

Molecular Screening for T315I and F317L Resistance Mutations in Iraqi Chronic Myeloid Leukemia Non-Responders Patients to Imatinib

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Abstract

The development of Imatinib Mesylate (IM), the first generation specific tyrosine kinase inhibitor (TKI) of BCR-ABL, has had a major impact in patients with Chronic Myeloid Leukemia (CML), establishing IM as the standard therapy for CML. Despite the clinical success obtained with the use of IM, primary resistance to IM and molecular evidence of persistent disease has been observed in 20-25% of IM treated patients. The existence of second generation TKIs (SGTKIs), which are effective in patients with IM resistance, makes identification of predictors of resistance to IM an important goal in CML. The aim of this prospective study was to determine prevalence of T315I, which confers full resistance to all available tyrosine-kinase inhibitors, and F317L mutations, which classified as resistant mutation to IM and dasatinib but sensitive to nilotinib, in IM non-responder CML patients from Iraq with highly sensitive Allele-Specific Oligonucleotide-PCR assay (ASO-PCR). Forty four CML patients were recruited from Baghdad Teaching Hospitalet-Hematology Unite from Feb. 2009 to Feb. 2013. Those patients were followed up (hematological, cytogenetically, and molecularly). At the time of sampling, thirty four CML patients in advance phase (accelerated phase (AP) or blast crisis (BC)) or loss their cytogenetic response were IM non-responders while ten CML patients in CP were IM responder, considered as T315I and F317L mutations negative control group. Ten IM non-responders patients out of those 34 patients were recruited to SGTKIs (Dasatinib and/or Nilotinib). The results shown that only T315I mutation was detected in one IM non-responder CML patient (3%). Although relation between response to SGTKIs and mutation status was non-significance ($P=0.100$), mutation is limited to only one patient who did not respond to either treatment forms. In conclusion, the uses of *bcr-abl* mutation screening test for T315I and F317L mutations in CML patients may influences the choice of specific SGTKIs after treatment with IM has failed.

Keywords: CML, imatinib resistance, T315I mutation, F317L mutation, allele specific oligonucleotides-PCR

1. Introduction

Treatment for patients with chronic myeloid leukemia (CML) has been greatly improved by the use of the BCR-ABL tyrosine-kinase inhibitors. However, relapse after an initial response is still a clinical problem (Marin et al., 2008). BCR-ABL1 kinase domain mutations represent the most frequent mechanism of resistance to TKIs therapy, being detected in 40%–90% of IM-resistant patients with CML in CP (Shah et al., 2002; Lowenberg, 2003; Hochhaus & La Rosee, 2004). Over 100 BCR-ABL single-point mutations have been reported in patients with CML resist to IM, which confer different levels of TKIs resistance (Quintas-Cardama & Cortes, 2009).

A threonine to isoleucine substitution at the 315 residue (T315I) of BCR-ABL protein, which forms a key H-bond interaction with TKIs, results in clinical insensitivity to imatinib, nilotinib, and dasatinib (Corbin et al., 2003; Lombardo et al., 2004; Shah et al., 2004; Weisberg et al., 2005; Kantarjian et al., 2006; Talpaz et al., 2006). Other mutations, however, can be inhibited at least to some extent by SGTKIs. A phenylalanine to lucien substitution at the 317 residue (F317L) of BCR-ABL protein is classified as resistant mutation to the Imatinib and dasatinib, but sensitive to nilotinib (O'Hare et al., 2007; Jabbour et al., 2008; Redaelli et al., 2009).

Since mutations are the major mechanism of TKI resistance, it is highly recommended to include a standard procedure for mutational analysis into the molecular follow-up of patients with CML treated with TKIs. Several

methods have been described for mutation detection in the ABL-kinase domain, but ASO-PCR is the most commonly used assay, shows an increased sensitivity for detecting known mutations (Iqbal et al., 2013). Early detection of the T315I and other mutations might be warranted due to their highly resistant to all available TKIs. The aim of this study was to determine prevalence of T315I and F317L mutations in IM non-responder CML patients from Iraq with highly sensitive ASO-PCR method.

