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RESEARCH ARTICLE

Incidence of T315I mutation in BCR/ABL-positive CML and ALL patients

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OBJECTIVES: Targeted therapy of Philadelphia-positive ALL and CML patients using imatinib (IM) has caused significant changes in treatment course and has increased the survival of patients. A small group of patients show resistance to IM. Acquired mutations in tyrosine kinase domain of BCR-ABL protein are a mechanism for development of resistance. T315I is one of the most common acquired mutations in this domain, which occurs in ATP binding site and inhibits the formation of hydrogen bond with IM. The aim of this study was to evaluate the prevalence of this mutation in BCR/ABL-positive CML and ALL patients.
METHODS: To conduct this study, 60 BCR-ABL-positive patients (including 50 CML and 10 ALL patients) who were subject to treatment with IM were selected. After taking the samples, presence of T315I mutation was assessed using ARMS-PCR on cDNA and its polymorphism was evaluated by sequencing.
RESULTS: The results showed that among 60 patients, only three patients had T315I mutation, which was detected using ARMS technique. The three patients bearing mutation were afflicted with CML and no significant association was found between blood parameters with duration of treatment in presence of mutation.
CONCLUSIONS: The mutation was found in three CML patients, which indicated lower likelihood and diagnostic value of this mutation in ALL patients. Given the negative direct sequencing results in T315I patients, it can be concluded that ARMS-PCR is a more sensitive technique when the number of cancer cells is low in patients during treatment.

Keywords BCR-ABL, T315I mutation, imatinib, CML, ALL

Introduction

Philadelphia (Ph) chromosome is one of the most well-known chromosomal abnormalities developed as a result of reciprocal translocation between chromosomes 22 and 9 [t(9; 22)(q34; q11)] through 3′ transfer of *Abl* proto-oncogene from 9q34 next to 5′ end of *Bcr* gene on 22q11.2. (La Starza et al., 2002; Iacobucci et al., 2012). This translocation gives rise to *Bcr-Abl* chimeric gene in which the first *Abl* exon substitutes for N-terminal exons of *Bcr* gene. Depending on the locus where the break has occurred, chimeric *Bcr-Abl* gene can have one, two, three or even more exons than *Bcr* gene, which results in the formation of chimeric proteins with different sizes (Fig. 1A). The presence of this chromosome has been reported in approximately 90%-95% of chronic myeloid leukemia (CML) patients, 5%-10% of adult ALL and 20%-25% of childhood acute lymphoblastic leukemia (ALL) patients (Faderl et al., 2003; Bhojwani et al., 2015). Tyrosine kinase inhibitors (TKIs) are used to treat BCR-ABL-positive patients. Imatinib Mesylate (IM, STI571) is the first and most commonly used TKI (Druker et al., 2006), which occupies the ATP binding site on BCR-ABL oncoprotein, inhibiting the phosphorylation of substrates and resulting in reduced tyrosine kinase activity of this protein as well as decreased proliferation of malignant cells (Druker et al., 1996; Chomel et al., 2010). Despite appropriate patient response to this drug, a small group of BCR/ABL-positive patients show primary or secondary resistance to IM therapy. During primary resistance, the patient shows no response to treatment, which is

