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TRACKING MOLECULAR RESPONSE OF TYROSINE KINASE INHIBITORS THERAPY IN CHRONIC MYELOID LEUKEMIA PATIENTS USING RT-PCR PROFILING OF BCR-ABL TRANSCRIPTS

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ABSTRACT

Chronic myeloid leukemia (CML) results from the formation of the Philadelphia chromosome by translocation between chromosomes 9 and 22, creating the BCR-ABL fusion oncogene. This study analyzed specific BCR-ABL transcript variants in 30 Pakistani CML patients to detect common fusion protein isoforms. RNA extraction, cDNA preparation, primer design and real-time PCR were utilized to profile three transcript variants (b3a2, b2a2, e1a2) which translate to fusion proteins p210 and p190. The b3a2 and b2a2 transcripts for p210 were detected in all 30 CML patients (100%). Additionally, 2 out of the 30 patients (6.6%) simultaneously exhibited e1a2 transcripts coding for p190. In conclusion, the major BCR-ABL fusion transcript identified in Pakistani CML cases is the b3a2/b2a2 subtype producing p210 fusion protein. A smaller subset shows co-expression of the minor e1a2 variant encoding p190. These findings help characterize the prevalence of distinct BCR-ABL isoforms within the native CML patient population. Further investigation of genotype-phenotype correlations may aid prognostic classification and treatment decisions for these patients.

Key Words: CML, PCR, real time PCR, Fusion transcripts, TKI's.

INTRODUCTION

CML (chronic myeloid leukemia) is a chronic disease describe as the reciprocal translocation between 9 and 22 chromosome called the Philadelphia (Ph) chromosomes, resulting the bcr-abl oncogene[1] . The chimeric oncoprotein bcr-abl initiates signaling pathways like P13K, MAPK, JAK/STAT that are elaborated the disease development, clonal instability and leukemogenesis. These signalling pathways increased the production of reactive oxygen species (ROS)[2] . There are three phases of CML i.e. chronic phase, accelerated phase and blast phase. The treatment of CML depends on tyrosine kinase inhibitors (TKIs). Imatinib drug is a 1st generation while dasatinib and nilotinib are the 2nd generation therapy, which is accepted as primary and secondary treatment of chronic myeloid leukemia patients. On the other hand, bosutinib and ponatinib are set aside for those patients that are intolerant to the above mentioned tyrosine kinase inhibitors[3] .

The great challenge for the treatment of CML disease is resistance of tyrosine kinase inhibitors. TKIs resistance may lead to the failure to get complete cytogenetic response (CCR) or may be disease development by dropping CCR that was showed initially by using TKIs[4] .

The Ph chromosome is a reciprocal translocation of chromosome 9 and 22 within long arms t(9;22)(q34;q11)[5] . About 5% chronic myeloid leukemia patients, the Ph chromosome known as a stamp of this disease. It is noticed that 5% of children and 15 to 30 % of grown person have ALL (acute lymphoid leukemia) while % of newly identified AML (acute myeloid leukemia) ([6,7]). In philadelphia translocation, Abl gene (3 segment) of 9q34 chromosome to the bcr gene (5 part) of 22q11 chromosome resulting a bcr-abl oncogene that set down to bcr-abl mRNA. The abl gene codes TKIs (tyrosine kinase inhibitors) with 145 kd molecular mass. It has eleven spans and exons above 230 kb (kilobase)[8]. The a2 to a11 exons of abl are transferred to the major cutoff region of bcr gene (M-bcr) within exon 22 and 16 on chromosome 22, that spreads above 5.8 kilobases (kb)[8]. The cutoff point in bcr may drop either between exon b2 and b3 of 5' or between exon b3 and b4 of 3'. A fusion gene (bcr-abl) is formed with b2a2 or b3a2 interaction and transcribed in 8.5 kilobases (kb) messenger RNA. This mRNA is decoded into bcr-abl chimeric protein of 210 kd[9] . When Philadelphia chromosomes or bcr-abl oncogene identified or these both are present in bone marrow or peripheral blood, it should be chronic myeloid leukemia confirmed. It is noted that 5 % patients Philadelphia chromosomes cannot be identified; it depends on bcr-abl transcript by using transcriptase PCR or fluorescent in situ hybridization (FISH) method[1] . In some cases of chronic myeloid leukemia patients, there cannot be identified philadelphia chromosomes or bcr-abl oncogene then these patients known as Philadelphia negative or bcr-abl negative and may shows as another disease. Measurable PCR is a best technique for left over disease after cytogenetic reduction due to its higher sensitivity. PCR performance have been identified as a standard technique for chronic myeloid leukemia due to best results for bcr-abl identification[10].

