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# Molecular Profiling of Chronic Myeloid Leukemia in Eastern India

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**Molecular breakpoint of the BCR-ABL fusion gene has been characterized for 122 chronic myeloid leukemia patients. Out of 122 cases, 33 b2a2, 69 b3a2, 2 e1a2, and 2 e19a2 cases have been detected. Six coexpressed both b2a2 and b3a2 transcripts. All the coexpressing samples had an A>G polymorphism at the putative splice branchpoint in intron 13. The T>C polymorphism in exon 13, reported to be linked to coexpression, was not present in all the coexpressing patients. No correlation of transcript type with platelet count was detected. Those expressing b2a2 transcript were diagnosed at relatively younger age and with higher white blood cell count, in agreement with other reports. However, the correlation was not statistically significant. Am. J. Hematol. 81:845–849, 2006.** © 2006 Wiley-Liss, Inc.

**Key words: CML; BCR-ABL fusion transcript; polymorphism; RT-PCR**

## INTRODUCTION

Chronic myeloid leukemia (CML) is probably the most extensively studied human malignancy. CML patients (90–95%) harbor the Philadelphia (Ph) chromosome, a shortened chromosome 22, resulting from a reciprocal translocation t(9;22)(q34;q11) between the long arms of chromosome 9 and 22, fusing ABL protooncogene on chromosome 9 with the BCR gene on chromosome 22. Product of the chimeric mRNA transcribed from the hybrid BCR-ABL fusion gene plays a causal role in pathogenesis of the disease. Ph chromosome is not exclusive to CML, and is found in 15–30% of adult and 2–5% of childhood acute lymphoblastic leukemia (ALL), and in <3% of patients with acute myeloid leukemia and occasionally in lymphoma and myeloma patients. The gene is leukemia-specific and can therefore be used as a sensitive marker of the disease and its progression. Ninety nine percent of CML patients have breakpoints that result in a fusion mRNA in which either BCR exon 13 (b2) or exon 14 (b3) (M-BCR) is fused to ABL exon a2 to form the b2a2 or b3a2 transcripts that get translated into an oncoprotein $p210^{BCR\text{-}ABL}$. In 60–80% of patients with Ph-positive ALL, the breakpoint occurs in the first intron of the BCR gene, in a region referred to as the minor breakpoint cluster region (m-BCR), producing the shorter isotype $p190^{BCR\text{-}ABL}$ from the e1a2 type mRNA. A third breakpoint μ-BCR results in e19a2 transcript, and occasionally other breakpoints yielding b2a3, b3a3, e1a3, e6a2, or e2a2 transcript has been detected [1].

Cytogentic analysis used to be the standard technique for monitoring treatment response. However, BCR-ABL transcripts can also be detected by reverse transcription polymerase chain reaction (RT-PCR) technique, which enables molecular characterization of the exact breakpoint and detection of residual leukemic cells at the level of one cell in a background of $10^5$–$10^6$ normal cells [2–4], and is



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currently used in many countries along with the quantitative PCR technique.

Coexpression of both the b2a2 and b3a2 transcripts by alternative splicing is detected in minority of patients (1.4–11%). Coexpression has been linked to two polymorphisms in the patient's DNA; one a thymine (T) to cytosine (C) polymorphism in the eighth position before the end of exon 13 of BCR gene, altering the third nucleotide of the relevant codon; and other, an adenine (A) to guanine (G) polymorphism within intron 13, occurring upstream of the 3′ splice site. The exonic change does not alter the amino acid sequence. The BCR intronic polymorphism, however, occurs at the invariant A of a sequence that is the best match to the poorly conserved branchpoint. It has been proposed that the change results in reduced efficiency of splicing of intron 13 of BCR and BCR-ABL allele [5–7] and use of the acceptor site at the end of intron 14, leading to coexpression of both the transcripts.

Various studies on the possible association of BCR-ABL chimeric mRNA type and variations in the clinical features observed during the course of the disease produced conflicting evidences and generated a debate protracting over several years [8–13], with some in favor [12] and some against [13].

In this article, an attempt has been made to establish the frequency of different type of BCR-ABL transcripts in CML patients of eastern India, in order to obtain more information on BCR-ABL chimeric mRNA type and its possible association with different clinical features observed during the course of CML at chronic phase and to find possible correlation between the polymorphisms at exon 13 and intron 13, with coexpression of b2a2 and b3a2 transcript.