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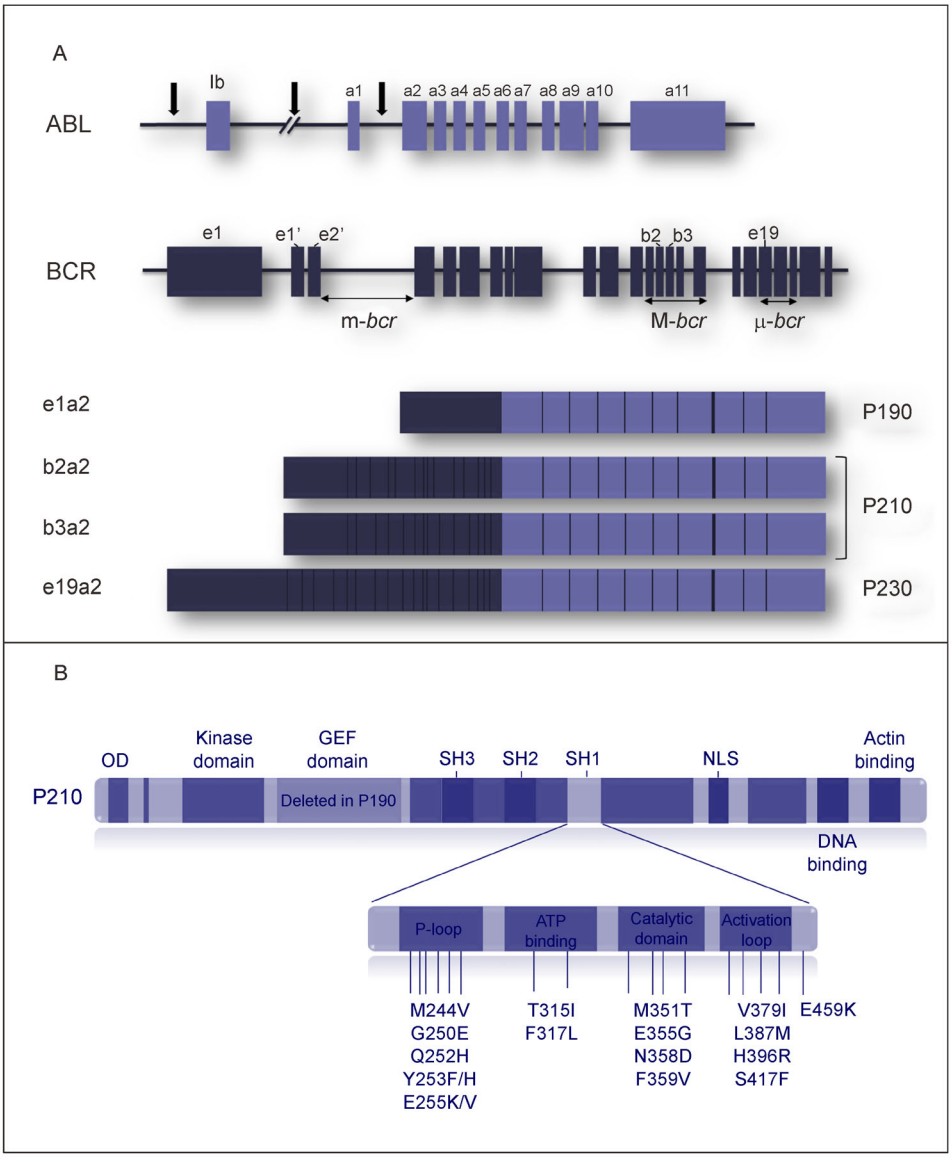

Figure 1 (A) The formation of the *Bar-Abl* fusion gene. (B) The most common mutations in BCR/ABL tyrosine kinase domain. (A) Depending on the locus where the break occurs in Bcr gene, chimeric *Bcr-Abl* gene bears two, three or even more exons than normal gene, which results in the formation of chimeric proteins of different sizes. (B) Several mutations occur in *Bcr-Abl* kinase domain. The most common sites include P-loop (codons 244-256), ATP binding site (codons 315 and F317), catalytic domain (codons 350-363) and activation loop (codons 381-407). T315I mutation occurs in ATP binding site where IM is found, causing resistance to treatment of patients with IM.

usually an inherited disorder. However, in secondary drug resistance, which is mainly acquired, patient's response to drug is gradually reduced. Acquired resistance can develop as a result of BCR-ABL dependent mechanisms (including *Bcr-Abl* gene amplification or *Abl* tyrosine kinase domain mutations) or BCR-ABL independent mechanisms (including changes in transporter genes and/or drug-metabolizing genes) (Hochhaus et al., 2002; Roche-Lestienne et al., 2003; Donato et al., 2004). In general, resistance to treatment by TKIs, especially in 40%-90% of acquired cases, occurs as a result of polymorphisms or mutations in BCR-ABL kinase domains (Jabbour and Soverini, 2009; Kagita et al., 2014). BCR-ABL kinase domain is highly conserved and different mutations occur in four loci of it, including P-loop (codons 244-256), IM binding site (codons 315 and F317), catalytic domain (codons 350-363) and activation loop (codons 381-407) (Fig. 1B) (Hughes eet al., 2006; Apperley, 2007). Studies show that different mutations in BCR-ABL tyrosine kinase cause varying degrees of drug resistance depending on the location and type of mutation. T315I missense mutation in BCR/ABL-

