

# Detection of a High Frequency of e1a3/BCR-ABL in Chronic Myeloid Leukemia Patients of Sulaimania Province by RT-PCR

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## Abstract

**Introduction:** In order to detect the mutation variants of BCR-ABL fusion genes that lead to leukemia in human, 30 blood samples from chronic myeloid leukemia patients and 10 blood samples from apparently healthy people (as control) were collected and tested hematologically, morphologically and by amplification of the fusion mRNA by RT-PCR. The e1a3 variant was detected by amplification of 309 bp from the fusion of exons 1 of bcr and exon 3 of abl gene.

**Materials and method:** Application of RT-PCR assay to detect the prevalence and frequency of e1a2 and e1a3 variants in CML patients and in routine diagnosis of the most common leukemia translocations.

**Results:** The RT-PCR showed that out of 30 patients, 23 (76.6%) were with e1a3 mutation variant and 7 (23.3%) were negative for e1a2 and e1a3 variants. All the 10 control samples showed no mutations related to these variants.

**Conclusion:** The e1a3 variant was detected by amplification of 309 bp from the fusion of exons 1 of bcr and exon 3 of abl gene and the RT-PCR showed that out of 30 patients, 23 (76.6%) were with e1a3 mutation variant and 7 (23.3%) were negative for e1a2 and e1a3 variants, while the 10 control samples showed no mutations related to these variants.

**Keywords:** Chronic myeloid leukemia (CML); e1a2; e1a3; RT-PCR

## Introduction

Chronic Myeloid Leukemia (CML) is a myeloproliferative disorder of hematopoietic stem cells [1]. It has been observed following exposure to high doses of ionizing radiation [2]. It is also believed that Abelson murine leukemia virus (A-MuLV) which is originated from recombination between Moloney murine leukemia virus (M-MuLV) and the cellular proto-oncogene c-abl [3] is usually defective in replication, but can transform host cells following re-infection [4] CML is generally associated with Philadelphia (Ph<sup>+</sup>) chromosome that is resulted from translocation between chromosomes 9q34.1 and 22q11.21<sup>8</sup>. On Ph<sup>+</sup> chromosome the bcr-abl fusion gene encodes a constitutively active tyrosine kinase [5], which causes malignant transformation of leukocytes [6]. Duplication of Ph<sup>+</sup> chromosome, an isochromosome 17 [7], as well as MYC gene over-expression as a consequence of trisomy 8 or gene amplification are also found [2].

Clinical symptoms are usually nonspecific, and include fatigue, weight loss, anorexia, headache [1], fever, bone pain and splenomegaly [7]. Up to 50% of patients may be asymptomatic [1]. Complete blood count (CBC) shows an increased number of white blood cells, low number of red blood cells or blood platelets [8]. Blood films show pathologic left shift with the appearance of a large number of granulocytes at all stages of differentiation including promyelocytes and myeloblasts [9]. Bone marrow shows marked hyper-cellularity due to myeloid hyperplasia [9].

The chimeric protein encoded by bcr-abl fusion gene varies depending on the breakpoint in the bcr gene. Three breakpoint cluster regions have been characterized to date; major (M-bcr), minor (m-bcr) and micro (mu-bcr) [10]. The majority of CML patients have p210 BCR-ABL (M-bcr), whose mRNA transcripts have b2a2 and/or b3a2 junction [11], which is associated with granulocytic leukemia. A small group has p190 BCR-ABL (m-bcr), whose mRNA transcripts have e1a2 and/or e1a3 variants [10], which is principally associated with monocytic leukemia [10]. CML resulting from a p230 BCR-ABL (mu-

bcr) is also rare, and has been associated with the CNL and/or with marked thrombocytosis [10].

Since the introduction of imatinib (Gleevec), the outlook of patients with CML has improved dramatically. Many patients can remain in remission for many years while on this drug [11]. Imatinib is a selective inhibitor not only for ABL kinase but also for PDGFR kinases and c-kit tyrosine kinases [12,13] and it exerts significant anti-leukemic activity in the majority of CML patients.

