

Frequency of Bcr-Abl Fusion Oncogene Splice Variants Associated with Chronic Myeloid Leukemia (CML)

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ABSTRACT

BCR-ABL fusion oncogene originates from the reciprocal translocation of chromosome 9 and 22 t(9;22) (q34;q11). It translates a chimeric protein, p210, characterized by constitutive activation of its tyrosine kinase, which triggers leukemogenic pathways resulting in onset of chronic myeloid leukemia (CML). In CML, the classic fusion is b2a2 or b3a2 fusing exon 13 (b2) or exon 14 (b3) of BCR to exon 2 (a2) of ABL. The type of bcr/abl transcripts may be associated with different prognosis and hence useful in therapeutic plan. This study was conducted to calculate the frequency of these splice variants as the frequencies of different fusion oncogenes associated with leukaemia can vary in different geographical regions due to interplay of genetic variation in different ethnic populations, diverse environmental factors and living style. A very sensitive nested RT-PCR was established to detect BCR-ABL splice variants in CML. Sensitivity of RT-PCR assay was of the order of 10⁻⁶. Thirty CML patients were subjected to BCR-ABL analysis. Out of 30 Pakistani patients, 19 (64%) expressed b3a2 while 11 (36%) expressed b2a2 transcript. This shows that BCR-ABL splice variants differ in their frequencies which may have an effect on biology and implications for prognosis and management of BCR-ABL positive Leukemias.

Keywords: BCR-ABL Positive Leukemia, Leukemia Cytogenetics, Philadelphia Chromosome, Chronic Myeloid Leukemia, BCR-ABL Alternative Splicing, BCR-ABL Splice Variants, Leukemia Alternative Splicing, Pharmacogenetics

1. Introduction

Cytogenetically chronic myeloid leukemia (CML) is characterized by the presence of Philadelphia (Ph) chromosome, the diagnostic hallmark of CML, which is present in majority of CML patients. It originates from the reciprocal translocation of chromosome 9 and 22 t(9;22) (q34;q11) [1]. This reciprocal translocation gives rise to BCR-ABL fusion oncogene, which translates a chimeric

protein, p210BCR-ABL that is characterized by constitutive activation of its tyrosine kinase activity. In CML, the classic fusion is b2a2 or b3a2 fusing exon 13 (b2) or exon 14 (b3) of BCR to exon 2 (a2) of ABL [2,3]. Both b3a2 and b2a2 transcripts can be formed as a result of alternative splicing. These transcripts lead to the production of an 8.5 kb transcript coding for a 210-KDa (p210) chimeric protein [4,5]. This constitutively active cytoplasmic tyrosine kinase does not block differentiation, but enhances proliferation and viability of myeloid lineage cells and leads to development of CML [6].

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Genetic abnormalities lead to formation of fusion oncogenes and this phenomenon is driven by the environmental factors and living style which differ in different geographical regions, the frequencies of different fusion oncogenes associated with leukaemia can vary in different ethnic groups [7], which can have a significant effect on the management and prognosis of this type of leukaemia [8,9]. This study was conducted to calculate the frequency of BCR-ABL gene splice variants associated with CML. RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) was chosen as the method of choice to detect BCR-ABL gene as this technique is one of the most sensitive methods for this purpose.

2. Materials and Methods

2.1. Sample Collection

Blood samples of 30 CML patients were collected from different hospitals of Lahore. Clinical features of the patients are given in **Table 1**. Samples were collected in EDTA vacutainer tubes as per manufacturer's instruction with patient number, age and time/date of collection written on the tubes. Sample collection and processing for analysis was always started within 24 hours to minimize mRNA degradation [10]. All the samples were stored at 20°C. The procedure for isolation of RNA was adapted from Chomczynski and Sacchi with slight modification [11,12].

2.2. RNA Extraction and cDNA Synthesis

Quantity of RNA was estimated spectrophotometrically [13], while the quality of RNA was checked by native

agarose gel electrophoresis and formaldehyde denaturing gel electrophoresis [14]. RNA was reverse-transcribed to cDNA for using as template in PCR reaction. RT-reaction protocol and other reaction conditions were adapted from Van-Dongen [15]. Briefly, 10 µL of RNA was added to 10 µL of RT-reaction mixture containing 5X RT buffer (20 mM Tris HCl, 50 mM KCl, pH 8.3, 10 mM DTT), 10 mM dNTPs, 10 mM random hexamer primers, RiboLock™ RNase inhibitor (20 units), M-MuLV Reverse Transcriptase (40 units) (Fermentas, USA) and DPCE-treated water. Reaction was carried out by incubating at room temperature for 10 min, 37°C for 60 min, 99°C for 3 min and held at 4°C in the last step in the PCR machine.

