Prognostic Implication of BCR-ABL Fusion Transcript Variants in Chronic Myeloid Leukemia (CML) Treated with Imatinib. A First of Its Kind Study on CML Patients of Kashmir

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Abstract

Background: The prognostic significance of the common BCR-ABL transcripts like e13a2 (b2a2) and e14a2 (b3a2) in Chronic myeloid leukemia (CML) has been reported from patients treated with different tyrosine kinase inhibitors but its impact on clinical response and overall survival remains still unexplored. The aim of this study was to evaluate the prognostic significance of different transcript types in a cohort of CML patients treated with imatinib.

Methods: A total 42 confirmed cases of Chronic Myeloid Leukemia (CML) patients were recruited into our cohort study and a multiplex Reverse Transcriptase-Polymerase Chain Reaction technique (RT-PCR) was used to detect 3 main transcript types ‘e1a2’, ‘e13a2’, and ‘e14a2’ found in CML. Results: Only two types of transcripts e13a2 (b2a2) and e14a2 (b3a2) were detected in our CML patients and none had the e1a2 type. All the patients were RT-PCR positive for either e13a2 or e14a2 fusion transcript demonstrating 100% concordance with their Ph+ve cytogenetic status at baseline. TLC count (range of 201-600x10³/µl) and platelet count (range of 201-900x10³/µl) at baseline were found to be associated more with the e14a2 (b3a2) than the e13a2 (b2a2) transcript type (p-value: 0.001). The two transcripts found did not relate significantly towards sex, age-group or indicated spleen size ranges as well as percentage ranges of blast cells. Conclusion: We conclude that there is no overall prognostic implication of either the e13a2 or the e14a2 transcript type across the spectrum of indicated clinical parameters evaluated. Even the overall survival analysis of the two transcript types revealed no prognostic association whatsoever.

Keywords: BCR-ABL- chronic myeloid leukemia- imatinib- survival- RT-PCR

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Introduction

Chronic Myeloid Leukemia, the first neoplasm in humans to be associated with a single, specific, acquired genetic lesion (Nowell et al., 1960), is one of the best-understood myeloproliferative disorders at the molecular level. It comprises ~20% of all adult leukemias and is diagnosed at a median age of approximately 50 years. The disease originates from the transformation of a hematopoietic stem cell with resultant expanding myelopoiesis that characteristically evolves through three phases when untreated: 1) a chronic phase of four to five years duration manifest by myeloid hyperplasia with circulating granulocytes that are present in all stages of maturation; 2) an accelerated phase of shorter duration during which myeloid elements begin to lose the ability to differentiate; and 3) inevitably, a blast phase of acute leukemia of myeloid (70%) or lymphoid (30%) phenotype. CML tends to be a disease of middle life with a slowly increasing age trend in Western countries. The median age at presentation reported from large cohort studies is 45–55 years (Kantarjian et al., 1993; Kantarjian et al., 1996; Kantarjian et al., 1998). In India, CML is seen prevalent in third and fourth decades (Deshmukh et al., 2005) and the median age of onset is 38–40 years (Bhutani et al., 2002) compared to about 50 years in the West. Here in Kashmir (North India), our institute happens to be the only referral centre for leukemias and on an average; we receive 3-4 patients with CML every month with almost even gender distribution. As per a study (Pandith et al., 2012), the leukemia rank fifth in order of occurrence among the cancers prevalent in Kashmir valley, wherein CML figures as the second most incident leukemia after Acute Lymphoid Leukemia (ALL).

