BCR-ABL kinase domain mutations, including 2 novel mutations in imatinib resistant Malaysian chronic myeloid leukemia patients—Frequency and clinical outcome

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\textbf{A B S T R A C T}

Discovery of imatinib mesylate (IM) as the targeted BCR-ABL protein tyrosine kinase inhibitor (TKI) has resulted in its use as the frontline therapy for chronic myeloid leukemia (CML) across the world. Although high response rates are observed in CML patients who receive IM treatment, a significant number of patients develop resistance to IM. Resistance to IM in patients has been associated with a heterogeneous array of mechanisms of which point mutations within the ABL tyrosine kinase domain (TKD) are the frequently documented. The types and frequencies of mutations reported in different population studies have shown wide variability. We screened 125 Malaysian CML patients on IM therapy who showed either TKI refractory or resistance to IM to investigate the frequency and pattern of BCR-ABL kinase domain mutations among Malaysian CML patients undergoing IM therapy and to determine the clinical significance. Mutational screening using denaturing high performance liquid chromatography (dHPLC) followed by DNA sequencing was performed on 125 IM resistant Malaysian CML patients. Mutations were detected in 28 patients (22.4%). Fifteen different types of mutations (T315I, E255K, G250E, M351T, F359C, G251E, V289F, E355G, N368S, L387M, H369R, A397P, E355A, D276G), including 2 novel mutations were identified, with T315I as the predominant type of mutation. The data generated from clinical and molecular parameters studied were correlated with the survival of CML patients. Patients with Y253H, M351T and E355G TKD mutations showed poorer prognosis compared to those without mutation. Interestingly, when the prognostic impact of the observed mutations was compared inter-individually, E355G and Y253H mutations were associated with more adverse prognosis and shorter survival ($P=0.025$ and $0.005$ respectively) than T315I mutation. Results suggest that apart from those mutations occurring in the three crucial regions (catalytic domain, P-loop and activation-loop), other rare mutations also may have high impact in the development of resistance and adverse prognosis. Presence of mutations in different regions of BCR-ABL TKD leads to different levels of resistance and early detection of emerging mutant clones may help in decision making for alternative treatment. Serial monitoring of BCR-ABL transcripts in CML patients allows appropriate selection of CML patients for BCR-ABL TKD mutation analysis associated with acquired TKI resistance. Identification of these KD mutations is essential in order to direct alternative treatments in such CML patients.

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1. Introduction

Chronic myeloid leukemia (CML) is caused by the reciprocal translocation, t(9;22)(q34;q11), involving chromosomes 9 and 22 which results in fusion of BCR and ABLI genes. As a consequence of this translocation, a novel, BCR-ABL fusion gene is created which results in expression of the constitutively active tyrosine kinase BCR-ABL protein. The constitutive activity of this tyrosine kinase
plays a central role in the pathogenesis of the disease. The expression of chimeric BCR-ABL protein with deregulated tyrosine kinase activity has been shown to be necessary and sufficient for the transformed phenotype of CML cells [1].

Imatinib mesylate (IM), the molecular targeted drug for the treatment of all phases of CML is a 2-phenylaminopyrimidine derivative that works by binding to the tyrosine kinase domain of BCR-ABL and blocking its function [2]. Targeted inhibition of BCR-ABL kinase with IM has become the frontline therapy for newly diagnosed patients with CML and other leukemias that express BCR-ABL kinase [3]. Despite the successful treatment results obtained with IM, achievement of prolonged response to IM is still a daunting problem, the chief obstacle being development of resistance to IM in a significant proportion of patients. Some of the chronic phase CML patients and most of the CML patients with late stage (accelerated or blast phase) develop resistance to IM and the disease progresses creating concern in the treatment of CML.

Among the multiple mechanisms of resistance identified, the dominant mechanism appears to be acquisition of point mutations in the kinase domain of BCR-ABL which results in altered affinity of IM for the BCR-ABL1 protein. BCR-ABL mutations had been reported to occur in approximately 50% of patients who develop resistance to IM [4].

Defining the mechanisms of resistance in large number of patients is the best option to begin with the clinical efforts to overcome IM resistance. As a first step, we conducted comprehensive BCR-ABL kinase domain mutation analysis of 40 Philadelphia (Ph) positive CML patients who demonstrated IM resistance. Mutations were detected in 32.5% (13/40) of patients [5]. Additional 85 IM resistant CML patients were subsequently screened for mutations. This report encompasses mutation screening results carried out in 125 Ph positive CML patients (inclusive of 40 patients already reported) who developed resistance to IM.

