following the course of disease in patients after bone marrow transplants or in patients in remission (Amiel *et al.*, 1994). Recently a procedure was described for the enzymatic amplification of genomic DNA sequences *in vitro* (Saiki *et al.*, 1985). We have extended and modified that method to include RNA as the starting template for what is called reverse transcriptase based polymerase chain reaction (RT-PCR) (Roth *et al.*, 1989; Martiat *et al.*, 1990). For the diagnosis of CML, a complementary DNA copy of the mRNA from patient blood or bone marrow cells is first synthesized, followed by polymerase chain reaction (PCR) amplification of only the diagnostic *BCR/abl* chimeric cDNA sequences. Products of the PCR reaction are then analyzed by direct sequencing analysis (Matero *et al.*, 1991). This PCR procedure complements existing diagnostic protocols, is much more sensitive, and provides information

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about the nature of the molecular defect without requiring large clinical samples or resorting to molecular cloning (Kawasaki *et al.*, 1988). In this study, the PCR analysis was compared with the cytogenetic study for the detection of Ph chromosome in CML patient samples, and the usefulness of the PCR technique was evaluated as a clinical laboratory tool for diagnosis of CML.

Materials and Methods

Material

Sixty eight patients with a clinical diagnosis of CML seen at our institution between Jan. 1994 and Dec. 1995 were entered in this study. All patients met at least three of four criteria for the diagnosis of CML: (1) splenomegaly, (2) leukocytosis with left shift and/or bone marrow myeloid hyperplasia, (3) a leukocyte alkaline phosphatase score less than 14, and (4) the presence of the Ph chromosome by karyotype (Amiel *et al.*, 1994). K562 cells were used as a standard for the development of a PCR method (Konopka *et al.*, 1984).

Cytogenetic analysis

Heparized peripheral blood cells were cultured for 48 h in RPMI 1640 culture medium (GIBCO, Gland Island, Gland Island, USA) supplemented with 15% fetal calf serum (GIBCO) and antibiotics without the addition of any mitogens. We processed the cells by the standard cytogenetic method and stained the chromosomes with 5% Giemsa solution (Rowley, 1973; Sanchez *et al.*, 1973). If available, 20 metaphases were analyzed and documented by photography. Chromosome abnormalities were identified and described according to the ISCN (Harnden and Klinger, 1985).

Synthesis of primers for RT-PCR

DNA sequences used for the CML studies are found in three refs (Heisterkamp *et al.*, 1985; Grosveld *et al.*, 1986; Shtivelman *et al.*, 1986). The sequences of the oligomers are listed in Table 1. Amplification primers were synthesized using a 392 DNA/RNA synthesizer and purified by an oligonucleotide purification cartridge (Applied Biosystem, Foster city, USA).

RNA extraction, cDNA synthesis and PCR amplification

Total RNA was extracted from nucleated cells using the method of Chomczynski and Sacchi (1987). Briefly, mononuclear cells were washed in phosphate-buffered saline, pelleted, and suspended in 4 M guanidinium isothiocyanate. The mixture was then heated to 60℃, and the DNA was sheared by repeated passage through an 18-gauge needle. Phenol at 60℃ and half volume of buffer (sodium acetate, Tris-HCl, and EDTA) was added. The mixture was repeatedly extracted with chloroform (×3), and the RNA was precipitated with cold ethanol. A final RNA preparation was obtained after further extraction with phenol/chloroform and precipitation with ethanol by centrifugation. The RNA was dissolved in sterile water. One microgram of total RNA in 40 μl water was mixed with 200 ng of primer and heated to 80℃ for 5 min, then rapidly cooled in ice. cDNA synthesis was carried out at 42℃ for 30 min after adjustment of the mixture to contain 10 mM of Tris-HCl pH 8.3, 1.5 mM of $MgCl_2$, 200 μM of each dNTP, 20 U of RNasin (Promega, Madison, USA) and 200 U of Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco-BRL Life Technologies, Cergy-Pontoise, France) (Sadamura *et al.*, 1992). The amplification was run using 5 μl of cDNA with a 45 μl mixture including 200 ng of each primer, 200 μM of each dNTP, 10 mM of Tris-HCl pH 8.3, 1.5 mM of $MgCl_2$ and 1 U of AmpliTaq™ (Perkin-Elmer Cetus, Norwalk, USA) by following one cycle (94℃ for 2 min) and 30 cycles (93℃ for 3 min, 65℃ for 2 min and 72℃ for 3 min). The PCR was carried out using an automated thermal cycler (GeneAmp PCR system 9600. Perkin Elmer Cetus, Norwalk, USA). The same PCR mixture without primer was used as a negative control for PCR. After the addition of 2 μl loading buffer 30% (v/v) glycerol stained with bromophenol blue and xylene cyanol), aliquots (5 μl) of the reaction products were loaded on 2% agarose gel electrophoresis in a glass plate apparatus (Bio Rad, Richmond, USA) for precise analysis of the amplified DNA products (Elder and Southern, 1983). Gels were examined under UV illumination and documented by photography (Markovits *et al.*, 1979).

