Contribution of BCR-ABL kinase domain mutations to imatinib mesylate resistance in Philadelphia chromosome positive Malaysian chronic myeloid leukemia patients

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Introduction

Imatinib mesylate (IM) is used as a front-line therapy for chronic myeloid leukemia (CML) as it is highly effective in the treatment and management of Philadelphia (Ph) positive CML patients. IM is a selective inhibitor of tyrosine kinase that binds competitively to the adenosine triphosphate (ATP) docking site of tyrosine kinase proteins, including ABL itself and the hybrid BCR-ABL proteins. It also inhibits other tyrosine kinases, such as platelet-derived growth factor receptors KIT and ARG.1 As IM binds to the ATP binding site at the tyrosine kinase active site, it inhibits the transfer of terminal phosphate from ATP to tyrosine residues on its substrates, thus inhibiting the enzyme activity of the tyrosine kinase protein semi-competitively. Mutations in the BCR-ABL kinase domain can cause resistance to IM by shifting its equilibrium toward the open or active conformation. Some other mutations interfere with a critical hydrogen bond that forms between the ABL 1 kinase domain and inhibitor molecule thus impairing the ability of the BCR-ABL protein to bind to the inhibitor molecule.2

Even though IM has become the gold standard in front-line treatment of CML, resistance to this drug is a daunting problem. Development of resistance to IM has been a setback for patients, as well as for treating physicians in the clinical management of CML patients. A patient with CML could display either a primary or secondary resistance to IM therapy. Primary resistance is defined as inability to achieve any initial landmark response, and secondary response is defined as achieving a good initial response but subsequently losing any relevant response.3

Development of resistance to IM is a multifactorial phenomenon in patients with CML and may be mediated by a range of different mechanisms. However, there are 2 broad mechanisms of resistance: BCR-ABL dependent and BCR-ABL independent pathways.4,5 BCR-ABL dependent pathways have been reported to be the most common cause of IM resistance which commonly involve mutations in the tyrosine kinase domain (TKD) of the BCR-ABL gene,6 as well as amplification and overexpression of the BCR-ABL gene locus.7 The present study was designed to clarify the mechanisms of resistance involving BCR-ABL dependent pathways.

So far, more than 100 mutations have been identified in the tyrosine kinase domain of the BCR-ABL gene.5 Different studies have reported a broad range of frequencies of mutations and this is probably due to the different composition of study cohorts. But no reports are available from Malaysia. Apart from BCR-ABL mutation, few studies have reported on the amplification of the BCR-ABL gene locus that is associated with IM resistance among CML patients.5 In these rare cases, the presence of multiple copies of the BCR-ABL gene in interphase nuclei were reported in IM resistant patients using fluorescence in situ hybridization (FISH). In this study, we investigated the frequency and pattern of BCR-ABL kinase domain mutations using dHPLC and BCR-ABL gene amplification by FISH on 40 Malaysian CML patients who showed resistance to IM.

Materials and Methods

Study subjects

The study was undertaken at Universiti Sains Malaysia Hospital from 2008 to 2011, after obtaining approval from the institute’s Research and Ethics Committee. The study included 50 CML patients from Malaysia. Of these, 23 patients were resistant to IM and were enrolled in the study. The patients included in the study were resistant to IM based on a good initial response but subsequently losing any relevant response.3

Methods

The methodology of our study was as follows: Blood samples were obtained from IM resistant CML patients using a 10 mL vacutainer tube with EDTA. The diluted blood sample was then centrifuged at 1200 g for 10 minutes to separate the platelet and red blood cells. The supernatant was removed and theuffy coat was used for cell isolation. The isolated cells were stained with Wright’s stain to confirm the white blood cells. The white blood cells were then centrifuged at 1200 g for 10 minutes and the supernatant was removed. The pelleted cells were processed using the proteinase K method to extract the DNA. The extracted DNA was then stored in the −20°C freezer until further use.