Imatinib drug shows significant action for chronic myeloid leukemia, it is known as regular treatment with low toxic effects. In phase 1, the patients who were taken 25-1000 milli gram doses of interferon alfa on regular bases resulted as unsuccessful[11] . The patients who were taken less than 50 milli gram per day shows lower response as compared to those of 98% patients who were taken minimum 300 milli gram per day resulted as a complete hematogenic response while 54%

of patients achieved a cytogenetic response. Letter, there was not recognized the toxicity of interferon alfa drug[11] . On the other hand, after examined phase 1 study it has been recommended imatinib drug 600 to 800 milligram per day for best effects [12]. Recently, there will be no any suggestion about the patients who were attained complete molecular response can securely cease this treatment. It was studied that the patients who were stopped imtinib drug treatment shows cytogenetic and molecular response promptly, also showed invisible continued level of bcr-abl [13].

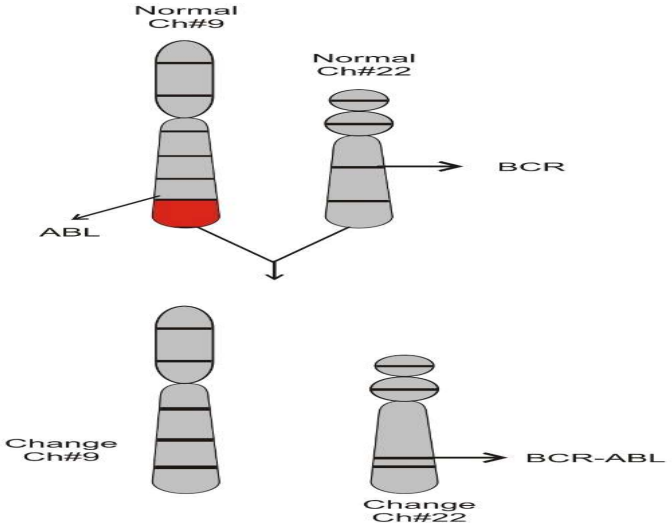


Figure 1: Translocation of chromosome # 9 and 22.

MATERIALS AND METHODS

Place of Work

The whole experimental work was done in the advance Biochemistry Lab, department of Biochemistry, Faculty of Science and Technology, University of Central Punjab, Lahore after the approval of University Board of advanced studies and research and human research ethical committee.

Inclusion Criteria

The following inclusion criteria was adopted in the present experimentations:

i. Patients should be CML.
ii. It will include both genders (Males or Females)
iii. Age limit elder than 18 years.

Exclusion Criteria

Following exclusion criteria was ensured in the present study:

i. Myeloma patients.
ii. Acute Myeloid Leukemia (AML) patients.
iii. Pregnant women.
iv. Patient with any other previous medical / chronic background / history.

Blood/Data Collection

Fresh venous blood sample (10.0 mL) of 30 diagnosed CML patients 10 healthy individuals (with no other previous medical / chronic background / history) was taken in fasting condition in clotted gel vial and EDTA vacutainers in Oncology Departments, Mayo and Jinnah hospital

Lahore. Enrolled participants in study were later categorized according to gender and age groups. These patients were observed on follow-up after 3 months. (i.e. Patients at dose 1 and after three months, i.e. dose 2). Written informed consent was obtained from all the patients or family members.