The sensitivity of the RT-PCR

Total RNA was extracted from serial 10 fold dilutions of K562 cells (10^6 to 1 cell) which had been premixed with approximately 10^6 normal peripheral blood leucocytes obtained from normal subjects (Konopka *et al.*, 1984; Shtivelman *et al.*, 1986). Total RNA was reverse transcripted into cDNA and amplified as described (see the 'Materials and Methods' section). 20 μl of PCR products were analysed by 2% agarose gel electrophoresis.

Direct sequencing analysis

Direct sequencing of the PCR product was analyzed using a commercial kit (USB, Sequencing Kit, Amersham Life Science, UK). Briefly, a 10 μl aliquot of PCR product was treated with 1 μl of exonuclease I (1.0 U/ml) for 15 min at 37℃ and at 80℃ for 15 min.

The same procedure was repeated with 1 μl of alkaline phosphatase (2.0 U/μl). A sample of 5 μl of PCR product was mixed with 4 μl of distilled water and 1 μl of primer (5 pmol/μl). After boiling for 2~3 min, the mixture was cooled as quickly as possible by placing the vial in ice for 5 min. Then 10 μl of DNA mixture was mixed with 2 μl of sequencing reaction buffer (80 mM of Tris-HCl pH 8.3, 140 mM of KCl and 10 mM of $MgCl_2$) to which was added 1 μl of 10 mM of dithiothreitol (DTT), 2 μl of diluted dGTP labelling mixture and 0.5 μl of α^{35}S-dATP (250 μCi/μl). The amplified PCR products were incubated to anneal with the primer at room temperature for 5 min and added to 2 μl each of four dideoxy mixtures. Ten units of DNA polymerase (USB sequencing Kit, Amersham Life Science, UK) were added to each dideoxy reaction, incubated at 37℃ for 20 min. The sequence information was obtained after electrophoresis of 2 μl of the final reaction mixtures onto a 7% polyacrylamide-urea sequencing gel followed by autoradiography (Feinberg and Vogelstein, 1984).

Results

Detection of Philadelphia chromosome by cytogenetic analysis

A total of 68 CML samples, chromosomal translocations (Ph chromosome) were found in 53 cases (77.9%) by cytogenetic analysis (Table 1). In fifteen Ph chromosome negative cases, four complicated abnormal cases were found (data not shown). All of the above four cases represented the *BCR/abl* transcripts by PCR study.

Detection of *BCR/abl* gene rearrangement using RT-PCR

Two types of spliced genes were found in *BCR/abl* gene rearrangement study (Fig. 1). When *BCR* exon

Table 1. Oligonucleotide primers for the detection of *BCR/abl* mRNA

Code	Sequences	Use
RC1	5′ ATGACCCCAACCTTTTCGT	RT primer
CM1	5′ GAGCTGCAATGCTGACCAACT	5′ primer
CM2	5′ CATCTGACTTTGAGCCTCAGGGTCT	3′ primer