Reverse transcription polymerase chain reaction (RT-PCR) assay is important to evaluate benefits of bone marrow transplantation and minimal residual disease (MRD) monitoring [14]. RT-PCR assay is rapid and accurate affording the ability to make crucial therapeutic decisions [14] the requirement for dividing cells is not needed and providing a sensitive marker for subsequent MRD testing [15]. In this case there is no need to wait for cytogenetic studies that require a longer time and has a lower sensitivity [14].

The bcr-abl fusion gene counts for the major molecular marker for leukemia in molecular pathology laboratories [14]. Recent WHO classification announced that genetic analysis of leukemia has prognostic and therapeutic implications, which require accurate genetic diagnosis that can be rapidly obtained by molecular approaches

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**Received** November 04, 2016; **Accepted** November 18, 2016; **Published** November 25, 2016

**Citation:** Salih GF (2016) Detection of a High Frequency of e1a3/BCR-ABL in Chronic Myeloid Leukemia Patients of Sulaimania Province by RT-PCR. J Leuk 4: 221. doi: [10.4172/2329-6917.1000221](https://doi.org/10.4172/2329-6917.1000221)

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[15]. RT-PCR assays are designed to detect specific fusions, and it would be extremely labor intensive to evaluate leukemia via a panel of individual monoplex assays. This can be circumvented by the use of multiplex RT-PCR assays [16].

This study aimed at detecting the prevalence of e1a3 and e1a2 variants in CML patients of Sulaimani province and the frequency of each variant through using RNA amplification technique by reverse transcription polymerase chain reaction (RT-PCR) and to apply RT-PCR assay in routine diagnosis of the most common leukemia translocation.

## Method

The genomic RNA was efficiently extracted by filter-based method. This method was used in RNA extraction of blood, which is rapid, safe and inexpensive [17]. Then all RNA samples (30 CML patients and 10 controls) were reversely transcribed to cDNA according to Bioneer RT PreMix protocol. Eight µl of genomic RNA, 2.5 µl of R-ABL (N6) [TGTGACTGGCGTGATGTAGTTGC] primer, 1.5 µl of R-actβ [GCATACCCCTCGTAGATGGG] as internal control and 3 µl of nuclease free D.W. were mixed in a 0.2 ml PCR tube and then the mixture was put in thermal cycler. RT1 reaction includes denaturation of genomic RNA at 65°C for 5 min. and then the reaction was hold at 4°C. The RT PreMix was dissolved with 5 µl of D.W. and then RT1 mixture was added to this mixture in a final volume 20 µl of RT2. RT2 reaction includes cDNA synthesis at 42°C for 1h, heat inactivation at 95°C for 5 min (according to Bioneer RT PreMix protocol), then the reaction was hold at 4°C.

The cDNA region from bcr-abl fusion gene was amplified by PCR using specific oligonucleotide primers. All 30 patient samples and 10 control samples were tested for the fusion using 1 µl 10 µM of F-BCR (N2) [ACCGCATGTTCCGGGACAAAAG] (0.5 µM), 1 µl 10 µM of R-ABL (N6) (0.5 µM), 0.5 µl 10 µM of F-actβ [TATGTGGGCGACGAGGCCCA] (0.25 µM), 0.5 µl 10 µM of R-actβ (0.25 µM) and 4 µl of cDNA sample were used in a final volume of 20 µl. Amplification includes initial denaturation for 4 min. at 94°C, then 35 cycles: each cycle consists of 94°C for 44 sec., 63°C for 30 sec. (according to primer manufacturer), and 72°C for 40 sec., final extension 72°C for 5 min. and then the reaction was hold at 4°C. The amplification products were analyzed on 2% of agarose gel stained with ethidium bromide and run at 90 V, 100 mA, 9 W for 90 min.