2. Methods

2.1. Study subjects

The study was undertaken at Hospital Universiti Sains Malaysia, during the period from June 2008 to September 2013, after getting approval from the Research and Ethics Committee of University Sains Malaysia and Ministry of Health, Malaysia (NMRR-10-1207-7183 and NMRR-10-1206-7127). For this comparative cross sectional study, 125 Philadelphia (Ph) chromosome positive Chronic Myeloid Leukaemia patients were recruited from four hospitals in Malaysia (Hospital University Sains Malaysia, Hospital Pulau Pinang, Hospital Ipoh and Pusat Perubatan Universiti Kebangsaan Malaysia) that altogether can be considered to represent CML patients of peninsular Malaysia covering the west, east and central regions of the country.

The Philadelphia chromosome positive Chronic Myeloid Leukaemia patients included were those in chronic, accelerated or blast phase, treated for at least 12 months, with IM dose of 400 mg as frontline treatment. Patients were enrolled by random sampling according to the phase 2 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 this study. The medical records of all patients were reviewed until September 2013. The basic demographic, disease characteristics and treatment management details were collected. For each patient, diagnosis was confirmed by hematological, cytogenetic as well as molecular analysis. The response to IM therapy was evaluated based on the measurement of hematologic, cytogenetic and molecular responses. Hematological response was evaluated every 3rd month of treatment and cytogenetic response was evaluated every 6th month of treatment.

The European LeukemiaNet 2013 [6] was used as a guideline for the assessment of clinical response of the CML patients. Complete hematologic remission was classified as when a patient showed transition of peripheral blood cell counts as well as bone marrow morphology back to normal in which total white blood cell count would be less than $10 \times 10^9$ L$^{-1}$ and platelet count would be less than $450 \times 10^9$ L$^{-1}$. Absence of peripheral blast, immature granulocytes like promyelocytes or myelocytes, less than 5% peripheral basophils and non palpable spleen were considered for co-defining the complete hematological remission [7]. Cytogenetic remission was categorized into complete, major, partial and non-response groups. A total disappearance of Ph chromosome in cytogenetic analysis confirmed the complete cytogenetic response (CCyR) while presence of less than 35% Ph$^+$ cells in bone marrow confirmed the 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 under minimal cytogenetic response group. Patients whose bone marrow showed more than 95% Ph$^+$ chromosome positivity were classified as no cytogenetic response to IM following Baccarani et al. [6,7].

According to LeukemiaNet 2013, primary resistance, also known as intrinsic resistance to IM is denoted by failure to achieve complete hematologic response (CHR) and/or having more than 95% Ph$^+$ chromosome within 3 months; having less than partial cytogenetic response within 6 months; and having incomplete hematologic response or no cytogenetic response (CyR) within 12 months. Secondary resistance or acquired resistance is defined as when patients initially respond to therapy, but eventually lose CHR, complete CyR as well as major molecular response (MMR) [8].

2.2. BCR-ABL mutations analysis

BCR-ABL mutation analyses were carried out in all CML patients employing denaturing high performance liquid chromatography (dHPLC) and direct DNA sequencing method. For this, total RNA was extracted from the peripheral blood by using QIAamp RNA blood mini extraction kit (QIAGEN, Germany) according to the manual with a slight modification, followed by cDNA synthesis using cDNA Synthesis Kit (Bioline, United Kingdom).

Subsequently, amplification of 3 overlapping fragments covering the entire tyrosine kinase domain was generated by nested PCR using the primers described by Soverini et al. [9]. The first PCR (BCR-A) with 1475 bp amplicon length was amplified from the synthesized cDNA, followed by the second and third PCR (ABL-B and ABL-C) with 393 bp and 482 bp amplicon length respectively, amplified from BCR-A amplicon. All the PCR were performed using AccuSure Mix (Bioline, United Kingdom) [9].

Then, the presence of sequence variation was screened by dHPLC (ProStar Helix System, Varian, USA). The PCR products of samples that showed altered dHPLC profile, indicative of mutation, were directly sequenced with both forward and reverse primers, after purification steps using PCR purification kit (QIAGEN, Germany) to characterize the mutation.

2.3. Bioinformatics analysis

An online program available at http://genetics.bwh.harvard.edu/pph2/, called 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 (CAAA34438) with ABL1 protein sequence of chimpanzee (Pan troglodytes: XP_001166213.2), pig (Sus scrofa; XP_003122293.3).
mouse (Mus musculus; NP_001106174.1), rat (Rattus norvegicus; NP_001094320.1), cow (Bos taurus; NP_001193789.1) and chicken (Gallus gallus; XP_001233812.1).