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The reciprocal t(9;22)(q34;q11) translocation is identified as the initial transforming event in the development of CML although some data suggest that it may be preceded by clonal hematopoiesis. The translocation yields a shortened chromosome 22 called the Philadelphia (Ph) chromosome (Rowley et al., 1973). The molecular basis of CML is the formation of the BCR-ABL fusion gene as a result of Ph translocation. The 5′part of the gene consists of BCR derived exons and the 3′end of ABL originating sequences. The breakpoints in ABL lie at the 5′part of the gene, upstream of exon 2 (termed a2). In the vast majority of CML patients (95%) and approximately one third of Ph+ ALL patients the BCR gene breaks in the 5.8 kb breakpoint cluster region (bcr) spanning exons 12 to 16 (formerly referred as exons b1-b5), termed the major bcr (M-bcr). As a result of alternative splicing, either b2a2 or b3a2 (also called e13a2 and e14a2, respectively) transcripts are formed. Two other breakpoint cluster regions in the BCR gene have also been characterized: the minor-bcr (m-bcr) and micro-bcr (µ-bcr) regions. Rarely, the break in BCR involving exons 1 and 2, produces the e1a2 transcript (190-kDa protein), or between exons 19 and 20, producing the e19a2 transcript (230-kDa protein) (Li et al., 1999; Faderl et al., 1999; Verschraegen et al., 1995, Langabeer et al., 2013). There are few reports who have studied the prognostic significance of the BCR-ABL1 transcripts (Prejzner et al., 2002; Tefferi et al., 1986). Improved response has been reported in patients carrying the e14a2 (b3a2) transcript compared with those with the e13a2 (b2a2) transcripts after treatment with standard-dose Imatinib (Lucas et al., 2009; de Lemos et al., 2005; Hanfein et al., 2014).

The aim of this study was to evaluate the prognostic significance of different transcript types in a cohort of CML patients treated with imatinib.

Materials and Methods

A total of 42 patients all of whom happened to be in chronic phase of CML were recruited into our cohort study after their screening from the Departments of Medical Oncology, Sher-i-Kashmir Institute of Medical Sciences (SKIMS) and Clinical Hematology, SKIMS. Informed consent from each patient as well as the approval from the ‘Institute Ethics Committee’ (IEC) of SKIMS was obtained prior to start of the research program. Patients were subjected to prospective evaluation and analysis with respect to the requirements of the objectives of research program at the Department of Immunology and Molecular Medicine, SKIMS. Patients were recruited from October 2013 to November 2014 and followed up till May 2016. The diagnosis of CML was based on characteristic peripheral blood smear analysis and complete blood profiling along with bone marrow examination findings of the patients. The mean age of the 42 patients was ~47 years and the time range of survival between diagnosis and the follow-up cut off i.e. May 2016 among our patients was from 12 months to 31 months with the mean survival time being 23.76 months.

3-4 ml of peripheral blood was collected from each CML patient into a lavender-top EDTA vacutainer.

Samples were put to density gradient centrifugation (Ficoll, Sigma) and the white cell component of the peripheral whole blood was subjected to Trizol (Ambrosio) RNA extraction. The extracted RNA was analysed for purity and integrity by DEPC-treated Gel electrophoresis. The RNA was reverse-transcribed by Maxima cDNA synthesis kit and subjected to Multiplex RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) or qualitative PCR analysis for fusion gene transcript genotyping. We adopted a multiplex RT-PCR protocol used by (Cross NCP et al., 1994) to detect 3 main transcript types of ‘e1a2’, ‘e13a2’, and ‘e14a2’ and the primer sequences (Eurofin Oligo) as detailed along with the expected transcript amplicons generated (Table 1). The thermal conditions used were as follows: Cyclic denaturation at 94°C for 35 seconds; Cyclic annealing at 61°C for 30 seconds; Cyclic extension at 72°C for 30 seconds and Final extension at 72°C for 7 minutes.

All the cyclic steps in the thermal profile were repeated 35 times. The step of initial denaturation was omitted as cDNA template synthesized as a single strand did not require initial double strand separation so crucial for DNA. Also, it circumvented the co-amplification of any contaminating DNA due to possible mispriming.

