Dual transcripts of *BCR-ABL* & different polymorphisms in chronic myeloid leukaemia patients

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Background & objectives: Chronic myeloid leukaemia is (CML) characterized by the presence of a hallmark chromosomal translocation, the Philadelphia chromosome. Although there are many reports available regarding the different variants of *BCR-ABL* in CML, we studied the co-expression of e13a2 and e14a2 transcripts and a few polymorphisms in CML patients.

Methods: Molecular genetics approach was adapted to screen for polymorphisms, mutation and translocation in *BCR*, *ABL* kinase domain and *BCR-ABL* breakpoint region in 73 CML patients.

Results: All eight patients with dual transcripts were found to harbour an exonic polymorphism (c.2700 T>C) and an intronic polymorphism (g.109366A>G) that were earlier reported to be associated with co-expression of both the transcripts. We also observed c.763G>A mutation in *ABL* kinase domain and two polymorphisms, c.2387 A>G and c.2736A>G in the *BCR* gene.

Interpretation & conclusions: Though our data support the previous findings that co-expression of *BCR-ABL* transcripts is due to the occurrence of exonic and intronic polymorphisms in the *BCR* gene, it also shows that the intronic polymorphism can arise without the linked exonic polymorphism. The occurrence of *ABL* kinase domain mutation is less frequent in Indian population.

Key words *ABL* kinase domain - bipolar disorder - chronic myeloid leukaemia - dual transcript - Philadelphia chromosome - polymorphism

Chronic myeloid leukaemia (CML), a clonal disorder of the haematopoietic stem cells is characterized by the presence of Philadelphia (Ph) chromosome. The Ph chromosome is generated as a result of reciprocal translocation between the long arms of chromosome 9 and 22 wherein, a part of Abelson (c-*ABL*) gene from chromosome 9 fuses with the Breakpoint Cluster Region gene (*BCR*) of chromosome 22, resulting in the formation of *BCR-ABL* oncogene. About 95 per cent of CML patients have Ph chromosome and it has been reported to be present in 25-30 per cent of acute lymphoid leukaemia (ALL) and occasionally in acute myeloid leukaemia (AML) patients[1]. The *BCR-ABL* oncogene is translated into BCR-ABL oncoprotein that shows constitutive tyrosine kinase activity[1]. Depending on the breakpoint in the *BCR* gene, different

transcripts of *BCR-ABL* oncogene are reported. It was observed that most of the breakpoints involve exon 13 or exon 14 of the *BCR* major (M-*BCR*) region which fuses with the exon 2 of *ABL* gene resulting in e13a2 or e14a2 mRNA that gets translated into p210 kDa BCR-ABL oncoprotein[1]. There are also reports with breakpoints in exon 1 of *BCR* minor (m-*BCR*) region giving rise to e1a2 transcript that gets translated into a p190 kDa oncoprotein. The p190 kDa oncoprotein is mostly related to ALL[1]. There are a few reports where the breakpoint occurs in the exon 19 of *BCR* micro (μ-*BCR*) region giving rise to e19a2 transcript which encodes for a 230 kDa oncoprotein which comprises CML with neutrophilic predominance (CML-N)[2,3]. Other transcripts such as b2a3, b3a3, e1a3, e6a2 or e2a2 have also been reported[4].

The occurrence of dual transcripts has been linked to two polymorphisms, a thymine (T) to cytosine (C) in exon 13 of *BCR* gene altering the third nucleotide in the relevant codon (AAT to AAC) resulting in a silent mutation without altering the amino acid (Asp to Asp) and the other one is adenine (A) to guanine (G) polymorphism within intron 13 of the *BCR* gene[5]. Branford *et al*[5] have earlier proposed a mechanism for the co-expression of both the transcripts. The intronic polymorphism that occurs at invariant A is a poorly conserved breakpoint that results in reduced efficiency of exon 14 skipping and alternative transcription of *BCR* and *BCR-ABL* alleles. This further results in the activation of a cryptic branchpoint that leads to reduced efficiency of *BCR* intron 13 splicing and *BCR-ABL* allele and alternatively leading to the use of acceptor site at the end of intron 14, hence resulting in the co-expression of both the transcripts[5].

Here we report the co-expression of e13a2 and e14a2 transcripts, a kinase domain mutation and a set of polymorphisms in the *BCR* gene, which are also known to be associated with other diseases, in CML patients.

