International Journal of Research and Review
Vol.7; Issue: 4; April 2020
Website: www.ijrrjournal.com
Original Research Article *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

BCR-ABL1 Transcript Type Does Not Alter Response to Imatinib in Chronic Myeloid Leukemia Patients

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ABSTRACT

Introduction: The site of the breakpoint in the *BCR* gene may influence the phenotype of the disease. In majority of CML. Earlier studies reported patients with b3a2 transcript had higher survival rates compared to b2a2 transcript. This study aimed to study the association between the frequencies of BCR‑ABL transcripts and response to imatinib in CML patients.
Aims: This study aimed to study the association between the frequencies of BCR‑ABL transcripts (b3a2 transcript, b2a2 transcript and b2a2/b3a3 transcripts) and response to imatinib in CML patients.
Methodology: In the beginning of the study, before initiation of Imatinib therapy,
detection of BCR-ABL1 fusion gene transcripts for confirmation of diagnosis of CML by Qualitative multiplex RT-PCR .After initiation of Imatinib therapy, follow-up and response monitoring of patients was done for 6 months to 12 months i.e. during the duration of the study, depending on the time point at which the patient was recruited in the study. Hematological response (Hb, TLC, DLC and platelet count) was assessed at regular intervals during the duration of the study. Molecular Response (BCR-ABL1/ABL1%) was assessed once, either at 6 months or at 12 months after beginning of imatinib therapy, depending on the time of recruitment of patient in the study.
Results: It was observed that 50 % of cases had b3a2 transcript, while 26.6 % cases had b2a2 transcript and remaining 23.3 % had b2a2/b3a3 transcripts. 93.3% of patients with b3a2 transcript had achieved complete hematological response by the end of three months. Also, 75 % of patients with b2a2 transcript had achieved CHR by the end of three months. Howover, 85.7% patients with both b2a2/b3a3 transcripts had achieved by the end of three months. This difference is found to be statistically significant (p=0.536).
Conclusions: No association was found between type of BCR-ABL transcript and achievement of hematological or molecular response in chronic myeloid leukemia patients treated with Imatinib.

Keywords: Chronic myeloid leukemia, Imatinib, BCR-ABL transcripts

INTRODUCTION

Chronic myeloid leukemia is a stem cell disorder of myeloid precursors which is characterized by the presence of Philadelphia chromosome (Ph).[1] The Ph chromosome is a shortened 22nd chromosome resulting from a reciprocal translocation between the long arms of chromosomes 9 and 22 t(9;22)(q34;q11)[2], leading to *BCR‑ABL1*fusion gene with constitutive tyrosine kinase activity. The site of the breakpoint in the *BCR* gene may influence the phenotype of the disease.[3] In majority of CML cases, the breakpoint is in the M‑BCR and an abnormal fusion protein p210BCR‑ABL (b2a2 and b3a2 isoforms)[4] is formed which has enhanced tyrosine kinase activity. Imatinib mesylate is a first‑generation tyrosine kinase inhibitor (TKI) used for treating Ph‑positive CML

cases[5]. Earlier studies reported patients with b3a2 transcript had higher survival rates compared to b2a2 transcript[6-9]. This study aimed to study the association between the frequencies of BCR‑ABL transcripts and response to imatinib in CML patients.

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AIMS:

This study aimed to study the association between the frequencies of BCR‑ABL transcripts(b3a2 transcript, b2a2 transcript and b2a2/b3a3 transcripts) and response to imatinib in CML patients.

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METHODOLOGY

The proposed study was carried out in the Department of Biochemistry in collaboration with the Department of Medicine and Department of Pathology, Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi.

STUDY DESIGN:

It was a hospital based prospective study.

SAMPLE SIZE: A sample size of convenience was taken, which included 30 cases of Chronic Myeloid Leukemia (CML) in chronic phase (CP-CML).

SELECTION OF CASES:

Inclusion criteria

- Newly diagnosed CML patients, in the age group 18-80 years, with diagnosis confirmed by qualitative PCR for BCR-ABL1 fusion gene, who were to be initiated on Imatinib therapy.

Exclusion criteria

- Chronic MyeloMonocytic Leukemia (CMML).
- BCR-ABL1 positive adult ALL patients.
- Other myeloproliferative disorders.
- Patients who had previously undergone any treatment for chronic myeloid leukemia.
- SELECTION OF CONTROLS: Age and sex matched 30 normal healthy volunteers.

