A Prognostic and Predictive Study of BCR-ABL Expression Based on Characterization of Fusion Transcripts

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Abstract BCR-ABL translocation is a key hallmark of chronic myeloid leukemia (CML). The chemistry involves transcription of a novel 8.5 kb mRNA with a b3a2 and/ or b2a2 junction. Though there is an improvement in survival using the TKIs, there are causes for the treatment failures. Thus, the study focuses on the causes underlying the failed response. This study comprises BCR-ABL expression in correlation with age, gender and transcript type and disease monitoring in follow-up samples. Eighty-seven chronic phase CML patients were enrolled in the study. Out of these 24 patients were followed further. Quantitative Real time PCR was performed to assess the BCR-ABL expression followed by gel electrophoresis of PCR products. The results were expressed as CN/ μ g RNA. The results obtained were correlated with SPSS 16.0 software. We found three different types of the expression that includes b2a2, b3a2 and co-expression (b2a2 + b3a2). Higher incidence of b2a2 with a relatively equal incidence of co-expression was observed. The BCR-ABL expression was higher in males, in young patients and in those exhibiting co-expression of the transcripts. Greater relapse was seen in females and in older patients. The patients with co-

expression of transcripts exhibited the highest expression; however these patients showed the best treatment response suggest the co-expression is the favourable parameter for CML patients. The transcript type and BCR-ABL expression are well correlated and hence can be considered as a prognostic as well as the predictive indicator considering the BCR-ABL expression for CML patients.

Keywords CML · BCR-ABL expression · b2a2 & b3a2 · Co-expression · qRT-PCR

Introduction

Chronic myeloid leukemia is a disorder of hematopoietic stem cell that results in increased myeloid cells within the peripheral blood, directing it to myeloid hyperplasia in bone-marrow which affects the erythroid cell as well as the platelet count [1]. This infirmity is a result of translocation that juxtaposes the ABL gene (Abelson) on the BCR gene (breakpoint cluster region) forming a BCR-ABL mutation also known as Philadelphia Chromosome, which primes to increased proliferation of leukemic cells [2]. The chimeric protein p210 thereby formed as a result of this translocation, constitutively leads to activation of tyrosine kinase activity [3].

This disorder is tri-phasic, mainly occurring in chronic phase presenting over production of mature granulocytes and less than 10% blasts in blood and bone-marrow. Untreated chronic phase invariably march towards acute phase having more than 20% blasts. However, the disease at times stays in between these two phases known as accelerated phase having roughly 10–20% of blasts. The disease is in its most aggressive nature when the patient suffers from blast crises [4].

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As a part of known fact, the molecular hallmark of CML is the exchange of genetic material between the long arms of chromosome 9 and chromosome 22. Based on the breakpoints in the BCR gene, the three main variants of BCR/ABL genes formed. These breakpoints are between exon 13 and 14 (b2) or exon 14 and 15 (b3) with breakpoints at intron 1 or 2 of the ABL gene. It gives rise to major fusion genes that transcribe either as b2a2 or b3a2 mRNA [5]. There are major chances of co-expression of these transcript types owing to alternative splicing, which might be due to existence of several leukemic cell lines with different BCR-ABL transcript expression. A small proportion of CML patients display a larger fusion transcript that is a result of union of BCR exon 19 and ABL exon 2 forming e19a2 junction. It is a rarely found transcript in the patients [6].

Imatinib (Gleevec; STI571), a potent tyrosine kinase inhibitor that strongly and specifically binds to the ATP binding pocket of BCR-ABL chimeric protein, is majorly used as standard therapy for all CML phases. Despite of the clinical success with imatinib demonstrating long-term survival for the majority of patients increasing to 90% with remission of 5–6 years [7], one-third of patients need an alternative therapy, frequently a second-generation TKI, such as dasatinib and nilotinib. Patients who need second-line therapy include those with imatinib intolerance or mainly primary or acquired imatinib resistance. Thus, exploiting overall potential of imatinib requires close attention to patient response to recognize suboptimal response and treatment failure as early as possible and to make timely adjustments to the therapeutic strategy [8].