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The study subjects were divided into two groups: Group A consisted of 11 patients with CML resistant to IM, and Group B consisted of 12 patients with IM resistant CML. The patients were divided into two groups based on the criteria for primary and secondary resistance to IM. The primary resistance criteria were met when the patients had never responded to IM therapy, while the secondary resistance criteria were met when the patients had responded to IM therapy but subsequently lost the response. The patients were further divided into two groups: Group A consisted of 11 patients with CML resistant to IM, and Group B consisted of 12 patients with IM resistant CML. The patients were divided into two groups based on the criteria for primary and secondary resistance to IM. The primary resistance criteria were met when the patients had never responded to IM therapy, while the secondary resistance criteria were met when the patients had responded to IM therapy but subsequently lost the response.
participants included 40 Philadelphia chromosome (Ph) positive CML patients in chronic, accelerated or blast phase, treated for at least six months with standard dose IM (400 mg) as front-line treatment according to the phase II extended access protocols, and who showed only suboptimal response or signs of clinical response to IM. Those CML patients who were Ph negative, and those who did not opt for IM treatment, were excluded from the study. The medical records of all patients were reviewed until June 2011. Basic demographic, disease characteristics, and treatment management details were collected. For each patient, diagnosis was confirmed by hematologic, cytogenetic, as well as molecular analysis. The response to IM therapy was evaluated on the basis of measurement of hematologic, cytogenetic and molecular responses. Hematologic response was evaluated every 3rd month of treatment and cytogenetic response was evaluated every 6th month of treatment.

According to European LeukemiaNet 2010, complete hematologic remission would show peripheral blood cell counts and bone marrow morphology returning to normal with total white blood cell count less than 10x10⁹/L and platelet count less than 450x10⁹/L. Complete hematologic remission was also defined by absence of peripheral blast, immature granulocytes such as promyelocytes or myelocytes, less than 5% peripheral basophils and non-palpable spleen.10 Cytogenetic remission was categorized into complete, major, partial and non-responders groups. A total disappearance of Ph chromosome in cytogenetic analysis confirmed complete cytogenetic response (CCyR) while presence of less than 35% Ph+ cells in bone marrow confirmed partial cytogenetic response (PCyR). Patients with minor cytogenetic response showed 36-65% of Ph+ cells in bone marrow while those who showed 66-95% Ph+ chromosome positivity were categorized as a minimal cytogenic response group. Patients whose bone marrow showed over 95% Ph+ chromosome were classified as non-responders to IM.18 Primary resistance, also known as intrinsic resistance to IM, is defined as having no hematologic response within three months, having incomplete hematologic response or no cytogenetic response within six months, and having less than partial cytogenetic response within 12 months. Secondary resistance or acquired resistance is defined as evolution of the disease from chronic phase to accelerated phase or blast phase, loss of hematologic response, loss of MCyR or CCyR.11

**BCR-ABL gene amplification**

The samples of patients who did not show any BCR-ABL mutations were investigated for the progressive amplification status of the **BCR-ABL** gene using FISH. For this, LSI **BCR-ABL** dual fluorescent probes (Vysis Inc., USA) which hybridize to the fusion regions and generate intense fluorescence signals were used. The copy number of fusion signals in a minimum of 200 interphase nuclei was determined for each sample.

**Bioinformatics analysis**

An online program (available at http://genetics.bwh.harvard.edu/php2/), PolyPhen-2, was used to predict the potential consequence of each mutation on the BCR-ABL protein structure. ClustalX program (version 2.0.12) was used for multiple alignment of **Homo sapiens** ABL1 protein sequence (CA344338) with ABL1 protein sequence of chimpanzee (**Pan troglodytes**; XP_001166213.2), pig (**Sus scrofa**; XP_00122293.3), mouse (**Mus musculus**; NP_001106174.1), rat (**Rattus norvegicus**; NP_001104320.1), cow (**Bos taurus**; NP_001193789.1) and chicken (**Gallus gallus**; XP_001233812.1).