positive patients is a well-known mutation in the mentioned gene (Gorre et al., 2001; Quintás-Cardama and Cortes, 2008), which is associated with poor prognosis and reduced overall survival in patients (Ernst et al., 2011). This mutation is a function of threonine substitution for isoleucine in position 315 of BCR-ABL protein as well as IM binding site on BCR-ABL protein, which leads to inhibition of IM binding and sustained protein kinase activity of BCR-ABL protein, proliferation, survival and resistance of malignant cells to apoptosis (Gorre et al., 2001; Soverini et al., 2007; Kimura et al., 2014) (Fig. 1). In addition, recent studies have shown that the use of second generation TKIs such as Nilotinib or Dasanitib has little effect in elimination of BCR-ABL leukemia cells in patients with T315I mutation (Branford et al., 2009; Maru, 2012). Therefore, currently available third generation TKI drugs such as Ponatinib can be effective against T315I (Shah et al., 2004; Weisberg et al., 2005; O'Hare et al., 2009; Maru, 2012). However, the second and third generation drugs are not able to eliminate malignant cells in quiescent phase and can only affect the circulating cells (La Rosée and Deininger, 2010). This study was conducted to determine the prevalence of T315I translocation in BCR-ABL-positive CML and ALL patients in DNA and cDNA level. Since this mutation is one of the most common causes of secondary resistance to IM therapy in this two patient groups, awareness of its prevalence as well as its assessment before and during treatment enables the prediction of response to treatment and can be useful to take appropriate therapeutic strategies tailored to patient's conditions.

Materials and methods

Patients and samples

The present study includes 60 BCR/ABL-positive patients (including 50 CML patients and 10 ALL patients) treated with standard imatinib dose of 400mg according to European Leukemia Net (ELN) guidelines. The study was approved by the institutional ethics committee of Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1394.342), and informed consent was obtained from each patient participating in the study. 5 ml peripheral blood (PB) sample was collected from each patient in EDTA-containing vials. Median age of CML and ALL patients was 45.19 (range 16–75 years) and 17.25 years (range 3–50 years), respectively. Out of 50 CML cases, 48% were male and 52% were female and in ALL group 80% were male and 20% were female. Evaluation of minimal residual disease (MRD) was used to evaluate the patients' response to treatment. Other clinical data of patients is shown in Table 1.

DNA extraction and direct sequencing technique

Total DNA was isolated from PB by Qiagen kit (Germany) based on manufacturer's instructions. DNA was spectrophotometrically quantified at 260 nm and was then used for mutational screening by direct sequencing technique using two sets of primers covering the *Abl* kinase domain region. The following specific primers were used: ABL1-ex6-

Table 1 Clinical characteristics of patients

		CML	ALL	*p*-value
Sex	Male	48.0%	80.0%	-
	Female	52.0%	20.0%	-
Age		45.19 (16.0-75.0)	17.25 (3.0-50.0)	-
WBC1*		42.3 (5.30-220.4)	20.0 (10.20-119.70)	0.82
WBC2**		6.19 (3.35-19.2)	8.3 (3.5-10.3)	0.249
Hb1*		10.65 (8.1-19.1)	10.7 (8.5-13.0)	0.654
Hb2**		11.6 (8.0-16.4)	10.0 (8.4-11.8)	0.019
PLT1*		389.0 (168.0-2166.0)	102.0 (30.0-274.0)	0.0001
PLT2**		189.0 (59.0-1289.0)	111.0 (60.0-297.0)	0.145
Organomegaly	No organomegaly	62.0%	55.6%	-
	Hepatomegaly	0.0%	11.1%	-
	Splenomegaly	30.0%	11.1%	-
	Hepato-Splenomegaly	8.0%	22.2%	-
Therapy duration	>1 year	51.2%	100.0%	-
	1 year	31.7%	0.0%	-
	<1 year	17.1%	0.0%	-
Therapy response	Optimal	52.3%	90.0%	-
	Failure	40.9%	10.0%	-
	Warning	6.8%	0.0%	-

* Counting parameters at the time of diagnosis.
**Counting parameters at the time of T315I mutation evaluate.

Forward (F): 5′-AGTCTCAGGATGCAGGTGCT-3′; ABL1-ex6- Reverse (R): 5′-AATGTGTTGCCAGCACTGAG-3′.

