Screening for 3435C>T and 2677G>T/A Polymorphisms of Multi-Drug Resistance (MDR1) Gene in Malay Patients with Leukemia

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Abstract

The prevalence of 2677G>T and 3435C>T polymorphisms of the multi-drug resistance (MDR1) gene was found to be different in many populations and it was significantly influenced by ethnicities. The mechanism on how these two single nucleotide polymorphisms (SNPs) play a role in regulating the MDR1 expression especially in leukemia patients is still unclear and controversial. The objective of this study was to evaluate the distribution of 3435C>T and 2677G>T/A polymorphisms among Malay patients diagnosed to have leukemia (n=101) by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based assays. The genotype frequencies for homozygous genotype GG, heterozygous genotype GT and genotype GA and homozygous mutation genotype TT or AA in exon 21 were reported as 36.9%, 48.5%, 5.8% and 8.7%, respectively. In exon 26, the frequencies were 34.0%, 50.5% and 15.5% for the homozygous wild type CC, heterozygous mutation CT and homozygous mutation TT, respectively. There were no association found between the distribution of SNPs in both exons with the types of leukemia and sex of patients. Therefore, further clinical studies in a larger sample size should be carried out in order to find the association between sex and types of leukemia in distribution of common SNPs in Malay patients with leukemia. The significant implication of these common SNPs to the level of P-gp expression in Malay patients with leukemia that might contribute to the chemotherapy resistance should be carried out as a future study.

Keywords: P-glycoprotein, Multi-drug resistance, SNPs, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic myeloid leukemia (CML)

Introduction

Leukemia remains a difficult disease to treat despite the improved clinical outcome by recent progress in chemotherapy. One major problem was the emergence of leukemic blast cells that were resistant to anticancer drugs, which eventually will lead to treatment failure. A representative cause of multi-drug resistance (MDR) was the expression of the MDR1 gene product, P-glycoprotein (P-gp) on the cell surface membrane[1]. The P-gp belongs to a family of ATP-binding cassette (ABC) transporters that shared sequence and structure homology. The MDR1 gene mapped at position 7q21.1 of chromosome 7 and characterized as ATP-binding cassette (ABC) drug pump[2].

Over expression of the MDR1 gene had been reported in several human cancers [3] as well as acute leukaemia [4]. The expression of P-gp had been reported in leukemia cells from about one-third of patients with acute myeloid leukemia (AML) at the time of diagnosis, and more than 50% of patients at relapsed; higher levels occurred in certain subtypes including secondary leukemia. Expression of P-gp is correlated with reduction in complete remission rates and a higher incidence of refractory disease [5]. In acute lymphoblastic leukemia (ALL), expression of P-gp was observed in 38% of cases [6]. It was reported that the expression of MDR1 gene was increased in the stage of relapsed or refractory disease compared to the patient in diagnosis stage.

Recently, several single nucleotide polymorphisms (SNPs) of MDR1 gene were identified with some consequential protein amino acid changes [7]. As determined by Cascorbi et al., [8], the three most frequent SNPs in the Caucasian population are located in exon 12, exon 21 and exon 26 at position 1236, 2677 and 3435 respectively. Moreover, it was shown in healthy volunteers that these changes are in linkage disequilibrium and may therefore be associated with transcriptional regulation of the MDR1 mRNA [9]. However, a mechanism on how these SNPs play a role in regulating the P-gp expression remains unclear.

To the best of knowledge, the 3435C>T and 2677G>T/A polymorphisms of MDR1 gene has not been assessed in Malay patients with leukemia. Therefore, the PCR-
RFLP based assays was developed to evaluate frequency of allelic variants of 3435C>T and 2677G>T/A polymorphisms in 101 Malay patients with leukemia admitted in Hospital Universiti Sains Malaysia (HUSM), Kota Bharu Kelantan, Malaysia. The results might provide basic information for treatment planning and valuable tool to individualize pharmacotherapy especially in Malay patients with leukemia.