STUDY PLAN:

➢ On inclusion in the study the patients underwent -

1. Detailed clinical examination (relevant findings like spleen size were noted).
2. Hematological laboratory tests :
   a. complete blood count (Hb , TLC, DLC ,total platelet count)
3. Molecular analysis

Sample collection: A peripheral blood sample, 5mL from cases and 3mL from controls, was collected for molecular studies, in an EDTA vial by venipuncture, after taking informed consent.

In the beginning of the study, before initiation of Imatinib therapy:

1. Detection of BCR-ABL1 fusion gene transcripts for confirmation of diagnosis of CML by Qualitative multiplex RT-PCR .
2. At follow up ,Quantitative REAL-TIME PCR for BCR-ABL1 fusion gene to assess molecular response was done once either at 6 months or at 12 months after initiation of imatinib therapy.

MOLECULAR ANALYSIS:

1. Multiplex RT –PCR for detection of BCR-ABL1 fusion gene transcripts:

Diagnosis of CML was confirmed by Multiplex RT-PCR which allows simultaneous detection of all the BCR-ABL1 fusion gene transcripts in addition to normal BCR gene as an internal control. cDNA synthesized from the total RNA was used in multiplex PCR[10] .The sequence of oligonucleotide primer sequences used for this multiplex PCR are as shown in Table 1 and Table 2.

TABLE 1 : Primer Sequences used for Multiplex RT-PCR for BCR-ABL1 fusion gene

Primer code	Primer Sequence
C5e	5'-ATAGGATCCTTTGCAACCGGGTCTGAA-3'
B2B	5'-ACAGAATTCCGCTGACCATCAATAAG-3'
BCR-C	5'-ACCGCATGTTCCGGGACAAAAG-3'
CA3	5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3'

TABLE 2 :PCR Cocktail preparation (1X) for Multiplex RT-PCR for BCR-ABL1 fusion gene

REAGENTS	VOLUME
cDNA	2µL
PCR Master mix	10µL
Four primers (CA3, C5e, BCR-C, and B2B), (25pmol/ µL)	0.3µL each
ddH2O	11.8µL
Total	25µL

TABLE 3: PCR conditions for Multiplex RT-PCR for BCR-ABL1 fusion gene

STEP	TEMPERATURE	DURATION
1.Initial denaturation	95°C	5 minutes
2.Denaturation	95°C	30 seconds
3.Annealing	68°C	30 seconds
4.Extension	72°C	30 seconds
Repeat step 2-4	Cycles	35
5.Final extension	72°C	10 minutes
6.Cooling	4°C	10 minutes

The expected band size for different BCR-ABL1 fusion transcripts were:

- 808bp - normal BCR
- 481 bp - e1a2
- 385 bp - b3a2
- 310 bp - b2a2
- 103bp – b2a3
- 209bp – b3a3

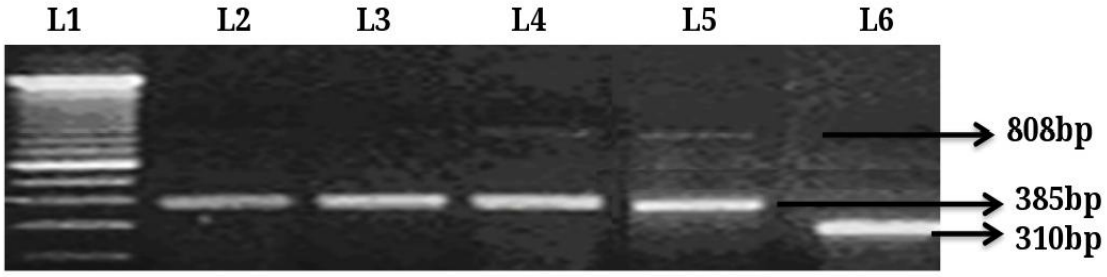

FIGURE 1: Ethidium Bromide stained gel electrophoresis image of BCR-ABL transcripts by multiplex RT-PCR. L1.100bp ladder, L2, L3, L4, L5. b3a2 transcript (385 bp). L6. b2a2 transcript (310bp).