Molecular Analysis

Extraction of RNA

Human genomic RNA was extracted from the collected blood samples of CML patients who were on TKIs therapies (1st dose and 2nd dose after 90 days) and Healthy Controls by the adopted protocol provided by the given kit method.

cDNA synthesis and qPCR analysis

Whole RNA was used to make the cDNA product, which was then be amplified using primers designed for transcript variants of BCR-ABL (b3a2, b2a2 and e1a2). The samples were analyzed using gene expression SYBR green assay. In qRT-PCR, the amplification of ABL gene was performed simultaneously in duplicate but in separate reaction as a internal control and to normalize BCR-ABL values. In each experiment, a non-template control was used. Each sample's mean across three replications was computed. The mean Ct (threshold cycle) values for each patient was used to assess the relative changes (percentage) in expression. The relatives' change in gene expression was calculated using the Ct (threshold cycle) approach and the formula $2^{-d\overline{C}t}$.

PCR Amplification for Sanger sequencing

The PCR was done in a 20µl reaction volume containing genomic DNA (10 ng), oligonucleotide primers (0.4ρM) each, PCR Buffer (1X), dNTPs (200 µM), MgCl2 (2mM) and *Taq* Polymerase (2U). The following PCR cycling conditions were carried out: 3 minutes for 1 cycle at at 95°C, 35 cycles for 30 seconds at 95°C with annealing temperatures 57°C for 30 seconds and 72°C for 30 seconds followed by 1 cycle for 7 minutes at 72°C.

Designing of primers for Real time PCR

The amplification primers for transcript variants of BCR-ABL (b3a2, b2a2 and e1a2) were formed by using the software (Primer 3) from the website (http://frodo.wi.mit.edu). Designed primers are given in table 1.

Table 1: The list of primer for transcript variants of BCR-ABL (b3a2, b2a2 and e1a2) for real time PCR analysis.

Name of primer	Primer Sequences (5'-3')	bp
ABL-R	**5'-TCCAACGAGCGGCTTCAC -3'**	18
BCRb2-F	**5'-TGCAGATGCTGACCAACTCG -3'**	20
BCRe1-F	**5'-ACCGCATGTTCCGGGACAAAA-3'**	21
ABL-F	**5'-GTCTGAGTGAAGCCGCTCGT -3'**	20

ABL-R	**5'-GGCCACAAAATCATACAGTGCA -3'**	22

Statistical Analysis

The relatives' change in gene expression was calculated using the Ct (threshold cycle) approach and the formula 2^{-dCt}. the correlation between drug dose and gene expression was determined by using t-test. A p-value of <0.05 was considered statistically significant.

RESULTS

BCR-ABL fusion transcripts analysis (Quantitatively) in CML patients.

The fusion transcripts (b3a2 and b2a2) of BCR-ABL for protein P210 and fusion transcript (e1a2) for protein P190 were analyzed in 30 Pakistani CML patients' samples using quantitative real time polymerase chain reaction (qRT-PCR) **(Figure 2).**