Material & Methods

A total of 73 consecutive patients diagnosed with CML[3,6] (70-chronic phase, 1-accelerated phase and 2-blast crisis phase) from January 2013 to March 2015, and five healthy controls (family members of patients who gave consent) were included in the study (38 patients from Sparsh Hospital & Critical Care Unit, Bhubaneswar and 35 from SCB Medical College, Cuttack). Written informed consent was obtained from all patients and the study was approved by the ethics committee of the Institute of Life Sciences, Bhubaneswar, Odisha, India. The inclusion criteria included patients with CML above the age of 18 yr. Pregnant women and terminally ill patients were excluded.

DNA extraction, RNA isolation and cDNA preparation: Peripheral blood samples (0.5-0.8 ml) were collected in an EDTA coated vacutainer tube. Genomic DNA was extracted from the peripheral blood of all control and CML patients using Genelute Blood genomic DNA kit (Sigma-Aldrich, USA) following the manufacturer's protocol. RNA was isolated from the peripheral blood of the control and patients using Ambion's mirVana miRNA isolation kit (Life technologies, USA) following the manufacture's instruction. Complementary DNA (cDNA) was prepared from 50-200 ng of RNA using First strand cDNA synthesis kit (Thermo Scientific, USA). PCR primers were designed to amplify the breakpoint region (*BCR-ABL*) [breakpoint region forward (F) and reverse (R)], e13a2 and e14a2 transcript specific forward primers (*BCR* exon 13 F and *BCR* exon 14 F) and a common *ABL* R primer, respectively to confirm the presence of dual transcript, *ABL* gene (*ABL* control F and R), *BCR* gene (*BCR* exon 10 F and 13 to check for polymorphisms in *BCR* region and *BCR* intron 13 F and *BCR* exon 14 R to check for the presence of intronic polymorphism) and the *ABL* kinase domain (First PCR-breakpoint region F and *ABL* exon 10 R followed by a second PCR using *ABL* kinase domain F and R primers). The list of primers used in the study is shown in Table I[5,7-11]. Polymerase chain reaction (PCR) was performed using the Eppendorf Mastercycler gradient (Eppendorf Scientific, Germany). For amplification of the breakpoint region (*BCR-ABL*), *BCR* gene, *ABL* gene and *ABL* kinase domain from the cDNA, the PCR mixture (15 μl) contained 100 mM Tris HCl (*p*H 8.8), 50 mM KCl, 1.5 mM MgCl$_2$, 0.1 per cent triton X-100, 10 mM dNTPs (deoxynucleotides), 10 pmol of the forward and reverse primers, 2 units of DyNAzyme II DNA polymerase (Thermo Scientific, USA). The products were resolved in agarose gel and the desired amplicons were purified using GenElute gel extraction kit (Sigma-Aldrich, USA) and sequenced on Applied Biosystems Genetic analyzer 3500 (Life Technologies, USA).

Table I. List of primers used in the study

| Gene	Primers used (5'-3')	Reference
ABL control F	GTATCATCTGACTTTGAGCC	7
ABL control R	GTACCAGGAGTGTTTCTCCA	7
Breakpoint region F	CGCACCGGCAAGAGTTACAC	8
Breakpoint region R	GGTTGGGGTCATTTTCACT	8
ABL Kinase domain F	TCCCCCAACTACGACAA	8
ABL Kinase domain R	AGAACTTGTTGTAGGCCAG	8
ABL exon 10 R	CCTGCAGCAAGGTACTCACAGC	8
BCR exon 10 F	AGCTTCCAGATGGTGGATGA	Self designed
BCR exon 13 R	CACACGAGTTGGTCAGCATC	10
BCR intron 13 F	GTCACCTGCCTCCCTTTCC	5
BCR exon 14 R	CGATGACATTCAGAAACCCATAG	5
BCR exon 13 F	GCATTCCGCTGACCATCAAT	9
BCR exon 14 F	TCCACTCAGCCACTGGATTTAA	11
ABL R	TGGGTCCAGCGAGAAGGTT	11
F, forward; R, reverse		

Mutation detection: Sequence data were analyzed using the Chromas Lite v 2.01 software (*http://www.technelysium.com.au/chromas_lite.html*) and blast searched using NCBI nucleotide Blast, aligned with reference Genbank cDNA sequence (*BCR* - NM_004327 and *ABL* - NM_005157) using EMBL-EBI clustal Omega multiple sequence alignment tool (*http://www.ebi.ac.uk/Tools/msa/clustalo/*). The disease causing potential of the altered sequences was evaluated using Mutation Taster software tool (*http://www.mutationtaster.org*). The mutations were checked with already available mutations and polymorphisms in the dbSNP database (*http://www.ncbi.nlm.nih.gov/SNP/*).

