Frequency of BCR-ABL Fusion Transcript in Iranian Patients with Chronic Myeloid Leukemia

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Abstract

Introduction: Reverse transcriptase-polymerase chain reaction (RT-PCR) assay is a useful tool for the detection of fusion transcript resulting from specific chromosomal translocation of the leukemia cells. A specific chromosomal abnormality, the Philadelphia chromosome (Ph), is present in 90% to 95% of CML patients.The aberration results from a reciprocal translocation between chromosome 9 and 22, creating a BCR-ABL fusion gene.There are two major forms of the BCR/ABL fusion gene, involving ABL exon 2, but including different exons of BCR gene. The transcripts b2a2 or b3a2 code for a p210 protein. Another fusion gene leads to the expression of an e1a2 transcript, which codes for a p190 protein. Another, less common fusion genes are b3a3 or b2a3 (p203) and e19a2 (p230). The incidence of one or other rearrangement in chronic myeloid leukemia (CML) patients varies in different reported series. In general, fusion transcripts are determined individually, a process which is labor intensive in order to detect all major fusion transcripts.

Methods: This study was designed to determine the frequency of different fusion genes in 75 iranian patients with CML. peripheral blood samples were analyzed by multiplex reverse transcriptase polymerase chain reaction (RT-PCR) from adult patients to detect all types of BCR-ABL transcripts of the t (9:22) and found that all cases were positive for some type of BCR/ABL rearrangement.

Results: Most of our patients showed b3a2 fusion gene (62%), while the remaining showed one of the transcripts of b2a2, b3a3, b2a3, e1a2 or coexpression of b3a2 and b2a2. The rate of coexpression of the b3a2 and b2a2 was 5%.

Conclusion: In contrast to the other reports, we did not see any coexpression of p210/p190. This may reflect either the sensitivity of the detection techniques used or the possibility of genetic differences between the populations studied. Coexpression may be due to alternative splicing or to phenotypic variation, with clinical course different from classical CML.

Key words: BCR-ABL, CML, Multiplex RT-PCR

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Introduction

The hallmark of chronic myelogenous leukemia is the t(9;22)(q34;q11). In this translocation, the 30 segment of the c-abl proto-oncogene on chromosome 9 is juxtaposed with the 50 segment of the bcr gene on chromosome 22.[1–4] Breaks in the c-abl gene typically occur in the first intron. Breaks in bcr generally occur in one of three regions: the major breakpoint cluster region (M-bcr), the minor breakpoint cluster region (m-bcr) and the micro breakpoint cluster region (m-bcr).[5] Breakpoints occurring in M-bcr involve introns 13 or 14 and join exon 13 (also known as b2) or 14 (also known as b3) with exon 2 of abl (a2) resulting in the fusion transcripts b2a2 and b3a2, respectively. These transcripts lead to the production of an 8.5 kb transcript coding for a 210 kDa (p210) chimeric protein.[6,7] both b3a2 and b2a2 transcripts can be formed as a result of alternative splicing (Okamoto et al., 1997; Lichty et al; 1998) Breakpoints in m-bcr involve the first intron of bcr and join exon 1 (e1) with a2 resulting in a smaller fusion transcript, e1a2, that codes for a 190 kDa (p190) protein.[8] Breakpoints in m-bcr involve intron 19 and result in the joining of

exon 19 (e19) of bcr with a2, e19a2, coding for a 230 kDa (p230) protein.[9] Although all bcr-abl fusion proteins display activated tyrosine kinase activity, the p190 form has been shown to have more transforming potential than p210 in vitro and in vivo.[10-12] Fusion transcripts b3a2 and b2a2 account for the majority of CML cases. The e1a2 fusion transcript is seen primarily in t(9;22)- positive acute lymphoblastic leukemia and lymphoid blast phase of chronic myelogenous leukemia, but rarely in chronic myelogenous leukemia in chronic phase. The rare e19a2 fusion transcript is found in cases of chronic myelogenous leukemia with prominent neutrophilic maturation.[13] These unusual neoplasms also have been reported in the literature by some investigators as chronic neutrophilic leukemia.[14]

This study was designed to determine the frequency of expression of the BCR/ABL rearrangement in 75 Iranian patients with CML. We interpreted the detection of two or more p190, p210, p201 encoding transcripts in the patient's samples.