Thus in this study, we retrospectively analysed BCR-ABL expression of 87 patients in correlation with clinical factors such as age, gender and transcript type. These patients were even followed for successively three years and the collected follow up samples were evaluated for disease monitoring.

Materials and Methods

Patients and Criteria

Eighty-seven patients with confirmed diagnosis of CML in chronic phase were enrolled for the study at The Gujarat Cancer and Research Institute, Civil hospital, Ahmedabad during 2014–2016. Four ml of venous blood sample was drawn in EDTA vacutainers (BD, U.S.A.) from the enrolled patients. The cohort included 58.6% males and 41.4% females. Informed consents of the patients were taken during sample collection. HIV/HBsAg positive patients and those possessing other myelo-proliferative disorder were excluded. Possible follow-up of these

patients were parallelly maintained at a regular interval of 6 months. However, we could track only twenty-four patients for their follow-up samples.

RNA Extraction

m-RNA was isolated from the peripheral blood by Qiagen RNA isolation kit (QIAamp RNA Blood Mini Kit #52304). The RNA extracted was quantified and qualified spectrophotometrically. The quality was assured by gel electrophoresis.

c-DNA Synthesis

1 µg of RNA extracted, was reverse transcribed to c-DNA in 50 µl reaction volumes with Mo-MuLV-reverse transcriptase (Applied Biosystems, high Capacity cDNA Reverse Transcription kit, cat #4368813), using random hexamer primers. The thermal cycler conditions were 25 °C for 10 min, 37 °C for 120 min followed by extension at 4 °C. The transcribed c-DNA is stored at – 80 °C.

Real Time PCR

Total 100 ng cDNA was subjected to quantitative Real Time PCR (Ipsogen, BCR-ABL1 M-BCR kit, Cat. #673113). The BCR-ABL copy numbers that were calculated against the standards were compared with control gene (ABL) to calculate the NCN to measure the disease response in CML patients. The cyclic conditions for real time PCR was 50 °C for 2 min, 95 °C for 10 min and 50 cycles with 95 °C for 15 s and 60 °C for 1 min. The results were expressed in terms of BCR-ABL copies per microgram of RNA (CN/µg RNA).

Gel Electrophoresis

The amplified product was run on the 1.5% agarose gel to check the type of transcript present in the sample lane with 50bps DNA ladder. The gel was visualized in Gel Documentation system (Bio-Rad, Gel Doc XR+).

Statistical Analysis

Various clinicopathological parameters were analyzed using SPSS 13.0 software. Student *t* test and Spearman (Non-Parametric) tests were used for correlation studies. The patients were tracked for successive four follow-ups and the results were correlated with various clinicopathological parameters.

Fig. 1 **a** Representative gel image showing b2a2 and negative transcript type. **b** Representative gel image showing b3a2 and coexpression (b2a2 & b3a2) transcript type

Results

Altogether, eighty-seven patients with CML in chronic phase at diagnosis were analyzed. These patients were consecutively followed for four successive follow ups. These patients were under treatment of Hydrea 500 mg, followed by Glivec/Imatinib in variable doses ranging from 400 to 800 mg depending on the response and clinical conditions.

Total Expression Analysis of the Cohort

Principally, expression of 87 patients with confirmed diagnosis of BCR-ABL in chronic phase was measured by real time PCR. The results obtained were denoted as BCR-ABL expression *Mean ± SE (CN/μg RNA)*.

Obtained results displayed, that there was a notified decreased expression in the first follow-up sample i.e. a decrease from $28,371.20 \pm 23,266.35$ ($n = 87$) to 4695.05 ± 1962.85 ($n = 70$). However gradually, patients showed a marked increase in the expression i.e. $29,114.13 \pm 27,973.26$ ($n = 43$), suggestive of the relapse and a resistance to the treatment.