**Table 1. Demographic, disease characteristics and treatment management of the chronic myeloid leukemia patients.**

| Demographic | N. patients (%) |
|-------------|----------------|
| Gender      |                |
| Male        | 16 (40)        |
| Female      | 24 (60)        |
| Age         |                |
| Median (range) | 43 (17-65)   |
| Male        | 41 (17-65)     |
| Female      | 45 (23-65)     |
| Race        |                |
| Malay       | 34 (85)        |
| Chinese     | 5 (12.5)       |
| Indian      | 1 (2.5)        |
| Treatment duration (month) |            |
| 13-24       | 1 (2.5)        |
| 25-36       | 3 (7.5)        |
| 37-48       | 6 (15)         |
| 49-60       | 14 (35)        |
| >61         | 16 (40)        |
| Disease characteristic and treatment management | |
| CML stage prior to IM | |
| Chronic phase | 37 (92.5)     |
| Accelerated phase | 3 (7.5)      |
| Blast phase   | -              |
| Treatment prior to IM |          |
| IFN          | 2 (5.0)        |
| Hydroxyurea  | 21 (52.5)      |
| No           | 17 (42.5)      |
| CML stage in response to IM |          |
| Chronic phase | 31 (77.5)     |
| Accelerated phase | 6 (15)       |
| Blast phase   | 3 (7.5)        |
| Hematologic response (HR) |          |
| Complete     | 34 (85)        |
| Suboptimal   | 5 (12.5)       |
| Loss of HR   | 1 (2.5)        |
| Cytogenetic response (CR) |          |
| No           | 10 (25.0)      |
| Major/ partial | 4 (10.0)      |
| Minor        | 11 (27.5)      |
| Minimal      | 10 (25.0)      |
| Complete but loss CyR | 5 (12.5)    |
| Resistance type |            |
| Primary resistance | 35 (87.5)    |
| Secondary resistance | 5 (12.5)   |

CML, chronic myeloid leukemia; IM, imatinib mesylate.
patients recruited was 2:3; mean age was 41 and 45 years, respectively. A slight predominance of the disease among females was observed.

In this cross-sectional study, out of 40 Philadelphia chromosome positive CML patients, mutations were detected in 13 (32.5%). Among these 13, 8 (70%) showed T315I mutation. Another 5 patients showed E255K (n=2), Y253H, M351T and V289F (1 each) mutations, respectively (Figure 1). The remaining 27 patients who did not show tyrosine kinase domain mutation were subjected to BCR-ABL gene amplification using FISH. However, none of these 27 IM resistant patients showed BCR-ABL gene amplification.

Cytogenetic analysis of the bone marrow samples was carried out in all these IM resistant CML patients of whom 13 showed additional chromosomal abnormalities apart from the Philadelphia chromosome. The additional chromosomal abnormalities included: trisomy 6 (n=1 patient), trisomy 8 (n=1), trisomy 10 (n=3), trisomy 16 (n=1) and trisomy 19 (n=2); monosomy 4 (n=2), del(15)(q22-26) (n=1), del(17)(q23-25) (n=1) and i(17q10) (n=1). However, a comparison of the ACA (with or without ACA) with mutation (with or without mutation) showed no significant difference (P=0.646).

**Discussion**

Reciprocal translocation between chromosomes 9 and 22, t(9;22)(q34;q11) is reported to be the main factor contributing to CML. This unique chromosome arrangement, known as Philadelphia chromosome translocation, generates a BCR-ABL fusion gene that encodes a constitutively active tyrosine kinase protein. BCR-ABL fusion gene has now become the universally accepted molecular signature and the transforming event in CML pathogenesis. The mRNA molecules transcribed from BCR-ABL fusion gene usually contain one of the two BCR-ABL junctions designated as b2a2 (e13a2) and b3a2 (e14a2). However, both mRNAs translate into p210 Kda fusion protein that may up-regulate the tyrosine kinase activity. This results in transformed cells that have growth factor independent proliferation along with decreased apoptosis, defective adhesion, as well as genomic instability.