Isolation of mononuclear cells

EDTA PB samples were layered onto Ficoll-Paque (Lymphodex, inno-Train, Germany) gradient and centrifuged at 400 g for 20 min at 24°C. Isolated mononuclear cells were twice washed with phosphate buffer saline (PBS).

RNA extraction and cDNA synthesis

Total RNA was extracted from 10^6 isolated cells using RNeasy Mini Kit (RIBO-prep, Russia) based on manufacturer's instructions. RNA was spectrophotometrically quantified at 260 nm. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA in 20 ml reaction mixture using a cDNA synthesis kit (Bioneer, Korea) according to the manufacturer's instructions. The mixture was incubated at 20°C for 30s, 42°C for 4 min and 55°C for 30s, followed by 95°C for 5 min performed at 8 cycles.

ARMS-PCR reaction

cDNA was used for ARMS-PCR reaction. The following specific primers were used in this technique: ABL kinase Forward (F):5′-CGCAACAAGCCCACTGTCT-3′; ABL Kinase Reverse (R):5′-TCCACTTCGTCTGAGATACTG-GATT-3′ and AS-T315:5′-CGTAGGTCATGAACTCAA-3′. 25μL PCR reaction mixture was prepared containing 5μL 10× buffer, 2 μL dNTP, 1 μL each primer, 3 μL $MgCl_2$ (50mM), 0.3 μL Taq polymerase Enzyme, 9.7 μL DW and 2 μL cDNA. The thermal cycling conditions were as follows: 5 min at 95°C for 1 cycles, 30 s at 95°C, 30s at 67°C, 40 s at 72°C followed by 35 cycles and 5 min at 72°C performed at 1 cycle. All the samples were run in duplicate to minimize handling errors.

Statistical analysis

Data were analyzed by SPSS software for Windows v.22.0. To compare demographic variables between the two groups, Mann–Whitney test was used to normalize the data. Chi-square test was used to compare the proportion of mutations in the two groups. $p < 0.05$ was considered statistically significant.

Results

Sixty BCR-ABL-positive patients treated with IM were evaluated for presence of T315I mutation in DNA and cDNA level. The sequencing results were normal for all the patients; in other words, none of the patients had this mutation in DNA level (Fig. 2). However, ARMS-PCR technique detected mutation in 4.6% of patients (3 from 60 patients) (Fig. 3), which indicates the presence of acquired mutation in cDNA level. Since these three patients were afflicted with CML, the mutation rate of 6% can be estimated in this group of patients, while it was not detected in ALL patients (Table 2). Demographic variables were compared between the two groups in terms of normality of data using Mann–Whitney test, which indicated a significant correlation between PLT1 ($p = 0.0001$) and Hb2 ($p = 0.019$) in the two groups. The two groups were matched in terms of treatment period and a significant difference was observed between the two groups according to chi-square test ($p = 0.062$) while the difference was not significant among the patients bearing mutation ($p = 0.418$) (Table 3). Also, no significant correlation was found between presences of mutation with response to treatment in T315I patients (Table 4).

Table 2 The T315I mutation rate in BCR/ABL positive patients

		Group		Total
		CML	ALL	
T315I mutation	Negative	(47) 94.0%	(10) 100.0%	(57) 95.4%
	Positive	(3) 6.0%	(0) 0.0%	(3) 4.6%
Total		(50) 100.0%	(10) 100.0%	(60) 100.0%

Table 3 T315I mutation and during treatment relationship in CML patients

		T315I		Total
		Negative	Positive	
Duration	> 1 year	58.3%	100.0%	60.8%
	< 1 year	14.6%	0.0%	13.7%
	1 year	27.1%	0.0%	25.5%
Total		100.0%	100.0%	100.0%
Chisquare(*p*-value)		2.056 (0.418)		

Table 4 T315I mutation and responses to treatment relationship in CML patients

		T315I		Total
		Negative	Positive	
Response	Optimal	(37) 58.8%	(2) 66.7%	59.3%
	Failure	(17) 35.3%	(1) 33.3%	35.2%
	Warning	(3) 5.9%	(0) 0.0%	5.6%
Total		100.0%	100.0%	100.0%
Chisquare (*p*-value)			0.209 (1.00)	

