Amplification of pfmdr1, pfcrt, pvmdr1, and K13 Propeller Polymorphisms Associated with Plasmodium falciparum and Plasmodium vivax Isolates from the China-Myanmar Border

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Malaria in the China-Myanmar border region is still severe; local transmission of both falciparum and vivax malaria persists, and there is a risk of geographically expanding antimalarial resistance. In this research, the pfmdr1, pfcrt, pvmdr1, and K13-propeller genotypes were determined in 26 Plasmodium falciparum and 64 Plasmodium vivax isolates from Yingsjiang county of Yunnan province. The pfmdr1 (11.5%), pfcrt (34.6%), and pvmdr1 (3.1%) mutations were prevalent at the China-Myanmar border. The indigenous samples exhibited prevalences of 14.3%, 28.6%, and 14.3% for pfmdr1, respectively, whereas the samples from Myanmar showed prevalences of 10.5%, 21.1%, and 5.3%, respectively. The most prevalent genotypes of pfmdr1 and pfcrt were Y437H and M74I, respectively. No pvmdr1 mutation occurred in the indigenous samples but was observed in two cases coming from Myanmar. In addition, we are the first to report on 10 patients (38.5%) with K13 mutations in the China-Myanmar border. The F446I allele is predominant (19.2%), and its prevalence was 28.6% in the indigenous samples of Yingjiang county and 15.8% in samples from Myanmar. The present data might be helpful for enrichment of the molecular surveillance of antimalarial resistance and useful for developing and updating guidance for the use of antimalarials in this region.
polymorphisms including surveillance of drug-resistant The results might provide basic evidence for further molecular resistance in the border counties of China remains unknown. Therefore, there is an urgent need to monitor drug resistance of vivax malaria in this area.

Several genetic polymorphisms can provide reliable data about the prevalence of drug resistance related P. falciparum and P. vivax. The P. falciparum chloroquine resistance transporter gene (pfcrt) T76 mutation and multidrug resistance 1 gene (pfmdr1) Y86 mutation have been linked to chloroquine and amodiaquine resistance (13, 14). Similarly, the P. vivax multidrug resistance 1 gene (pvmdr1) F976 mutation, which was shown to be associated with reduced susceptibility to chloroquine, was selected to evaluate the resistance of P. vivax (15). As for artemisinin resistance, the slowly clearing infections were strongly associated with single point mutations in the "propeller" region of the P. falciparum kelch protein gene on chromosome 13 (kelch13) (16). Recently, the WHO stated that artemisinin resistance should be suspected when ≥5% of patients carry K13 resistance-associated mutations (17).

Here we report an assessment of antimalarial resistance marker polymorphisms including pfmdr1, pfcrt, pvmdr1, and K13 propeller in samples collected from the China-Myanmar border region. The results might provide basic evidence for further molecular surveillance of drug-resistant P. falciparum and P. vivax strains in this region.

MATERIALS AND METHODS

Study site. The study was conducted in Yingjiang county, one of the 18 counties at the China-Myanmar border, in western Yunnan province. It has a long borderline of 214.6 km with the Kachin state of Myanmar, which is a tier II area according to the Global Plan for Artemisinin Resistance Containment (GPARC) by the WHO (17). The population of Yingjiang county is 307,960, and cross-border trade, logging, quarry, and plantation activities are frequent. In 2013, there were 72 malaria cases, including 18 indigenous cases in this county, which accounted for 21.2% of the total indigenous cases in China.

Sample collection. The blood samples at enrollment from confirmed malaria cases were collected from January 2013 to July 2014 in Yingjiang county. Approximately 200 μl of finger-prick blood was spotted on a piece of Whatman filter paper (3MM) and air dried. The samples were labeled with study numbers, names, and dates and stored at −20°C until DNA extraction.

Preparation of DNA template from blood samples. Parasite genomic DNA from all blood spot samples collected in microcentrifuge tubes was extracted by use of a QIAamp DNA blood kit (Qiagen, Valencia, CA), following the dried blood spot protocol provided in the kit. The known polymorphisms pfmdr1 and pfcrt were assessed. Also, we investigated the mutation of the PF3D7_1343700 kelch propeller domain (PF13_0238, also called K13 propeller), a molecular marker of artemisinin resistance. Sequences were evaluated using nested PCR followed by restriction fragment length polymorphism (RFLP) analysis, as described previously (14, 18). The pvmdr1 single nucleotide polymorphisms (SNPs) at 976 were detected using a DNA template mismatch primer method (19). Polymorphisms were analyzed by Shanghai DNA Biotechnologies Co., Ltd. (Shanghai, China). Sequences were analyzed by the BLAST program (http://blast.ncbi.nlm.nih.gov/). Multiple nucleotide sequence alignments and analysis were performed using the BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/BioEdit/bioted.html).