In the present study, we aimed to examine the frequency distribution and nature of ABL kinase domain mutation in CML patients treated with IM. The ability to detect mutations in the BCR-ABL gene varies depending on the technique used. In the present study, we used the dHPLC technique for mutation analysis, followed by sequencing to characterize the mutations. The dHPLC assay provided a much

### Table 2. Cytogenetic and molecular test results of the chronic myeloid leukemia patients.

| Cytogenetic analysis (Bone marrow) | 27 (67.5) |
|-----------------------------------|----------|
| Additional chromosomal abnormalities | 13 (32.5) |

**Mutation at BCR-ABL gene**

| Absent | 27 (67.5) |
| Present | 13 (32.5) |

**Type of BCR-ABL TKD**

| T315I | 8 (61.5) |
| E255K | 2 (15.4) |
| M351T | 1 (7.7) |
| Y253H | 1 (7.7) |
| V289F | 1 (7.7) |

**Occurrence of mutation**

| Primary resistance | 12 (92.3) |
| Secondary resistance | 1 (7.7) |

**BCR-ABL gene fusion transcript**

| b3a2 | 31 (77.5) |
| b2a2 | 7 (17.5) |
| ela2 | 2 (5.0) |

Figure 1. Sequencing analysis results of BCR-ABL gene showing: a) M351T mutation; and b) T315I mutation. The circles indicate the nucleotide triplet that encodes the amino acid.
faster and less expensive method for mutation screening. Taking advantage of the dHPLC based assay for ABL mutational screening, as well as a sequencing technique, we screened 40 patients who showed resistance to IM. Our results showed that ABL kinase mutations are a relevant mechanism of resistance to IM in 32.5% of Malaysian CML patients.

Worldwide, researchers have detected quite a number of mutations within the BCR-ABL fusion domain among IM resistant CML patients. In the present study on 40 Malaysian CML patients showing IM resistance, we detected a total of five different point mutations all leading to amino acid substitutions. Most of these mutations were found to be located at the functional fraction of the BCR-ABL fusion protein. Point mutations in ABL kinase domain have been characterized into two groups:13 1) mutations that impede contact between BCR-ABL and IM; and 2) mutations that alter the spatial conformation of the protein.7 The BCR-ABL structure contains two flexible loop structures, the adenosine triphosphate-binding phosphate loop and the activation loop, which have specific arrangement in the inactive conformation of BCR-ABL that stabilizes the structure.5 According to Litzow, IM mutations in these loops destabilize their arrangement such that the kinase domain cannot assume the inactive conformation required for IM binding.3

Mutations located in the IM contact point were reported to significantly reduce IM sensitivity. Normally, these amino acids make hydrogen bonds with IM; therefore, the mutations occurring in one of the amino acids might be causing resistance.2 This type of mutation was found in 8 of our patients who showed T315I mutation. From the literature reviewed, T315I was found to be the most common mutation reported worldwide and it also happened to be the most common mutation (70%) among the IM resistant CML patients in the present study.14-16 In T315I mutation, a polar amino acid that participated in hydrogen bonds (threonine) is substituted with isoleucine, which is the hydrophobic amino acid. Thus, this single amino acid substitution has been reported to interfere with a critical hydrogen bond that forms between the ABL 1 kinase domain and IM.2 This allocated binding prevented the IM inhibition of BCR-ABL and conferred resistance to IM.

The detection of BCR-ABL T315I mutation in the present study is clinically relevant. PolyPhen2 with a score of 0.999 using the HumDiv model (http://genetics.bwh.harvard.edu/pph2/) predicted the T315I mutation to be possibly damaging. Indeed, residue 315 seems to be critical for binding most adenosine triphosphate-competitive kinase inhibitors because BCR-ABL T315I confers resistance to 2nd generation tyrosine kinase inhibitors such as nilotinib and many other drugs.17

Other than T315I mutation, V289F mutation is also located at the IM binding site. In this mutation, valine, a very hydrophobic amino acid at position 289 is substituted with phenylalanine that is also a very hydrophobic amino acid. Like T315I, V289F was also predicted to be possibly damaging when analyzed using PolyPhen2 program with a score of 0.999 using the HumDiv model (http://genetics.bwh.harvard.edu/pph2/).