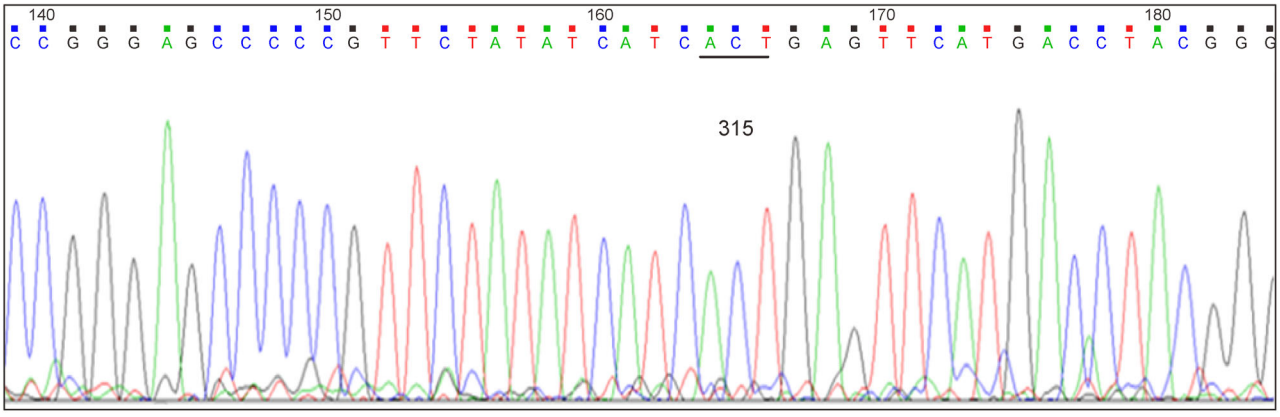

Figure 2 Evaluation of T315I mutation by direct sequencing technique in BCR-ABL-positive patients.

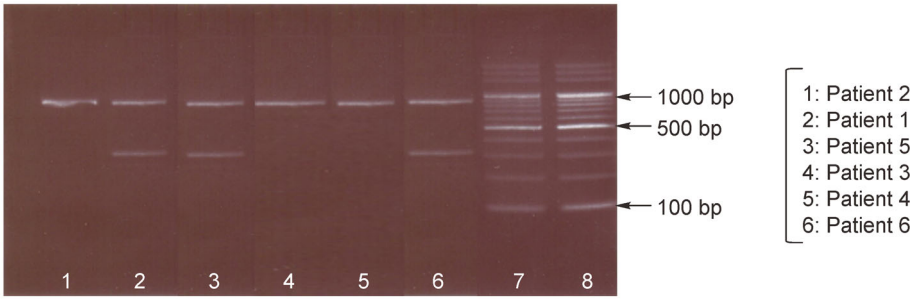

Figure 3 Evaluation of T315I mutation by ARMS-PCR techniques in BCR-ABL-positive patients.

Discussion

T315I missense mutation is one of the most common acquired mutations in IM-resistant CML patients, which occurs in IM binding site on BCR-ABL protein, inhibiting IM binding to protein and causing sustained protein tyrosine kinase activity of BCR-ABL (Kimura et al., 2014). BCR-ABL protein activates different cell signaling pathways (Fig. 4), causing proliferation, survival and resistance to apoptosis of malignant cells (Chomel et al., 2010; Ernst et al., 2011). Recent studies have evaluated the prevalence of different mutations in tyrosine kinase domain in BCR/ABL-positive patients who are resistant to treatment and it was observed that the highest incidence of mutation in this domain was related to T315I (Khorashad et al., 2013). In this study, 60 BCR-ABL-positive patients were evaluated for the presence of T315I mutation in DNA and cDNA level, and no mutation was detected in DNA level. However, assessment of T315I mutation in cDNA level indicated the presence of mutation in three CML patients (6%) treated with 400 mg dose of IM. Several studies have shown that the prevalence of this mutation in CML patients is 2%-20%, and the extent of this range is dependent upon detection methods, patient cohort characteristics and treatment response of patients(Jabbour et al., 2008; Nicolini et al., 2009).

