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Molecular Screening for E255K and F359V Mutations in Non-Responders Iraqi Chronic Myeloid Leukemia Patients to Imatinib Mesylate Therapy

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Abstract

Mutations in the kinase domain (KD) of BCR-ABL are the most prevalent mechanism of acquired resistance to first generation of tyrosine kinase inhibitor Imatinib Mesylate (IM) in chronic myeloid leukemia (CML) patients. Dasatinib and Nilotinib, the second generation of tyrosine kinase inhibitors (SGTKI), have been approved for second-line treatment of CML patients who demonstrate resistance to IM. The identification of E255K and F359Vmutations, which are considered highly resistant to SGTKI nilotinib, lead to a clear-cut decision in the choice of the appropriate SGTKI dasatinib. The aim of this study is to assess the frequency of mutations E255K and F359V in Iraqi CML patients who showed criteria of failure or suboptimal response to IM by using Allele Specific Oligonucleotid-Polymerase Chain Reaction (ASO-PCR). In this cross-section study, 70 patients were diagnosed clinically and hematologically as CML, who attended the Baghdad Medical City /Teaching Hospital/Hematology Unite, during the period between September 2011 to June 2012. They were on IM in different doses (400-800 mg/day) for at least one and a half year. Those patients were classified according to their responsiveness to IM into four groups according to European Leukemia Net. Also, 10 healthy age match subjects were included who served as technical negative control. Peripheral blood (PB) samples were taken from each subject. DNA was extracted using commercial available DNA extraction kit. Molecular screening for the presence of E255K and F359V mutations were done using ASO-PCR. The mean value of age of CML patients was 40.5 ± 2.56 years. Male to female ratio of CML patients was 1.12:1. The result of molecular screening for two mutations showed that all seventy CML patients were negative for both mutations. We conclude that the presence of other variants of studied mutations, another types of point mutations or other mechanisms for resistance to IM such as over expression of BCR-ABL, drug influx and efflux could be the causes of IM resistance.

Keywords: chronic myeloid leukemia, imatinib, treatment resistance, allele-specific oligonucleotide polymerase chain reaction

1. Introduction

Chronic myeloid leukemia (CML) is a clonal disease that results from an acquired genetic change in pluripotential hematopoietic stem cells. This altered stem cell proliferates and generates a population of differentiated cells that gradually displace normal haemopoeisis and leads to greatly expanded total myeloid mass. The hallmark of this disease is a unique chromosome, known as the Philadelphia chromosome (Ph), which is found in 95% of CML patients (Goldman & Melo, 2003; Vaidya, Ghosh & Vundinti, 2011). The current management of CML has been essentially transformed by the introduction of targeted therapy in the form of selective tyrosine kinase inhibitor (TKI). CML patients outcomes have dramatically improved to the extent that first line-TKI, imatinib mesylate (IM), which is considered as the first-line agent for nearly all patients presenting with CML, regardless the phase of the disease. Impressive clinical response is obtained in the majority of patients in chronic phase (CP). However, the clinical response in a number of patients will not be sustained and approximately 20% to 40% of patients expressed drug resistance (Milojkovic & Apperely, 2009). Development of resistance to IM and other TKIs is believed to be a consequence of the interaction of multiple factors including, kinase domain mutations, mutations outside KD, treatment compliance, bioavailability, pharmacodynamics and genetic changes (Quintas-Cardama,