Another mutation is M351T. In this mutation at 351, amino acid methionine, a very hydrophobic amino acid, is substituted with a less hydrophobic amino acid (threonine). Using the PolyPhen2 program, this type of mutation was predicted to be possibly damaging with a score of 1.00 using the HumDiv model. In a study on a Singaporean population, Ang et al. reported that M351T mutation was uncommon in Asian IM resistant CML patients, including Chinese, Malay, Indian and other Asian ethnic origins, as no M351T mutation was found in their study.18 However, interestingly, one of our patients (of Malay ethnic race) was found to have M351T mutation within the IM binding site of BCR-ABL kinase domain.

Mutations were also found in the P-loop of the BCR-ABL fusion domain. The residues of nucleotide binding loop (p-loop) were reported to be from 247 to 256.19 In our study, 2 patients showed different p-loop mutations which were Y253H and E255K mutations, respectively. Both of these mutations were predicted to be possibly damaging by using the PolyPhen2 program. Patients with mutated p-loop BCR-ABL domain have been reported to be 70 to 100-fold less sensitive to IM compared to native BCR-ABL.20 Other than that, mutations at the activation loop (a-loop) that include the residues from 381 until 402 in ABL have also been reported to cause different levels of IM sensitivity among CML patients.21 However, none of our patients harbored this type of mutation.

Location of a mutation in a protein sequence may reflect the effect to the protein functions. Due to evolution, some proteins that play a similar biological function in different species may have significantly different protein sequences. However, part of the protein sequences in most species is preserved to retain the 3-dimensional structure, as well as the structure that is critical for conducting the biological function. Thus, these parts of protein sequences are usually conserved across species.22 The multi-alignment of human ABL1 protein with its orthologs among various species, using the ClustalX program (version 2.0.12), showed that all five mutations (T315I, M351T, E255K, Y253H and V289F) that we identified in our patients are conserved among species in a highly conserved block (Figure 2). From this, it is reasonable to presume that these mutations may lead to alteration of the BCR-ABL protein structure or important structure for its biological function that may affect the action of IM on this protein.

The RT-PCR amplification of the TKD domain of the BCR-ABL gene in this study was performed by employing the primers described by Soverini et al. which covers only codon 206-421.12 Even though most of BCR-ABL mutations reported worldwide occurred in the TKD, mutations in the region of codon 421-500 which
encode C-terminal lobe (i.e. E453K, Y456C and K459Q) have also been reported.23 Apart from the c-terminal, it has been reported that mutations could also occur at the SH2-SH3 domain (i.e. A196V, R47C and K84N).24 Unfortunately, in the current study, these 2 regions were not included in mutation screening. Had these 2 regions also been screened for mutations, it is probable that the mutation frequency would have been higher. Thus, for a better overview of the BCR-ABL mutation, apart from the tyrosine kinase domain, both c-terminal and SH2-SH3 domain regions should also be taken into consideration. From these results, it is clear that the BCR-ABL mutations contributed at least in part to resistance to IM in 32.5% of our patients. However, in those patients without kinase domain mutations, it is likely that additional factors may also contribute to resistance. In the remaining 27 patients who did not show any mutations, the contribution of BCR-ABL gene amplification in mediating resistance was investigated using FISH. But none of these 27 patients showed any BCR-ABL gene amplification. It is presumed that in these 27 CML patients, the mechanism of resistance to IM might be due to BCR-ABL independent pathways. Another possibility could be the lower sensitivity of the FISH method. The FISH probes used are significantly larger than the BCR-ABL fusion region. Consequently, the gene amplification of BCR-ABL might not always be visible. Therefore, the usage of quantitative PCR performed on DNA is highly recommended to detect BCR-ABL gene amplification. Our results showed that BCR-ABL mutations are not the only major mechanisms of resistance to IM in Malaysian CML patients. Additional factors besides kinase domain mutations may also contribute to resistance to IM and this means we need to find out what other predominant mechanisms of IM resistance are involved.

Conclusions

Different mutations confer different levels of resistance and, therefore, detection as well as characterization of TKD mutations is highly important in order to guide therapy in CML patients. Knowing the exact mutations responsible for IM resistance will help to select the most suitable TKIs for CML patients and improve their management. Furthermore, early detection of such mutations may allow timely treatment intervention to prevent or overcome resistance.

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