In our study, two patients with T315I mutation (66.7%) showed favorable response to treatment, and only one of them was resistant to treatment (33.3%). Unlike previous studies,

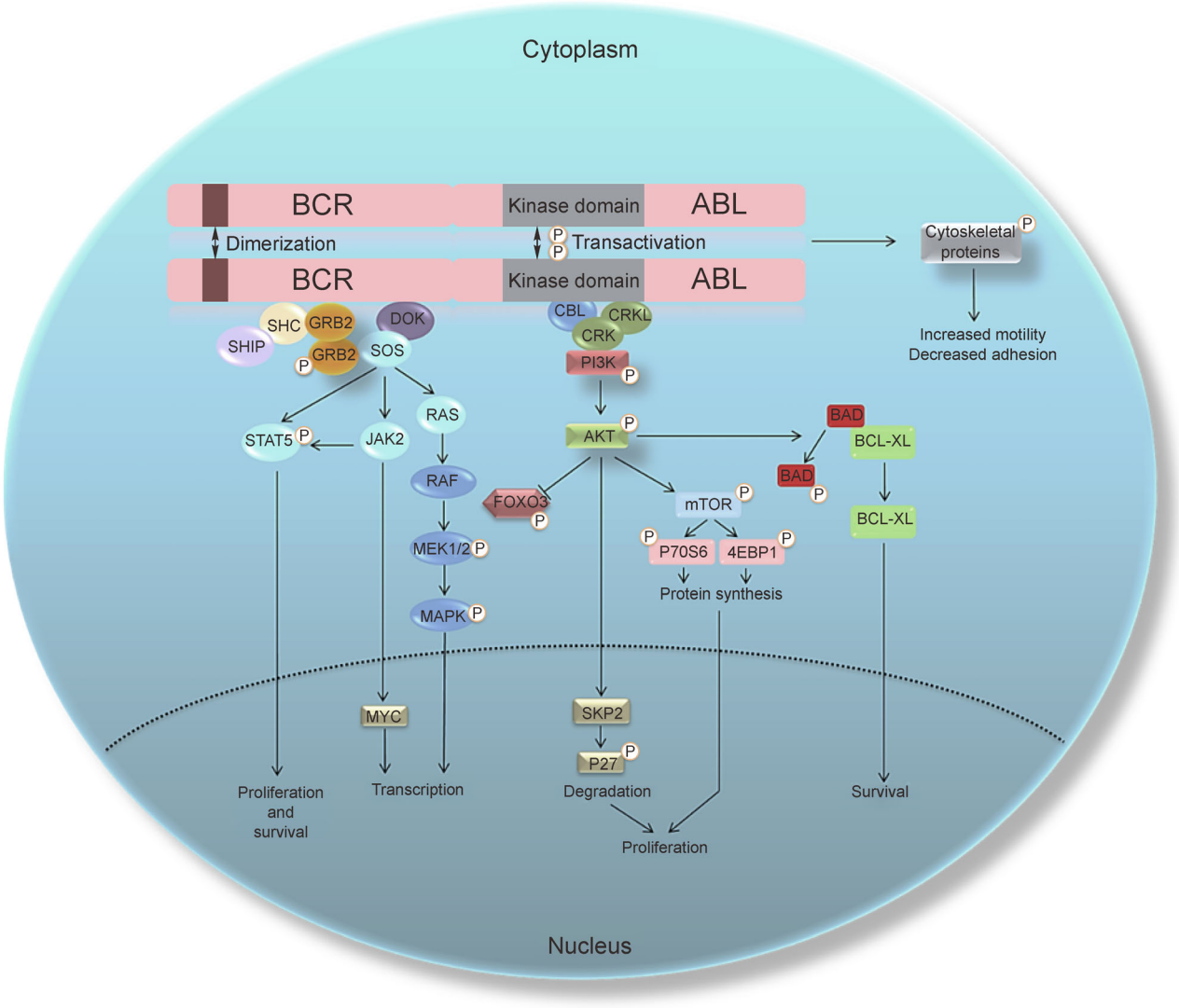

Figure 4 Activation of different signaling molecules by BCR-ABL fusion protein. BCR-ABL protein activates several signaling pathways that enhance cell proliferation and genetic instability, inhibit apoptosis and reduce cell adhesion. The most important signaling molecules activated by BCR-ABL fusion protein include Myc, Ras, MAP/ERK, JAK/STAT, NFκB, PI-3kinase and c-Jun. Therefore, BCR-ABL can act as an oncoprotein via activation of these signaling pathways.