2.4. Statistical analysis

Fisher’s exact test was used to test categorical differences, and student t-test was performed to check differences of measurable variation among patients. Unconditional logistic regression analysis was used to assess the relationship between tyrosine kinase domain mutation and the CML patients’ response to IM as well as the cytogenetic response by calculating the odd ratios (ORs) and 95% confidence interval (CI). The tests were conducted by SPSS software (version 20.0) and all P-values were two-sided. Survival analysis was done by calculating the overall survival that included the interval between the initiation of imatinib treatment and death regardless of duration of discontinuation of imatinib treatment. For overall survival comparison, Kaplan–Meier curves for each category were plotted and compared using the log-rank test.

3. Results

In the present study, mutational analysis was performed on 125 IM resistant, Malaysian CML patients. The demographic profile, disease characteristics, cytogenetic response and molecular response details of these 125 CML patients were recorded and are shown in Tables 1 and 2.

Interestingly, from all the mutations identified (Table 3), 2 mutations appeared to be novel mutations which have not been reported so far, in any cancer database namely Catalogue of Somatic Mutations in Cancer (http://www.sanger.ac.uk/genetics/CGP/cosmic/), International Cancer Genome Consortium (http://www.icgc.org/) and Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php). So, these two mutations can be considered as novel mutations. For nucleotide positioned 251, a substitution from Gly (G) to Asp (D) was reported [10]. However, in our study, instead of G251D, we identified a new mutation of G251E, involving a substitution from Gly (G) to Glu (E).

Association risk of the tyrosine kinase domain mutation with cytogenetic response after 12 months of IM treatment among the IM resistant CML patients was evaluated. Presence of tyrosine kinase domain mutation was found to be associated with a higher risk for having no cytogenetic response among the CML patients (OR, 3.2; 95% CI, 1.267–8.083; P-value, 0.014).

Table 2
Disease characteristics and treatment management of the IM resistant CML patients.

<table>
<thead>
<tr>
<th>Disease characteristic and treatment management</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML phase during sample collection</td>
<td></td>
</tr>
<tr>
<td>Chronic phase</td>
<td>89(71.2%)</td>
</tr>
<tr>
<td>Accelerated phase</td>
<td>24(19.2%)</td>
</tr>
<tr>
<td>Blast phase</td>
<td>12(9.6%)</td>
</tr>
<tr>
<td>Hematologic response (HR)</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>109(87.2%)</td>
</tr>
<tr>
<td>Suboptimal</td>
<td>3(2.4%)</td>
</tr>
<tr>
<td>Loss of HR</td>
<td>13(10.4%)</td>
</tr>
<tr>
<td>Cytogenetic response (CyR)</td>
<td></td>
</tr>
<tr>
<td>CCyR</td>
<td>27(21.6%)</td>
</tr>
<tr>
<td>MCyR</td>
<td>22(17.6%)</td>
</tr>
<tr>
<td>mCyR</td>
<td>20(16.0%)</td>
</tr>
<tr>
<td>nCyR</td>
<td>32(25.6%)</td>
</tr>
<tr>
<td>Complete but loss CyR</td>
<td>24(19.2%)</td>
</tr>
<tr>
<td>IM response</td>
<td></td>
</tr>
<tr>
<td>Suboptimal response</td>
<td>43(34.4%)</td>
</tr>
<tr>
<td>Primary resistance</td>
<td>52(41.6%)</td>
</tr>
<tr>
<td>Secondary resistance</td>
<td>30(24.0%)</td>
</tr>
</tbody>
</table>

3.1. Survival analysis

The data generated from clinical and molecular parameters studied were correlated with the prognosis and survival of CML patients. In the Kaplan–Meier survival analysis, patients in chronic phase showed significantly higher overall survival compared to patients in accelerated phase (P = 0.001) and patients in blast crisis (P < 0.001). When considering the cytogenetic response of the patients against IM treatment, patients who achieved complete cytogenetic response showed significantly higher overall survival compared to patients who did not achieve any cytogenetic response (P = 0.005).