Kantarjian & Cortes, 2009). Mutations within the KD of BCR-ABL account for 30% to 50% of IM resistance cases. Point mutations that change the amino acid of the contact site or the specific KD conformation to which IM binds attenuate it inhibitory property (Cang & Liu, 2008). More than 100 point mutations have been reported (OHare, Eide & Deininger, 2007; Cang & Liu, 2008). Some of these mutations have important role to clinician in the decision of change treatment from IM to SGTKIs, such as (E255K/V, F359V/I/C). Mutations are less sensitive to nilotinib and sensitive to dasatinib, and that can give idea about the importance to look for these mutations after IM failure or suboptimal response to IM (Tatar et al., 2011; Wongboonma, Thongnoppakhun, & Auewaraku, 2011). Screening for BCR-ABL mutations could be done using several molecular methods (Deininger et al., 2004; Soverini et al., 2004; Kang et al., 2006; Ernst et al., 2009; Jones et al., 2009). Allele Specific oligonucleotides-Polymerase chain reaction (ASO-PCR) is considered as a very sensitive (0.5%) (Wongboonma, Thongnoppakhun & Auewaraku, 2011) and rapid method used for monitoring CML patients who fail to respond to TKIs or lose their responsiveness (Kang et al., 2006; Ernst et al., 2009).

2. Methods

2.1 Patients and Controls

In this cross-section study, seventy patients were recruited from Baghdad Medical City/Hematology Unite, in the period between September 2011 to June 2012. They were diagnosed clinically and hematologically as CML. Patients were classified according to their responsiveness to IM into four groups according to European Leukemia Net (ELN 2009) (Baccarani, Cortes, & Pane, 2009): 1-Failure group which included 22 patients, whose florescence in situ hybridization (FISH) for BCR-ABL gene was more than 35% despite normal WBCs count while using IM (600-800 mg/day) for at least one and a half year. 2-Suboptimal responders group which included 16 patients, whose FISH for BCR-ABL gene was reading 1-35% with normal WBCs count and they were on IM for one and a half year. 3-Advanced disease group (patients in accelerated phase or Blast crisis) which included14 patients, those patients have high WBCs count and blast cells more than 10% in PB and/or bone marrow while using IM (800 mg/day) for at least one and a half year. 4-Optimal responder which included 18 patients receiving IM 400mg/day and whose FISH reading is <1% with normal WBCs count and they were includes as mutation negative control group. Ten healthy age matched subjects were included in ASO-PCR procedure as healthy negative control. The study protocol was approved by The Ethical Committee of Hematology.

2.2 Molecular Screening for Resistance Mutations Using ASO-PCR

DNA was extracted from 300 µl PB using DNA isolation kit (Promega, USA) following manufacturer information. Point mutations result in substitution of Glutamine (E) to Lysine (K) at position 255 and Phenyl alanine (F) to Valine (V) at position 359 of BCR-ABL tyrosine kinase were screened by ASO-PCR (Iqbal, Siddiqui, & Qureshi, 2004). Patient´s DNA and DNA from healthy individuals were amplified by using ASO-primers as well as internal control-primers in separate reaction mixtures. Mutation specific primers (ASO) and internal control primers (IC) sequences were given in Table 1. Briefly, three master mixes (each of 30 µl) were prepared, one for mutation E255K detection, the second for mutation F359V detection and third for IC as in the following: 1X PCR buffer, 200 µM of dNTPs (promega, USA), 25 pmol of each primers, 1.5 U/reaction of Taq DNA polymerase (Promega, USA). Then, 2 µl (equivalent to 100ng) of DNA was added for each ASO reaction tube and IC reaction tube. PCR reaction tubes were transferred to thermal cycler (Eppendorf-thermal cycler, Germany), which was programmed as following: pre-denaturation at 94 ºC for 4 minutes (X1),{95ºC for 2minutes, 63 ºC for E255K or 60 ºC for F359V,for 1 minutes, 72 ºC for1 minutes} (X30) and final extension at 72 ºC for 10 minutes (X1). PCR products were electrophoresed in 2.5% agarose gel.

2.3 Results Interpretation

Expected results are PCR products of 453 bp in positive reaction of E255K, 345 bp in positive reaction of F359V and 158 bp in reaction of IC.

