RESEARCH ARTICLE

Pvmdrl Polymorphisms of Plasmodium vivax Isolates from Malaria Endemic Areas in Southern Provinces of Thailand

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Abstract

Since chloroquine is the mainstay for treatment of Plasmodium vivax infection, monitoring of chloroquine drug resistance marker provides useful information for effective malaria control program. The aim of the study was to investigate the genetic polymorphisms of pvmdrl in Plasmodium vivax isolates collected from Southern Thailand. A total of 70 P. vivax isolates were collected by finger-prick from patients. DNA from dried blood spot samples were extracted and analyzed for pvmdrl polymorphisms. Seven non-synonymous and one synonymous mutations were identified in pvmdrl gene. Five haplotypes of pvmdrl were observed with different frequencies. However, 21 isolates (30%) carried 4 mutations of 515R, 698S, 908L, and 958M. Amino acid Y976F and F1076L mutations, key point mutations associated with P. vivax chloroquine resistance, were detected in 17 (24.3%) and 38 (54.3%) isolates, respectively. The prevalence and pattern of mutations of pvmdrl obtained from this study suggest the spreading of chloroquine resistance alleles in P. vivax isolates from Southern part of Thailand. The monitoring of chloroquine drug resistance marker in P. vivax can provide useful information for early warning system and for developing the appropriate drug policy.

Keywords: Plasmodium vivax, pvmdrl, chloroquine resistance
ความหลากหลายของ pvmdr1 ของเชื้อพลาสโมเดียมไวแวกซ์จากพื้นที่ระบาด
ของเชื้อมาลาเรียในจังหวัดภาคใต้ของประเทศไทย

กาญจนา รังษีหิรัญรัตน์ 1, จิราภรณ์ คุ้มทรัพย์ 2, วรรณนา ขยันธัญกุล 3, นพครฐา ผึ้งสิน 4, เกศรา ณ บางช่าง 3

1 วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพมหานคร
2 คณะเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ ปทุมธานี
3 วิทยาลัยแพทยศาสตร์นานาชาติจุฬาภรณ์ มหาวิทยาลัยธรรมศาสตร์ ปทุมธานี
4 ภาควิชาวิทยาการสุขภาพ วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า กรุงเทพมหานคร

บทคัดย่อ

เนื่องจากคลอโรควินถูกใช้เป็นยาหลักในการรักษาการติดเชื้อมาลาเรียนานั้น ดังนั้น การใช้ตัวติดตามการค้นหาคลอโรควินซึ่งมีประโยชน์สำหรับการควบคุมเชื้อมาลาเรีย
วัตถุประสงค์ของการศึกษาครั้งนี้เพื่อศึกษาความหลากหลายของรูปแบบยีน pvmdr1 ในเชื้อ
พลาสโมเดียมไวแวกซ์ที่เก็บจากภาคใต้ของประเทศไทย จำนวน 70 ตัวอย่าง ด้วยวิธีเก็บจาก
ปลายนิ้วของผู้ป่วย น้ำมันเอทีที่เก็บบนกระดาษกรองมาสกัดและวิเคราะห์หาความหลากหลาย
ของยีน pvmdr1 ผลการทดลองพบการกลายพันธุ์ยีนชนิด non-synonymous 7 ตัวแหน่ง และชนิด
synonymous 1 ตัวแหน่ง โดยยีน pvmdr1 5 รูปแบบที่พบมีความถี่แตกต่างกัน เชื้อมาลาเรีย
จำนวน 21 (30%) ตัวอย่าง พบการกลายพันธุ์ 4 ตัวแหน่ง (515R, 698S, 908L, 958M) โดยพบ
การกลายพันธุ์ยีนชนิด Y976F และ F1076L ซึ่งเป็นตัวแหน่งสำคัญที่เกี่ยวข้องกับการตีนคลอโรควินในเชื้อมาลาเรียนานั้น
จำนวน 17 (24.3%) และ 38 (54.3%) ตัวอย่าง ตามลำดับ ความถี่และรูปแบบของ pvmdr1 ที่พบในครั้งนี้แสดงให้เห็นว่ามีการกระจายของเชื้อตีนคลอโรควินในเชื้อมาลาเรียนานั้น
ในพื้นที่ที่มีการระบาดในภาคใต้ของประเทศไทย และการติดตามการตีนคลอโรควินของเชื้อมาลาเรียสามารถใช้เป็นข้อมูลในการเฝ้าระวังและ
เป็นแนวทางในการใช้ยาต้านมาลาเรีย