Based on the presence of mutation at tyrosine kinase domain of BCR-ABL1 gene, mutation free patients showed significantly higher overall survival compared to patients who showed the presence of M351T, Y253H and E355G mutation (P = 0.028, P < 0.001 and P = 0.022, respectively). Thus, it is reasonable to suggest that the classification of IM resistant CML patients based on the presence and type of BCR-ABL mutations may be associated with disease outcome. Interestingly, among the Malaysian CML patients studied, mutations such as Y253H and E355G were associated with lower survival than T315I mutation (P = 0.005 and P = 0.025 respectively) (Fig. 2).

Table 3
BCR-ABL TKD mutation analysis results of the CML patients.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation at BCR-ABL gene</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>97(77.6)</td>
</tr>
<tr>
<td>Present</td>
<td>28(22.4)</td>
</tr>
<tr>
<td>Type of mutation</td>
<td></td>
</tr>
<tr>
<td>T315I</td>
<td>9(7.2)</td>
</tr>
<tr>
<td>E255K</td>
<td>4(3.2)</td>
</tr>
<tr>
<td>G250E</td>
<td>2(1.6)</td>
</tr>
<tr>
<td>M351T</td>
<td>2(1.6)</td>
</tr>
<tr>
<td>F359C</td>
<td>2(1.6)</td>
</tr>
<tr>
<td>G251Ea</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>Y253H</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>V289F</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>E355G</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>N368S</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>L387M</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>H396R</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>A397P</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>E355A</td>
<td>1(0.8)</td>
</tr>
<tr>
<td>D276G</td>
<td>1(0.8)</td>
</tr>
</tbody>
</table>

a Novel mutations.
Fig. 1. The multi-alignment of human ABL1 protein with its orthologs of various species by using ClustalX program version 2.0.12 and the position of each mutation in the tyrosine kinase domain. All the mutations detected are highly conserved among different species and located in conserved block of amino acids.

Fig. 2. Overall survival of IM resistant CML patients based on the presence of mutation in TKD of BCR-ABL gene.

4. Discussion

The development of resistance to IM in CML patients has been associated with a heterogeneous array of mechanisms. These include BCR-ABL dependent pathways and BCR-ABL independent pathways. BCR-ABL dependent pathways have been reported as one of the most frequent mechanism that contribute to IM resistance among CML patients treated with Imatinib [11]. The BCR-ABL dependent pathway mostly involves the TKD mutations of the BCR-ABL1 gene [12,13]. Druker et al. [15] demonstrated that blocking of the BCR-ABL1 protein into the inactive conformation by Imatinib prevents the transfer of phosphate from AMP (ATP) to substrates and inhibits the downstream signaling pathways. However, according to Corbin et al. [14], some mutations seem to disrupt critical contact point between Imatinib and BCR-ABL1. In addition, other mutations appear to induce a transition from the inactive to the active state, a conformation which imatinib would be unable to bind [14,15].

In this study, 28 (22.4%) Malaysian IM resistant CML patients showed the presence of mutation in their tyrosine kinase domain.
of BCR-ABL 1 gene. On comparing the mutation frequency of the present study with the frequency reported in other study populations, a lower frequency was observed among IM resistant Malaysian CML patients (Table 4).

According to Kim et al. [18], the frequency of mutations is correlated with the proportion of patients in different phases of the disease. In the study by Kim et al. [18], 25% of the recruited IM resistant CML patients were in chronic phase (CP), whereas the rest 75% of CML patients were in accelerated phase (AP) or blast phase (BP). Consequently, Kim et al. [18] observed a high (63%) frequency of mutation among their IM resistant CML patients. In the present study, 75% of the patients studied were in CP and only 25% were in AP and BP. Thus, the relatively low overall frequency of mutations in our study compared to other published studies may be explained by the predominance of chronic phase CML patients included for mutation analysis. A low percentage of TKD mutation (19%) was also observed by Brandford et al. [16] in IM resistant Australian CML patients. The low percentage of TKD mutations observed in Brandford et al.’s study (2003) also could be due to the majority of patients screened being in the CP. The present study results as well as other quoted reports (Table 4) are in agreement with Brandford et al. [21] who reported that the estimated percentages of IM resistant patients in CML-CP, CML-AP who carried clinically relevant mutations would be approximately 18% and 31% respectively whereas it would be 29–52% in patients with CML BP [21].

Interestingly, among the nine different types of mutations identified, two (G251E and N368S) were novel mutations and had not been reported previously. The G251E mutation is located in the P-loop region where ATP binds. Several studies [18,22,23] have reported that mutation in the P-loop region play an important role in the development of resistance to IM among the CML patients and have 70-fold to 100-fold less sensitivity to IM compared to native BCR-ABL [22]. Although one of the novel mutations, N368S, is located outside the three crucial regions (IM binding site, P-loop and a-loop) on the BCR-ABL kinase domain, still it was predicted to be clinically relevant via in-silico analysis.