## Results

Ten local healthy subjects were tested for hemoglobin (Hb), white blood cells (WBC), and platelet count and bcr-abl fusion gene. They included 6 males and 4 females; their age ranged 18-62 years. All showed normal ranges of Hb, WBC and platelet count [18] and they were negative for e1a2 and e1a3 fusion variants (Table 1). Results for 30 subjects diagnosed clinically as CML patients showed different Hb, WBC and platelet count (Table 2).

From the specific primer typing, the prevalence of the two studied fusion variants (e1a2 and e1a3) and their distribution among CML patients of Sulaimani province is shown in Table 3. The only variant detected with a primer set F-BCR (N2) and R-ABL (N6) was e1a3 variant 76.6% while e1a2 appeared in 0% of total patients.

Blood film of patients on diagnosis showed leukocytosis and a pathologic left shift [7] with the appearance of immature granulocytopoietic cells at all stages of differentiation including

promyelocytes and myeloblasts [7]. Basophilia and eosinophilia were present in the peripheral blood and confirmed the diagnosis (Figures 1A and 1B) [9]. In addition, individual granulocytes showed qualitative changes such as anisocytosis [7].

No.	A	S	Hb* g/dL	WBC**x10 <sup>3</sup> /µl	Plt.***x10 <sup>3</sup> /µl	bcr-abl: e1a2, e1a3
1	21	M	14.8	5.0	166	-ve
2	34	M	14.3	6.2	265	-ve
3	56	M	15	7.2	304	-ve
4	18	M	16.4	7.0	293	-ve
5	46	F	13.6	6.2	224	-ve
6	62	F	12	5.2	228	-ve
7	33	F	12.3	6.6	234	-ve
8	26	M	14.1	8.5	448	-ve
9	12	M	12.5	7.3	294	-ve
10	19	F	13	5.8	374	-ve
M			13.8	6.5	283	

\*: Normal range of Hb for female (12.0-16.0 g/dL)

\*\*: Normal range of Hb for male (14.0-18.0 g/dL)

\*\*\*: Normal range of WBC (4.3-10.0x10<sup>3</sup> cells/ µl)

\*\*\*\*: Normal range of platelet (140-440x10<sup>3</sup> cells/ µl)

m: male; f: female; M: Mean; -ve: Negative

**Table 1:** Hb, WBC, platelet and bcr-abl fusion gene of control samples, those with normal CBC.

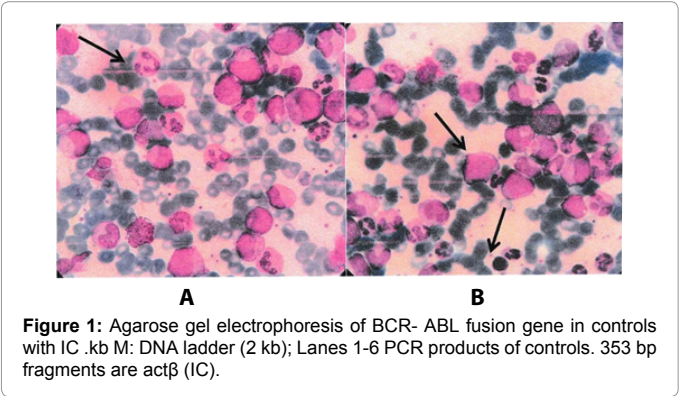
No.	A	S	Hb* g/dL	WBC***x10 <sup>3</sup> / µl	Plt.***x10 <sup>3</sup> / µl	bcr-abl: e1a3	Therapy
1	35	M	12.9	4.7	164	+ve	Glivec
2	46	F	10.4	6.2	477	+ve	Glivec
3	68	F	13.2	5.7	152	+ve	Glivec
4	54	M	13.8	6.5	148	+ve	Glivec
5	64	M	7.8	189	95	+ve	-
6	45	M	14	6.5	202	+ve	Glivec
7	53	F	13.3	4.5	175	-ve ★	Glivec
8	62	M	6.6	1.7	63	-ve ★	Glivec
9	42	F	8.9	122	126	+ve	-
10	35	M	15	7.0	242	+ve	Glivec
11	33	F	13.8	5.5	182	-ve ★	Glivec
12	52	M	11.4	5.8	140	-ve ★	Glivec
13	65	F	11.6	3.7	133	+ve	Glivec
14	57	F	12	5.6	149	+ve	Glivec
15	47	M	12.2	4.8	153	+ve	Glivec
16	54	F	11.3	9.7	284	+ve	Glivec
17	60	M	12.1	11.6	504	+ve	Glivec
18	45	F	10.3	8.3	500	+ve	Glivec
19	41	F	10.1	5.1	129	+ve	Glivec
20	65	M	12.8	7.4	255	+ve	Glivec
21	45	F	10.1	8.5	218	+ve	Glivec
22	58	F	10.9	7.7	128	+ve	Glivec
23	50	M	12.6	5.5	119	+ve	Glivec
24	52	F	12.5	6.5	21	+ve	Glivec
25	36	M	13.4	5.6	187	-ve ★	Glivec
26	51	M	11.2	6.3	145	-ve ★	Glivec
27	65	F	13	5.5	147	+ve	Glivec
28	47	M	14.2	6.5	212	+ve	Glivec
29	65	F	7.2	1.8	72	-ve ★	Glivec
30	50	F	11	5.8	143	+ve	Glivec