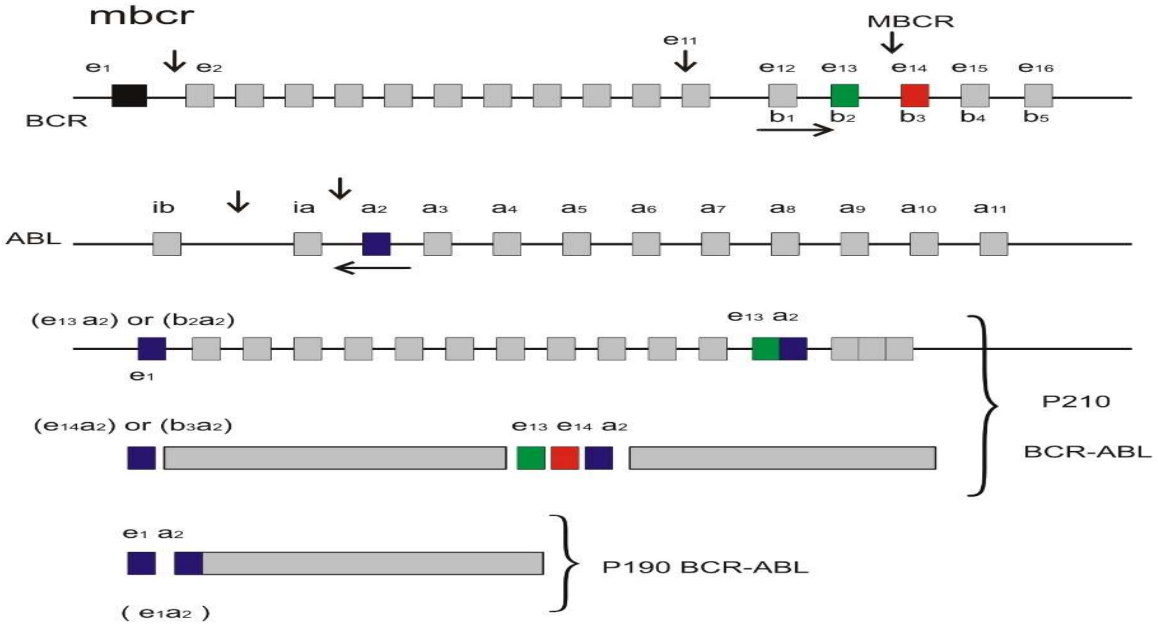


Figure 2: Fusion transcripts (b3a2 and b2a2) of BCR-ABL for protein P210 and fusion transcript (e1a2) for protein P190.

All 30 CML patients showed the existence of transcripts (b3a2 and b2a2) and 2 patients showed the coexistence of transcript variant (e1a2) out of 30 patients (6.6%).

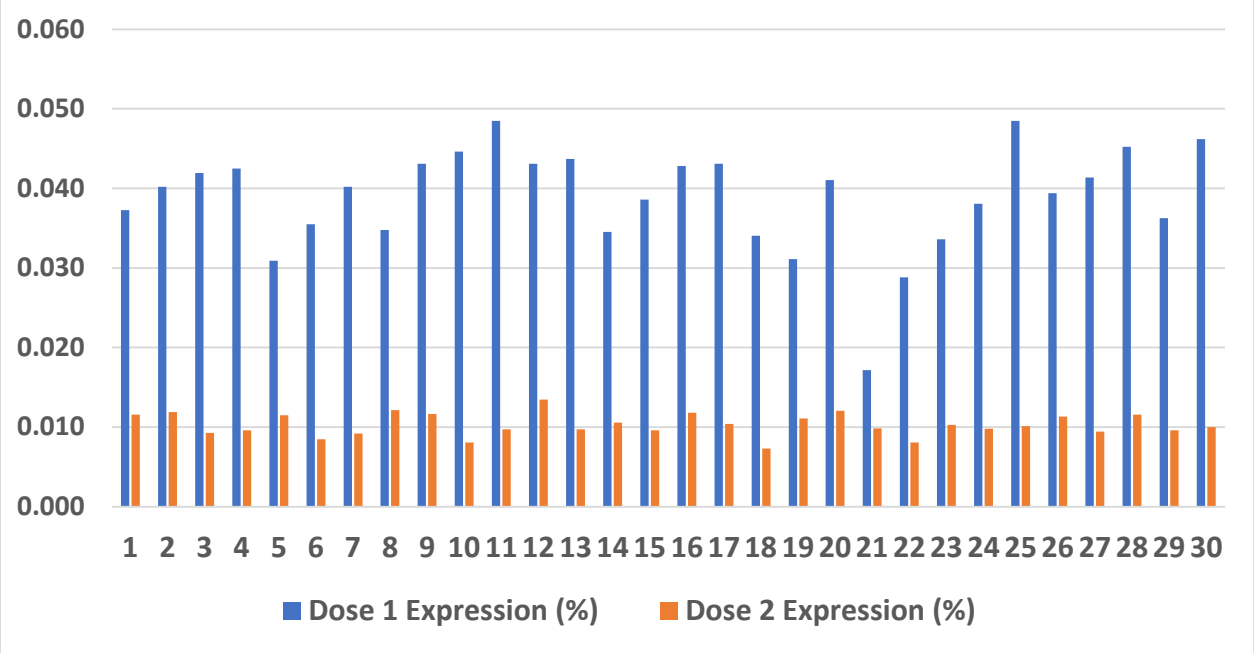


Figure 3: Graphical representation of the percent expression of fusion transcripts (b3a2 and b2a2) of BCR-ABL for protein P210 relative to abl gene for dose 1 & dose 2.