2. Quantitative REAL-TIME PCR for BCR-ABL1 fusion gene

A peripheral blood sample was collected at follow-up either at 6 months or at 12 months after initiation of imatinib therapy to assess the molecular response. Molecular response was assessed by calculating BCR-ABL1/ABL1 ratio. This was done by quantification of BCR-ABL1 p210b2a2 or b3a2 transcripts. *Ipsogen* BCR-ABL1 Mbcr kit(from QIAGEN, Netherlands,) was used for this purpose and protocol followed was as per the manufacturer's instructions.

PRINCIPLE :

Total RNA is reverse-transcribed and the generated cDNA is amplified by PCR using a pair of specific primers and a specific internal double-dye probe(FAM-TAMRA).the probe binds to the amplicon during each annealing step of the PCR. When the Taq DNA polymerase extends the DNA segment from the primer bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'→3' exonuclease activity of the Taq DNA polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them and leading to an increase in fluorescence from the FAM and a decrease in fluorescence from the TAMRA.

TABLE 4 A:PCR Cocktail preparation(1X) for quantitative RT-PCR for BCR-ABL1 fusion gene

COMPONENT	VOLUME (µL)
TaqMan Universal PCR master mix	12.5µL
Primers and probe mix	1µL
Nuclease-free PCR grade water	6.5µL
Sample (cDNA ,100ng RNA equivalent)	5µL
Total volume	25µL

TABLE 4B :PCR conditions for quantitative RT-PCR for BCR-ABL1 fusion gene

Mode of analysis	Quantitation
Hold	Temperature:50°C Time: 2 mins
Hold 2	Temperature : 95 °C Time : 10 mins
Cycling	50 times 95 °C for 15 secs 60 °C for 1 min with acquisition of FAM fluorescence in channel Green: single
Extension	Temperature :72 °C Time: 10 mins

Interpretation of results:

Data analysis principle:

Using TaqMan technology, the number of PCR cycles necessary to detect a signal above the threshold is called the threshold cycle (Ct) and is inversely proportional to the amount of target present at the beginning of the reaction.

Using standards with a known number of molecules, a standard curve is established to determine the precise amount of target present in the test sample. The *ipsogen* standard curves used 3 plasmid standard dilutions for the ABL1Control Gene and 5 standard dilutions for the BCR-ABL1Fusion Gene, in order to ensure accurate standard curves.

Standard curve and quality criteria:

For each gene (ABL1 and BCR-ABL1), raw Ct values obtained from plasmid standard dilutions were plotted according to the log copy number (3,4 and 5 for C1,C2 C3 ; and 1,2,3,4,5 for F1, F2, F3, F4, F5).

Normalized copy number (NCN)

The ABL1 standard curve equation was used to transform raw Ct values for the unknown samples into ABL1 copy numbers ($ABL1_{CN}$)

The BCR-ABL1 standard curve equation was used to transform raw Ct values for the unknown samples into BCR-ABL1 copy numbers ($BCR\text{-}ABL1Mbcr_{CN}$)

Ratio of these CN values gives the normalized copy number (NCN):

$NCN = [\ BCR\text{-}ABL1Mbcr_{CN} / ABL1_{CN}\ ] * 100$

FOLLOW-UP

After initiation of Imatinib therapy, follow-up and response monitoring of patients was done for 6 months to 12 months i.e. during the duration of the study, depending on the time point at which the patient was recruited in the study.

Hematological response (Hb, TLC, DLC and platelet count) was assessed at regular intervals during the duration of the study.

Molecular Response (BCR-ABL1/ABL1 %) was assessed once, either at 6 months or at 12 months after beginning of imatinib therapy, depending on the time of recruitment of patient in the study.

STATISTICAL ANALYSIS

All statistical analysis was performed using SPSS software22.0. Variables were presented as number and percentage. Fisher exact test, was used to estimate the statistical significance of differences observed between the groups. $p<0.05$ was taken as statistically significant.

RESULTS

The age of the cases ranged from 18 to 80 years and that of controls ranged from 20 to 75 years. The mean age for cases was 39.70 ± 18.04 years while that of controls was 39.27 ± 16.00 years. There was no significant difference found in age distribution of cases and controls. The cases included 22 males and 8 females. On the other hand there were 19 males and 11 females in the control group. This difference was not significantly different.