2. Methods

2.1 Patients

Forty four CML patients were included in this study which extended from Feb 2009 to Feb 2013. Patient's samples were collected from Baghdad Teaching Hospital- Hematology Unite. Those patients were followed up hematological, cytogenetically, and molecularly. At the time of sampling, 34 CML patients in advance phase (accelerated phase or blast crises phase) or loss their cytogenetic response were IM non-responders while 10 CML patients in CP were IM responder, considered as T315I and F317L mutations negative control group. Patients clinical characteristics are given in Table 1. Ten IM non-responders patients out of those 34 patients were recruited to SGTkIs (Dasatinib and/or Nilotinib), Table 2. The treatment strategy for the remaining 24 IM non-responders patients was IM(600-800) mg/day Haematological, cytogenetic, and molecular responses were defined according to the European Leukemia Net (Baccarani et al., 2006). The study protocol was approved by the Ethical Committee of Hematology.

2.2 ASO-PCR

Peripheral blood (PB) samples (44) were collected from patients. DNA was extracted from PB using commercial available DNA extraction Kit (Promega, USA) following manufacture instructions. ASO-PCR was performed in 30µl reaction (Kang et al., 2006; Wongboonma et al., 2012). Briefly, three master mixes were prepared, one for T315I mutation detection, the second for F317L mutation detection and the third one for wild type *bcr-abl* detection (WT). PCR master mixes were prepared as following (per one reaction): 6µl of 5X PCR buffer, 1.5µl of 200µM dNTPs (Promega, USA), 2µl (equivalent to 25 pmol) of T315I mutation forward primers: (5'-gcccccgttctatcatcat-3') or F317L mutation forward primer: (5'- ccgttctatcatcactgagttg-3), or normal *bcr-abl* specific forward primers: (5'- gcc ccc gtt cta tat cat cac -3') and 2µl (equivalent to 25 pmol) of common reverse primer: (5'-ggatgaagttttctctccag-3') (Alpha,USA), 1.5 Units of Go Tag DNA polymerase (Promega, USA) and 12.2µl of dH₂O. Two microliters (equivalent to 100ng) of patient's DNA was added for each mutation reactions and WT reaction. Also, cDNA that reverse transcribed from extracted RNA of K562 cell line (Ampion, USA) was used as standard mutation negative control and no-template control (NTC) reaction tube (using dH₂O) were added to each of T315I, F317L and WT reactions.

First, the ASO-PCR was optimized by varying annealing temperature (Ta) (52° to 69°C). The PCR profile was as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1min, and final extension at 72°C for 5 min. The products were assessed on a 2.5 % agarose gel and staining with ethidium bromide. Molecular size of PCR products of T315I mutant, F317L mutation, and WT were 158 bp, 167 bp, and 158bp, respectively. Analysis of each sample was repeated at least twice in independent ASO-PCR reaction.

2.3 Statistical Analysis

Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007. Numeric data were expressed as median, mean, Standard deviation and range. Nominal data were expressed as number and percent. Chi-Square test was used to study association between nominal data. Fischer Exact test was used when Chi-Square test is not valid. P-value was considered significant when it is less than 0.05.

Table 1. Characteristics of studied patients

| | IM Non-responder CML patient Total No.=34 | | IM responder CML patient Total No.=10 | P value |
|-----------------------------------|--|------------|--|---------|
| Age (year) | 39.88 (\pm 2.24) | | 37.40 (\pm 3.60) | 0.588 |
| Gender | Male | 14(42.42%) | 5(50%) | --- |
| | Female | 19(57.58%) | 5(50%) | --- |
| Stage | Advance phase | 19(57.58%) | 0(0%) | --- |
| | Loss of Cytogenetic response | 14(42.42%) | 0(0%) | ---- |
| | Chronic phase | 0(0%) | 10(100) | ---- |
| Disease duration (year) | 6.12 (\pm 0.49) | | 3.67 (\pm 0.75) | 0.017 |
| Treatment dose(mg/day) | 666.67 (\pm 28.43) | | 400(\pm 0.00) | <0.001 |
| WBCs count (x 10 ⁹ /L) | 15.20(\pm 3.15) | | 5.20(\pm 0.30) | 0.003 |
| FISH (%) | 68.45(\pm 3.18) | | 0.00(\pm 0.00) | <0.001 |

- CML=Chronic myeloid leukemia, IM=Imatinib, FISH=Fluorescent in situ hybridization.