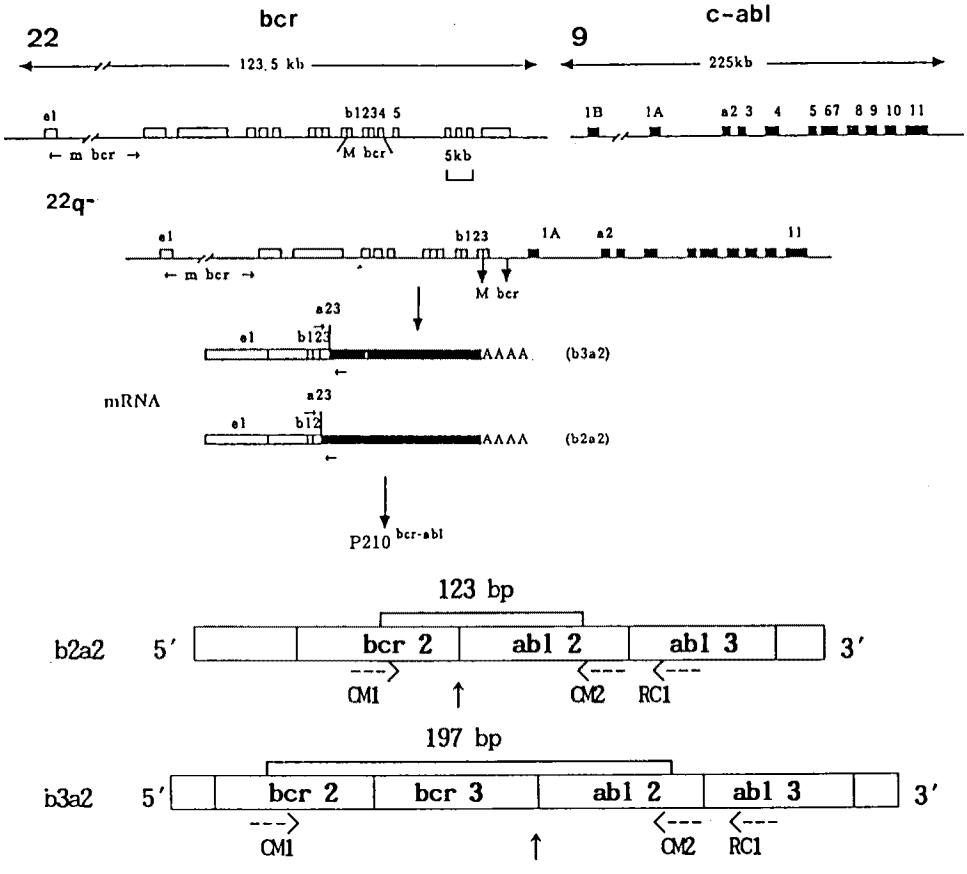

Fig. 1. Diagram of the *BCR/abl* gene rearrangements. The BCR and ABL genes are shown at the top, with the exons, of coding regions, shown in boxes. Region mbcr and M bcr are the 75 kb first intron and the 5.8 kb breakpoint cluster region, respectively. M bcr yields two PCR products, b2a2 and b3a2. CM1, CM2 and RC1 represent 5′, 3′ and reverse transcription primer, respectively.

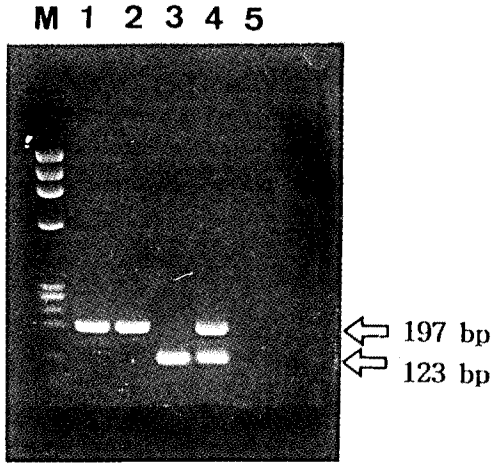

Fig. 2. Detection of *BCR/abl* mRNA by RT-PCR. Two kinds of bands were observed. Lane M: Molecular weight size marker (PhiX 174/*Hae*III, Promega Co., USA) (1353, 1078, 872, 603, 310, 281, 234, 194 bp); lane 1: K562 cells for the positive control; lane 2: *BCR/abl* fusion gene: b3a2; lane 3: *BCR/abl* fusion gene: b2a2; lane 4: *BCR/abl* fusion gene: b3a2/b2a2; lane 5: Negative control.