To estimate the effect of the novel mutations on the outcome of the CML patients, in-silico analyses were performed on the mutations. The G251E as well as N368S mutations were predicted to be possibly damaging by using PolyPhen2 with a score of 0.898 for both mutations, using HumDiv model (http://genetics.bwh.harvard.edu/pph2/). So, both BCR-ABL G251E and N368S mutations in the present study was predicted to be clinically relevant. Hence, it is reasonable to suggest that BCR-ABL mutations that have no clinical significance might be quite rare. However, these novel mutations were unable to be classified as acquired mutations because patient sample at the point of resistance only was available and not at the point of diagnosis. Thus, the presence of these mutations at the time of diagnosis could not be proved.

The multi-alignment of human ABL1 protein with its orthologs among various species by using ClustalX program version 2.0.12 revealed that both G251E and N368S mutations are conserved among species in a highly conserved block. Fig. 1 shows the multi-alignment of human ABL1 protein with its orthologs of various species by using ClustalX program version 2.0.12 and the position of each mutation in the tyrosine kinase domain that were discovered among the patients. 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 Imatinib on this protein. However, the real impact of the two novel mutations identified, in mediating resistance is yet to be determined as further functional studies are warranted to elucidate this issue.

On survival analysis, IM treated CML patients with BCR-ABL mutations, especially those with T315I, M351T, Y253H and E355G TKD mutations showed shorter survival period compared to those with other types of mutations and those without mutations. Location of T315I mutation at the IM binding site of tyrosine kinase strongly explains the shorter survival and poor prognosis of CML patients with T315I mutation. Moreover, there are reports stating that patients with T315I mutation are not only resistant to IM but also to other second and third generation of TKI like Dasatinib [24], Nilotinib [25] and Bosutinib [26]. Thus, T315I mutation confers true resistance to TKI and thereby promotes shortening of patients’ survival.

However, when inter-individual comparison of patients with these four mutations that were associated with shorter survival was made, patients with Y253H and E355G TKD mutations showed worst outcome compared to patients with T315I mutation (P = 0.005 and P = 0.025 respectively). In this present study, the overall survival of patients with T315I mutation was not the worst as being generally expected. This could be attributed to the fact that some of the patients identified with T315I mutation had been subjected to bone marrow transplantation. During the overall survival analysis, the patients’ status was censored in consequence to the bone marrow transplant. This must have resulted in the overall survival being higher than expected, for the patients with T315I mutation.

The location of Y253H mutation at the nucleotide binding loop (P-loop) highlights its impact on IM resistance development, which also explains the reason for poor disease outcome. Withdrawal of IM is the best option for patients with Y253H mutation which also confers a true resistant phenotype to IM. Although E355G is not located within the three crucial regions of tyrosine kinase domain, it is probable that it still could be having some effect on the binding toward IM as E355G is located near the activation loop. Thus, the results suggest that apart from those mutations occurring in the three crucial regions (catalytic domain, P-loop and activation-loop), mutations in other regions also may have adverse impact resulting in the development of resistance and poor outcome.

Evaluating CML patients with clinical signs of resistance for BCR-ABL mutations is an important component of disease monitoring, in determining how patients respond to treatment and to understand the BCR-ABL mutation as the cause of resistance. Not only the presence of mutations, but also the actual amino acid change should be
investigated in CML patients displaying resistance to IM in order to optimize therapeutic response. Certain specific mutations in BCR-ABL have been linked with poor outcome and hence mutation screening is clinically relevant to identify CML patients who are likely to have poor outcome and to facilitate selection of subsequent therapy. Furthermore, presence of mutations in different regions of BCR-ABL TKD leads to different levels of resistance. The type of mutation potentially can indicate whether second or third generation BCR-ABL inhibitor or alternative therapeutic strategies should be given to such IM resistant patients and also can help to identify those who need IM dose escalation.

Conflict of interest statement

All authors have no conflict of interest to report.

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Contributions. M.H.E. carried out the lab works, acquisition of data, analysis and interpretation of data, drafting the article, revised it critically for intellectual content and final approval of the version to be submitted; A.A.B., A.H., R.H., G.A.S., P.M., and F.A.W. supplied the acquisition of data and revised the article; R.A. provided the conception and design of the study, revised the article critically for important intellectual content and final approval of the version to be submitted.

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