คำสำคัญ: พลาสโมเดียมไวแวกซ์, pvmdr1, การตีนคลอโรควิน
Introduction

Combination of chloroquine and mefloquine is the first-line treatment for *Plasmodium vivax* since 1946. Recently, treatment failure of chloroquine in *P. vivax* has been reported in some malaria endemic countries. Thailand is highly endemic to *Plasmodium falciparum* but recent reports suggest that malaria due to *P. vivax* is increasingly, especially in Southern peninsula of Thailand. Reduced *in vitro* parasite’s susceptibility to chloroquine as well as other antimalarial drugs has been demonstrated in the malaria parasites along the international borders of Thailand. Several studies have been proposed to investigate genetic diversity of *P. vivax*. However, this species has been less well studied when compare to *P. falciparum*. Similar approaches have been adopted to investigate *P. vivax* at the molecular level. Although the molecular mechanisms underlying chloroquine resistance in *P. vivax* remain unclear, similar molecular mechanisms of multi-loci genes in *P. falciparum* have been proposed to be involved in *P. vivax* chloroquine resistant phenotype. The *P. vivax* multidrug resistance (*pvmdr*) and putative transporter (*pvcrt-o*) genes, which are orthologous to *pfmdr1* and *pfcrt* genes, have been identified as chloroquine resistance markers in *P. vivax*. The mutant alleles of both genes were suggested to be associated with chloroquine resistance in *P. vivax* in Southeast Asia both *in vivo* and *in vitro*. Sequence analysis of *pvmdr1* and *pvcrt-o* demonstrated that *pvmdr1* gene contained at least 24 single nucleotide polymorphisms (SNPs), whereas *pvcrt-o* gene contained 5 SNPs. However, sequence analysis of *pvmdr1* SNPs at homologous positions of *pfmdr1* did not reveal any polymorphism as that found in *P. falciparum*, suggesting the different mechanisms of chloroquine resistance between *P. vivax* and *P. falciparum*. The aim of the present study was to investigate the genetic polymorphisms of *pvmdr1* in *P. vivax* isolates collected from Southern Thailand.

Materials and Methods

Sample collection

*P. vivax* field isolates in this study were collected from malaria patients attending malaria clinics in Southern part of Thailand along Thailand-Myanmar and Thailand Malaysia borders. The study protocol was reviewed and approved by the Ethics Review Committee for Research Involving Human Research Subject, Health Science Group, Chulalongkorn University. Written informed consent for study participation was obtained from each patient prior to participation. Blood films were stained with Giemsa stain and examined by light microscope for confirmation of *P. vivax* infection. Approximately 100-200 μL of blood samples from finger-prick were spotted onto filter paper and stored in small plastic zip lock bags prior to extraction of parasite DNA for identification of *pvmdr1* polymorphisms.

Identification of *pvmdr1* polymorphisms

Genomic DNA was individually extracted from dried blood spots on filter paper using a QIAGEN DNA extraction mini-kit (Qiagen, Germany) and used as a template for amplification by polymerase chain reaction (PCR). The *pvmdr1* gene
was amplified by nested PCR using specific primers as previously described. The outer PCR was carried out in the following reaction mixture with a total volume of 20 μL: 0.2 μM of each primer, 2.5 mM MgCl₂, 100 mM KCl, 20 mM Tris-HCl (pH 8.0), 100 μM deoxynucleotide triphosphates (dNTPs), 10-20 μg of genomic DNA, and 0.5 unit of Taq DNA polymerase. The nested PCR was carried out in a PCR mixture similar to that used for the outer PCR. One μL of outer PCR product was used as DNA template. The outer and nested PCR cycling parameters were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and then followed by final extension at 72°C for 5 minutes. The PCR products were fractioned by 1.5% agarose gel electrophoresis, purified with QIAquick PCR purification kit (Qiagen, Germany), and sequenced by automated DNA sequencer (ABI system, Singapore).