Table 2. Responsiveness to SGTkIs in ten IM non- responders CML patients

| Patient Code No. | Treatment response | | | | | | Follow-up duration (Year) |
|---------------------|--------------------|-----|-----|-----------|-----|-----|------------------------------|
| | Nilotinib | | | Dasatinib | | | |
| | CHR | CyR | MMR | CHR | CyR | MMR | |
| 1 | X | - | - | X | X | - | 3 |
| 2 | X | X | - | - | - | - | 3 |
| 7 | X | X | - | √ | X | - | 3 |
| 18 | √ | - | - | √ | - | - | 1.5 |
| 25 | √ | - | - | - | - | - | 1 |
| 26 | √ | - | - | - | - | - | 2 |
| 27 | √ | √ | √ | - | - | - | 3 |
| 29 | √ | - | - | - | - | - | 2 |
| 30 | √ | √ | √ | - | - | - | 3 |
| 33 | √ | - | - | - | - | - | 1 |

-CHR: Complete hematological response, CyR: Complete cytogenetic response, MMR: Major molecular response.

X: No response.

√: Response.

-: Not identified

3. Results

3.1 Mutation Detection

Only T315I mutation was detected in one IM non-responder CML patient (3%), as shown in Figure 1. This patient did not exhibit any response along the disease duration (7ys), even after IM dose escalation (600-800 mg/day) and the treatment switched over to nilotinib and then dasatinib (Table 2, patient cod No-1-).

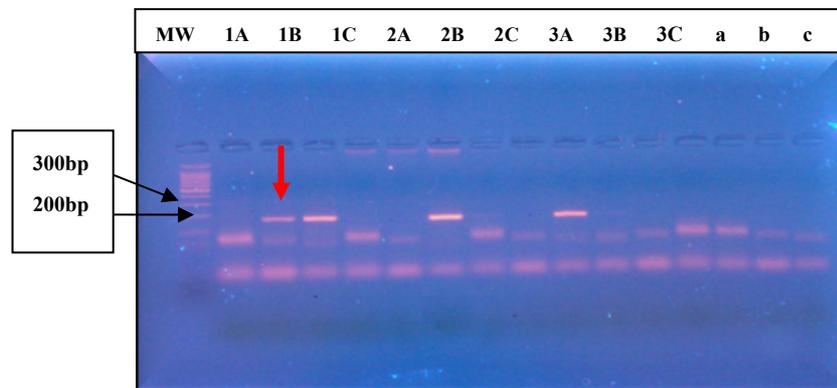


Figure 1. ASO-PCR amplification results

1, 2 & 3: Number of patients. A & B: PCR amplified products from mutated allele (T315I and F317L, respectively). C: PCR amplified product from wild allele. Lane (a, b, & c): No template control for master mixes .MW: Molecular weight marker of DNA Ladder. Electrophoresis was carried in 2.5% agarose gel at (4V/cm) for 60 mints.

3.2 Relation between Response to SGTkIs and Mutations

Table 3 shows the response to SGTkIs in IM non-responders CML patients and the presence of resistance mutations. Although p -value is not significant ($P=0.100$), mutation is limited to only one patient who did not respond to either treatment forms. Other nine patients treated with nilotinib and/or dasatinib shown at least CHR and 2/9 shown CCyR and MMR. Also, those nine patient did not shown any of studied mutations before using SGTkIs.

Table 3. Relationship between the presence of resistance mutations and response to SGTkIs in IM non-responder CML patients

| Mutation | | IM non- responder Patients No.(%) | IM responder patients No.(%) | P value |
|----------|----------|--------------------------------------|---------------------------------|---------|
| T315I | Positive | 1(3.03) | 0(0) | 0.100 |
| | Negative | 30(90.91) | 10(100) | |
| F317L | Positive | 0(0) | 0(0) | |
| | Negative | 32(96.97) | 10(100) | |

4. Discussion

Mutations considered clinically resistant to nilotinib and/or dasatinib were detected in more than 40% of patients who acquire mutations during IM therapy, and associated with a poor response to nilotinib and/or dasatinib therapy (Branford et al., 2009; Müller et al., 2009). Detecting these mutations at IM failure facilitates selection of the most appropriate alternative therapy. Therefore, mutation analysis is always required before changing to other TKIs or other therapy. If an inappropriate TKI is selected, there is a high risk of treatment failure with clonal expansion of the resistant mutants (Baccarani et al., 2009).