Methods

A total of 75 CML patients underging treatment from June 2004 to October 2005 were enrolled in this study at the time of diagnosis. The diagnosis of CML was established according to clinical presentation and morphological criteria of BMA, and was confirmed by cytogenetic assay for t(9;22) and multiplex RT-PCR analysis for BCR-ABL transcripts.The median age was 40 years (range 9-55 years), 45% were female and 55% male. Seven patients were in accelerated phase, 12 were in blast crisis and the remaining were in chronic phase.

RNA Isolation and cDNA synthesis: Mononuclear cells (MNC) were isolated from 10 to 20 ml peripheral blood (PB) or 1–3 ml bone marrow aspirate (BMA) by density gradient centrifugation on Ficol-Hypaque. Total RNA was extracted from about 106 MNC by TRIZOL (Gibco BRL, Gaitherburg, MD).The integrity of RNA was determined by gel electrophoresis prior to reverse transcription. For cDNA synthesis, the concentration of RNA was first measured by a spectrophotometric method and then the cDNA was synthesized using first strand cDNA synthesis kit (Fermentas UAB, Lithuania). One micro gram RNA was reversely transcribed with 10U/µl MMLV, in 1x RT buffer, 25ng/µl random hexamer primer, 25µM dNTP, 0.01M DTT and 2U/µl RNasin. At 75ºC for 2 min, 42 ° C for 1 hr, and 75 ° C for 10 min.

Multiplex RT-PCR conditions: The cDNA product was amplified with 1U/µl Taq polymerase, 240µM dNTP, 1.8 M MgCl2 and 0.6 µM of the four primers (CA3-, C5e-, BCR-C and B2B). Multiplex RT-PCR assay was performed on a Hybaid PCR machine with the program of 10sec at 100 ºC, 1 min at 96 ºC, 3 min at 60 ºC, 2min at 72 ºC, 10 sec at 100 ºC, 20 sec at 97 ºC, 25sec at 58 ºC, 25 sec at 60 ºC, 10 sec at 78 ºC, 50 sec at 73 ºC, 31 times to step 5 and 10 min at 73 ºC . The sequence of oligonucleotide primers used in multiplex RT-PCR for BCR-ABL fusion transcripts as the target gene and BCR transcripts as internal control are shown in Table 1.[5]

In the PCR protocol, cDNA synthesized from K562 cells (b3a2), and from patients with b2a2 cell types were used as positive controls; and sterile water was used as negative control. Normal BCR transcript was used as an internal control. The PCR products were run on a 2% agarose gel with ethidium bromide to analyze the size of the amplicons.

Results

The primer combinations in multiplex RT-PCR allowed simultaneous detection of all known types of BCR-ABL and BCR transcripts in one reaction simultaneously.

The expected bands were as follows: 808bp, normal BCR; 481 bp, e1a2; 385 bp ,b3a2; 310bp, b2a2; 209bp, b3a3 and 103 bp, b2a3(Cross, Lin & Goldman,1994;Cotter,1996; Burmeister et al.,2000). The quality of RNA and efficiency of cDNA synthesis were analyzed by amplification of BCR gene as an internal control. The amplified product (808bp) from the BCR gene was the only band detected in BCR/ABL negative patients.; the absence of this bands indicated procedural failure. The results of multiplex RT PCR for some different patients are shown in Figure 2.

