■ BRIEF COMMUNICATION

Prevalence of Drug Resistance-Associated Gene Mutations in *Plasmodium vivax* in Central China

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Abstract: Resistance of *Plasmodium* spp. to anti-malarial drugs is the primary obstacle in the fight against malaria, and molecular markers for the drug resistance have been applied as an adjunct in the surveillance of the resistance. In this study, we investigated the prevalence of mutations in *pvmdr1, pvcrt-o, pvdhfr,* and *pvdhps* genes in temperate-zone *P. vivax* parasites from central China. A total of 26 isolates were selected, including 8 which were previously shown to have a lower susceptibility to chloroquine in vitro. For *pvmdr1, pvcrt-o,* and *pvdhps* genes, no resistance-conferring mutations were discovered. However, a highly prevalent (69.2%), single-point mutation (S117N) was found in *pvdhfr* gene. In addition, tandem repeat polymorphisms existed in *pvdhfr* and *pvdhps* genes, which warranted further studies in relation to the parasite resistance to antifolate drugs. The study further suggests that *P. vivax* populations in central China may still be relatively susceptible to chloroquine and sulfadoxine-pyrimethamine.

Key words: *Plasmodium vivax*, drug-resistance, mutation, central China

Among the 5 species of *Plasmodium* parasites that affect humans, *Plasmodium vivax* is the most widely distributed and causes a serious public health burden [1,2]. In central China, the transmission of malaria is entirely due to *P. vivax*. Since 2000, vivax malaria has demonstrated resilience to eradication and has become increasingly prevalent in this region, especially in areas along the Huai River [3]. Control measures of vivax malaria are confounded by 2 major factors; dormant hypnozoite stages in the liver, and the emergence of drug resistance. Chemotherapy remains a key factor in the fight against malaria. The tools that are used to monitor drug efficacy include clinical trials, in vitro studies, and molecular markers for detection, each of which made important contributions to a more complete understanding of anti-malarial drug resistance. In a previous study, the susceptibility of *P. vivax* to anti-malarial drugs was observed by in vitro testing [4], though further investiga-

tions are needed to assess the predictive value of molecular markers in central China.

Molecular markers of drug-resistant *P. vivax*, including mutations in multidrug resistance 1 gene (*pvmdr1*) and putative transporter protein CG10 gene (*pvcg10* or *pvcrt-o*), which are orthologous to *pfmdr1* and *pfcrt-o* genes, respectively, have been identified as possible genetic markers of chloroquine-resistance (CQR) [5,6]. The genotypes of dihydrofolate reductase (*pvdhfr*) and dihydropteroate synthase (*pvdhps*) have been determined, and their association with the clinical response to sulfadoxine-pyrimethamine (SP) has been confirmed [7-10]. The sequences which result in amino acid changes of these genes vary depending on the geographic area, and an increasing number of specific mutations results in higher levels of resistance. Chloroquine resistance is associated with single nucleotide polymorphisms (SNPs) in *pvmdr1* at codon (c) 976 (Y976F) [11] and *pvcrt-o* at c10 (K10 insertion). Also, SNPs at c57 (F57L), c58 (S58R), c61 (T61M), and c117 (S117N) in *pvdhfr* are associated with pyrimethamine resistance [12,13], while sulfadoxine resistance is associated with SNPs at c382 (S382A/C), c383 (383G), and c553 (A553G) [12]. This study aimed to fill some of the gaps in our knowledge of susceptibility to commonly used an-

ti-malarials, and hence, the prevalence of genetic polymorphisms in *P. vivax* drug resistance-associated genes, including *pvmdr1*, *pvcrt-o*, *pvdhfr*, and *pvdhps*, were evaluated using isolates from central China.