Simultaneously, the RNA samples after normalization to the concentration of approximately 500 ng were subjected to the integrated c-DNA synthesis and real-time amplification for the fusion transcript load at baseline using Taqman probe based BCR-ABL transcript quantitation kit (Geno-Sen’s Genome Diagnostics Pvt. Ltd.) on the Agilent Stratagene Mx-3000-P real-time PCR platform to ascertain any load-dependent correlation with the fusion transcript types.

Results

Patients included 18 males (42.85%) and 24 females (57.14%) within the age range of 07-75 years. 19 (45.23%) cases belonged to age group of ≤45 years and the rest 23 (54.76%) were >45 years of age. All the patients had raised TLC counts with different grades of Leukocytosis as well as Splenomegaly.

Only two types of transcripts, e13a2 (b2a2) and e14a2 (b3a2) were detected in our CML patients and none had the e1a2 type (Table 2). The e13a2 and e14a2 transcript types were amplified as 310 bp and 385 bp PCR amplicons respectively and were gel documented as demonstrated in a representative gel picture below (Figure 1).

The respective frequency of the two detected transcript types of e13a2 and e14a2 was 26.19% and 73.81% respectively. We attempted to evaluate the transcript distribution across the several clinico-pathological characteristics (Table 2), which included Gender, Age, Spleen size, TLC as well as Platelet count along with the percentage of Blast cells, Promyelocytes, Myelocytes and Metamyelocytes as also the quantitative transcript load assessment at the baseline.

There was no association found as far as the Gender, Age, Spleen size and Blast cell percentage were concerned. The two transcripts happened to be randomly distributed among the said categories and did not demonstrate any
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The case with the platelet count, wherein the count range of 201-900x10^3/µl (between 2 lacs to 9 lacs) was found to be more significant (p-value: 0.0001) as patients with such a high platelet count were predominantly found to harbor the transcript type e14a2 (b3a2) (Table 2). Likewise, the % age range of 11-20 for Promyelocytes, 05-40 for Myelocytes and Metamyelocytes and >80% for transcript load at baseline (Table 2) were found to be more associated bias towards a particular sex, age-group or indicated spleen size ranges as well as % age ranges of Blast cells. However, the TLC count, particularly the range of 201-600x10^3/µl (between 2 lacs to 6 lacs) at baseline was found to be associated more with the e14a2 (b3a2) than the e13a2 (b2a2) transcript type (p-value: 0.001). In other words most patients with such a TLC count were found to harbor the e14a2 transcript. Similar was the case with the platelet count, wherein the count range of 201-900x10^3/µl (between 2 lacs to 9 lacs) was found to be more significant (p-value: 0.0001) as patients with such a high platelet count were predominantly found to harbor the transcript type e14a2 (Table 2). Likewise, the % age range of 11-20 for Promyelocytes, 05-40 for Myelocytes and Metamyelocytes and >80% for transcript load at baseline (Table 2) were found to be more associated

![Figure 1](image1.png)

**Figure 1.** Gel Electrophoresis Documentation of BCR-ABL Transcripts in CML.

Table 1. Primer Sequences Used in Multiplex RT-PCR of BCR-ABL Transcripts.

| Transcript      | Primers     | Primer sequences (5’-3’) | Amplicon size (bp) |
|-----------------|-------------|--------------------------|--------------------|
| Normal BCR      | B2B+C5e     | 5'-ACAGAATTCCGCTGACCATCAATAAG-3’ | 808                |
|                 |             | 5'-ATAGGATCCCTTTGCACAACCGGGTCTGAA-3’ |                   |
| e1a2            | BCR-C+CA3   | 5'-ACCAGCATGTTCCGGGACAAAAAG-3’ | 481                |
|                 |             | 5'-TGTGTGACTGGCGATGTAGTATGTTGCTTG-3’ |                   |
| e13a2 (b2a2)    | B2B+CA3     | 5'-ACAGAATTCCGCTGACCAATAAG-3’ | 310                |
|                 |             | 5'-TGTGTGACTGGCGATGTAGTATGTTGCTTG-3’ |                   |
| e14a2 (b3a2)    | B2B+CA3     | 5'-ACAGAATTCCGCTGACCAATAAG-3’ | 385                |
|                 |             | 5'-TGTGTGACTGGCGATGTAGTATGTTGCTTG-3’ |                   |