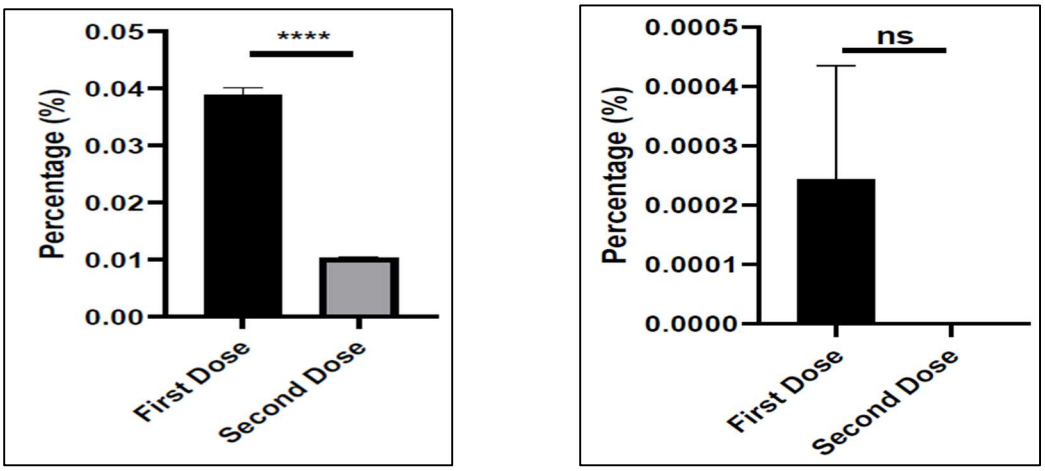
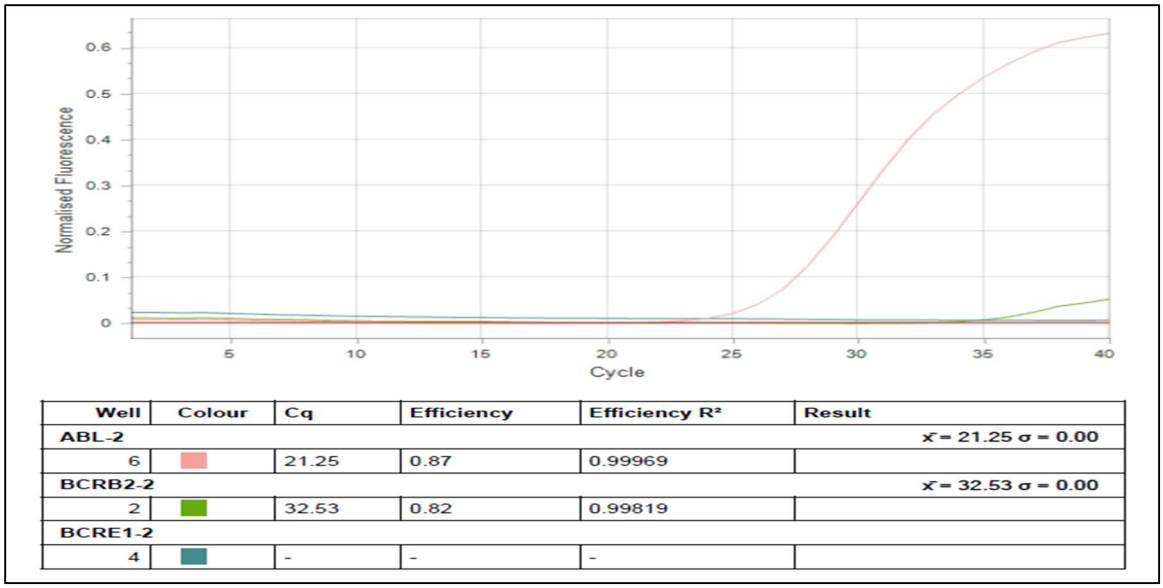


Figure 4: Graphical representation of the combined percent expression of fusion transcripts (b3a2 and b2a2) of BCR-ABL for protein P210 relative to abl gene for all patients (dose 1 & dose 2).

