Polymorphisms of Molecular Markers of Antimalarial Drug Resistance and Relationship with Artesunate-Mefloquine Combination Therapy in Patients with Uncomplicated *Plasmodium falciparum* Malaria in Thailand

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Abstract. The aim of this study was to investigate the association between genetic polymorphisms of *Plasmodium falciparum* chloroquine resistance transporter (*pfCRT*), *P. falciparum* multidrug resistance 1 (*pfMDR1*), and *P. falciparum* ATPase (*pfATP6*) and clinical outcome after a three-day mefloquine-artesunate combination therapy in 134 patients with uncomplicated *Plasmodium falciparum* malaria in an area with multidrug resistance along the Thailand-Myanmar border. Analysis of gene mutation and amplification were performed by nested real-time polymerase chain reaction and SYBR Green I real-time polymerase chain reaction, respectively. The mutation for *pfCRT* (codons 76, 220, 271, 326, 356, and 371) was found in all isolates (100%), whereas no mutation of *pfMDR1* (codon 86) and *pfATP6* (codons 37, 693, 769, 898) was found. The *PfMDR1* copy number was significantly higher in isolates with recrudescence (median number = 2.44) compared with a sensitive response (median number = 1.44). The gene copy number was also found to be significantly higher in paired isolates collected before treatment and at the time of recrudescence. All isolates carried one *pfATP6* gene copy.

INTRODUCTION

Malaria remains an important infectious disease in Thailand despite an intensive control program. The emergence and spread of multidrug-resistant *Plasmodium falciparum* is the key factor that contributes to difficulty in the control of this disease. The antimalarial drug chloroquine had long been the drug of choice for the treatment of acute uncomplicated *P. falciparum* malaria in Thailand before the establishment of chloroquine resistance in the late 1950s. The development and spreading of resistance of *P. falciparum* to the next first-line treatment with sulfadoxine-pyrimethamine and mefloquine monotherapy further aggravated the situation. Currently, the Malaria Control Program of Thailand recommends the artemisinin-based combination therapy (ACT), a three-day artemisinate-mefloquine combination for uncomplicated *P. falciparum* malaria throughout the country. Nevertheless, recent reports of high failure rates associated with this treatment regimen along the Thailand-Cambodia border suggest the possibility of clinical resistance of *P. falciparum* to this combination.

The two genes encoding the parasite transporter proteins *P. falciparum* chloroquine resistance transporter (*pfCRT*) and *P. falciparum* multidrug resistance 1 (*pfMDR1*), which are located in membranes of digestive vacuoles, are proposed to play a role as key contributors of resistance of *P. falciparum* to antimalarial drugs. Several distinct sequence diversity of point mutations among different geographic areas were detected in individual chloroquine-resistant lines/isolates, particularly the *pfCRT* K76T, which showed a strong association with chloroquine resistance. For *pfMDR1*, N86Y mutation was found to be associated with chloroquine resistance *in vitro* in field isolates or laboratory lines. Amplification of *pfMDR1* has also been predicted as a predictor of treatment failure after ACT. Apart from *pfCRT* and *pfMDR1*, the sarco/endoplasmic reticulum Ca²⁺-ATPase ortholog of *P. falciparum* (PFATP6) was suggested to be involved in the mechanism of action and resistance of the parasite to artemisinins. L263E, S769N, E431K and A623E single nucleotide polymorphism (SNPs) in PFATP6 was proposed to be associated with reduced parasite sensitivity to artemisinins.

The aim of this study was to investigate the association between polymorphisms (gene mutation and amplification) of *pfCRT*, *pfMDR1*, and *pfATP6* and clinical outcome after a three-day artesunate-mefloquine combination therapy in patients with uncomplicated *P. falciparum* malaria in an area with multidrug resistance *P. falciparum* along the Thailand-Myanmar border.