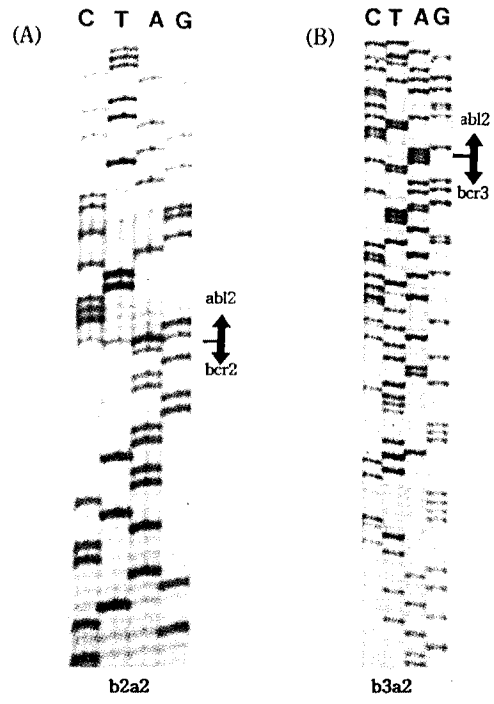

Fig. 3. Direct sequence analysis of the PCR product of *BCR/abl* rearrangement. RNA samples were amplified as described in methods. The PCR products were annealed with primer and subjected to four reactions containing α-^{35}S dATP and dideoxy nucleoside triphosphates, designated as C, T, A and G.

3 is present in the chimeric mRNA, a 197 bp fragment is amplified (b3a2). On the other hand, a 123 bp fragment is expected when exon 2 of *BCR* is joined to *ABL* exon 2 (b2a2) (Fig. 2). In a total of 68 CML cases, the *BCR/abl* transcripts were found in sixty two cases (91.2%). This include 44 cases of b3a2 (64.7%), 17 cases of b2a2 (25.0%) and one case of both b3a2 and b2a2 (1.5%) (Table 2). Among 15 Ph negative CML by cytogenetic analysis, nine cases represent the *BCR/abl* transcripts by PCR. The identity of the *BCR/abl* transcripts were confirmed by the direct sequencing of the amplified RNA products. Fig. 3 (A), (B) represent the direct sequencing analysis of the b2a2 and b3a2 junctions, respectively.

The sensitivity of the RT-PCR

Serial dilutions were practiced to detect the sensitivity of PCR for *BCR/abl* gene rearrangement with K562 cells as described in the Method section. The amplified PCR band detected to the 1×10^5 dilution of 1 μg of RNA from the Ph chromosome positive K562 cell line still provided an easily detectable signal in this assay (Fig. 4).

Discussion

The Ph chromosome is the derivative of a translocation between the long arms of chromosome 9 and 22 [t(9:22)(q34:q11)] (Rowley, 1973). As a result of this translocation, most of the *ABL* oncogene, located on chromosome 9, is juxtaposed next to part of the *BCR* gene, located on chromosome 22, creating a new *BCR/abl* fusion gene (Fig.1) (Heisterkamp *et al.*, 1983; Konopka and Witte, 1985). In most cases of CML, the breakpoint is within a small 5.8 kb segment between bcr exons 2 and 3 or exons 3 and 4 in the center of the *BCR* gene, which is called major breakpoint clus-

Table 2. The result of cytogenetic studies and RT-PCR analysis in 68 chronic myelocytic leukemia patients

Chromosome translocation[a] (Ph chromosome)	*BCR/abl* rearrangement[b]				Total
	b3a2	b2a2	b3a2/b2a2	Negative	
Ph(+)	36(52.9%)	16(23.5%)	1(1.5%)	–	53(77.9%)
Ph(−)	8(11.8%)	1(1.5%)	–	6(8.8%)	15(22.1%)
Total	44(64.7%)	17(25.0%)	1(1.5%)	6(8.8%)	68(100%)

[a] Cytogenetic study, [b] RT-PCR analysis.