Based on the completed wild-type sequences, including nucleotides, the amino acid sequences and SNPs reported in the target genes were analyzed; GenBank accession nos. AY571984 for *pvmdr1* [14], AF314649 for *pvcrt-o* [15], X98123 for *pvdhfr* [8], and AY186730 for *pvdhps* [10]. According to the reported SNP results, the target sequences of each gene were selected for PCR amplification and sequencing, covering the resistance-conferring mutations of each gene. Samples, determined to be positive for *P. vivax* by microscopy, were collected from local hospitals and Centers for Disease Control and Prevention (CDC) in central China from 2005 to 2008. Ethical approval was obtained from the ethical review committees at the National Institute of Parasitic Diseases, Chinese CDC, and the Walter Reed Army Institute of Research, USA. Filter papers with whole blood deposits were used for DNA elution by methods described previously [16]. To avoid cross-contamination, punch was cleaned in 70% ethanol and then punched a clean filter paper 3 times before cutting a new sample. During the process, measures were taken to prevent cross-contamination.

Amplifications of target gene fragments were performed by PCR using 2 µl of 10×PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2.5 mM MgCl$_2$, 0.2 mM of each dNTP, 0.25 µM of each primer, 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California, USA), and 1 µl of genomic DNA or the amplicon from the first PCR. Amplifications were carried out in a G-Storm GSI thermal cycler (Gene Technologies Ltd, Somerton, England) in a final reaction volume of 20 µl. The cycle conditions were an initial denaturation at

94℃ for 10 min, followed by 35 cycles of denaturation at 94℃ for 50 sec, annealing at primer dependent temperature for 1 min, and extension at 72℃ for 1-1.5 min. The gene-specific primers for amplifications were used as described previously (Table 1). Among them, primers set F1/R1 were used for the first-round PCR to amplify the *pvdhps* gene, and F2/R2 were used for the second-round PCR. The purified PCR products were sequenced with the sequencing primer using the ABI PRISM 310 Genetic Analyzer and a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The deduced amino acid sequences were aligned and analyzed with Lasergene® software (DNASTAR, Madison, Wisconsin, USA). Amino acid sequences were compared with the reference wild-type sequences, where insertions and deletions were verified manually.

The *pvmdr1* has an open-reading frame (ORF) of 4,392 base pairs (bp) encoding a protein of 1,464 amino acids (aa). *Pvcrt-o* coding sequence extends across 14 exons, which range from 45 to 266 bp, each of the exon contains part of the ORF. The whole *pvdhfr-ts* gene was composed of an exon with 1,872 bp (623 aa) and a short tandem repeat region (262-309 bp). The DHFR and TS domains of the gene with a linkage sequence of 100 aa encode 237 and 286 aa residues, respectively. The *pvdhps* gene consists of 2,591 bp, including the ORF sequence 2,196 bp (731 aa) which includes 3 exons, nucleotides 1-127, 374-2326, and 2,476-2,591 bp, respectively. The reported codon mutations of each gene were indicated, and resistance-conferring mutations were marked with red color (Fig. 1).

Based on the reported results, fragments of *pvmdr1* (expected amplicon sizes are 604 bp), *pvcrt-o* (1,186 bp), *pvdhfr* (716 bp), and *pvdhps* (1,301 bp) were selected for amplification (Fig. 1). A total of 26 *P. vivax* single infection samples were selected for this study, including 8 samples collected in Bengbu, Anhui

Table 1. Sequence and features of primers used in this study

Gene	Primer sequence (5′→3′)	Position (bp)[a]	Use	Amplicon size (bp)	Annealing temp. (°C)	Reference
Pvmdr1	F: GGATAGTCATGCCCCAGGATTG	2751--2772	PCR/Seq	604	62	[9]
	R: CATCAACTTCCCGGCGTAGC	3335--3354	PCR			
Pvcrt-o	F: AAGAGCCGTCTAGCCATCC	–99--–81	PCR/Seq	1,186	61	[35]
	R: AGTTTCCCTCTACACCCG	1069--1086	PCR			
Pvdhfr	F: ATGGAGGACCTTTCAGATGTATT	1--23	PCR/Seq	716	58	[11]
	R:CCACCTTGCTGTAAACCAAAAAGTCCAGAG	686--715	PCR			
Pvdhps	F1:AGGAAGCCATTCGCTCAAC	1121--1139	PCR	1,700	56	[34]
	R1:GGAACGCTGCAAACAACAC	+211--+229	PCR			
	F2:GGTTTATTTGTCGATCCTGTG	1300--1320	PCR/Seq	1,301	56	[14]
	R2:GAGATTACCCTAAGGTTGATGTATC	2576--+9	PCR/Seq			