In qRT-PCR, the amplification of ABL gene was performed simultaneously in duplicate but in separate reaction as a internal control and to normalize BCR-ABL values. In this study cohort of CML patients, e1a2 (P190) BCR-ABL transcripts showed a low response to TKI therapies. The qRT-PCR was done in all (n = 30) CML patients on 1st dose and 2nd dose (after 90 days) of TKIs therapies (imatinib and nilotinib). Among 30 patients all 30 patients exhibit remarkably decrease in the expression of BCR-abl gene after 2nd dose of tyrosine kinase inhibitors.

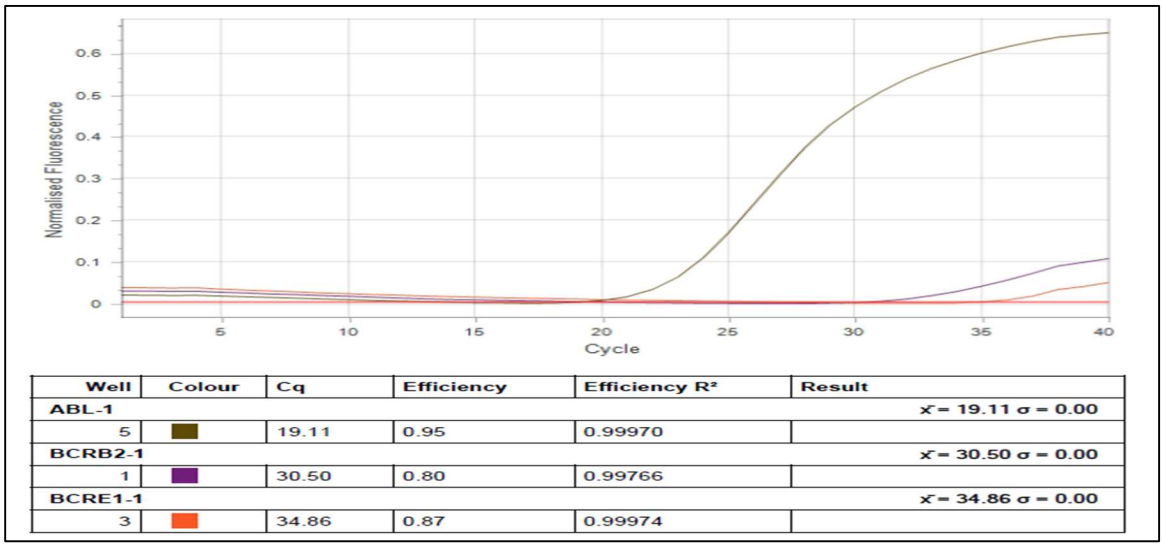
B2a2 (BCRB2)



Well	Colour	Cq	Efficiency	Efficiency R²	Result
ABL-2					x̄ = 21.25 σ = 0.00
6		21.25	0.87	0.99969	
BCRB2-2					x̄ = 32.53 σ = 0.00
2		32.53	0.82	0.99819	
BCRE1-2					
4		-	-	-	

Figure 5: Combined expression profile of abl gene and fusion transcripts **(b3a2 & b2a2)** generated by qRT-PCR for CML patients (Expression profile of abl gene (detected), Expression profile of **(b3a2 & b2a2)** (detected)**,** Expression profile of e1a2 (not detected)**.**