Results & Discussion

Of the 73 CML patients evaluated, 32 (44 %) were positive for e13a2 and 33 patients were positive for e14a2 (45%) transcript and eight (11%) expressed both e13a2 and e14a2. Of these eight patients, five were in chronic phase (CP), one in accelerate phase (AP) and two in blast crisis phase (BC) (Fig. 1). We investigated for the occurrence of associated polymorphisms in *BCR* gene in these patients. The exonic polymorphism (2700 T>C, N900N) was observed in 18 of 32 (56.25%) e13a2 patients (homozygous), 5 of 33 (15.15%) e14a2 patients (homozygous) and all eight (100%) patients with dual transcripts (6/8 homozygous condition and 2/8 heterozygous condition) (Fig. 2A). The intronic polymorphism (g.109366A>G) was also observed in

all eight patients with dual transcripts (1/8 homozygous condition and 7/8 heterozygous condition) (Fig. 2B). In addition, a few other polymorphisms were also observed in the *BCR* gene of the patients with dual transcripts; N796S (rs140504) polymorphism in all and Y910C (rs35537221) polymorphism in one patient (Fig. 2C, 2D). As three patients were in the advanced phase of CML, we also looked for possible kinase domain mutation in these patients. An E255K mutation was observed in one of the blast crisis patients (Fig. 2E). This patient also had the N796S and Y910C polymorphism. The list of polymorphisms and mutations that were observed is summarized in Table II (Fig. 2F).

Co-expression of e13a2 and e14a2 transcripts has been reported in certain population groups: 13 per cent of Mexican, 2.5 per cent of Mexican Mestizos, 5.36 per cent of east Indian, 4.6 per cent of Sudanese and 4.38 per cent of north Indian population[9, 12-15]. The occurrence of dual transcripts is not phase specific as it has been observed in all phases of the disease. Branford *et al*[5], have shown earlier that the presence of *BCR*'*s* intronic polymorphism is associated with the activation of cryptic splice site that most likely results in reduced efficiency of RNA splicing and the skipping of exon 14 in *BCR* and *BCR-ABL*. Mondal *et al*[9] reported six patients with dual transcripts from eastern India. They observed intronic polymorphism in
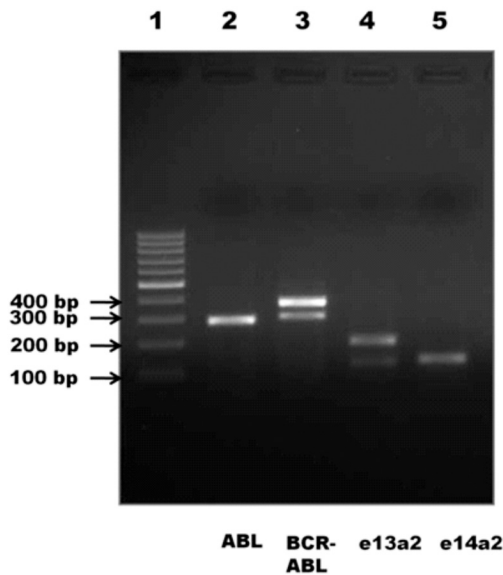
Fig. 1. Agarose gel electrophoresis of dual transcript samples. Lane 1- 100 bp ladder, lane 2- *ABL* control (288 bp), lane 3- breakpoint region (*BCR-ABL*) (e13a2-308 bp and e14a2-383 bp), lanes 4 and 5- e13a2 (123 bp and 198 bp) and e14a2 (129 bp) transcript specific PCR, respectively.

all six individuals at least in heterozygous condition, however, the exonic polymorphism was not observed in all the patients. Although our study included only 73 patients, but the frequency of co-expression of both e13a2 and e14a2 was more when compared to other study groups from India. Our study also shows that the intronic polymorphism arises without the linked exonic polymorphism in the Indian population.

Although the acquisition of *BCR-ABL* is enough for the initiation of CML, the occurrence of additional secondary mutations is required for the progression of the disease from the initial chronic phase to the fatal blast crisis phase. Several study groups have reported the presence of *ABL* kinase domain mutations as one of the molecular mechanisms for drug resistance and disease progression[16]. Kuila *et al*[8], have earlier reported that the occurrence of *ABL* kinase domain mutation in Indian population is less frequent when compared to other populations.