[a]The position of the primer is given according to the nucleotide sequence of the target gene. Primers anchored outside the open reading frame were marked as–(extended at 5′ end) and + (3′ end). Seq, sequencing; F, forward primer; R, reverse primer.
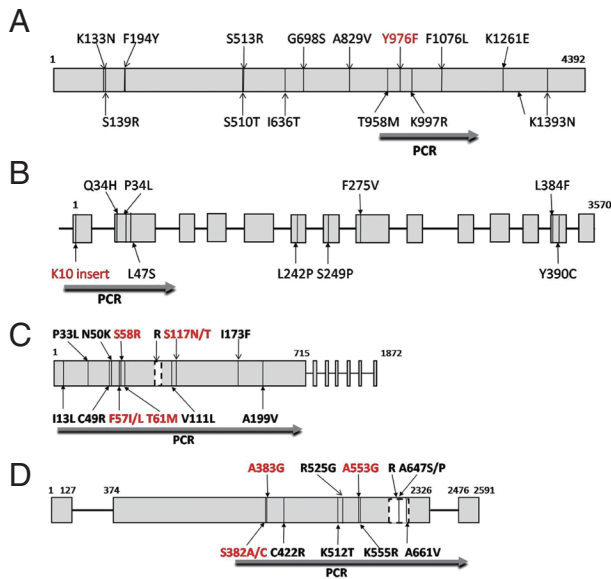
Fig. 1. Schematic diagrams of amino acid point mutation positions and sequencing regions of *pvmdr1* (A), *pvcrt-o* (B), *pvdhfr* (C), and *pvdhps* (D) genes. Previously reported point mutations are indicated, and resistance-conferring mutations are marked in red. B and D; boxes indicate exons, and lines indicate introns. R; repeat region GGDN/TSGGDN/THGGDN (C) and GEAKLTN-GEGKLTN-GEAKLTN-GEGKLTN-GEAKLTN-GEGKLTN-GDAK-LTN-GDSKLTN-GEAKLTN (D).

Table 2. Prevalence of single nucleotide polymorphisms and tandem repeat genotypes in *pvmdr1, pvcrt-o, pvdhfr,* and *pvdhps* genes in central China

Genotype in each drug resistant gene	No. of isolates sequenced/no. of total isolate selected (%)
Pvmdr1	
Wild-type Y976 codon	26/26 (100)
Mutant L1076 codon	26/26 (100)
Pvcrt-o	
Wild-type without K10 insert	22/22 (100)
Pvdhfr	
Wild-type genotype (IPCNFSTVSIA)	8/26 (30.8)
Mutant N117 codon	18/26 (69.2)
Mutant tandem repeat[a]	20/26 (76.9)
Pvdhps	
Wild-type genotype (SACKAVA)	23/23 (100)
Mutant tandem repeat[b]	23/23 (100)

[a]Including 2 types; deletion of 292-309 base pairs (65.4%, 17/26) and mutation at codon 99 (11.5%, 3/26).
[b]Including 2 types with the sequences of GEAKLTN-GEGKLTN-GEAK-LTN- GEGKLTN-GDAKLTN-GDSKLTN-GDSKLT-GEAKLTN (4.3%, 1/23) and GEAKLTN-GEGKLTN-GEGKLTN-GEAKLTN-GEGKLTN- GDAKLTN-GDSKLTN-GEAKLTN (95.7%, 22/23).

Province during 2005-2006, which had shown lower susceptibility to chloroquine in a previous in vitro study [4], as well as 18 further samples collected in Suining, Jiangsu Province during 2008. The history of anti-malarial drugs used in these 2 sites was similar, and Suining is located in the border of the 2 provinces. The *pvmdr1* gene was successfully sequenced in all 26 isolates.