Materials and Methods:

Patients and samples collection:
A total of 101 Malay patients diagnosed to have leukemia (ALL, AML and CML), who were admitted in HUSM were enrolled in this study. Written consent was taken from all patients and for patients aged 16 and below, consent was taken from legal guardians. The study was approved by the local Ethics Committee of the School of Medical Sciences (Reference No: USM/PPSP®/EthicsCom./2004 (124.3[4]), Universiti Sains Malaysia, Kota Bharu Kelantan, Malaysia.

During the study period, out of 101 patients, 35 (34.7%) were diagnosed as AML, 48 (47.5%) as ALL and 18 (17.8%) as CML. Five out of 48 (10.4%) ALL patients were diagnosed to have relapsed. There were 58 (57.4%) males and 43 (42.6%) females. Age of the patients ranged from 1 to 75 years.

Table 1: List of primers, type of enzymes used and fragment sizes for PCR-RFLP assays

<table>
<thead>
<tr>
<th>Exon</th>
<th>Accession Code</th>
<th>Primer sequences</th>
<th>Restriction enzyme</th>
<th>Cutting site</th>
<th>Recognition sequences</th>
<th>SNPs</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>#M29440</td>
<td>F: 5’ GTT TTG CAG GCT ATA GGT TCC 3’ R: 5’ TTT AGT TTG ACT CAC CTT CCC G 3’</td>
<td>BanI</td>
<td>2677</td>
<td>5’ GG/PuPyCC 3’</td>
<td>G&gt;T</td>
<td>228, 209, 19</td>
</tr>
<tr>
<td>21</td>
<td>#M29440</td>
<td>F: 5’ GTT TTG CAG GCT ATA GGT TCC 3’ R: 5’ TTT AGT TTG ACT CAC CTT 3’</td>
<td>BsrI</td>
<td>2677</td>
<td>5’ ACTGGN/ 3’</td>
<td>G&gt;A</td>
<td>228, 210, 18</td>
</tr>
<tr>
<td>21</td>
<td>#M29440</td>
<td>F: 5’ GTT TTG CAG GCT ATA GGT TCC 3’ R: 5’ TTT AGT TTG ACT CAC CTT 3’</td>
<td>BseYI</td>
<td>2677</td>
<td>5’ CCCAGC 3’</td>
<td>Wild type (GG)</td>
<td>205, 23</td>
</tr>
<tr>
<td>26</td>
<td>#M29445</td>
<td>F: 5’ GAT CTG TGA ACT CTT TTC A 3’ R: 5’ GAA GAG AGA CTT ACA TTA GCC 3’</td>
<td>MboI</td>
<td>3435</td>
<td>5’/GATC 3’</td>
<td>C&gt;T</td>
<td>244, 172, 72</td>
</tr>
</tbody>
</table>

DNA Isolation:
Venous blood (200 µl) was obtained and genomic DNA was extracted using DNA Extraction Kit (QIAGEN, Germany). All procedures were followed as suggested by the manufacturer. The concentration and purity of DNA samples were estimated spectrophotometrically.