MATERIALS AND METHODS

Study site and patients. The study was a part of a clinical trial to monitor the clinical efficacy of the three-day artesunate-mefloquine combination therapy conducted during March 2009–December 2009 at Mae Tao Clinic for migrant workers located along the Thailand-Myanmar border in Tak Province. The study protocol was approved by the Ethics Committee of the Ministry of Public Health of Thailand. A total of 134 Burmese patients greater 15 years of age who came to the clinic with typical symptoms of malaria and had a blood smear positive for *P. falciparum* and treated to the clinic during the three day course of treatment or until signs and symptoms of malaria disappeared and were requested to return for follow-up on days 7, 14, 21, 28, and 42 or at any time if fever or symptoms suggestive of malaria developed. Treatment outcome was categorized according to the 2003 World Health Organization definition, i.e., adequate
clinical and parasitologic response, early treatment failure, late clinical failure, and late parasitologic failure. 15

Sample collection and DNA extraction. Blood samples were collected on 3MM filter paper (Whatman, Springfield Mill, United Kingdom) before treatment and at the time of treatment failure (parasite reappearance). Parasite DNA was extracted from dried blood spots by using the Chelex resin modified technique. 16 The extracted DNA was used for analysis of pfcr, pfmdr1, and pfatp6 mutations and determination of pfmdr1 and pfatp6 copy number. Reappearance of parasitemia caused by recrudescence and reinfection was differentiated by nested polymerase chain reaction (PCR) amplification of the three polymorphic genes for merozoite surface protein 1, merozoite surface protein 2, and glutamate-rich protein from paired dried blood spot samples collected pre-treatment and post-treatment. 17

Detection of pfcr, pfmdr1, and pfatp6 polymorphisms by PCR–restriction fragment length polymorphism. Polymerase chain reaction–restriction fragment length polymorphism was used to detect mutations of pfcr (codons 76, 220, 271, 326, 356, and 371), 18 pfmdr1 (codon 86), 19 and pfatp6 (codons 37, 693, 769, and 898). 20 The PCR was performed in a total volume of 25 μL with 0.4 μM of each primer, 2.5 mM MgCl2, 100 μM deoxynucleotides (dNTPs), 1× PCR buffer (100 mM KCl, 20 mM Tris-HCl, pH 8.0), 0.5 units of Taq DNA polymerase (Fermentas, Vilnius, Lithuania), and 2 μL of genomic DNA. DNA of G112 (chloroquine sensitive) and K1 (chloroquine resistance) P. falciparum served as control clones.

Quantification of pfmdr1 and pfatp6 gene copy number by real-time PCR. The Pfatp6 and pfmdr1 gene copy numbers were determined by SYBR Green I real-time PCR (iCycler; Bio-Rad, Hercules, CA) and using the default thermocycler program: 10 minutes of pre-incubation at 95°C, followed by 40 cycles for 15 seconds at 95°C and 1 minute at 60°C. The oligonucleotide primers used were those designed by Ferreira and others with modifications. 21 Individual real-time PCR was carried out in a 25-μL reaction volume in a 96-well plate containing 1× PCR buffer (10 μM MgCl2, 200 μM dNTPs, 1 μM each of sense and antisense primers, and 12.5 μL of Platinum™ PCR SuperMix (Invitrogen, Carlsbad, CA).

The 2-ΔΔCt method of relative quantification was adapted to estimate the copy number of P. falciparum genes. The genomic DNA extracted from P. falciparum 3D7 clone, which harbors a single copy of each gene, was used as a calibrator, and Pf-β-actin 1 served as the housekeeping gene in all experiments. Dd2 genomic DNA carrying four copies of pfmdr1 was used as a second calibrator. The threshold cycle (Ct) was determined as the increase in reporter signal that was first detected above baseline. Results were analyzed by a comparative Ct method based on the assumption that the targets (pfatp6 and pfmdr1) and reference (Pf-β-actin 1) genes were amplified with the same efficiency within an appropriate range of DNA concentrations. The ΔΔCt calculation for the relative quantification of targets was ΔΔCt = (Ct, target gene – Ct, Pf-β-actin 1) – (Ct, target gene – Ct, Pf-β-actin 1), where x represents unknown sample and y represents P. falciparum 3D7 clone. Results for each sample were expressed as an N-fold change in x target gene copies, normalized to Pf-β-actin 1 relative to the copy number of the target gene in P. falciparum 3D7 clone, according to equation amount of target = 2-ΔΔCt. A minimum of two experiments were carried out for each gene and each sample. In each experiment, each individual sample was analyzed in duplicate wells and the Ct of each well was recorded at the end of the reaction.