In this study, we detected T315I mutation in 3.03% (1/34) IM non-responder CML-AP patient, but we did not detect F317L in any studied petitions. That concerned with the finding of Quintas-Cardama et al. (2011), who referred to that T315I mutation was detected in 3.38% (7/207) CML patients in different phases after IM failure using semi-nested reverse transcriptase-PCR followed by direct sequencing technique. Also, using the same technique and sets of primers that used in this study, Kang et al. (2006) were reviled that 2.63% (1/38) of patients included in their study were positive to T315I mutation. Arechavaleta et al. (2011) study referred to that 4.6% (3/65) of IM resistance CML patients were positive to this mutation using Allele Specific-Reverse Transcriptase-PCR technique. In Mir et al. (2009) study, they did not detect T315I or F317L mutation between

12 IM resistance CML patients but they detected F317L mutation in 4/12 after treatment with dasatinib for 10ms. The patient, who T315I positive in this study, was screened for T315I mutation after 6ms in (400 mg/d) (using the same experimental conditions in our previous study (Dhahi et al., 2011). She was in CP and she did not achieved CHR, CyR or MR. At that time, she was negative for T315I mutation and progress to AP. After IM dose escalation to 800mg/d for 12ms, again she did not achieved any responsiveness and she became positive for T315I mutation at this period. Even that, she was treated with nilotinib in 2009 and she had transient HR response for one month, so treatment was switchover to dasatinib from 2010-2013, with no any responsiveness.

The number of samples was too small in this study, because only those patients whom selected for our study were had not gap in treatment. Other patients the excluded from this study were either had multiple gaps in treatment or die.

Even we didn't detect these two mutations in other patients included in this study; there is a possibility of the presence of T315 and F317 mutations variants. T315A/M and F317I/V/C/R considered as highly resistant mutations (Shah et al., 2007; Soverini et al., 2009). For instance, the dasatinib-resistant T315A mutation displays substantially reduced transformation potency relative to wild-type BCR-ABL in vitro. However, T315A in the context of other mutations had an increased transformation potency (Oehler et al., 2008). Recent study refereed to that T315V showed high resistance level to four TKIs (imatinib, bosutinib, dasatinib, and nilotinib). Also, F317R is moderately resistant to IM and nilotinib and highly resistant to bosutinib and dasatinib. The gatekeeper T315V mutation and F317R involve residues with previously unidentified amino acid substitutions. It is likely that the T315V mutation (GTT codon) evolved from the previously identified T315A clone (GCT codon) as a consequence of a second mutational event occurred in the same codon (Redaelli et al., 2012). The targeting of more than a single pathogenic event in a highly heterogeneous cancer could produce better results (Mologni et al., 2010).

A study was referred to the possibility of presence of multiple mutations in TKIs resistance patients rather than T315I. In Kwan et al. (2009) study, they identified 2% of patients had multiple mutations resistant to both nilotinib and dasatinib, not T315I, using mass spectrometry technique.

IM non-responders patients included in this study whom treated with SGTkIs (except T315I positive patient) were showed a type of response to dasatinib or /and nilotinib. Two patients out of Ten achieved CHR, CCyR and MMR after nilotinib treatment.

However, low-level mutations after IM treatment has failed could be present below the detection limit of technique used, even its highly sensitive. Sensitive detection of low-level mutations that confer clinical resistance to nilotinib and/or dasatinib is highly predictive of their rapid clonal expansion under the selective pressure of nilotinib or dasatinib therapy (Hughes et al., 2009; Redaelli et al., 2009).

Recently, new compounds were developed to overcome resistance generated by the T315I mutant. Among them ponatinib, a pan BCR/ABL inhibitor (O'Hare et al., 2009; Zhou et al., 2011) and the switch pocket inhibitor DCC-2036 were reported as potently active against the T315I mutation (Chan et al., 2011).

In conclusion, the uses of *bcr-abl* mutation screening test for T315I and F317L mutations in CML patients may influences the choice of specific SGTkIs after treatment with IM has failed. We plan for future steps such as study large group of IM non-responder CML patients and use sequencing technique to screen tyrosine kinase domain for other types of mutations and mutation variants.

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