PCR-RFLP assays:
The reaction mixture for PCR amplification consisted of DNA template, 0.4 µM of each primers for both exons as listed in TABLE 1 (primers used in this study were followed as suggested by Ilmer et al., [10] and Cascorbi et al., [8], 3.0 mM M₃Cl₂ (for exon 26) and 2.5 mM M₃Cl₂ (for exon 21), 200 µM deoxynucleotide triphosphate (dNTPs), 10X AmpliTaq Gold PCR buffer and 1 unit AmpliTaq Gold (Applied Biosystems). PCR grade water was added to a final volume of 50 µl. PCR amplification conditions were as follows: initial denaturation at 95°C for 2 minutes, followed 32 cycles for denaturation at 95°C for 15 s, annealing at 60°C (exon 26) and 53°C (exon 21) for 30 s and extension at 72°C for 30 s. The terminal extension was performed at 72°C for 7 minutes. The specific products were analyzed on standard 1.7% agarose gels stained with SyBr Green (Amresco, Ohio). The type of enzymes used and DNA fragments generated after digestion are shown in TABLE 1; separated on 4% agarose gel, stained with SyBr Green and visualized under UV light.
In principle, there were three different enzymes used in exon 21 in order to identify the type of allelic involved for homozygous wild type, heterozygous for genotype GA and heterozygous for genotype GT. Two different reverse primers used resulting two different recognition sites for BsrI and BanI enzymes. The additional base G was added at the 3’ end of the reverse primer in order to produce cutting site for BanI enzyme. In order to screen for homozygous wild type for genotype GG, samples were digested with BseY1 enzyme and the sizes of the fragments generated were 205 bp and 23 bp. Samples showed persistent single band after digestion with BseY1 enzyme were classified having whether heteroduplexes GT or GA or contains homozygous mutation for genotype TT or AA at position 2677. These samples were then digested with BanI enzyme in order to screen for heterozygous genotype GT. In this case, three fragments generated 228 bp, 209 bp and 19 bp in sizes. Those samples did not digest with BanI enzyme was subjected to BsrI enzyme in order to screen for heterozygous genotype GA. The fragments generated after 16 hours incubation were 228 bp, 210 bp and 18 bp.

The PCR products for exon 26 contain the recognition site for MboI enzyme. In exon 26, all samples were subjected to RFLP assays using MboI enzyme where 3 bands observed indicates the present of heterozygous mutation at position 3435 with the sizes of 244 bp, 172 bp and 72 bp. If the samples do not contain any mutation, the sizes of fragments generated were 172 bp and 72 bp while 1 band indicates the samples were homozygous mutation where the sizes of the fragments were 244 bp.

### Table 2: The distribution of common polymorphisms in exon 21 and exon 26 of MDR1 gene according to the types of leukemia and sex of the patients. These results were obtained based on the number of fragments generated after digestion with specific enzymes during PCR-RFLP based assays.

<table>
<thead>
<tr>
<th>Genotype frequency (%)</th>
<th>Exon 21</th>
<th>Exon 26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CT</td>
</tr>
<tr>
<td>AML (n=35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>(28.6)</td>
<td>(57.1)</td>
<td>(5.7)</td>
</tr>
<tr>
<td>GT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>(45.8)</td>
<td>(39.6)</td>
<td>(8.3)</td>
</tr>
<tr>
<td>TT/AA</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(33.3)</td>
<td>(50.0)</td>
</tr>
<tr>
<td>ALL (n=48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>(45.8)</td>
<td>(39.6)</td>
<td>(8.3)</td>
</tr>
<tr>
<td>GT</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>(33.3)</td>
<td>(50.0)</td>
<td>(16.7)</td>
</tr>
<tr>
<td>GA</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>(6.9)</td>
<td>(6.9)</td>
<td></td>
</tr>
<tr>
<td>TT/AA</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(7.9)</td>
<td>(7.9)</td>
</tr>
<tr>
<td>CML (n=18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>(33.3)</td>
<td>(50.0)</td>
<td>(16.7)</td>
</tr>
<tr>
<td>GT</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>(6.9)</td>
<td>(6.9)</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>(3.2)</td>
<td>(3.2)</td>
<td>(3.2)</td>
</tr>
<tr>
<td>TT/AA</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(7.9)</td>
<td>(7.9)</td>
</tr>
</tbody>
</table>

*Statistical analysis was done using Fisher Exact’s test
**Statistical analysis was done using chi-square test

### Statistical analysis

Frequency and percentage were calculated for all the variables. The association between distribution of SNPs in both exons with type of leukaemia’s and sex of patients were analyzed using chi-square and Fisher Exact’s test where p<0.05 considered as statistically significant.