**Data and sequence analysis**

Each fragment was sequenced in both the forward and reverse directions and assembled using Bioedit version 7.1.3. (Ibis BioSciences, Carsbad, California, USA). DNA fragments were aligned using the ClustalW multiple sequence alignment (freely available from http://www.genome.jp/tools/clustalw). Nucleotide sequence alignments of all isolates were compared with the wild-type sequence from GenBank for *pvmdr1* (Accession no. AY618622) using Bioedit version 7.1.3.

**Results**

A total of 78 *P. vivax* isolates were collected for analysis of *pvmdr1* genetic polymorphisms. The *pvmdr1* gene was successfully sequenced in 70 *P. vivax* isolates. Among all of the studied mutations of *pvmdr1*, no N91Y, Y189F, S510T, I636T, A829V, K997R, L1022L, S1071C, N1079D and D1291Y mutations were found. Numbers of *pvmdr1* mutant alleles at amino acid residual at codon indicated were identified as shown in Table 1. Seven non-synonymous and one synonymous mutations were identified in *pvmdr1* gene. All isolates contained at least 4 to 6 mutations. Among these, 5 haplotypes of *pvmdr1* were observed, of which the majority of the isolates (21/70) carried 4 mutations (515R, 698S, 908L, 958M). Nineteen isolates (19/70) carried 5 mutations (515R, 698S, 908L, 958M, 1076L) and 17 isolates (17/70) carried 6 mutations (515R, 698S, 908L, 958M, 976F, 1076L). Eleven isolates (11/70) carried 5 mutations of 513R, 515R, 698S, 908L, 958M and 2 isolates (2/70) carried 6 mutations of 513R, 515R, 698S, 908L, 958M, 1076L. Isolates carrying 1076L were observed in 38 isolates (54.3%), whereas isolates carrying both 976F and 1076L were observed in 17 isolates (24.3%) (Table 2).
Table 1. Prevalence of pvmdrl mutant alleles.

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Number (%) of pvmdrl mutant alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>N91Y (aac/tac)</td>
<td>N91Y = 0 (0)</td>
</tr>
<tr>
<td>Y189F (tac/ttc)</td>
<td>Y189F = 0 (0)</td>
</tr>
<tr>
<td>S510T (agc/acc)</td>
<td>S510T = 0 (0)</td>
</tr>
<tr>
<td>S513R (agt/aga)</td>
<td>S513R = 13 (18.6)</td>
</tr>
<tr>
<td>S515R (agc/agg)</td>
<td>S515R = 70 (100)</td>
</tr>
<tr>
<td>T529 (aca/aga)*</td>
<td>T529 (aca) = 6 (8.6), T529 (acg) = 64 (91.4)</td>
</tr>
<tr>
<td>I636T (atc/acc)</td>
<td>I636T = 0 (0)</td>
</tr>
<tr>
<td>G698S (gcc/ace)</td>
<td>G698S = 70 (100)</td>
</tr>
<tr>
<td>A829V (gcc/gtc)</td>
<td>A829V = 0 (0)</td>
</tr>
<tr>
<td>M908L (agt/ctg)</td>
<td>M908L = 70 (100)</td>
</tr>
<tr>
<td>T958M (acg/atg)</td>
<td>T958M = 70 (100)</td>
</tr>
<tr>
<td>Y976F (tac/ttc)</td>
<td>Y976F = 17 (24.3)</td>
</tr>
<tr>
<td>K997R (aag/agg)</td>
<td>K997R = 0 (0)</td>
</tr>
<tr>
<td>L1022 (cta/taa)*</td>
<td>L1022 (cta) = 0 (0)</td>
</tr>
<tr>
<td>S1071C (agt/tgt)</td>
<td>S1071C = 0 (0)</td>
</tr>
<tr>
<td>F1076L (ttt/ctt)</td>
<td>F1076L = 38 (54.3)</td>
</tr>
<tr>
<td>N1079D (aac/gac)</td>
<td>N1079D = 0 (0)</td>
</tr>
<tr>
<td>D1291Y (gat/tat)</td>
<td>D1291Y = 0 (0)</td>
</tr>
</tbody>
</table>