Data analysis. Data were analyzed using Microsoft Excel 2003 and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The Fisher exact test was used to assess the differences in the gene polymorphisms between indigenous cases and cases from Myanmar. The P values were calculated, and results were considered statistically significant when P was <0.05.

Ethical considerations. The study was reviewed and approved by the ethical committee of the Chinese Centre for Disease Control and Prevention (China CDC).

RESULTS

Study samples. A total of 90 malaria cases were included in this study: 64 P. vivax and 26 P. falciparum. The P. vivax cases were composed of 18 indigenous cases and 46 cases from Myanmar, while the P. falciparum cases were composed of 7 indigenous cases and 19 cases from Myanmar (Fig. 1).

pfmdr1. The pfmdr1 gene was sequenced successfully in 3 isolates of all P. falciparum samples (11.5%, 3/26) that covered codons 86 and 184. Among all of the mutational types, no N1042D, S1034C, and D1246Y mutations were found. Mutations at codon N86Y (11.5%, 3/26) were common; Y86Y184 was the most prevalent (66.7%, 2/3) of all haplotypes (Tables 1 and 2). Further, one patient harboring the N86Y mutation was found in Nabang, China; the other two patients with this mutation were from Myanmar (P = 1.0000) (Table 1).

pfcrt. Sequencing of the pfcrt gene was successful in 9 isolates (34.6%, 9/26) that covered codons 74, 75, and 76. Of all three mutated codons, K76T was the most prevalent (23.1%, 6/26) (Table 1). Two patients with the mutated genotype K76T were ob-

### TABLE 1 Selection of P. falciparum and P. vivax polymorphisms

<table>
<thead>
<tr>
<th>SNP</th>
<th>In China No.</th>
<th>Total no.</th>
<th>%</th>
<th>From Myanmar No.</th>
<th>Total no.</th>
<th>%</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfmdr1 N86Y (n = 3)</td>
<td>1</td>
<td>7</td>
<td>14.3</td>
<td>2</td>
<td>19</td>
<td>10.5</td>
<td>1.0000</td>
</tr>
<tr>
<td>pfmdr1 Y184F (n = 1)</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>19</td>
<td>5.3</td>
<td>NS</td>
</tr>
<tr>
<td>pfcrt M74I (n = 2)</td>
<td>1</td>
<td>7</td>
<td>14.3</td>
<td>1</td>
<td>19</td>
<td>5.3</td>
<td>0.4738</td>
</tr>
<tr>
<td>pfcrt N75E (n = 2)</td>
<td>0</td>
<td>7</td>
<td>0</td>
<td>2</td>
<td>19</td>
<td>10.5</td>
<td>NS</td>
</tr>
<tr>
<td>pfcrt K76T (n = 6)</td>
<td>2</td>
<td>7</td>
<td>28.6</td>
<td>4</td>
<td>19</td>
<td>21.1</td>
<td>1.0000</td>
</tr>
<tr>
<td>pvmdr1 Y976F (n = 2)</td>
<td>0</td>
<td>18</td>
<td>0</td>
<td>2</td>
<td>46</td>
<td>4.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

*NS, not significant.
erved in Nabang, China; the other four patients with K76T were from Myanmar (P = 5.0000). Four different pf geneotypes were found, among which M74N75T76 was the most common (55.6%, 5/9) (Table 2). One patient harboring I74N75K76 was detected in Tongbigan, China, and the other one was returning from Myanmar (P = 0.4738). Another two haplotypes, including M74E75K76 and M25E75T76, were all in patients from Myanmar.

**pfmdr1.** Regarding pfmdr1, 64 P. vivax samples were assayed. Only 2 patients (3.1%, 2/64) harbored the Y976F allele, and the patients were returning from Myanmar (Table 2).

K13 propeller. To investigate the K13 propeller polymorphism, all 26 P. falciparum samples were assayed and sequenced. Ten patients (38.5%, 10/26) with five different point mutations, including two reported mutation sites and three unreported polymorphism, all 26

**DISCUSSION**

Yunnan province is located in southern China, and malaria is one of the most important public health problems (20). The incidence of malaria transmission is more severe in the China-Myanmar border counties. The total number of malaria cases in Yunnan province according to the annual reported data was 576 in 2013, including 460 P. vivax cases, 106 P. falciparum cases, and 10 cases of other species. Most of them (n = 463, 80.4%) were observed in the 18 China-Myanmar border counties. The situation in Yingjiang county was the most severe, with an incidence rate of 2.3 per 10,000 people, and 18 local transmission cases were reported, representing 21.2% of the total local cases in China, with 54 other cases imported from Myanmar. Based on these facts, it was selected as the study site.