Data analysis. The association between treatment outcome (sensitive response, recrudescence) and mutations of pfcr, pfmdr1, or pfatp6 and copy number of pfmdr1 and pfatp6 were determined by using the chi-square test. Difference in pfmdr1 gene copy number in isolates with sensitive and recrudescence response was assessed by using the Mann-Whitney U test (unpaired samples) or Wilcoxon-ranked sign test (paired samples). All statistical tests were performed at statistical significance level of α = 0.05 (SPSS version 12.0, SPSS, Inc., Chicago, IL).

RESULTS

A total of 134 patients with P. falciparum monoinfection were enrolled in the study. Baseline demographic and clinical data have been reported. 22 On the basis of the 42-day follow-up, 89 (66.4%) patients had successful treatment (adequate clinical and parasitologic response), and 45 (33.6%) patients had reappearance of P. falciparum parasitemia (treatment failure) during 28–42 days follow-up (early treatment failure, late clinical failure, and late parasitologic failure). Among the 45 patients with treatment failure, 16 (11.9%) of 134 patients and 29 (21.6%) of 134 patients were identified as having re-infection and recrudescence, respectively.

Association between treatment response and mutations of pfcr, pfmdr1, or pfatp6. The mutation of pfcr was found in all isolates (100%), whereas no mutation of pfmdr1 and pfatp6 was observed at any amino acid codons under investigation (Table 1). There was no association between treatment outcome and mutations for all three genes. Paired samples collected before treatment and at the time of recrudescence showed the same patterns of gene polymorphisms.

Association between treatment response and copy number of pfmdr1 and pfatp6. The pfmdr1 gene copy number observed in 117 isolates ranged from 1 to 8 copies, and most isolates had only one gene copy. The distribution of pfmdr1 copy number in patients with sensitive response (87 isolates) and treatment failure (30 isolates with recrudescence and re-infection) is shown in Table 2. The pfmdr1 copy number was significantly higher in isolates with recrudescence compared with those

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Prevalence of mutations in isolates collected from malaria patients with sensitive, treatment failure, and recrudescence (before treatment and at the time of recrudescence) responses, Thailand*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mutation</strong></td>
<td><strong>Sensitive (n = 89)</strong></td>
</tr>
<tr>
<td>Pfcr K76T</td>
<td>89 (100.0)</td>
</tr>
<tr>
<td>Pfcr A220S</td>
<td>88 (98.9)</td>
</tr>
<tr>
<td>Pfcr Q271E</td>
<td>89 (100.0)</td>
</tr>
<tr>
<td>Pfcr A326S</td>
<td>89 (100.0)</td>
</tr>
<tr>
<td>Pfcr S356T</td>
<td>89 (100.0)</td>
</tr>
<tr>
<td>Pfr B371I</td>
<td>89 (100.0)</td>
</tr>
<tr>
<td>Pfmdr1 N86Y</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Pfatp6 R37K</td>
<td>ND</td>
</tr>
<tr>
<td>Pfatp6 G639D</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Pfatp6 S679N</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Pfatp6 I898L</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

* Pfcr = Plasmodium falciparum chloroquine resistance transporter; Pfmdr1 = P. falciparum multidrug resistance 1; Pfatp6 = P. falciparum ATPase; ND = not determined.
† Analysis was conducted for 28 isolates.
with sensitive responses ($P = 0.007$). Median (95% confidence interval) values of copy number in the groups with sensitive and recrudescence response were 1.44 (1.26–1.61) and 2.44 (1.51–3.38), respectively. The corresponding proportions of patients who carried $\geq 3$ gene copies were 0.13 and 0.39, respectively. Furthermore, gene copy number was also found to be significantly higher in paired isolates ($n = 18$) obtained before treatment and at the time of recrudescence ($P = 0.002$). The relationship between time of treatment follow-up and percentage of patients without recrudescence who carried 1, 2 and $\geq 3$ pfmdr1 gene copies is shown in Figure 1. A significant decrease in the rate of successful response after day 7 of treatment was observed in patients who carried isolates with $\geq 3$ gene copies. No association between pfatp6 gene copy number and treatment response was found; all isolates carried one gene.