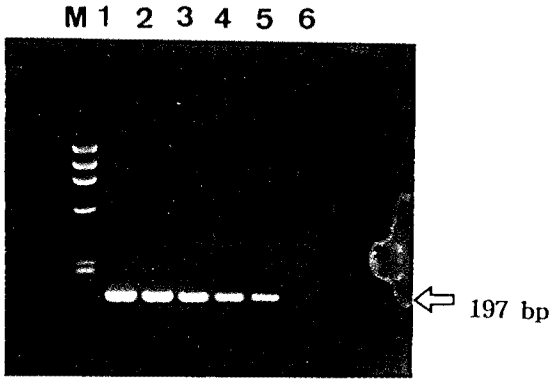

Fig. 4. Sensitivity of RT-PCR in the detection of *BCR/abl* gene arrangement. Total RNA from K562 cell was sequentially diluted and subjected to 2% agarose gel electrophoresis. Lane M : Molecular weight size marker (PhiX 174/*Hae*III, Promega Co., USA) (1353, 1078, 872, 603, 310, 281, 234, 194 bp); lane 1 : 1×10; lane 2 : 1×10^2; lane 3 : 1×10^3; lane 4 : 1×10^4; lane 5 : 1×10^5; lane 6 : 1×10^6.

ter region (M-bcr) (Fig. 1) (Mills *et al.*, 1991). This region encompasses four exons (exons 12 through 15, but often referred to as exons b1 through b4). Thus, two distinct species of mRNA may be encoded by this form of *BCR/abl* transcript. A gene that includes *BCR* exon b2 may give rise to either an mRNA in which exon b3 is spliced to *ABL* exon 2 (b3a2). A gene from which *BCR* exon b2 has been excluded encodes an mRNA in which *BCR* exon b2 is spliced to *ABL* exon 2 (b2a2) (Daley *et al.*, 1990). These types of hybrid mRNA and protein differ in size by 75 bases and 25 amino acids, respectively (Shtivelman *et al.*, 1986). Transcription of this chimeric *BCR/abl* gene leads to the formation of an 8.5 kb mRNA and a p210 bcr-abl protein (Ben *et al.*, 1986) (Table 3). Both the mRNA and protein are unique to M-bcr-positive cells (Benn *et al.*, 1987). This fusion protein exhibits strong tyrosine kinase enzymatic activity that has been shown to transform hematopoietic cells *in vitro* and may contribute directly to the cell proliferation and differentiation seen in CML (Hunter and Cooper, 1985; MaLaughlin *et al.*, 1987; Stam *et al.*, 1987; Kurzrock *et al.*, 1988; Daley *et al.*, 1990; Benz *et al.*, 1994) (Table 3). A second type of the *BCR/abl* gene rearrangement which is found in ALL (Acute Lymphoblastic Leukemia) patients results from breakpoints within a 10.8 kb region of the *BCR* gene's first intron, which was called m-bcr (minor bcr) (Romero *et al.*, 1989). This alternate rearrangement codes for a fusion protein of 190 kD (Bernarde *et al.*, 1987; Hooberman and Westbrook, 1989) (Fig. 1).

Historically, cytogenetic finding of the Ph chromosome has been the standard in the diagnosis of CML and the only technique for detection of translocations (Rowley, 1973; Sanchez *et al.*, 1973). In recent years, advances in molecular techniques, and better understanding of the molecular biology of the Ph chromosome, have altered substantially the methodology for detecting the Ph chromosome. The detection of the *BCR/abl* fusion gene in CML may be accomplished by using Southern blot hybridization studies with *BCR*-specific genomic probes (Heisterkamp *et al.*, 1985; Benn *et al.*, 1987). The use of these studies has enhanced our understanding of the pathobiology of CML and has permitted identification of *BCR/abl* translocations from many cases of so-called Ph negative CML (Martiat *et al.*, 1990). PCR, however, offers more sensitivity than conventional Southern blotting techniques (Erlich *et al.*, 1991) and can be designed to be highly specific for the *BCR/abl* region. Since the breakpoint on chromosome 9 (c-abl oncogene) in patients with the Ph chromosome may occur over a 100 kb region, PCR detection based on DNA as a whole may not be successful due to this large molecular distance. This problem, however, can be easily overcome with PCR based on a RNA template, which encompasses a much shorter molecular distance and gives a consistent *BCR/abl* junction. The RNA can then be converted to cDNA by a reverse transcriptase reaction and thus readied for the PCR amplification procedure (Roth *et al.*, 1989). Previous studies have shown the applicability of PCR in detecting the *BCR/abl* gene in patients with CML (Kawasaki *et al.*, 1988; Morgan *et al.*, 1989).