* indicates synonymous mutation

Table 2. Haplotype frequencies of pvmdrl.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>S513R</th>
<th>S515R</th>
<th>G698S</th>
<th>M908L</th>
<th>T958M</th>
<th>Y976F</th>
<th>F1076L</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>L</td>
<td>M</td>
<td>Y</td>
<td>F</td>
<td>21 (30)</td>
</tr>
<tr>
<td>2</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>L</td>
<td>M</td>
<td>Y</td>
<td>L</td>
<td>19 (27.1)</td>
</tr>
<tr>
<td>3</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>L</td>
<td>M</td>
<td>F</td>
<td>L</td>
<td>17 (24.3)</td>
</tr>
<tr>
<td>4</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>L</td>
<td>M</td>
<td>Y</td>
<td>F</td>
<td>11 (15.7)</td>
</tr>
<tr>
<td>5</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>L</td>
<td>M</td>
<td>Y</td>
<td>L</td>
<td>2 (2.9)</td>
</tr>
</tbody>
</table>

Discussion

Plasmodium vivax control is now being problematic due to drug resistance. The first case of chloroquine resistance in P. vivax has been reported since 1989 in Indonesia\(^2\) and further sporadic cases were subsequently observed in Oceanian and other Asian countries.\(^8,18\) In Thailand, the proportion of P. vivax infection has become increasing and a trend of gradual decline of in vitro sensitivity to chloroquine has been reported in some areas.\(^19\) Since the effective in vitro culture systems for P. vivax are not available and more complicated when compared with P. falciparum. Thus, monitoring of genetic polymorphisms associated with drug resistance markers provides an understanding of molecular mechanism underlying chloroquine resistance in P. vivax infection.
Unlike chloroquine-resistant *P. falciparum*, the mechanism of chloroquine resistance in *P. vivax* remains unclear. The orthologous *P. falciparum* genes linked to chloroquine resistance have been used to identify chloroquine resistance gene in *P. vivax*.\textsuperscript{12-13, 17, 20} The *pvmdr1* gene was found to be more polymorphic than *pvcrto*.\textsuperscript{21} No wild-type haplotype was found in our study. Seven non-synonymous and one synonymous mutations were observed in *pvmdr1*. Y976F and F1076L mutations, possible genetic markers for chloroquine resistance in *P. vivax*, were detected in 24.3% and 54.3% of *P. vivax* isolates which are lower than those observed in 9 *P. vivax* Thai isolates from Mae Sod (44% and 56%).\textsuperscript{13} The observation of high frequency of F1076L mutation (54.3%) in our present study was similar to that reported in a previous study in the isolates from Papua New Guinea. However, the frequency of Y976F *pvmdr1* mutation (24.3%) was much lower than the Papua New Guinea isolates (100%). None of both mutations was found in the isolates collected from Korea.\textsuperscript{14}

This finding may suggest that Y976F and F1076L mutations in *P. vivax* were spread and distributed from Southeast Asia and high prevalence of F1076L mutation may indicate a trend of rapid aggravation of chloroquine resistance *P. vivax* in this region. Apart from the mutations at both codons, one non-synonymous mutation at codon T529 (aca = 8.6%, acg = 91.4%) was also detected in our study. The commonly found mutations at codon S515R, G698S, M908L and T958M were also detected in all *P. vivax* isolates. Sequence analysis of *pvmdr1* gene has shown no mutation at codons 91, 189, 1071, 1079, and 1291 homologous to *pfmdr1* at codons 86, 184, 1034, 1042, and 1246, respectively, which are associated with chloroquine resistance in *P. falciparum*. Five haplotypes of *pvmdr1* were observed with different frequencies. However, majority of isolates (30%) carried 4 mutations (515R, 698S, 908L and 958M). It is interesting that polymorphism in *pvmdr1* gene was higher than that in *pfmdr1* gene, which might be one factor that contributed to *in vitro* resistant parasites.\textsuperscript{22} Identification of differences in the orthologous gene between *P. falciparum* and *P. vivax* is important for comparing genetic determinants of chloroquine resistance in both malaria species. The mechanism of chloroquine resistance in both *P. falciparum* and *P. vivax* is probably similar, but the development of resistance may be different.

**Conclusion**

We have analyzed the polymorphisms in *pvmdr1*, a chloroquine drug resistant gene, in 70 *P. vivax* isolates collected from Southern part of Thailand. Seven non-synonymous and one synonymous mutations were identified. Five haplotypes of *pvmdr1* were observed with different frequency. The finding suggested that monitoring of chloroquine drug resistance marker in *P. vivax* is essential for early warning system and expedites the appropriate drug policy.
Acknowledgements

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References