**DISCUSSION**

Polymorphisms of pfcrf and pfmdr1 genes have been proposed as molecular markers of chloroquine resistance, and they also influence the susceptibility of *P. falciparum* to mefloquine, quinine, halofantrine, and artemisinins.$^{22–24}$ Thailand is well documented as the foci of chloroquine resistance since the 1960s.$^{1,2,25–27}$ It was suggested that the replacement of chloroquine by other antimalarial drugs (antifolates, mefloquine, and artemisinins) in past decades may revert chloroquine-resistant parasites to chloroquine-sensitive parasites.$^{28,29}$ Artesunate-mefloquine has been used for treatment of uncomplicated *P. falciparum* malaria in Thailand since 1996, but the decrease in treatment efficacy has been reported in areas along the Thailand-Myanmar and Thailand-Cambodia borders.$^{4,5,14,30}$

Our study, which was conducted 30 years after chloroquine had been withdrawn from clinical use, still showed a pfcrf mutation frequency of virtually 100%. This finding may be caused by the continued use of chloroquine as first-line treatment for *P. vivax* malaria in Thailand for almost six decades. In contrast, all isolates showed wild-type pfmdr1 genotype. Investigation of pfcrf K76T, A220S, Q271E, N326S, I356T, and R371I and pfmdr1 N86Y mutation of isolates from Thailand has showed distinct patterns and prevalence depending on the period under investigation and study areas with selection history, but the K76T change appears necessary for the resistance phenotype and is the most reliable molecular marker of chloroquine resistance among the various mutations.$^{31}$ The prevalence of pfcrf and pfmdr1 mutations was found to be 100% and 9%, respectively, in isolates obtained during 1988–1993 and 2003 from the Thailand-Cambodia border.$^{32}$ Isolates obtained from the Thailand-Myanmar border during 2002–2004 showed a pfcrf 76T mutation frequency of 99% and a pfmdr1 86Y mutation frequency of 22.2%,$^{33}$ and isolates obtained from the same area during 2010 showed a pfcrf 76T mutation frequency of 96% and a pfmdr1 86Y mutation frequency of 8%.$^{34}$

**Table 2**

<table>
<thead>
<tr>
<th>Treatment outcome, no. (%)</th>
<th>Pfmdr1 copy number</th>
<th>Sensitive† (n = 87)</th>
<th>Re-infection (n = 9)</th>
<th>Pre-treatment (n = 21)</th>
<th>Post-treatment (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64 (73.6)</td>
<td>8 (88.9)</td>
<td>12 (57.1)</td>
<td>4 (22.2)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12 (13.8)</td>
<td>1 (11.1)</td>
<td>2 (9.5)</td>
<td>2 (11.1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>7 (8.0)</td>
<td>0 (0.0)</td>
<td>1 (4.8)</td>
<td>4 (22.2)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4 (4.6)</td>
<td>0 (0.0)</td>
<td>3 (14.3)</td>
<td>5 (27.8)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (4.8)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>2 (9.5)</td>
<td>2 (11.1)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>1 (5.6)</td>
<td></td>
</tr>
</tbody>
</table>

*Pfmdr1 = P. falciparum multidrug resistance 1.
†Statistically significant difference from pre-treatment recrudescence ($P = 0.007$).
‡Statistically significant difference between pre-treatment and post-treatment recrudescence ($P = 0.007$).