2.3. RT-PCR Amplifications

PCR primers and nested PCR protocols for the detection of BCR-ABL fusion gene in CML patients were adopted from Radich [16]. The sequences of primers are given in **Table 2**.

The cDNA product was PCR amplified in two rounds with 1 U/µL of Taq DNA Polymerase, 125 mM primers, 10x PCR buffer with KCl, 25 mM MgCl₂ and 10 mM dNTP Mix. Sterile water was used as negative control. Thermal cycling conditions for nested PCR were: Preliminary denaturation at 95°C for 2 min followed by 35 cycles of denaturation of double stranded DNA at 95°C for 5 sec, annealing at 55°C for 10 sec and extension at 72°C for 15 sec, followed by a final extension at 72°C for 3 minutes. Round 2 was carried out with the same conditions. The PCR products were run on a 2% agarose gel with ethidium bromide to analyze the size of the amplicons.

3. Results

Primer combinations used in amplification allowed to amplify both types of transcripts in a single reaction. Quality of RNA was analyzed on formaldehyde gel electrophoresis (**Figure 1**) and efficiency of cDNA synthesis on agarose gel electrophoresis. Two types of PCR products were detected as 305bp and 234bp for b3a2 and b2a2 respectively (**Figure 2**). The frequencies of both fusion transcripts were found to be 63.33% and 36.66% for b3a2 and b2a2 respectively. The sensitivity assay was

Table 1. Clinical features of CML patients.

Characteristics	Patient Number (n = 30)
Age (years)	Mean = 35 Range = 12-70
Gender	
Male	21
Female	9
Splenomegaly	26
Hemoglobin (< 10 g/dL)	16
WBC Count (/mm ³)	
50,000 - 100,000	5
> 100,000	25
Platelet Count (/mm ³)	
100,000 - 450,000	22
> 450,000	8
Mode of Diagnosis	
Ph+	30
BCR-ABL+	30

Table 2. Primers used for detection of fusion gene BCR-ABL in CML by RT-PCR.

PCR Round	Primer	Primer Sequence (5'-3')
1 st Round	CML C	5'-GAAGTGTTCAGAAGCITCTCC-3'
	CML D	5'-TGATTATAGCCTAAGACCCGGA-3'
2 nd Round	CML A	5'-TGGAGCTGCAGATGCTGACCAACTCG-3'
	CML B	5'-ATCTCCACTGGCCACAAAATCATACA-3'

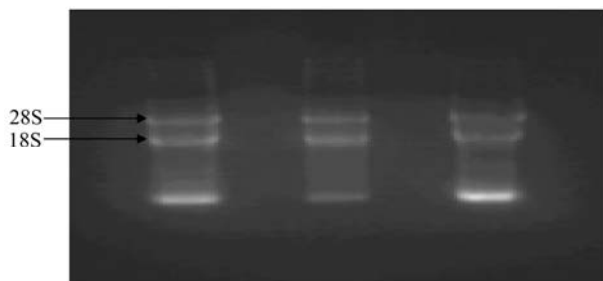


Figure 1. Formaldehyde gel showing bands of RNA from 3 different samples of CML patients.

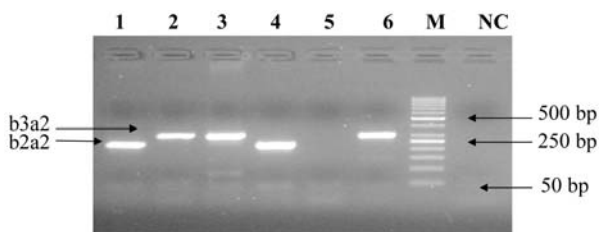


Figure 2. 2% agarose gel containing Ethidium bromide showing two types of characteristics BCR-ABL transcripts b2a2 (1,4) and b3a2 (2,3,6) (234bp and 305bp respectively), M: 50bp Ladder, NC: Negative Control.

also performed by generating 10-fold serial dilutions to evaluate the sensitivity of RT-PCR for detection of these fusion transcripts. Using these dilutions, detection limit of the nested PCR was found upto 10^{-6} of cDNA for each sample (Figure 3).