We were able for the reliable detection of typical p210 transcripts, such b2a2 or b3a2 and atypical types, such as transcripts lacking ABL exon a2 (b2a3 and b3a3), or p190BCR/ABL transcripts, such as e1a2 in 75 patients at the

time of presentation. The Fig3,illustrate the diagram of several BCR-ABL fusion transcripts detected among our patients with their frequency. Using multiplex RT-PCR, the majority of the patients (82.6%) expressed one of the p210BCR-ABL rearrangements (b3a2 or b2a2). Table 2 shows clinical data for the patients according to BCR/ABL transcript type. The frequency of different fusions post BMT by RT-PCR were 62.6% b3a2, 20% b2a2, 1.4% e1a2, 16% rare type fusions. The median age for all of the cases was over 40 years, while for patients expressing both b3a2/b2a2, the median age was 25.

Usually one and sometimes two types of mRNA can be expressed in the same patients. For example one of our patients showed both b3a2 and b2a2 fusion genes the other showed b3a2, b2a2 and also b3a3 (p203BCR-ABL). These cases were diagnosed as chronic-phase CML, with cytogenetic abnormalities in addition to t (9;22) and high risk clinical data.In about 9.4%, exon a2 is not expressed, instate exon a3 is expressed, leading to b2a3 or b3a3.

The sensitivity of assay was evaluated by generating 10 fold serial dilution series of K562 RNA concentration equivalent of 1 to 10^5 cells. Using these dilutions, as few as 1 cell in a background of 10^4 normal cell was detected. The specificity of the assay was evaluated by testing 10 non-CML samples. No detected signal was found with these primer sets. The integrity of the RNA in each sample was confirmed by simultaneous BCR amplification.

Discussion

RT-PCR is useful for analyzing the transcriptional activity of genes and gene isoforms. Multiplex RT-PCR is similar to conventional PCR but includes more than one pair of primers, so that all the known bcr-abl transcripts can be detected. For diagnostic samples, the use of multiplex PCR has been suggested to detect simultaneously several kinds of BCR-ABL and BCR transcripts as internal controls in one reaction by using three BCR and one ABL primers. Multiplex RT-PCR was successfully developed for the first time in our Hematology, Oncology and BMT Research Center for the rapid detection of BCR-ABL transcripts and also to distinguish various BCR-ABL breakpoints in CML patients. The sensitivity of the primer combinations used in our multiplex PCR give a sensitivity of between 10^{-3} and 10^{-4} and can detect all types of fusion transcript in one PCR reaction.

This method allowed us a reliable detection of typical BCR-ABL transcripts, such as b2a2 and b3a2, and atypical types, such as transcripts lacking ABL exon a2 (b2a3 and b3a3), or transcripts resulting from BCR breakoints outside M-bcr, such as e1a2 or e6a2.

BCR-ABL gene rearrangement studies in 75 Iranian Ph+ CML patients showed the frequency of b2a2 and b3a2 transcripts to be 20.4% and 62% respectively. In a study by Reiter et al, 1998 the incidence of b2a2 and b3a2 transcripts in Ph+ CML patients was 31.6% and 68.4% respectively. Lee et al, 1996 found that the frequency of b2a2 and b3a2 transcripts to be 30.2% and 67.9% respectively. Thus in Iranian CML patients, the frequency of b3a2 transcripts was found to be about 3 times higher than that of b2a2.

Although relatively few studies are currently available regarding the significance of bcr-abl transcript type, some preliminary reports suggest that knowledge of transcript type may have clinical meaning or help us to further understand the pathogenesis of t(9;22)-positive leukemias. For example, Perego et al[15] reported that chronic myelogenous leukemia patients with b3a2 transcripts had higher platelet counts than those with b2a2 transcripts. In another study of chronic phase chronic myelogenous leukemia patients, Prejzner[16] suggested that patients with b3a2 transcripts had longer survival than those with b2a2 transcripts. However, We did not observe significant differences between these breakpoint groups with respect to the clinical finding. There are also no differences in the prognosis of patients with b2a2 or b3a2 transcripts.