Transcript Type Identification

The type of transcript of BCR-ABL gene expressed in the patients was identified by agarose gel electrophoresis of the PCR products. The bands were obtained at 146bps for b3a2 and 72bps for b2a2. Dual bands were seen in case of co-

expression of the transcripts. Gel identification indicated that 26.4% patients expressed b3a2 type transcript while 34.5% patients showed b2a2 expression. In 32.2% patient's co-expression of both the transcripts was seen. Even more, there were 6.7% patients that were negative for any of these transcripts type (Fig. 1a, b).

Expression Analysis of BCR-ABL in Co-relation with the Clinicopathological Parameters

Further the overall expression of 87 patients was correlated with the prognostic factors i.e. Age, Gender and Transcript type (Table 1).

In accordance with age and gender, the results obtained exhibited that there was higher BCR-ABL expression in male patients and those with age less than 40 years. In case

Table 1 BCR-ABL expression and clinical parameters

Parameters	Type	N (87)	BCR-ABL Mean $\pm$ SE (CN/μg RNA)
Transcript	b3a2	23	2247.47 ± 564.36
	b2a2	30	2517.6 ± 891.47
	Co-expression	28	$83,576.57 \pm 72,040.06$
	Negative	6	155.16 ± 89.91
Gender	Male	51	$43,711.29 \pm 39,568.97$
	Female	36	6639.41 ± 4766.82
Age	< 40	46	$46,550.45 \pm 43,858.94$
	> 40	41	7974.97 ± 4688.73

of transcript type, higher expression was seen in patients with co-expression of b3a2 and b2a2. However, the results obtained were not significant.

The BCR-ABL expression was correlated with prognostic factors using Spearman correlation. We could notify a positive correlation between the transcript type and the copies of the transcripts expressed. (Spearman $r = 0.22$, $p = 0.03$). This led us to study the transcript type and its correlation with clinical parameters.

Transcript Type in Correlation with Age and Gender

The correlative studies have previously emphasized much on the importance of transcript type with the BCR-ABL expression. Hence we studied effect of transcript type i.e. b3a2, b2a2, co-expression and negative transcript with the stratified age and gender of the patients. The results obtained for each transcript type are shown in the in the table below (Table 2). However the correlation obtained was not significant with both age and gender in all transcript types.

Correlation of Relapse with BCR-ABL Expression in Follow-Up Studies (N = 24)

Results showed evident response of treatment at second follow-up, i.e. a decrease expression of BCR-ABL gene was seen to be 0.082 times. There was a marked increase of BCR-ABL gene expression by 5.5 times at fourth follow-up as compared to third follow-up. The results depict that the patients might have developed a resistance to the conventional therapy given during the disease phase (Fig. 2).

Fig. 2 BCR-ABL expression in follow-up patients

The relapse was so-forth measured taking into consideration clinical parameters i.e. age, gender and transcript type.

Patients with age more than 40 years displayed poor response to the treatment i.e. initially 1.5 times decrease was noted in the expression but after the third follow-up, the expression increased 9 times from the third follow-up (Fig. 3a). However, expression was relatively stable with treatment in younger patients. The results might be an indication of the fact that the cells of older patients are more susceptible to be resistant as compared to younger patients.

In regards to gender, relatively females showed higher relapse as compared to males. In females a relapse of 12 times was seen from the third follow-up while in males there was a marked increase of 2 times in the disease expression from the third follow-up (Fig. 3b).

One more aspect that was taken into scrutiny while studying the relapse conditions was the transcript type

Table 2 BCR-ABL transcript type and clinical parameters

Transcript	Parameter		N (30)	Mean $\pm$ SE (copies/ μ g RNA)
b2a2	Age	< 40	14	3634.21 $\pm$ 1848.55
		> 40	16	1540.56 $\pm$ 391.36
	Gender	M	18	2677.50 $\pm$ 1442.77
		F	12	2277.75 $\pm$ 635.38
b3a2	Age	< 40	11	1931.09 $\pm$ 607.40
		> 40	12	2537.50 $\pm$ 94.36
	Gender	M	14	2474.0 $\pm$ 870.25
		F	9	1895.11 $\pm$ 546.37
Co-expression	Age	< 40	19	108,905.26 $\pm$ 106,173.62
		> 40	09	30,104.89 $\pm$ 20,491.89
	Gender	M	16	134,106.19 $\pm$ 125,851.24
		F	12	16,203.75 $\pm$ 14,274.72
Negative	Age	< 40	04	232.75 $\pm$ 119.12
		> 40	02	–
	Gender	M	03	248.67 $\pm$ 166.95
		F	03	61.6 $\pm$ 61.66