### Results

As shown in TABLE 2, the genotype frequency for homozygous mutation found lower (11.4%) in exon 26 for AML patients while it was similar to ALL and CML patients. The genotype frequency for homozygous wild type found higher in exon 21 and exon 26 with 45.8% and 35.0%, respectively in ALL patients. Heterozygous for genotype GA at position 2677 in exon 21 was only found among Malay patients with AML and ALL with frequencies 5.7% and 8.3%, respectively while none of this genotype found in patients with CML. Overall, there was no association found between types of leukemia and sex of patients with the distribution of SNPs in both exons where p>0.05.

### Discussion

Even though there is increased number of new technologies available for identification of specific genes contributing to diseases, it still lack of knowledge on how these genes function and play a role in developing a disease. However, many of researchers are interested to study function of single base changes at the specific location of whole coding region and their contribution to the disease development. Most human diseases influenced...
Screening for polymorphisms of multi-drug resistance gene

by genes can be traced to SNPs, especially in important traits such as how diseases develop and how one responds to a pharmaceutical treatment. Variations in genome sequences underlie differences in one’s susceptibility or protection from all kinds of diseases, age of onset and severity of illness [11].

The 3435C>T polymorphism located in exon 26 is the most popular SNP in the MDR1 gene that have thoroughly been studied by many researchers in various field of diseases. This polymorphism has been reported as a silent SNP which does not result in amino acid change and it also been reported that the frequency of this SNP is significantly influenced by ethnicities [12]. In this type of polymorphism, T-allele has been found to be associated with reduced P-gp expression while the increased of C-allele frequency had been reported to give an impact on high expression level of P-gp. There was also been reported to play an important role in defence mechanism against several toxins including bacteria and viral particles [7]. Since this silent SNP is frequently correlated with the level of P-gp expression, many researchers are interested to study on how this SNP can influence the regulation and expression of P-gp.

In exon 26, the frequencies were 34.0%, 50.5% and 15.5% for the homozygous wild type CC, heterozygous mutation CT and homozygous mutation TT, respectively. The frequency of polymorphisms at position 3435 in exon 26 was compared to the data published by Tang et al., [13, 14] for Malay healthy volunteers in Asian population. We found the frequency for C-allele in our study quite similar to the frequency observed by Tang et al., [13] which 59.2% and 58.1%, respectively while lower compared to the Tang et al., [14] with the frequency of 63.0%. On the other hand, the frequency for T-allele was found to be higher (40.8%) compared to Tang et al [14] with frequency 37.0% and quite similar with Tang et al., [13] with frequency 41.9%.

In terms of distribution of common alleles in exon 21, Tang et al., [14] reported the frequency for G-allele, T-allele and A-allele at position 2677 in percentage of 57.5%, 36.0% and 6.5%, respectively though Tang et al., [13] reported the frequency for G-allele, T-allele and A-allele in percentage of 46.7%, 41.3% and 13.0%, respectively. However, in this study, the genotype frequencies were reported instead of reporting the allele’s frequencies where the frequency for homozygous genotype GG, heterozygous genotype GT and genotype GA and homozygous mutation genotype TT and AA were reported as 36.9%, 48.5%, 5.8% and 8.7%, respectively.

The 3435C>T polymorphism is found to be common in all ethnicities; however the frequency is dependent on racial background [9]. For example, 62% of European Americans and only 13% of African Americans carry at least one such allele. Besides that, this polymorphism also found to be linked to other non-synonymous polymorphism in exon 21 (2677G>T) and synonymous polymorphism in exon 12 (1236C>T). In the same study, both polymorphisms in exon 26 and exon 21 were found to be associated with altered fexofenadine disposition, especially when both homozygous groups (CC and TT) were compared. They found that subjects for homozygous wild type in both exon 21 (homozygous GG) and exon 26 (homozygous CC) had significantly higher of fexofenadine AUC (plasma concentration time) values than homozygous TT subjects.