**Table 2:** Hb, WBC, platelet and bcr-abl fusion gene of those diagnosed clinically as CML patients.

Subjects	Number of Subjects	Ela 3 variant No. (%)	Ela 2 variant No. (%)	Negative Samples*
Males	14	10 (33.3)	0	4 (13.3)
Females	16	13 (43.3)	0	3 (10)
Total	30	(76.6)	0	7 (23.3)

\*: No of Mutations For the two variants ela3 and ela2 was detected

**Table 3:** Distribution of bcr-abl fusion variants among 30 CML patients of Sulaimani province.



Discussion

Out of 30 patients, 2 were new cases and appeared to have leukocytosis [18], and they were positive for e1a3 fusion because they were newly diagnosed and they did not take CML treatment [2]. In this case, the hemoglobin and platelet of patients were decreased in favor of granulocytopenia [7]. The BCR-ABL negative cases that form 23.3% of total patients may have molecular remission or may be of other variants that occur between different exons of both genes and require specific primer set to diagnose. Out of 30 patients, two had leukocytopenia, low Hb, and platelet count and were negative for e1a2 and e1a3 fusion variants because they might take overdose of Glive. The other five negative cases have good prognosis, which means that they have response to Glivec and didn't contain a mutation that affect ATP binding site of ABL kinase [19].

Although the primers used in this research were also used by Rosline et al. [20] but they did not get e1a3 variant with respect to the 309 bp fragments produced (Figures 2 and 3). They rather got e1a2/ 481 bp (as they determined) by using the same primer set; however, the fusion was at different site, e1a2/ BCR exon1-ABL exon2 while e1a3/ BCR exon1-ABL exon3. This means that the reverse primer anneals with exon 3 of ABL gene.