![Figure 2](image2.png)

**Figure 2.** Kaplan Meier Analysis for Imatinib Resistance with Respect to Harboring e13a2 or e14a2 Transcript Type.

![Figure 3](image3.png)

**Figure 3.** Kaplan Meier Analysis for Overall Survival with Respect to Harboring e13a2 or e14a2 Transcript Type.
with the e14a2 transcript type compared to the e13a2 type with the respective p-values of 0.0001, 0.001 and 0.0001 (Table 2).

Subsequently, the two transcript types were evaluated against different cytogenetic responses (Complete cytogenic response CCR, Partial cytogenic response, PCR and No cytogenic response, NCR) at 3 Months, 6 Months and 1 Year. Even though there were more patients with PCR at 3 months and with CCR at 6 months and 1 Year harboring e14a2 transcript, the overall association was insignificant (Table 3) and the different cytogenetic responses at the three designated follow up durations seemed to be working out independent of the presence of either of the fusion gene transcripts.

Further, we evaluated the imatinib treatment outcome of patients on the basis of harboring a particular transcript type by Kaplan Meier analysis. There were 31 and 11 patients having e14a2 and e13a2 transcripts respectively and of the 08 events of imatinib resistance, 07 (22.58%) had e14a2 (b3a2) transcript and just 01 (9.09%) had e13a2 (b2a2) transcript. In other words the %age success of imatinib treatment is 77.4% and 90.09% with e14a2 and e13a2 respectively (Figure 2). Although, there are numerically more patients with e14a2 transcript showing imatinib resistance, the overall Kaplan Meier analysis is insignificant - (Log rank 0.843). This was probably due to more patients i.e. 31/42 (73.81%) harboring e14a2 than only 11/42 (26.19%) having e13a2 transcripts and

Table 2. Distribution of BCR-ABL Fusion Gene Transcripts Across the Indicated Patient Parameters at Baseline.

| Patient Parameters (Baseline) | Total n=42 | BCR-ABL Transcript type | p-value |
|-------------------------------|-----------|-------------------------|---------|
|                               |           | e1a2 | e13a2 | e14a2 |       |
| Gender                        |           |      |       |       | 0.8   |
| Males                         | 18        | 0    | 4     | 14    |       |
| Females                       | 24        | 0    | 7     | 17    |       |
| Age                           |           |      |       |       | 0.74  |
| ≤ 45                          | 19        | 0    | 4     | 15    |       |
| > 45                          | 23        | 0    | 7     | 16    |       |
| Spleen size (bcm)             |           |      |       |       | 0.5   |
| Not palpable                  | 2         | 0    | 1     | 1     |       |
| 01-15 cm                      | 27        | 0    | 9     | 18    |       |
| >15 cm                        | 13        | 0    | 1     | 12    |       |
| TLC (x1000)                   |           |      |       |       | 0.001 |
| 11-200                        | 34        | 0    | 10    | 24    |       |
| 201-600                       | 7         | 0    | 1     | 6     |       |
| >600                          | 1         | 0    | 0     | 1     |       |
| Platelet count (x1000)        |           |      |       |       | 0.0001|
| 40-200                        | 19        | 0    | 7     | 12    |       |
| 201-900                       | 21        | 0    | 3     | 18    |       |
| >900                          | 2         | 0    | 1     | 1     |       |
| Blast cells %                 |           |      |       |       | 0.5   |
| 01-10                         | 40        | 0    | 11    | 29    |       |
| 11-20                         | 1         | 0    | 0     | 1     |       |
| >20                           | 1         | 0    | 0     | 1     |       |
| Promyelocytes %               |           |      |       |       | 0.0001|
| 01-10                         | 26        | 0    | 8     | 18    |       |
| 11-20                         | 14        | 0    | 3     | 11    |       |
| >20                           | 2         | 0    | 0     | 2     |       |
| Myelo+Meta Myelocytes %       |           |      |       |       | 0.001 |
| 05-40                         | 23        | 0    | 3     | 20    |       |
| 41-65                         | 17        | 0    | 7     | 10    |       |
| >65                           | 2         | 0    | 1     | 1     |       |
| Transcript load (q PCR) Baseline |     |      |       |       | 0.001 |
| 0-40%                         | 11        | 0    | 5     | 6     |       |
| 40-80%                        | 20        | 0    | 6     | 14    |       |
| >80%                          | 11        | 0    | 0     | 11    |       |