The Ph chromosome is found in greater than 90% of patients with CML and a recombinant event is found in 95% of Ph chromosome positive patients with CML, 30~50% of Ph chromosome negative CML and 20% of adult patients with ALL (Kurzrock *et al.*, 1988; Martiat *et al.*, 1990). In this study, the Ph chromosome was only found in 53 cases by cytogenetic analysis. The reason why only 77.9% of Ph chromosomes were found by cytogenetic analysis in our experiments may be due to two factors. One is a masking of the Ph chromosome with a complicated structural abnormality and the other is a small number of abnormal cells in cytogenetic culture analysis. The *BCR/abl* transcripts were found in 62 cases (91.2%) of total and observed in over 60% of Ph chromosome negative cases. The b3a2 is more frequent than b2a2 (over double) (Table 2). The site of the breakpoint is only an indicator of the type of splice site present in the hybrid *BCR/abl* mRNA. Recent reports have suggested a correlation between the type of mRNA expressed and the disease phase or duration (Mill *et al.*, 1991). However this cannot explain different conclusions regarding the relationship between the location of the breakpoint and the duration of chronic phase in different groups of patients

unless, as this analysis suggests, there exist as yet unrecognized, systematic differences in other factor(s) between the groups (Benz *et al.*, 1994). Our finding of the *BCR/abl* fusion gene in all 53 patients who demonstrate the Ph chromosome positive and in 9 of 15 patients who have a clinical diagnosis of CML without the Ph chromosome supports the high sensitivity of PCR analysis (Table 2). The value of PCR for diagnosis and detection of CML can be compared as follows with that of the cytogenetic examination. (i) Cytogenetic examination will not be able to diagnose 5% to 10% of cases of CML but PCR techniques can provide additional information suggestive of impending acute transformation (Groffen *et al.*, 1984). In about half of the cases of Ph chromosome negative CML, the translocation is masked at a karyotypic level, but the molecular rearrangement has still occurred (Mills *et al.*, 1991). The PCR method is also applicable to the study of any type of RNA found in the cell, whether it is of normal or abnormal origin. (ii) PCR takes only 1~2 days to get the results, while cytogenetic analysis takes 3~4 weeks at least. PCR could be carried out from the scraps of bone marrow slides stored for several years (Benz *et al.*, 1994). (iii) Sample volume of PCR reaction is sufficient for 1~2 ml of peripheral blood. In cytogenetic analysis, however, 10~15 ml of fresh heparin blood is required. (iv) The Ph chromosome carries the *BCR/abl* fusion and a $9q^+$ chromosome. However, the Ph chromosome arises through more complex translocations in which one or more chromosomes are involved in addition of chromosomes 9 and 22. In these cases, Ph chromosome might be easily masked and is difficult to detect clearly by cytogenetic analysis, while PCR technique is possible to detect the *BCR/abl* fusion gene. (v) Finally, PCR amplification of coding sequences in genomic DNA discloses the exon-intron structure of the gene, and therefore PCR can provide information on the splicing type of *BCR/abl* rearrangement. (Bernards *et al.*, 1987; Yoffe *et al.*, 1987). In the sensitivity test of PCR, CML cells are readily detectable even when diluted 1 in 10^5 with normal blood (Fig. 5). One microgram of RNA is roughly equivalent to the amount contained in the cytoplasm of 10^5 K562 cells (10 pg of cytoplasmic RNA per cell). Therefore, the $1:10^5$ dilution contain the RNA from about one K562 cell. Since just 1/10th of the reaction mixture was used for analysis, the positive signal represents the amplified product of less than one cell equivalent. This result demonstrates that diagnosis is feasible even when the leukemic cells are present in extremely small numbers. A similar finding was reported for amplification studies of the *BCR/abl* fusion gene (Kawasaki *et al.*, 1988).

As a consequence, analysis of clinical specimens in CML using PCR can be performed in a relatively short time and is highly sensitive ($1:10^5$) (Fig. 4). This approach is simple and can routinely be used in most cases on blood and bone marrow cells of patients at the first stages of clinical presentation. PCR has allowed for the detection of a Ph chromosome in patients in whom the cytogenetics are negative despite a clinical presentation of CML. It also can moniotr minimal residual disease after bone marrow transplantation (Arthur *et al.*, 1988; Shtalrid *et al.*, 1988; Lange *et al.*, 1989; Morgan *et al.*, 1989).

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