Resistance to antimalarial drugs has been a long-standing problem in the GMS. MDR P. falciparum strains that have emerged in the Thai-Cambodian border region, as well as the emerging resistance to chloroquine (CQ), to sulfadoxine-pyrimethamine (SP), and then to mefloquine (MQ), are gradually spreading in the tropical world (21). Malaria in the China-Myanmar border region is a topic of regional and national public health concern. The development and spread of MDR P. falciparum have led to the adoption of artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated P. falciparum malaria in this region to improve treatment outcomes. However, widespread artemisinin resistance has been observed in the GMS (4, 22); this poses a great threat of resistant parasites being brought into China with the migrant population, immediately affecting therapy efficacy. In the China-Myanmar border area, artemisinins have been used for more than 30 years, mostly as monotherapies prior to 2005. Earlier *in vitro* assays had already detected a trend of declining sensitivity to artemisinins in the border area of Yunnan province (23, 24). Therefore, an understanding of whether artemisinin-resistant parasites have spread to the neighboring regions or emerged elsewhere in this area is essential for coordinating containment efforts.

In addition, P. vivax malaria persists in areas of Yunnan province along the China-Myanmar border (25), and CQ was adopted more than 50 years ago in China as the first-line drug used for treatment of the blood-stage *P. vivax* infection. Although high-level resistance of *P. vivax* to CQ and SP was reported more than a decade ago in the north of Myanmar (26, 27), the resistance in the border counties of China remains unknown.

The malaria parasite encodes many transporters, and some of them such as *pfmdr1* and *pfcrt* have been strongly connected with antimalarial drug resistance (28). The *pfmdr1* genotype is correlated with resistance of *P. falciparum* to CQ, MQ, and artemisinins, whereas knockdown of *pfmdr1* expression leads to increased susceptibility (29). The N86Y *pfmdr1* mutation that confers CQ resistance is also associated with decreased sensitivity to artemisin-
Resistance to common antimalarial drugs has been reported for *P. vivax* in the GMS, including Myanmar and Vietnam, and also in Indonesia (38–40). A trend for a gradual decline in the *in vitro* sensitivity of this parasite to CQ had also been reported during 2005 to 2008 around the China-Myanmar border and in central China (41, 42). Our findings revealed that the presence of the *pfmdr1* Y976F mutation in Yingjiang county in China is consistent with previous reports of declining sensitivity to CQ; this was also observed in Myanmar and Xishuangbanna (Yunnan province), which exhibit high frequencies of the *pfmdr1* Y976F allele (43, 44). The long history of CQ use and the frequent population movement across the borders may contribute to the CQ-resistant *P. vivax* strains detected in Yingjiang county. Close surveillance at sentinel sites in this region should continue so that the emergence and spread of *P. vivax* resistance can be carefully monitored.

Artemisinin and its derivatives have been used for falciparum malaria treatment in China since the late 1970s (45). *In vitro* assays showed that the susceptibility of *P. falciparum* to artemisinins was declining in China, but no evidence of the artemisinin resistance has been detected (46). In our study, five nonsynonymous mutations were found, and three of them were not reported previously. Furthermore, our study showed that F446I was the predominant allele (19.2%, 5/26), and two cases with this mutation were found in the port city of Nabang, China. Another mutation, K13 propeller A676D, was also observed in China, China (Fig. 2). However, the C580Y allele, which was widely found in Cambodia (16), was not found in this study. Nevertheless, our results indicate that the mutated K13 propeller gene alleles exist in the China-Myanmar border area, and their presence should raise concerns regarding the risks of emerging artemisinin resistance in the GMS. We recommend further clinical trials associated with K13 propeller mutations, which might be useful for identifying additional genetic loci involved in monitoring the threat of artemisinin resistance.

The prevalence of the K13 propeller polymorphism detected in Yingjiang county indicates that ACTs should be used in the China-Myanmar border area, and rational use of antimalarials against *P. falciparum* strains imported from Southeast Asia should be adopted. In addition, routine monitoring and surveillance, as recommended by the WHO Global Plan for Artemisinin Resistance Containment, should continuously be strengthened. Additional clinical investigations to complement sentinel surveillance, in-
including either analysis of the drug markers or risk factors or new approaches to monitor resistance, are required.

In conclusion, the present data might be helpful for enrichment of molecular surveillance of antimalarial resistance and for developing and updating guidance for the use of antimalarials in the region.

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REFERENCES