## MATERIALS AND METHODS

### Patients

This study includes 122 patients (82 male, 40 female) who were under treatment in the hematology department at two medical centers in Kolkata from 2001 to 2005. The diagnosis of CML was established on the basis of cytochemical analysis of bone marrow aspirates. All patients except one were in the first chronic phase (CP) of the disease. Most of the patients at present are treated with imatinib mesylate. Written informed consent was obtained from all the patients or their family members.

### Sample Collection and Isolation of Genomic DNA and RNA

Bone marrow (1–2 ml) in sodium heparin tube or peripheral blood (5 ml) in EDTA tubes was collected from the CML patients. Genomic DNA was isolated from whole blood/bone marrow samples, as described earlier [14]. Total RNA was isolated from peripheral blood and/or from bone marrow aspirate after lysis of red blood cells by QIA amp kit (Qiagen, Germany) following manufacturer's protocol.

### Characterization of Junctional Breakpoint by RT-PCR

cDNA synthesis was performed in a total volume of 20 μl, with 400 ng to 1 μg of RNA and other reagents according to manufacturer's protocol (TaqMan Reverse Transcription Reagent Kit, Applied Biosystem, USA). PCR in a reaction volume of 50 μl containing 10 μl of RT products, 5.5 mM $MgCl_2$, 200 μM of each dNTPs, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystem, USA), primer concentration and sequence were as described earlier [3,4]. The positive controls were derived from RNA sample of b2a2- and e1a2-positive CML patients, who has been confirmed by sequence analysis, and K562 cell line RNA was used as control for b3a2. The negative control was normal lymphocyte RNA. The PCR products were visualized directly on ethidium bromide-stained 3% agarose gel. ABL gene was used as internal control.

### Genotyping of BCR Exon 13 Polymorphism

The T to C polymorphism of BCR exon 13 at eighth position before the junctional region of BCR-ABL transcript was detected by PCR, using allele specific primer (BCR (M): GCATTCCGCTGACCATCAAC, BCR (N): GCATTCCGCTGACCATCAAT and common reverse primer BCR (R): GCATAGCTCTTCCTTCTGAA). The allele-specific primers amplified a 158 bp fragment when the corresponding allele was present. The 25-μl PCR reaction mixture contained 300-ng genomic DNA, 1× Buffer (MBI Fermentus, Lithuania), 1.5 mM $MgCl_2$, 5 pmol of each primer, 200 μM dNTP, and 1.25 U Taq polymerase (MBI Fermentus). After denaturation for 5 min at 95°C, PCR was performed: 30 cycles for 45 sec at 95°C, 45 sec at 60°C, and 1 min at 72°C. The last elongation step was extended to 5 min at 72°C. A 323-bp fragment of β-globin cluster amplified by primers (F: AGTGCTGCAAGAAGAACAACTACC and R: CTCTGCATCATGGGCAGTGAGCTC) was used as internal control (Fig. 1). The fragments were resolved by 2% agarose gel electrophoresis. The protocol was validated using positive and negative controls, which have been genotyped by direct sequence analysis.