Compared with Salvador I as the reference wild-type, mutant alleles at position 1076 (F1076L) were present in all isolates, wild-type c976 (Y976) was observed in all isolates, and no other non-synonymous or synonymous mutations were found (Table 2). The *pvcrt-o* gene was successfully sequenced in 84.6% (22/26) of the selected isolates, including 8 samples collected in 2005-2006 and 15 samples collected in 2008. Of the target fragment of the *pvcrt-o* gene that was sequenced, only wild-type was observed, and the K10 insertion (AAG) did not exist in any of the isolates tested (Table 2). As *pvmdr1*, the *pvdhfr* gene was also successfully sequenced and analyzed from all 26 selected isolates. Compared with the wild-type sequence, non-synonymous mutations were detected only at residues 99 and 117. The point mutation at c99 was localized in the short tandem repeat region. The central tandem repeat region be-

tween amino acid positions 88 and 105 of the *pvdhfr* gene also exhibited size polymorphism in a number of 18 bp repeats.

A total of 23.1% of the samples showed a pattern in the repeat region similar to that in the wild-type. The remaining samples identified as having deletion mutations and previously reported H99S mutations accounted for 65.4% and 11.5%, respectively. The deletion genotype within the *pvdhfr* tandem repeat region had the deletion at position 98-103 (THGGDN). The *pvdhfr* mutant allele at c117 (S117N) was observed in 18 isolates (69.2%) (Table 2). The *pvdhps* gene was successfully amplified and sequenced from 23 parasite isolates, including 6 samples collected in 2005-2006 and 17 samples collected in 2008. In contrast to the *pvdhfr* gene, no SNP was present in *pvdhps* except for the tandem repeat variation (spanning 603-665 aa). Length polymorphism of the sequence was observed for a variable number of tandem repeat unit G(E/D)(A/G/S) KLTN, and the samples were identified as 2 kinds of mutation types. The 2 repeat haplotypes were reported previously [17]. The most common haplotype represented 95.7% (22/23) of the analyzed samples and appeared to be different from the reference wild-type strain by only 1 mutational step. Another haplotype was only present in 1 sample, which was different to the wild-type in the tandem repeat unit and number (Table 2). The sample with the rare type of tandem repeat in the *dhps* gene was identified as having the wild-type *dhfr* tandem repeat

region. No relationship was observed between the mutations in the sequenced genes and the period of isolate collection.

As the most frequent and widely distributed cause of recurring malaria, *P. vivax* infection represents a considerable part of malarial disease as well as an economic burden in China, especially in the central region. In China, the action plan for malaria elimination was initiated by the Chinese government in 2010; however, the predominant malaria parasite *P. vivax* is among the biggest challenges facing the elimination programs. Several possible factors co-contributed to the reemergence of the disease, including emergence of drug resistance and global warming, and hence, it is important to increase research efforts for understanding the epidemiology of *P. vivax* in this area.

According to the anti-malarial drug policy of China, the first-line therapies for vivax malaria treatment are currently chloroquine plus primaquine during the past 60 years. Clinical resistance to pyrimethamine, used for malaria prophylaxis, was first reported in temperate-zone provinces in the late 1970s [18,19], though recent studies suggest that this family of drugs may find a role in the future treatment of *P. vivax* malaria in certain regions [20,21]. With the emergence of CQR *P. vivax* strains in many malarious regions, including the report of treatment failure by chloroquine in 4 cases of *P. vivax* malaria in Yunnan Province [22], it became a much higher priority to monitor drug resistance and develop new drugs for future treatment of *P. vivax* malaria. However, there has been no conclusive evidence showing the emergence and spread of CQR *P. vivax* in central China until now.

In a previous study, we investigated anti-malarial susceptibility of *P. vivax* in this temperate-zone by in vitro testing [4], and a variety of anti-malarial drugs with potential clinical use in China were tested. The results indicated that for *P. vivax* isolates from this area, the IC_{50}s of chloroquine were lower than those of isolates from South Korea and Thailand. Reduced susceptibility to pyrimethamine was also observed, compared to isolates from Thailand, but a greater sensitivity was observed in isolates from South Korea. Four patients from the same area were enrolled in the study, each of which had parasites with higher in vitro IC_{50}s, suggesting the presence of clinical resistance to chloroquine in this area. The molecular data for *pvmdr1*, *pvcrt-o*, *pvdhfr*, and *pvdhps* in this study could provide useful information about drug resistance in *P. vivax* isolates from central China.