**Figure 1.** Relationship between *Plasmodium falciparum* multidrug resistance 1 gene copy number (1, 2, and $\geq 3$) and time to recrudescence in patients with uncomplicated *P. falciparum* malaria after treatment with a three-day combination of artesunate-mefloquine.
The patterns and prevalence of pfCRT and pfMDR1 mutations are also markedly different among malaria-endemic areas of the world. In Saudi Arabia (Asia), pfCRT 76T and pfMDR1 86Y had prevalences of 100% and 40%, respectively.35 In Senegal (Africa), the prevalence of pfCRT 76 mutation was 81.6%.36

Gene amplification and the N86 allele of pfmdr1 have been suggested as candidate markers for resistance of P. falciparum to mefloquine, for reduced susceptibility to ACT, and for induced resistance of parasites to mefloquine-artemisinin.7,37,38 However, the present study showed no relationship between the pfmdr1 N86Y mutation and treatment efficacy of artesunate-mefloquine. Conversely, amplification of the pfmdr1 gene was found to be a significant marker of treatment failure for ACT. The increase in pfmdr1 copy number, particularly isolates carrying ≥3 copies, can reliably predict artesunate-mefloquine treatment failure.4 Significant association was found between isolates obtained from patients with successful treatment outcome and recrudescence, with gene amplification of 7% (9 of 117) and 28.2% (33 of 117), respectively. In addition, paired isolates obtained at recrudescence also showed significant increase in gene copy numbers compared with the pre-treatment isolates. This finding coincides with the observation of a significant prolongation of parasite clearance time and fever clearance time in this group of patients with recrudescence compared with patients with a sensitive response: median (95% CI) for parasite clearance time = 32.0 (20.0–48.0) versus 24.0 (14.0–32.0) hours and median (95% CI) for fever clearance time = 30.0 (22.0–42.0) versus 26.0 (18.0–36.0) hours (P < 0.005).14

Results from another study also support the link between pfmdr1 gene amplification and reduced susceptibility of parasite isolates to mefloquine, artesunate, and quinine (Prompradit and others, unpublished data). Using mefloquine monotherapy in the past might have led to selection of parasites carrying more than one pfmdr1 copy number in mefloquine-resistant isolates. Amplification of Pfmdr1 is commonly found in malaria-endemic area of Thailand, which differs from samples obtained in western Asia,39 South America,40 and western Africa,41 where almost all parasite isolates carry only one gene copy. In view of the observations of Vinayak and others,42 the Y184F polymorphism of pfmdr1 should also be further investigated.

The mechanism of action of artemisinins remains controversial and debatable. One of the proposed mechanisms is the interaction with the sarcoplasmic reticulum Ca2+-ATPase 6 (PfATP6). This enzyme, when expressed in Xenopus oocytes, was specifically inhibited by artemisinin derivatives containing an endoperoxide bridge.43 In addition, the activity of the enzyme was greatly influenced by the introduction of several gene mutations. The analysis of naturally occurring polymorphisms in PfATP6 in field isolates from French Guiana suggested that a polymorphism at codon 769 may be associated with reduced susceptibility of these isolates to artemether in vitro.13 However, other studies failed to detect codon 769 polymorphism in the field isolates.44 On the basis of results from our study, there appears to be no link between either amplification or mutation of pfATP6 and response to artesunate-mefloquine treatment. In accordance with information for isolates from Cambodia, French Guiana, and Senegal, none of the isolates observed in our study carried single nucleotide polymorphisms of pfATP6 at codon 769 and at other codons (37, 693, and 898).12 In a recent study, artemisinin resistance in western Cambodia was found not to be linked with candidate genes, i.e., mutations and/or amplification of pfmdr1, pfatp6, pfCRT, or upstream binding protein-1.46

In conclusion, amplification of pfmdr1 appears to be the candidate molecular marker of reduced susceptibility of P. falciparum isolates to mefloquine-arterisin combination therapy in a multidrug resistance area along the Thailand-Myanmar border. Results may also imply emerging resistance of P. falciparum isolates to artesunate-mefloquine combination therapy in Thailand.

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