Table 1. Sequences of primers used in ASO-PCR

Primer type	Forward primer (5'→3')	Reverse primer (5'→3')
E255K-ASO	GCGGGGGCCAGTACGGGA	GCCAATGAAGCCCTCGGAC
F359V-ASO	GAGTACCTAGAGAAGAAAAACG	ATGCCCAAAGCTGGCTTTG
Internal control (IC)	GCCCCCGTTCTATATCATCAC	GGATGAAGTTTTTCTTCTCCAG

2.4 Statistical Analysis

Data were analyzed using SPSS program (Statistical package for social Sciences) version 16 and Microsoft Office Excel 2007. Numeric data were expressed as mean ± SD. ANOVA test was used comparison mean more than 2 groups. Pearsons, S correlation coefficient was used to test correlation between two numerical variables. *P* less than 0.05 was considered significant.

3. Results

The mean age of CML patients was 40.5 ± 2.56 SD years at the diagnosis time, ranging from (25-75) years, Male to female ratio of CML patients was 1.12:1. The mean time of IM treatment was 18 months. The mean of drug dose among different groups of CML patients was the highest in the advanced group (800 mg/day). The mean of WBCs count among different groups of CML patients were highest in the advanced group patients (61.06±10.47) x10/L. The mean of FISH reading among different groups of CML patients were highest in the advanced group 74% as shown in Table 2.

Table 2. The mean of different parameters in different CML patients groups

Groups of Patients	Age (Year) *P*=0.021	Duration of disease (Year), *P* =0.001	Drug dose (mg/day) *P* =0.001	WBCs count (×10/L) *P* =0.001	FISH (%) *P* =0.001
Failure	45.5	4.97	718	6.54	67.40
Advanced	37.7	5.78	800	61.06	74.00
Suboptimal	34.6	4.68	600	7.25	18.87
Optimal	43.1	7.75	400	7.75	0

Screening analysis for the two resistant mutations E255K and F359V using ASO-PCR technique in all groups of CML patients were negative and no mutations were detected in healthy individuals and optimal responders that were included as disease control group, Figure 1.

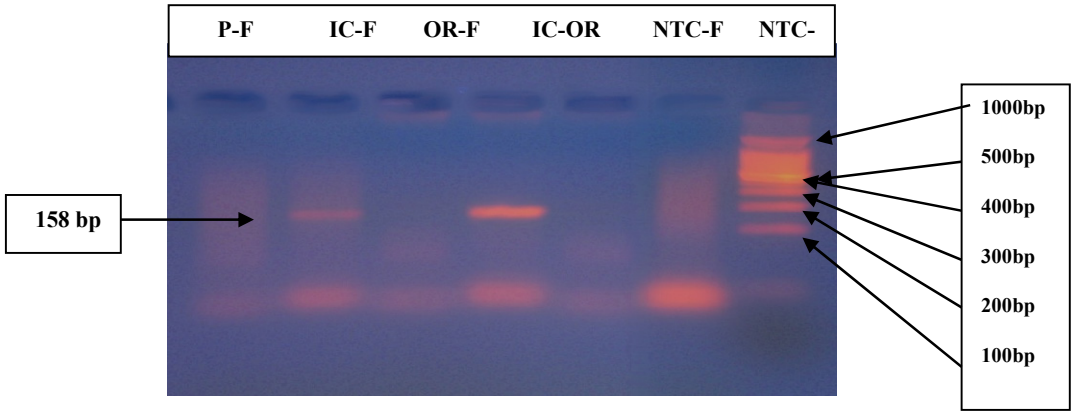

Figure 1. Allele Specific Oligonucleotide-PCR analysis of F359V mutations in CML patients

Lane P-F**:** PCR amplified product of F359Vmutation using DNA from non-responder CML patient.

Lane OR-F**:** PCR amplified product of F359Vmutation using DNA from optimal responder CML patient.

Lane IC-OR: Internal control of F359V mutation.

Lane NTC-F: No template control for mutation F359V.

Lane NTC-IC: No template control for internal control.