Diagnosis of CML was confirmed by Multiplex RT-PCR which allowed simultaneous detection of all the BCR/ABL fusion gene transcripts.

The frequencies of different transcripts detected are shown in Table 5.

TABLE 5: Frequencies of BCR-ABL transcripts detected

	b3a2 transcript	b2a2 transcript	b2a2/b3a3 transcripts
No. of cases (%)	15 (50)	8 (26.6)	7 (23.3)

Association between type of transcript and presence of complete hematological response (CHR) to imatinib was studied and no significant difference was found (Table 6).

TABLE 6 : CORRELATION BETWEEN TYPE OF TRANSCRIPT AND HEMATOLOGICAL RESPONSE TO IMATINIB

Type of transcript	CHR at 3 months present, n	CHR at 3 months absent , n	p-value
b3a2 transcript	14(93.3%)	1(6.7%)	0.536
b2a2 transcript	6(75%)	2(25%)	
b2a2/b3a3 transcripts	6(85.7%)	1(14.2%)	
Total	26	4	

Moreover, correlation between type of transcript and presence of molecular response to imatinib was studied and no significant difference was found (Table 7)

TABLE 7 : CORRELATION BETWEEN TYPE OF TRANSCRIPT AND HEMATOLOGICAL RESPONSE TO IMATINIB

Type of transcript	Optimal response, n=	Warning,n=	Failure ,n=	p-value
b3a2 transcript	10(66.7%)	2(13.3%)	3(20%)	p=0.685
b2a2 transcript	4(50%)	2(25%)	2(25%)	
b2a2/b3a3 transcripts	3(42.8%)	3(42.8%)	1(14.4%)	
Total	17	7	6	

DISCUSSION

It was observed that 50 % of cases had b3a2 transcript, while 26.6 % cases had b2a2 transcript and remaining 23.3 % had b2a2/b3a3 transcripts. With respect to hematological response, it was seen that 93.3% of patients with b3a2 transcript had achieved complete hematological response by the end of three months. Also, 75 % of patients with b2a2 transcript had achieved CHR by the end of three months. However, 85.7% patients with both b2a2/b3a3 transcripts had achieved by the end of three months. This difference is not found to be statistically significant (p=0.536) as shown in table 6.

With respect to molecular response, it was seen that 66.7% of patients with b3a2 transcript had achieved optimal molecular response. Also, 50 % of patients with b2a2 transcript had achieved molecular response. However, 42.8% patients with both b2a2/b3a3 transcripts had achieved optimal molecular response. On the other hand, 20 % patients with b3a2 transcript had molecular response failure. 25% of b2a2 transcript and 14.4 % of patients with b2a2/b3a3 transcripts had failure molecular response. This difference was not found to be statistically significant (p=0.685) as shown in table 7.

Cross *et al.*[13] described a multiplex‐PCR assay for the identification of *BCR‐ABL1* fusion transcripts, which allows rapid, specific, and simultaneous detection of the three *BCR‐ABL1* fusion transcripts in patients with CML and acute lymphocytic leukemia. In the present study, we aimed to find out the frequency of *BCR‐ABL1* transcripts and their association with response to imatinib treatment. However, several studies showed controversial reports between the role of fusion transcripts and prognosis.[17] Earlier studies reported patients with b3a2 transcript had higher survival rates compared to b2a2 transcript.[11,12] Sharma *et al.*[14] and Adler *et al.*[15-16] reported that patients with b3a2 had bad prognosis than patients with b2a2 transcripts and response to imatinib therapy. Sailaja Kagita et al[18] found that patients with b3a2 transcripts might be associated with poor response and worse prognosis in CML with imatinib treatment.

However, in the present study, no significant association was found between type of transcript and the achievement of hematological or molecular response to Imatinib. This might be attributed to a limited sample size of 30 cases. Thus, it is recommended that results may be confirmed in a bigger sample preferably at multiple centres.

CONCLUSION

In this study, no association was found between type of BCR-ABL transcript and achievement of hematological and molecular response in chronic myeloid leukemia patients treated with Imatinib.

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How to cite this article: Bhutani N, Arora D, Bhutani N. BCR-ABL1 transcript type does not alter response to imatinib in chronic myeloid leukemia patients. International Journal of Research and Review. 2020; 7(4): 363-368.

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