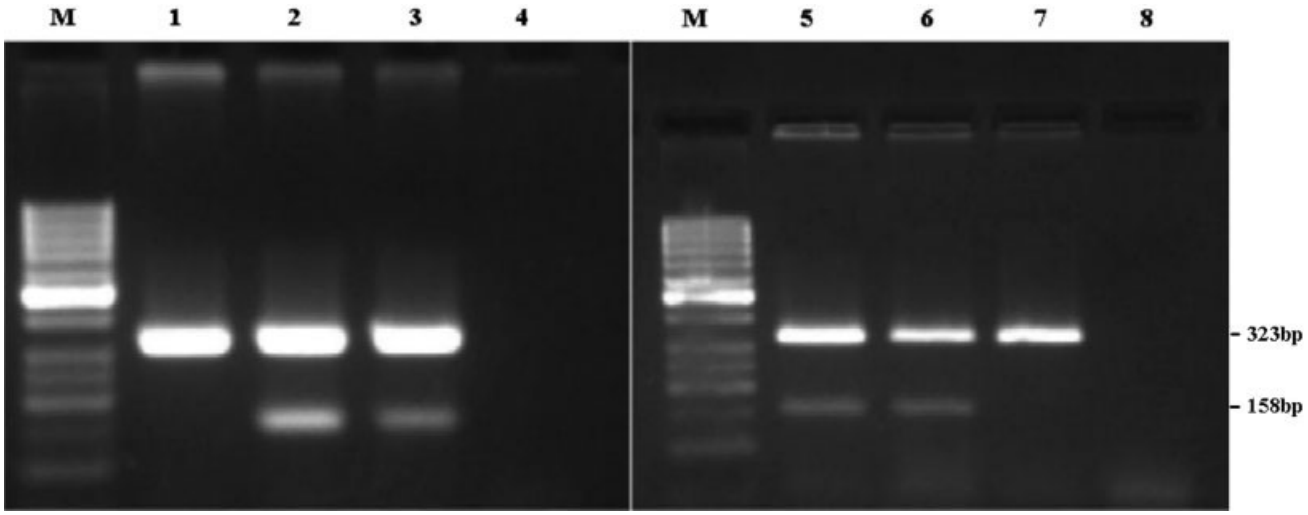


**Fig. 1. Lane M, 50-bp ladder marker; Lanes 1–4, mutation specific primer; Lanes 5–8, normal specific primer. Lanes 1 and 5, normal; Lanes 2 and 6, polymorphism heterozygote; Lanes 3 and 7, polymorphism homozygote; Lanes 4 and 8, no template control.**

### Genotyping of BCR Intron 13 Polymorphism

The A > G polymorphism destroys a Sau3A1 restriction site that was used for typing it. A 101-bp DNA fragment amplified using forward primer IntBCR13 (5′ GTCACCTGCCTCCCTTTCC) and reverse primer ExBCR14 (5′ ACGATGACATTCAGAAACCCATAG) was digested with Sau3A1 and the products were visualized by agarose gel electrophoresis.

### Sequencing Reaction

The BCR-ABL junction sequence, as well as the intron 13 and exon 13 polymorphisms, was initially determined by sequencing relevant products using Thermosequenase cycle sequencing kit Version 2.0 (Amersham, USA). Once the RT-PCR and PCR technique had been validated, the consecutive patients were classified on the basis of the PCR product size.

## RESULTS

Of the 122 CML patients, 112 (91.8%) patients were positive for one or more of the four junctional types tested. The b2a2 variant of BCR-ABL transcript was detected in 33 (29.47%) patients, b3a2 in 69 (61.61%) patients, both b2a2 and b3a2 transcripts were detected in six (5.36%) patients. Only two (1.78%) patients expressed e1a2 transcript, and two (1.78%) had e19a2 transcript.

One of the e1a2 expressing patients, a 58-year-old male had a very short period of CP and progressed to blast crisis and died. Other patient, a male aged 35 years, was treated with hydroxyurea, and progressed toward blast crisis stage in about 5 years. He was then treated with imatinib mesylate and is well maintained for the last 1 year. The e19a2 expressing patients have been described in detail elsewhere.

The clinical data, i.e., sex, age, white blood cell (WBC) count, and platelet count at diagnosis before the start of chemotherapy, were compared among patients with b3a2 and b2a2 junctional breakpoints (Table I).

Coexpression of b2a2 and b3a2 transcripts has been correlated with two polymorphisms, one at intron 13 and other at exon 13. In the present study, typing of the six patients coexpressing both the transcripts showed that all of them harbored the polymorphism at the intron, but not the one at the exon. Specifically, 4 coexpressing patients had GG, and 2 had GA genotype at the intron. At the exon, 2 were TT, 3 were TC, and 1 was of CC genotype (Table II).

Some essential thrombocythemia (ET) patients are Ph-positive, and they mostly express the b3a2 transcript [15–17] and only a few the b2a2 transcript. One of our b2a2 expressing patients had ET with several features resembling CML. Clinical data of the patient are as follows: age, 30 years; sex, male; Hb, 7.7 g/dl; RBC, $3.3 \times 10^6/mm^3$; WBC, $12.5 \times 10^9/l$; differential count neutrophils, 83%; lymphocytes, 6%; monocytes, 2%; eosinophils, 1%; basophils, 8%; platelets, $2{,}245 \times 10^9/l$; MCV, 74.5 fl; MCH, 22.6 pg; MCHC, 30.3 g/dl; reticulocytes, 1%. In bone marrow, megakaryocytes increased in