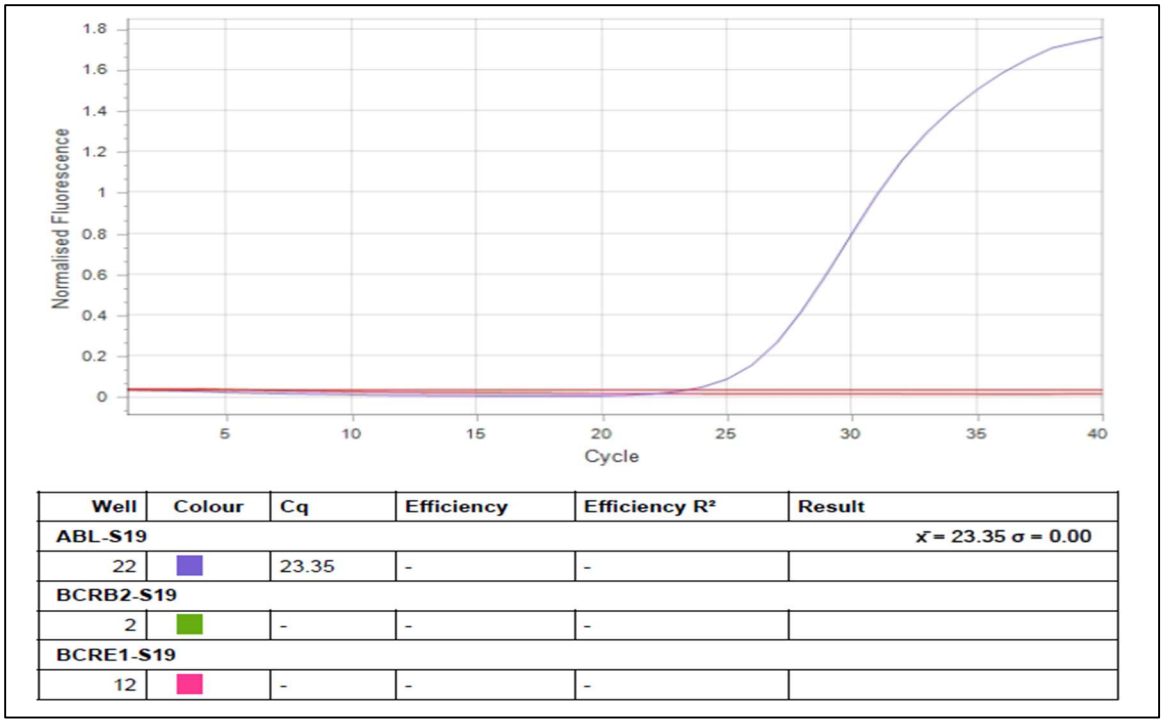
E1a2 (BCRE1)



Well	Colour	Cq	Efficiency	Efficiency R²	Result
ABL-1					x̄ = 19.11 σ = 0.00
5		19.11	0.95	0.99970	
BCRB2-1					x̄ = 30.50 σ = 0.00
1		30.50	0.80	0.99766	
BCRE1-1					x̄ = 34.86 σ = 0.00
3		34.86	0.87	0.99974	

Figure 6: Combined expression profile of abl gene and fusion transcripts **(b3a2 & b2a2) and e1a2** generated by qRT-PCR for CML patients (Expression profile of abl gene (detected), Expression profile of **(b3a2 & b2a2)** (detected)**,** Expression profile of e1a2 (detected)**.**

Healthy



Well	Colour	Cq	Efficiency	Efficiency R²	Result
ABL-S19					$\bar{x}$ = 23.35 σ = 0.00
22		23.35	-	-	
BCRB2-S19					
2		-	-	-	
BCRE1-S19					
12		-	-	-	

Figure 7: Combined expression profile of abl gene and fusion transcripts generated by qRT-PCR for CML patients (Expression profile of abl gene (detected), Expression profile of **(b3a2 & b2a2)** (not detected)**,** Expression profile of e1a2 (not detected)**.**

DISCUSSION

To detect multiple transcripts, RT-PCR is used with a primer particular for b1 exon (e12) of the gene (BCR) and a reverse primer specific for exon 2 (ABL). The current technique has the advantage of simplifying and reducing the RT-PCR technique for detecting these transcripts in a single assay. The requirement for distinct primers (sequence-specific) was abolished, increasing the PCR's complexity, labor, and expense. When compared to statistics from the western literature, which shows older mean ages (66, 52, and 59 years) at detection in CML patients, this is significantly younger[14,15,16]. This conclusion, however, is similar to previous Asian investigations[17]. Diagnosis at Young age in CML patients is typically connected with low- and middle-income nations, and environmental variables as well as underreporting of the elderly population of these nations could explain this[18].