Although the mechanisms of CQR in *P. vivax* are not very clear, 2 main transporters have been studied with regard to

CQR in *P. vivax*, including *pvcrt-o* and *pvmdr1*, indicating that the amino acid polymorphisms were associated with chloroquine resistance [9,10]. In our study, *pvmdr1* Y976F mutation and K10 insertion in the *pvcrt-o* gene were not found in all of the isolates tested, including those which had shown higher chloroquine IC_{50} values through an in vitro drug sensitivity study. Thus, it is indicated that CQR may not yet be prevalent in central China as had been previously thought. The point mutation *pvmdr1* F1076L, which has been suggested to be a neutral allele [23], existed in all isolates tested.

The resistance mechanism of *P. vivax* to antifolates has been proposed to be similar to that of *P. falciparum*, which has been linked to mutations at homologous positions in *pvdhfr* and *pvdhps* [11-14]. Results from in vitro drug assays and clinical assessments are generally agreeable with this assumption [12, 24-27], suggesting that molecular genotyping data for *pvdhfr* and *pvdhps* could provide useful information about SP resistance in *P. vivax*. Our study showed that mutant *pvdhfr* genotypes were present at relatively high levels, but the resistance-conferring mutations only occurred at a single c117N. Two mutant genotypes at residue 117 (S117N and S117T) have been reported in areas with extensive SP drug treatment [10,28] and only S117N was observed in this study. The S117N mutation has been hypothesized to represent the first step in the drug-resistance selection process in the parasite [29]. In contrast, S117T has been associated with highly mutated *pvdhfr*, which may be a key mutation for subsequent acquisition of additional mutations and development of high resistance to SP [11,29]. Consistent with these, highly mutated *pvdhfr* (double, triple, or quadruple mutations) was not observed in this study. Similar to a previous report, no resistance-conferring mutations were found in *pvdhps* gene among all of the isolates examined in this study. It has been proposed that the development of resistance mutations in *dhfr* and *dhps* of *P. vivax* is asynchronous, and mutations in *dhfr* appear to be selected before those in *dhps* [25,30]. The present study showed that resistance-conferring mutations were found in *pvdhfr* but not in *pvdhps* and thus further validated this hypothesis. The results suggest that the *P. vivax* parasites in central China may be still relatively susceptibility to SP.

A tandem repeat sequence is one of the unique features in the *pvdhfr*, which is absent in *P. falciparum* and *P. chabaudi*. The isolates examined in this study were typically separated into 2 types, deletion (nucleotides 292-309) and mutation H99S in *pvdhfr*, with the exception of 6 wild-type isolates. Both types of

the tandem repetitive sequence did not appear to be clearly associated with a specific genetic profile, and no relationship was found between the type of c117 and the repeat. A similar repeat motif has also been described in *pvdhps* gene [31,32], which has been predicted not to directly bind sulfa drugs, and therefore is unlikely to be involved in resistance to sulfa drugs in *P. vivax*. No wild-type of the tandem repeat sequence was determined in the *pvdhps* gene in this study. They were typically separated in 2 types also, although 1 type (95.7%) was mainly observed. Both of these tandem repeat sequences showed size polymorphisms, which may indicate a lack of SP-selective sweep in these parasite populations, and warrants further studies of the parasite resistance to antifolate drugs.

Resistance-conferring mutations in *pvmdr1*, *pvcrt-o*, and *pvdhps* genes were not discovered, but a single point mutation in *pvdhfr* gene was common to both studies. Comparing the results with isolates from South-East Asia, temperate *P. vivax* strains show much lower polymorphisms in these drug-resistance genes, consistent with the drugs pressure in these areas [28,33]. Although the number of samples used in this study was limited, isolates which had shown a likelihood of drugs resistance were selected, and the results were shown to be comparable to other studies in central China [17,25,34]. Analysis of the SNPs of drug-resistance genes has been proven to be useful and important in monitoring drug resistance in malaria endemic countries and should be investigated thoroughly.

ACKNOWLEDGMENTS

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