**TABLE I. Clinical Parameters at Diagnosis of CML Patients in Chronic Phase (CP) Expressing the b2a2 or b3a2 Type of the BCR-ABL Rearranged mRNA**

| | | | Stratification of the patients on the basis of WBC count | | | |
|---|---|---|---|---|---|---|
| | | | WBC < 100 ($10^9$/l) | | WBC > 100 ($10^9$/l) | |
| Parameter | b2a2 ($n = 17$) | b3a2 ($n = 31$) | b2a2 ($n = 8$) | b3a2 ($n = 11$) | b2a2 ($n = 9$) | b3a2 ($n = 20$) |
| Male/female | 15/2 | 21/10 | 7/1 | 8/3 | 8/1 | 13/7 |
| Age (yrs) | | | | | | |
| Mean ± SEM | 34.2 ± 3.2 | 38.5 ± 2.7 | 40.7 ± 4.9 | 44.3 ± 2.5 | 29.2 ± 3.8 | 35.2 ± 3.8 |
| Median | 30.5 (21–61)[a] | 40 (6–71) | 34 (30–61) | 46 (32–57) | 27 (21–58) | 37.5 (6–71) |
| WBC ($10^9$/l) | | | | | | |
| Mean ± SEM | 161 ± 34.6 | 162.3 ± 38.0 | | | | |
| Median | 162 (12.5–448) | 120 (15–1220) | | | | |
| Platelets ($10^9$/l) | | | | | | |
| Mean ± SEM | 419 ± 107.3 | 378.2 ± 77.7 | 537 ± 248.5 | 307 ± 72.7 | 338 ± 60 | 437 ± 122 |
| Median | 373 (57–2245) | 250 (59–2262) | 381 (57–2245) | 250 (145–1007) | 340 (87–728) | 250 (59–2262) |

[a]Values in parentheses indicate ranges.

**TABLE II. Six Patients Expressed Both b2a2 and b3a2 Type of BCR-ABL Transcript**

| Patients no. | Sex (M/F) | Age (yrs) | WBC ($10^9$/l) | Platelet ($10^9$/l) | BCR exon 13 polymorphism (T > C) | BCR intron 13 polymorphism (A > G) |
|---|---|---|---|---|---|---|
| 1 | F | 3 | 50 | 250 | TT | GG |
| 2 | F | 28 | 35 | 198 | CC | GG |
| 3 | M | 58 | 50 | 150 | TC | AG |
| 4 | M | 45 | 157 | 250 | TC | GG |
| 5 | F | 6 | 172 | 598 | TT | AG |
| 6 | M | 53 | 337 | 650 | TC | GG |

number, and both active and inactive forms were seen.

## DISCUSSION

The significance of the different types of transcript, and whether they have any correlation with the age at diagnosis, sex, race, clinical parameters, and outcome of the disease has been analyzed in many studies.

The distribution of transcript type has been studied in the European, and some other populations [11,18,19], with the ratio of b2a2 to b3a2 transcripts being roughly of the order of 40:60. However, one study on Ecuadorian population registered a very different ratio of 95:5 [20]. Our data, which is probably the first such report on Indian population is consistent with the studies on Caucasian population and does not show any large deviation.

It has been reported that the subgroup of patients who express b2a2 mRNA in CP CML had a high WBC count at the time of diagnosis and were younger with short CP duration [11]. Our b2a2 carrying patients were also relatively younger, with WBC count on the higher side. However, statistical significance could not be reached because of the small sample size and the time elapsed, since the start of the study is too short to distinguish CP duration between the two groups.

Two different groups reported that patients with 3′ M-BCR breakpoint [21,22] or with b3a2 transcripts [23] had significantly higher number of platelets. However, a third series [24,25] showed no correlation between the type of BCR-ABL transcript and platelet counts. A randomized CML trial in United Kingdom also failed to detect a correlation between either genomic or RNA findings and platelet numbers [13]. Our data also showed no correlation between the transcript type and platelet counts at diagnosis. However, when the UK series was divided into two groups according to the WBC counts, it was observed that, in patients with $<100 \times 10^9$ WBC/L, b3a2 transcript carriers had significantly higher platelet counts. Such a correlation was not observed (Table I) in our data even when the patients were subgrouped according to WBC count.

Coexpression of b2a2 and b3a2 transcript has been linked to two polymorphisms, T > C at exon 13 and A > G at intron 13. Analysis of the six patients coexpressing both transcripts in this study showed that the exonic polymorphism is not obligatory for coexpression. However, the intronic polymorphism was detected in all these six patients, at least in heterozygous state. Considering that the BCR intronic polymor-

phism occurs at the invariant A of the splice branchpoint, it is most likely that the polymorphism results in reduced efficiency of intron 13 splicing and alternative transcription of the normal BCR and BCR-ABL alleles, as suggested by other authors [7].

In summary, this work is the first description of distribution of different BCR-ABL fusion transcripts in CML patients of Indian origin and their clinical parameters. Lack of the exon 13 polymorphism in some coexpressing patients of this study shows that the polymorphism does not have any role in dual expression of b3a2 and b2a2 transcripts and the intron 13 polymorphism at the putative branchpoint is necessary and sufficient for dual expression.

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