In this study we found a low incidence of CML patients (5%) expressing more than one type of mRNA. For example, one patient showed coexpression of b3a2 and b2a2 fusion genes. The other patient had a coexpression of three type of fusion genes, b3a2, b2a2 and b3a3 (P203 BCR-ABL). Coexpression of more than one type of fusion transcript in a patient could be due to alternative splicing or for rare type due to existence of several leukemia cell lines with different BCR-ABL transcript expression.

Table 1: Sequence of oligonucleotide primers of multiplex RT-PCR for BCR-ABL transcript as the target gene and BCR transcripts as internal control.

C5e-	5' ATAG-GATCCTTTGCAACCGGGTCTGAA3'
B2B	5' ACAGAATTCCGCTGACCAT-CAATAAG3'
BCR-C	5' ACCGCATGTTCCGGGACAAAAG3'
CA3-	5' TGTTGACTGGCGTGATGTAGTTGCTTGG 3'

Table 2: Patient's clinical data based on BCR/ABL fusion transcript types

Rearrangement	Case (%)	Median age	Gender	WBC	PLTS
b2a2	15 (20%)	32	6/9	200(17-800)	413 (18-900)
b3a2	47 (62.6 %)	38	30/17	215(21-775)	481(100-1200)
b3a3/b2a2	2 (2.6%)	25	2/0	148(110-207)	467 (364-570)
e1a2	1 (1.4%)	28	1/0	400	700
b3a3/b2a3	7 (9.4%)	42	4/3	399(340-462)	690(350-1500)
e19a2	3 (4%)	49	2/1	320(240-400)	865(840-1090)

Figure 1: Schematic diagram of the position of specific primers on the exons of BCR and ABL used in the multiplex RT-PCR assay for the detection of major fusion transcript in patients with CML.

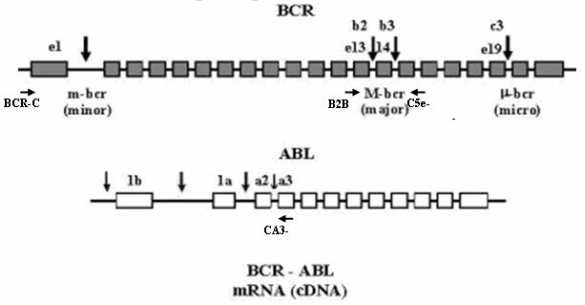


Figure 2: Gel of Multiplex PCR results. M:100bp marker; Line 1, K562 a b3a2-positive cell line; Line 2 and 3 ,from chronic phase CML patients expressing b3a2; Line 4, from chronic phase CML patients expressing b2a2, Line 5, a CML patient in remission which is negative after BMT; Line 6, normal and Line 7 H2O negative control. Lines 1-7 present the 808bp band product, as an internal control, from the BCR gene and indicate the quality of the RNA and efficiency of the cDNA synthesis.

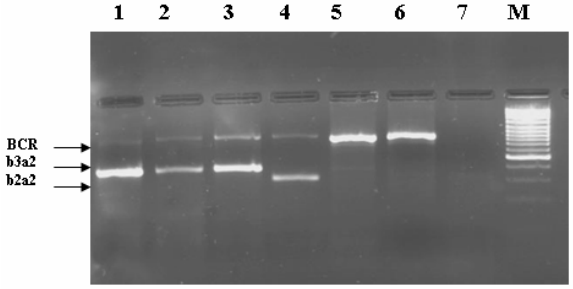
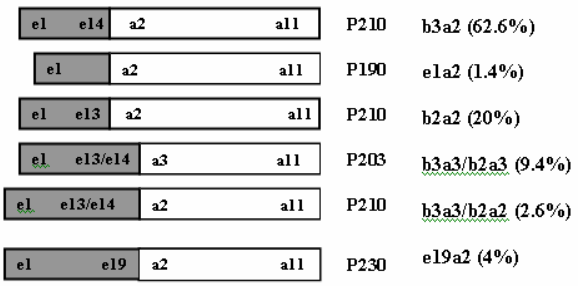


Figure 3: The diagram of several BCR-ABL fusion transcripts and their frequency in Iranian CML patients



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