Figure 1: Electrophoresis patterns for 2677G>T/A polymorphism in exon 21 evaluated by PCR-RFLP based assay
Lanes 1 and 2 : Shows digestion with BanI enzyme with the expected sizes 228, 209 bp
Lanes 3 and 4 : Shows digestion with BsrI enzyme with expected sizes 228, 210 bp
Lane 5 : Undigested PCR product (228 bp)
Lane 6 : Shows sample uncut sample (228 bp)
Lane 7 : Shows 100 bp DNA ladder

Figure 2: Electrophoresis patterns for 3435C>T polymorphism in exon 26 evaluated by PCR-RFLP based assay
Lane 1 : Shows undigested PCR product (244 bp)
Lane 3 : Shows sample with homozygous wild type CC (172 and 72 bp)
Lanes 2, 4, 6, 7 : Shows sample with heterozygous mutation CT (244, 172 and 72 bp)
Lane 8 : Shows sample with homozygous mutation TT (244 bp)
Lane 5 : Shows 100 bp DNA ladder
Interestingly, several hypotheses have been made in order to make an initial conclusion on how this 3435C>T polymorphism can influence the P-gp activity and function. Firstly, the 3435C>T transition might have an impact on post-transcriptional modifications of the mRNA or secondly it might link to a sequence that is important for mRNA processing [15]. In this study, the correlation between 3435C>T and 2677G>T/A polymorphisms (P<0.01) in terms of distribution of variations was thought to be interesting as preliminary finding that these two exons might play an important role in contributing to the mRNA or P-gp regulation and expression in Malay leukaemia patients.

As mentioned earlier, 3435C>T polymorphism might be linked with other polymorphisms in 1236C>T and 2677G>T/A. Ilmer et al., [10] had strongly agreed in their study where in 405 patients with AML, three most frequent SNPs were found in exon 12, exon 21 and exon 26. They found that patients with the homozygous mutant genotype in exon 12 and exon 26 showed a lower median age and was associated with poor risk cytogenetic aberrations. On the other hand, there was a significant association of the homozygous wild type genotype in exon 21 and exon 26 with lower MDR1 expression, whereas the heterozygous variants showed highest MDR1 values at all three investigated gene loci. The homozygous wild type in exon 21 was associated with lower overall survival (OS) with P<0.05 and worse OS is likely linkage disequilibrium of the investigated SNPs. It was indicated that combined polymorphisms could affect the regulation of MDR1 and mRNA expression.

However, the mechanism on how this linkage plays a role in regulating the P-gp activity remains unclear. Unlike what has been reported by Hoffmeyer et al., [7], the linkage between polymorphisms in exon 21 and 26 is not completely implying that these polymorphisms can act independently in regulating the P-gp expression and function. Lütsch et al., [16] discovered that polymorphisms for 1236C>T, 3435C>T and 2677G>T occur together at a frequency of 62%, which was identical to the value of 62% as previously reported by Kim et al., [9] in Caucasian population. Whereas, in Japanese population, the 3435C>T and 2677G>T SNPs were reported to be linked in 94% of cases.

In this study, combined polymorphisms was defined when in these two exons, polymorphisms happened concurrently whether homozygous wild type, heterozygous mutation or homozygous mutation. Therefore, when these polymorphisms were combined and 39% link were found between 3435C>T and 2677G>T SNPs while 1% link were found between 3435C>T and 2677G>A. On the other hand, there were 24% linked found between 3435C>C and 2677G>G among these studied samples. Thus, we would like to suggest that study involves combination of these SNPs would be giving a valuable results especially in initiating treatment modalities and assessing patients’ response to therapy especially those receiving P-gp substrate.

In other related study, Efferth et al., [17] showed no significant correlation for drug resistance and prognosis between homozygous genotype CC and heterozygous genotype CT with MDR1 mRNA expression of cell lines in ALL patients. They also found that there was no relationship between the response of the cell lines to doxorubicin and the 3435C>T genotypes.