4. Discussion

RT-PCR is one of the most sensitive techniques to detect BCR-ABL transcripts associated with CML [17]. Real time quantitative PCR is increasingly used to assess treatment response in patients with CML. When the level of residual leukaemia falls below the level of detection by bone marrow cytogenetic analysis, PCR based assays like the real time quantitative PCR or nested reverse transcriptase PCR are the methods of choice for further monitoring [18]. In this study nested PCR was opted as

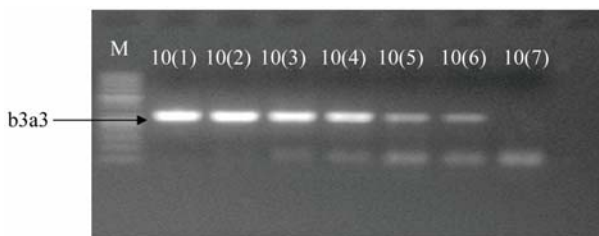


Figure 3. Amplification of BCR-ABL fusion transcript b3a2 (305 bp) from serial dilutions of cDNA ($10^0 - 10^{-7}$) showing a very good sensitivity of nested PCR for the detection of this transcript. M; 50 bp ladder.

the method of choice due to its sensitivity to detect both b3a2 and b2a2 fusion transcripts in a single reaction. In the sensitivity assay of nested PCR, very high-quality results were obtained and this technique showed sensitivity upto 10^{-6} .

In this study, the frequency of b3a2 and b2a2 was found to be 63.33% and 36.66% respectively. The frequency of b3a2 is two times higher than b2a2 with the ratio of 2:1 in our population. The coexpression of transcripts was not found in any of the patients. Spleen was grossly enlarged in most of the patients and mildly enlarged in some but without any correlation to transcript type. Data has been published about the frequency of different types of fusion oncogenes associated with acute lymphocytic leukemia (ALL) in Pakistan [19]. The frequency of BCR-ABL fusion oncogene in Pakistani childhood ALL patients was reported to be 49%, which is higher as compared to other reports from around the world [19]. Frequency of different fusion oncogenes in Pakistani ALL patients is different from other geographic regions [9]. The frequency of fusion oncogenes associated with different leukemia types was compared with western populations and significant differences were observed due to geographical, racial and ethnic differences [7]. In a study carried out by Verschraegen, the frequencies of b2a2 and b3a2 transcripts were 30.2% and 67.9% respectively [20]. Reiter found the incidence of b2a2 and b3a2 transcripts in CML patients to be 31.6% and 68.4% respectively [21]. In Korean CML patients, frequency of b2a2 and b3a2 was collectively found to be 98.18%, corresponding to 67.66% b3a2, and 32.34% b2a2 transcripts [22] where the number of patients with b3a2 was twice the number of patients with b2a2. Frequencies of fusion transcripts in Iranian CML patients were found to be 21% and 62% for b2a2 and b3a2 respectively [23]. The frequency of b3a2 transcripts was found to be almost three times higher than that of b2a2 [23].

Several authors have reported that CML patients with b2a2 BCR-ABL splice variants have a better prognosis and response to imatinib. De-Lemos *et al.* [24] reported that statistically significant difference was found for the levels of expression of transcripts b2a2 and b3a2 at six months of imatinib treatment, which shows that b2a2 may have a better molecular response than b3a2. Verma *et al.* [25] reported that rare variants like e1a2 have an inferior response to imatinib. Sharma *et al.* [26] found that 59% patients with b2a2 type achieved complete cytogenetic response (CGR) as compared to 28% patients with b3a2 ($p = 0.04$) while in 24 patients with minor or no CGR, 25% had b2a2 compared to 75% b3a2 type ($p = 0.04$). Moreover, expression of BCR-ABL/ABL% was higher in b3a2 patients compared to b2a2 ($p = 0.120$).

They also found that pre-treatment characteristics like mean spleen size, mean hemoglobin, mean, and mean platelets counts were not significantly different in the b3a2 vs. b2a2 transcripts groups [26], which supports our findings. These observations highlight the need for more extensive studies in different ethnic groups on the role of different BCR-ABL splice variants in biology [27] and treatment response of the BCR-ABL positive leukemia patients, as acquired BCR-ABL point mutations and other factors associated with imatinib resistance do not explain the reason of imatinib resistance in all BCR-ABL positive patients [28-30]. Studies related to the differences in clinical features and response to treatment in CML and philadelphia positive acute leukemia patients are specially required with the advent and FDA approval of other tyrosine kinase inhibitors (TKIs) like nilotinib and dasatinib for front-line treatment of BCR-ABL positive CML and ALL.

In conclusion, frequencies of BCR-ABL splice variants can vary in different geographical regions due to interplay of natural genetic variations in different ethnic populations, diverse environmental factors and living style. Moreover, the knowledge about the rate of occurrence of these transcripts associated with CML can be of very significance as it can lend a hand to further understand the pathobiology of t(9;22)-positive leukemic cells. Moreover, it will also assist in prognosis, treatment and management of these CML transcripts types.

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6. Conflict of Interest Statement

The authors have no potential conflict of interest.

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