MW: Molecular weight marker, DNA ladder 100 bp.

Electrophoresis was carried in 2.5% agarose gel at (3V/cm) for 60 minutes.

4. Discussion

Mutations in the kinase domain of BCR-ABL are the most prevalent mechanism of acquired IM resistance in patient with CML. Although the majority of CML patients treated with IM show at least, significant hematologic response, resistance to IM is still a problem, mainly in patients within the accelerated or blast crisis phases of the disease. It has become clear that optimal treatment of patients based on appropriate monitoring of therapeutic effect, which has emerged as a relevant and sensitive part of the therapeutic strategy. The correct monitoring of CML treatment, which include conventional cytogenetic karyotyping, FISH, molecular evaluation of residual disease and the search for BCR-ABL kinase domain mutations, should be performed at regular intervals. Also, response definitions should be standardized (Quintas-Cardama, Kantarjian, & Cortes, 2009).

In this study, the mean age of CML patients at time of diagnosis was 40.45 ± 2.56 years (Mean ± SD), and that consistent with the results obtained by two previous Iraqi studies (AL-Rawi, 2001; Ismail, Yahya, & Farid, 2003) but another Iraqi study found that the mean age was 34.71 ± 1.02 years (Dhahii ,2008).

Moreover the Male to female ratio was 1.12:1 which was consistent with the ratio mentioned by Al-Rawi study (AL-Rawi, 2001) in which the incidence of CML was slightly higher in male than female, but it differs from what was mentioned by Dhahii study1:1.62 (Dhahii, 2008), which is mostly due to the unstable security situation in Iraq in 2006 and 2007 when females could attend hospitals for receiving therapy more than males.

This study focused on two point mutations, E255K and F359V. Neither E255K nor F359V were detected in all of 70 CML patients using ASO-PCR technique. This is consistent with the result obtained by Willis et al study, who did not detect these two mutations in 66 patients by using ASO-PCR technique (Willis et al., 2005). However, Sorel study detected the E255K mutation in only one out of 59 IM resistant patients by using Double-Gradient–Denaturing-Gradient Gel Electrophoresis (Sorel et al., 2005). Another study by Elass, Osman & AL-Gadir (2011) detected E255K in one patient from 50 IM resistant CML patients by using ASO-PCR technique.

In an Egyptian study, mutation screening was performed by using allele specific oligonucleotide polymerase chain reaction (ASO-PCR) in 42 non-optimal responders; 28 resistant patients, 44 optimal and suboptimal responders. BCR-ABL mutations were detected in 16/28 resistant patients (57%) and in none of the 44 optimal or suboptimal responders (0%) (Elnahass et al., 2013).

In an Indian study, 4 and 3 cases of E255K and F359V mutations respectively were detected in 100 BCR-ABL positive imatinib, nilotinib and dasatinib resistant cases (Mitra et al., 2013).

In a Polish study, Direct sequencing analysis of BCR-ABL gene was performed in 92 patients treated with IM for more than 3 months. Co-existence of F359V and Y253F mutations were detected in one patient with lymphoid blast crisis of CML (Lewandowski et al., 2009).

Differences in the frequency of mutation detection may be attributed to several factors; differences in the sensitivities of the techniques, in the time point of analysis, in phase of the disease and eventually due to differences in the genetic make-up of patient populations (Gorre et al., 2001).

Other mechanisms can also cause resistance to imatinib in the absence of mutations in the KD. Wu et al studied LYN kinase (Lck/Yes novel tyrosine kinase), which regulates cell survival, and responsiveness of CML cells to TKIs. The study showed that IM treatment suppresses LYN phosphorylation in cells from IM-sensitive CML patients. Thus, disruption in LYN kinase may be involved in IM resistance in CML patients with BCR-ABL negative mutations (Wu et al., 2008). Apperley *et al* suggested that drugs influx and efflux may play a role in IM resistance CML patients with TK negative mutations (Apperley, 2007).

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