Fig. 3 a Age and BCR-ABL expression pattern in follow-up samples. b Gender and BCR-ABL expression pattern in follow-up samples

exhibited by the patients. Abrupt response was seen with relapse condition in patients with varied transcript type in relation to treatment response. However, better molecular response was seen with treatment in patients expressing both the transcripts together i.e. co-expressing patients showed lower relapse rate as compared to their individual expression which remained relatively the most stable with the treatment given (Fig. 4).

Discussion

The study retrospectively analysed laboratory data from 87 patients with CML in chronic phase and studied the association of varied clinicopathological parameters with the expression of BCR-ABL in response to treatment. Further the study includes the follow-up samples to assess the treatment response in CML patients. Considerably higher BCR-ABL expression was obtained in all patients. While analysing the follow samples, after the initial considerable effect of treatment the relapse was seen at the third follow-up sample suggest the resistance of the given therapy.

The observation of the relapse prompted us to study the causative factors and the expression trend. One major factor could be speculated was the presence of varied transcript type. Higher number of b2a2 expression was seen in this cohort. The results obtained coincide with the

study of Muddathir et al. [7]. Whereas some studies showed the contradictory results [9–11]. The differences in the results are due to diversified geographical locations and ethnic group taken under the study.

Over and above the absence of these transcripts (Negative) in some patients might be a suspicion of presence of some other rarer form of transcript like e19a2 [9, 10]. Thus, these negative transcripts might be further analysed for these rarer transcripts with the coordinative specific primers.

Various factors like age, gender and transcript type were correlated with the expression of the BCR-ABL gene. The incidence was higher in males compared to females. Supportively, the overall expression studies linearly showed higher expression of BCR-ABL gene in male patients as compared to females [12]. While the follow-up studies showed the higher relapse rate in females compared to males which is contradicting the three studies that showed females take longer time to relapse and hence the better response to the treatment as compared to males [7, 12, 13]. This might be projecting the effect of hormones or other genetic/environmental factors on the disease course.

Higher expression was noted in younger patients while increased relapse rate was observed in older age group. One of the hypotheses could be the aging effect. Berger et al. [14] demonstrate that the patients of age above

Fig. 4 Transcript type and of BCR-ABL expression pattern in follow-up samples

55 years showed higher risk of the disease, hereby contradicting our study.

CML cells mainly exhibit mRNA transcripts with b3a2 or b2a2 junction or, both b3a2 and b2a2 (co-expression) formed as a result of alternative splicing [9]. However, the study notifies greater incidence of b2a2 with an equal contribution of dual transcripts. While successive monitoring of the disease during follow-up, an abrupt expression pattern in b2a2 and b3a2 was observed. The related studies done earlier have shown worse prognosis in presence of b2a2 transcript. One explanation is that b2a2-expressing patients may have higher BCR-ABL kinase activity [15]. In an apparent contradiction to our findings, another study has reported that b2a2 patients had a better molecular response at 6 months [16]. However, a study by Prejzner et al. [17], showed longer survival in patients with b3a2 transcripts as compared to b2a2 transcript may be caused by the less aggressive course of the accelerated or blastic phase.

In accordance with the treatment effect, better results were seen with co-expressive transcripts with least relapse rate. To best of our knowledge, no other study has correlated the transcript type, clinical parameter and relapse pattern in CML patients and hence this is first of its kind. However, this study being carried out in small cohort; necessitates further investigation of the potential effects of the nature of the BCR-ABL break points in large group of

patients. Moreover, monitoring transcript type in every follow-up patient might be an indication of clonal switch between b3a2 to b2a2 or vice versa. This aspect can so-forth be useful in treatment planning and better management of CML patients.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest to the submitted manuscript.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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