Hashimoto *et al*[17], in a study in 171 Japanese patients reported that N796S polymorphism was
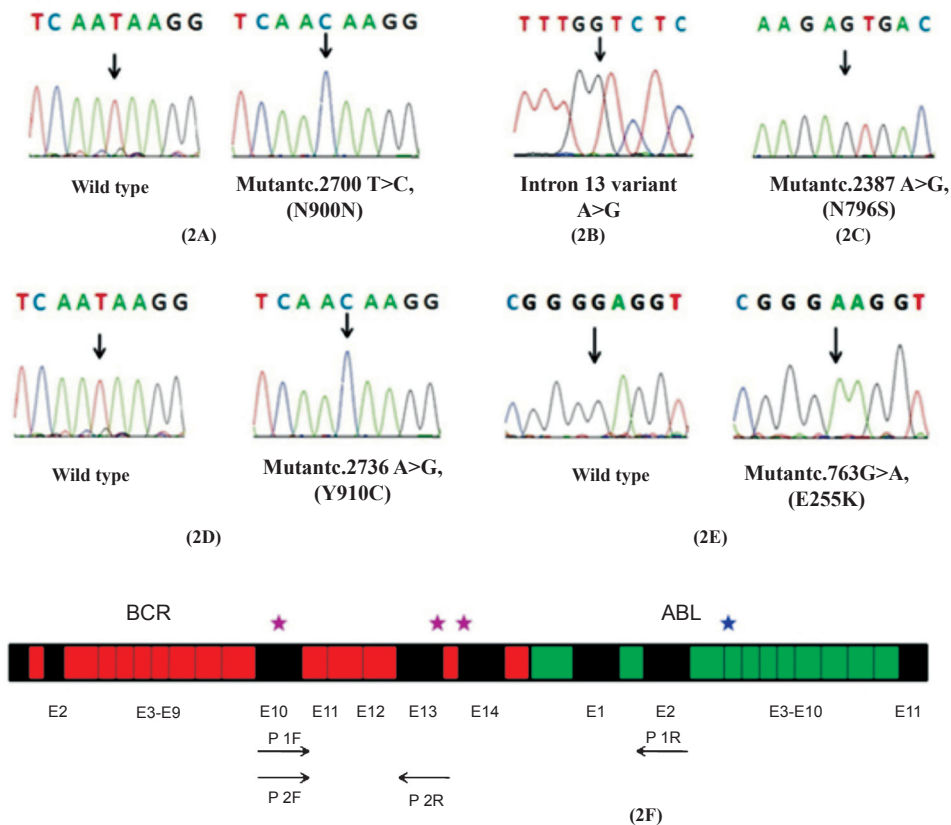
Fig. 2. Electropherogram of wild type and mutant alleles of patients 1- 8. The mutation sites are indicated by arrows **(Fig. 2A-2E)** and schematic representation of the various polymorphisms, mutations are represented by asterisks (*) and the positions of the primers are indicated by arrows in Fig. **2F**.

Table II. Representation of mutation and single nucleotide polymorphism observed in patients with dual transcripts

	Patient 1	Patients 2, 3 and 4	Patient 5	Patient 6	Patient 7	Patient 8
Kinase domain mutation	E255K (c.763G>A)	-	-	-	-	-
Transcript	e13a2, e14a2	e13a2, e14a2	e13a2, e14a2	e13a2, e14a2	e13a2, e14a2	e13a2, e14a2
Disease status	CML-BC	CML-CP	CML-CP	CML-AP	CML-BC	CML-CP
c.2387 A>G (N796S) SNP	Yes	Yes	Yes	Yes	Yes	Yes
c.2736A>G (Y910C) SNP	Yes	No	No	No	No	No
c.2700 T>C (N900N) SNP	Homozygous (C/C)	Homozygous (C/C)	Homozygous (C/C)	Homozygous (C/C)	Heterozygous (C/T)	Heterozygous (C/T)
Intron 13 variant g.109366A>G	Heterozygous (A/G)	Heterozygous (A/G)	Heterozygous (A/G)	Heterozygous (A/G)	Homozygous (G/G)	Homozygous (G/G)

BC, blast crisis phase; CP, chronic phase; AP, accelerated phase; SNP, single nucleotide polymorphism

strongly associated with bipolar II disorder. Another study reported that bipolar disorder was associated with interferon-alpha treatment[18]. Although interferon-alpha was used in CML therapy, we found this particular polymorphism in control samples also. Thus, this polymorphism may not have any association with interferon alpha treatment or bipolar disorder. We also observed Y910C (rs35537221) polymorphism in one patient. Though this polymorphism was predicted to be disease causing from mutation taster prediction tool, it occurs in 1000 Genome Project (TGP) and thus it is likely to be a polymorphism. No possible disease was found to be associated with this polymorphism but it was reported as a germ line variant by Greenman *et al*[19]. Isokpehi *et al*[20] have also reported it as a non-synonymous polymorphism that can be used as a candidate marker for arsenic responsiveness in protein targets.

In conclusion, our study shows the frequency of dual transcripts in 11 per cent of our patients. It also suggests that the intronic polymorphism can arise without the linked exonic polymorphism.

Acknowledgment

***Conflicts of Interest*:** None.

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