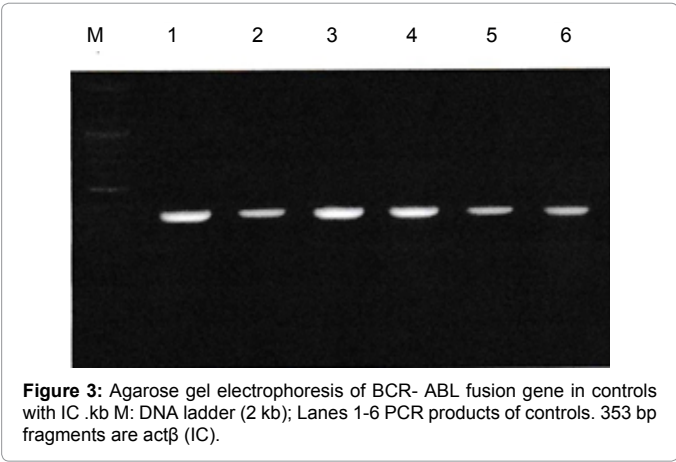
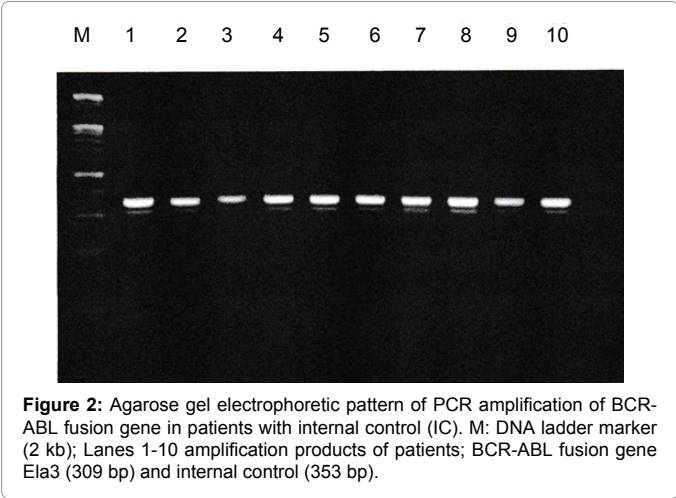
Heterozygous detection is reliable by RT-PCR method; specific primer sets for normal bcr and abl loci are required as well as the used primer set for bcr-abl fusion gene [21]. Gene silencing that caused by alteration of the methylation pattern of DNA on the related chromosome leads to prevent transcription [22] but it does not affect its DNA detection.

Out of 30 clinically diagnosed CML patients, 7 were negative for the two variants. The seven negative subjects have appeared to be negative for the two variants studied and to confirm their negativity another primer set is required. So either they are other variants or they are negative due to imatinib treatment leading to affect their fusion gene detection [23].

CML is one of the most common human cancers, being present in

high frequencies especially in countries with high radiation rate [24,25]. It is a common hematologic cancer worldwide especially in the regions after nuclear disaster [11]. More than 50 different point mutations in Abl kinase domain have been described [26]. However, many of these mutations are relatively rare, and the most common, affecting residues Gly250, Tyr253, Glu255, Thr315, Met351 and Phe359, account for 60–70% of all mutations [27]. With regard to the incidence, the frequency of CML by order is white, black, American Indian/ Alaska Native, Hispanic and Asian/ Pacific Islander respectively [28]. In north India the b3a2 BCR-ABL transcripts were most commonly detected (66.82%) followed by b2a2 (28.84%), b3a2+b2a2 (3.36%), b3a2+e19a2 (0.48%) and b2a2+e19a2 (0.48%) (29). In Pakistani patients, 64% expressed b3a2 while 36% expressed b2a2 transcript [29,30]. In Iran the majority of the patients express one of the p10 BCR-ABL transcripts (b3a2, 62% and b2a2, 20%), while the remaining showed one of the transcripts of b3a3, b2a3, e1a2 or co-expression of b3a2 and b2a2. The rate of co-expression of the b3a2 and b2a2 was 5% 29. In Turkey the most frequent type of transcriptions was p10 76%, p190 16.5% and p190 with p10 transcripts 7.5%. The most common type of p10 was b3a2 transcript (54%), p190 was e1a3+e1a2 (36%), p10+p190 was b3a2+e1a3 (40%), and b3a2+e1a3+e1a2 (40%) [31]. The prevalence of e1a3 is common (76.6%) in Sulaimani patients (Table 3). Sulaimani province mostly inhabited by Kurdish people and in general e1a3 is a common variant in Kurdish population of Sulaimani province.

The new cases appeared to have leukocytosis, with anemia and





thrombocytopenia, in favor of granulocytopoiesis that dominated the cell picture [7]. In blastic phase, the blast cells were found in greater frequency, they were in excess of 20% of the circulating leukocytes [9,32]. During this phase, smears showed elevated number of blast cells that resulted from malignant transformation of bone marrow stem cells [5,32]. For those patients that have response to treatment, these cells were little or absent.

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