Table 3. Distribution of BCR-ABL Fusion Gene Transcripts Across CCR, PCR and NCR at the Indicated Follow-up Durations.

| Follow-up Duration | BCR-ABL Transcript | Total | Cytogenetic response (PB) | P-value |
|--------------------|--------------------|-------|---------------------------|---------|
|                    |                    |       | CCR | PCR | NCR |       |
| 3 Months           | e13a2 (b2a2)       | 11    | 1   | 9   | 1   | 0.9   |
|                    | e14a2 (b3a2)       | 31    | 5   | 21  | 5   |       |
| 6 Months           | e13a2 (b2a2)       | 11    | 10  | 0   | 1   | 0.7   |
|                    | e14a2 (b3a2)       | 31    | 25  | 3   | 3   |       |
| 1 year             | e13a2 (b2a2)       | 11    | 10  | 1   | 0   | 0.9   |
|                    | e14a2 (b3a2)       | 31    | 27  | 4   | 0   |       |
small number of resistance events. Refer to Kaplan Meier analysis (Figure 2).

As the Kaplan Meier analysis for the two transcript types with respect to imatinib resistance at 3 months showed no significance in survival probability, we proceeded to compare their overall survival outcome as well. The calculated estimated mean survival time for e13a2 and e14a2 transcripts was 27.63 and 29.80 months respectively with the overall mean survival of 29.71 months (Figure 3).

There were total 03 events of death, which included 02 with e14a2 and 01 with e13a2. The survival probabilities in remaining 10 of 11 patients with e13a2 were 90.9% and that of 29 remaining e14a2 was 93.5%. There was no statistical significance noted in terms of the association of either of the transcripts with the overall survival outcome (Log rank P < 0.800). This was a reiteration of the scenario reflected in Kaplan Meier analysis with respect to imatinib resistance (Figure 3).

Discussion

As a first of its kind endeavour in our region (Kashmir-North India), we undertook this objective to analyse the frequency of different transcripts in CML patients of our region and determine whether there is any difference in several clinico-pathological characteristics with regard to these BCR-ABL transcripts at diagnosis in our patients as well as analyze the differences thereof in the imatinib treatment response by monitoring their cytogenetic as well as molecular response to the drug.

All the 42 CML patients reported either having b3a2 or b2a2 transcript type and there was no co-expression of the two variants found. The frequency of b3a2 and b2a2 found to be 73.81% and 26.19% respectively, which is somewhat different from the data derived in similar studies performed in the Caucasian population (Verschraegen et al., 1995). Similarly, based on different sample sizes, most studies have reported transcript frequencies, where the predominant type has been b3a2 with the respective ratios of 60-70% of b3a2:30-40% of b2a2, like 68.4% and 31.6% (Reiter et al., 1998); 67.9% and 30.2% (Verschraegen et al., 1995); 62% and 21% (Yaghmaie et al., 2008). However, there have been quite a few reports where the predominant type has been b2a2, documenting 56.5% patients with b2a2 type compared to 26% having b3a2 (Irshad et al., 2012). In many South American countries like Mexico, of the 83.00% of M-BCR transcripts among 250 patients 48.00% and 35.00% registered b2a2 and b3a2 respectively (Arana-Trejo et al., 2002). Similarly, the frequency found in Argentinean population was 41.7% for b2a2 and 37.5% for b3a2 (Sastre et al., 2007) along with an Ecuadorian population reporting 5% for b3a2 and 95% for b2a2 (Paz-y-Mino et al., 2002). While, such differences can happen due to differences in the sensitivities of the techniques used, the ethnic differences among different populations should also strongly be taken into consideration (Paz-y-Mino et al., 2002).