this study showed that T315I mutation is not only found in refractory patients but in patients with favorable response to treatment, which may be used as a prognostic factor for disease relapse or drug resistance in the future. On the other hand, the above results indicated no significant relationship between mutation, treatment period of patients, response to treatment, blood parameters and clinical signs in T315I positive patients. Therefore, evaluation of this mutation is recommended in all the patients in different phases of disease. The difference between sequencing results and ARMS may be due to the fact that the genome of normal and malignant cells is simultaneously separated in direct sequencing technique and T315I mutation domain is most probably missed since normal cells are predominant. However, due to amplification of *Bcr-Abl* fusion as well as using T315I-specific primer in ARMS technique, only malignant cells are evaluated for mutation, which increases the likelihood of mutation detection. Therefore, even though sequencing is a confirmatory method, it is worthless in these patients, especially when there is a scanty population of malignant cells. In addition, this difference could indicate the acquired nature of mutation since if it was hereditary, it should have been found in all normal and malignant cell populations of the three patients. Therefore, these two techniques can be used together to differentiate the inherited or acquired nature of mutation. Other studies have dealt with the prevalence of this mutation, such as the study of Tanaka et al. in 2011 on 131 patients, including 97 CML patient in chronic phase (CML-

CP), 6 patients in the advanced phase (CML-AP), 11 patients in blastic crisis (CML-BC) and 17 ALL-Ph+ patients. This study showed that 32 patients had 17 different types of point mutations in *Bcr-Abl* fusion gene. Among the detected mutations, T315I was the most frequent in CML-BC and ALL-Ph+ patients, which was associated with poor prognosis (Tanaka et al., 2011). However, this mutation was observed in none of the ALL-Ph+ patients in our study. In another study by Kagita et al. in 2014 on 63 CML patients who were resistant to 400 mg dose of IM, 15 exon mutations were detected in BCR-ABL kinase domain in 29 patients (46.03%), among whom 7 cases (24.13%) had various types of mutations. The most frequent mutations observed in these patients were as follows: T315I (12 cases), T240R (7 cases), A397A (4 cases), F359I and L387W (3 cases), L248R and I242F (2 cases) as well as one case of each of M244V, G250E, Y253Stop, E255V, T272T, F311I, M351T and L364L mutations. Out of 15 exon mutations observed, 11 mutations were non-synonymous associated with dysfunction of the mentioned protein, 3 cases were synonymous mutation and there was one case of termination mutation. P-loop and T315I mutations were observed in 51.72% of patients (15 out of 29) and other mutations in 48.27% of patients (14 out of 29). This study also showed that T315I-positive patients had a significantly higher expression level of BCR-ABL compared to T315I-negative ones ($p = 0.01$). The level of BCR-ABL was higher in patients bearing P-loop and T315I mutations compared to other mutations ($p = 0.001$) (Kagita et al., 2014). In an analytic epidemiologic study, Ernst et al. (2008) assessed the *BCR-ABL* kinase domain mutations in 911 CML patients who were resistant to IM, and indicated mutation in this domain in 50% of patients (456 out of 911). The main mutations observed in translocated BCR-ABL alleles were as follows: 58758 G/A (T240T), 68708 T/G (F311V), 68722 T/G (T315 I), 68736 A/G (Y320C), 58778 A/G (K247R) and 74901 A/G (E499E). Furthermore, T315I mutation was observed in a 45-year-old man in chronic phase of CML who showed a good response to therapy with 600 mg IM during 37 months (Ernst et al., 2008).

Since the majority of the mentioned studies have taken advantage of RT-PCR and sequencing to assess T315I mutation, we have used ARMS-PCR technique due to lower testing costs, simple procedure and quick results.

The findings of this study indicate that T315I mutation in BCR-ABL-positive patients has nothing to do with treatment period and response to treatment in patients. In addition to treatment-resistant patients, T315I mutation should be assessed in patients with a favorable response to therapy, which can be associated with the relapse of their disease in future and requires comprehensive studies in a longer period of time. Assessment of mutations associated with IM resistance has considerable importance for treatment, and different IM doses or treatment shift to second or third generation TKIs may increase patient survival in case of detection of such mutations.

Abbreviations

JAK-STAT: Janus kinases/signal transducers and activators of transcription; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor κB; PI-3 kinase: Phosphatidylinositol 3-kinase.

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Compliance with ethics guidelines

The authors declare that they have no conflict of interest. All the procedures performed in the studies involving human participants were in accordance with the ethical standards of local ethics committee of the Ahvaz Jundishapur University of Medical Sciences (IR.AJUMS.REC.1394.342) as well as 1964 Helsinki declaration. Written informed consent was obtained from all patients and normal subjects.

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