Males were found to have a higher prevalence of CML (63%) than females (37%) and almost identical to previously reported local data[19] as well as research's from around the world[20] . Male predominance may be due to the fact that haematological neoplasms are more common in males than females due to genetic and hormonal variations[18] . Current study found that the most leading transcript in CML patients was e14a2 (b3a2). Comparable frequencies of e13a2, e14a2, and their co-expression also found in some studies, although there was also variation from these results. The prevalence of common transcripts in CML patients from various populations has been described in Table 2.

Table 2: BCR-ABL transcripts in different countries according to different studies.

Country	e14a2 (b3a2)	e13a2 (b2a2)	e14a2 (b3a2) + e13a2 (b2a2)	Others
Current Study	49.5%	43.9%	6.6%	-------
Bulgaria (Balatzenko et al., 2011)	54%	45%	0%	-----
India (Mir et al., 2015)	68%	24%	0%	-----
Iran (Yaghmaie et al., 2008)	63%	20%	3%	14%
Malaysia (Hassan et al., 2008)	69%	31%	0%	------
Brazil (Filho et al., 2019)	48%	36%	16%	-----
Korea (Goh et al., 2006)	68%	32%	0%	------

Other consist e1a2, e19a2, b3a3/b2a2, b3a3/b2a3, b3a3 and e1a3.

A research conducted in India and Bulgaria found the e14a2:e13a2 proportion of the two primary transcripts of the M-BCR region to be 1.3:1, which is nearly identical to the ratio observed in the current study[21] . However, research from our local community show a larger ratio of the two transcripts than what is presented here[22] . E14a2 levels were found to be two times higher (ratio 2:1) than e13a2 levels in CML patients in Brazil, Malaysia, and Korea[23,24], whereas reports from Iran and India found e14a2 transcripts to be nearly three times higher than e13a2 transcripts e13a2[25,26]. Several studies have also shown that e13a2 is more common in CML patients than the more common e14a2 transcript[27,28]. The differences in the frequency of common transcripts between various populations and locations of the world can be explained by distinctions in natural genetic factors, environmental elements, and life styles among ethnic groups[29] .

Only two (6.6%) of the 30 patients studied in current study had co-expression of e13a2/e14a2. Co-expression of the both transcripts e13a2 and e14a2 in CML patients has been observed in varying degrees around the world. In a Brazilian investigation, the prevalence of this co-expression was shown to be as much as 16%[29]. Nevertheless, in our local community, the probability of co-expression was observed to be quite low, which is consistent with the current study's findings[30] . In the current study, the largest rate (63%) of CML patients had a total leukocyte count of >100×109/L at the time of presentation, indicating a higher tumor volume at the time of diagnosis. The majority of research participants (58%) also had platelet level within the normal range. Both of these outcomes are consistent with past research. In this work, a remarkable association of male gender was detected in the e14a2 transcript while noting a putative affiliation between BCR-ABL variations and clinical parameters in CML patients. Current result is consistent with studies undertaken in Iran, India, and Iraq[31,32]. By splitting haemoglobin levels, TLC, and platelets level

into different ranges, the correlation of fusion transcripts was also studied. Although e14a2 had a stronger correlation with increased platelets level than e13a2, it was not statistically important (P = 0.09). Similarly, no significant correlation could be identified in either transcript group by stratifying the patients based on WBC and haemoglobin levels. Data from past investigations on the relationship between haematological factors at diagnosis and transcript type remain contentious[33,34].

CONCLUSION:

In this Present study, 100% (30) of the CML patients had typical transcripts of BCR-ABL with a predominance of the subtype (e14a2). When compared to the e14a2 group, patients with the transcript (e13a2) had a considerably higher mean leukocyte count. Identification of the type of transcript is not only useful for closely monitoring illness progression in patients, but it can also aid in the selection of new therapy regimens that target certain transcripts. Further studies with a greater sample size is required to identify the probable contribution of transcript types of BCR-ABL and illness exhibition, which could aid to the diagnosis and disease controlling.

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CONFLICT OF INTERESTS

Authors declared that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors have equally contributed in research work and manuscript proofreading. All authors have read and agreed to the version of the manuscript.

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