Though the role of BCR-ABL transcript variants in the prognosis had always been a controversial issue, we attempted to evaluate the transcript distribution across several clinico-pathological characteristics including Gender, Age, Spleen size, TLC as well as Platelet count along with the percentage of Blast cells, Promyelocytes, Myelocytes and Metamyelocytes as also the quantitative transcript load at baseline. Although some authors reported no such role on the part of the transcript variants, others found data referring to the transcript variants to have prognostic values (Rozman et al., 1995; Opalka et al., 1992; Melo, 1996).

Our study did not reveal any significant transcript association as far as gender, age or baseline spleen size and blast cell percentage were concerned, which is similar to several studies in literature (Shepherd et al., 1992; Rozman et al., 1995; Perego et al., 2000; de Lemos et al., 2005) in respect of the size of spleen. In one study, a higher leukocyte count in b2a2 patients was reported (Martinez-Mancilla et al., 2002). However, in our patients, the TLC count, particularly the range of 201-600x10^9/µl (between 2 lacs to 6 lacs) at baseline was found to be associated more with the e14a2 (b3a2) than the e13a2 (b2a2) transcript type (p-value: 0.001), which is similar to one finding with however not as significant as ours (Polampalli et al., 2008). Further, some of the studies failed to show any correlation between platelet count and BCR-ABL transcript variants (Shepherd et al., 1995). Others recognized higher platelet count in patients carrying b3a2 (Perego et al., 2000; Balatzenko et al., 2011), which is consistent with our finding, wherein the count range of 201-900x10^9/µl (between 2 lacs to 9 lacs) was significantly associated with the transcript type e14a2 (P-value: 0.0001). Additionally, we found percentage ranges of 11-20 for Promyelocytes, 05-40 for Myelocytes and Metamyelocytes and >80% for transcript load at baseline as more associated with the e14a2 transcript type compared to the e13a2 type with the respective p-values of 0.0001, 0.001 and 0.0001. However, the significance could be superficial on account of very small number of patients contributing to the apparent significance and needs to be investigated further on a larger pool of patient samples.

As far as the impact of M-BCR transcript types (b3a2 and b2a2) on imatinib treatment response is concerned, we did not find any significant difference between the two. 26 of the 31 (83.87%) b3a2 patients and 10 of 11 (90.90%) b2a2 patients registered complete cytogenetic response at 1 year along with 24 of 31 (77.41%) b3a2 patients and 10 of 11 (90.90%) b2a2 patients simultaneously registering equivalent of ≤3 log reduction in transcript load at the same time. We did the Kaplan Meier analysis to evaluate the imatinib treatment and overall survival probabilities and found that there was no significant difference between the two. This was in agreement with some studies (Yaghmaie et al., 2008), but in disagreement with the findings of some others (Preetesh et al., 2016; Claire et al., 2009), where they reported better response outcomes in e14a2 type compared to the e13a2.

In conclusion, while a particular TLC, Platelet and Promyelocyte range showed a statistical correlation with the e14a2 transcript type, there has been no overall prognostic implication of either the e13a2 or the e14a2 transcript type across the spectrum of indicated clinical parameters evaluated. Even the overall survival analysis of
the two transcript types revealed no prognostic association whatsoever.

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