Recent report indicates that 3435C>T polymorphism may be of clinical relevance where there was an association between MDR1 3435C>T polymorphism and CNS (central nervous system) relapsed in childhood ALL patients. There was 7-fold risk reduction of CNS relapsed observed in the group of patients with intermediate or high risk of treatment failure for patients with the TT and CT genotypes. This data was consistent with study carried out by Jamroziak et al., [15] where increased frequency of C-allele carriers among Polish children with ALL who relapsed.

However, a number of large-scale genotype-phenotype correlations are required in order to understand a correlation between SNPs and clinical outcome of Malay leukaemia patients as future study. Laboratory testing of common polymorphisms affecting activity of transporters and enzymes involved in metabolism of a given drug might be useful to reduce toxicity and to increase efficacy of therapy by individual dosage adjustment [18].

As mentioned earlier, the corresponding of C-allele was associated with increased of P-gp levels [7]. In this study, the genotype frequency for homozygous CC found to be higher in ALL and very low in AML patients. However, when the distribution of genotype frequency is compared according to the type of leukemia, there was no significant difference. High frequency of homozygous CC in our ALL patients might have an association with chemotherapy response in these patients. This hypothesis however had been proved by Ilmer et al., [10] where they found that higher frequency of homozygous CC in patients with ALL was found to be associated with worse prognosis in these children and lower overall survival time in patients under age of 60 years old. However, larger sample size is required to obtain statistically significant and further clinical study should be carried out to come to this conclusion.

The TT genotype in exon 26 was found to be associated with occurrence of ALL and the C-allele carriers were found to be reduced risk of developing ALL [15]. The homozygous genotype CC was associated with poor outcome for the treatment. The T-allele carriers were generally linked to weaker expression of P-gp in normal tissues. Those homozygous for T-allele was found to have low intestinal expression of P-gp resulting in higher intracellular concentrations of mutagens and eventually leading to transformation of normal cell to cancer cells [19]. In contrast, homozygous for T-allele was found to
be associated with higher MDR1 mRNA levels in AML blasts cells [10]. The P-gp expression was found to be an independent predictor of complete remission achievement in adult with ALL. Furthermore, a hypothetic lower exposure of ALL cells to P-gp transported drugs in the CC homozygous individuals might be a consequence of increased renal clearance, active secretion from enterocytes into the gut [20].

Studies pertaining to linkage between 2677G>T and 3435C>T polymorphisms are gaining increasing significance especially as biomarkers to individualize pharmacotherapy in leukemia patients. This polymorphism might play a role in patients who are not responsive to drug treatment. Furthermore, P-gp is essential in building blood-brain barrier (BBB) and as transporters among other tissues as well as an important prognostic factor in several tumour diseases [8]. On the contrary, there was no significant difference when subjects were compared according to the gender and age of the patients where Jamroziak et al., [21] suggested that gender as well as other prognostic features might be treatment-dependent.

Even though the study on 2677G>T/A and 3435C>T polymorphisms and their effect on P-gp expression and regulation is still uncertain, further clinical correlation should be studied since there was evidence that this polymorphism may play a role in treatment and prognosis of patients with leukemia.

**Conclusion**

The clinical applications of genotyping studies demand such methodology that would be rapid, simple and cost-effective. Therefore, this study evaluated the PCR-RFLP based assay as an effective method to detect known polymorphisms at position 2677 and 3435 of MDR1 gene. Even though this study only reported preliminary result, the results could serve as a basis knowledge for large scale correlation studies on relevance of 3435C>T and 2677G>T/A genotypes in therapy of leukemia in Malay population that was treated with drugs belongs to P-gp substrates as future studies. The clinical finding should be carried out to find a significant correlation between chemotherapy resistances with the increasing of genotype CC in ALL subjects as well as patient with different types of leukemia in Malays.

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