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

Poonuch Muhamad, Papichaya Phompradit, Wannapa Sornjai, Thunyaluk Maensathian, Wanna Chaijaroenkul, Ronnatrai Rueangweerayut, and Kesara Na-Bangchang*

Thailand Center of Excellence on Drug Discovery and Development, Thammasat University, Rangsit Campus, Klong Luang District, Pathumthani, Thailand; Mae-Sot General Hospital, Mae-Sot, Tak Province, Thailand

Abstract. The aim of this study was to investigate the association between genetic polymorphisms of *Plasmodium falciparum* chloroquine resistance transporter (*pfcr*), *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 *pfcr* (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.¹ 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.² 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.³ Currently, the Malaria Control Program of Thailand recommends the artemisinin-based combination therapy (ACT), a three-day artesunate-mefloquine combination for uncomplicated *P. falciparum* malaria throughout the country.³ 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.^{4,5}

The two genes encoding the parasite transporter proteins *P. falciparum* chloroquine resistance transporter (*pfcr*) 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 *pfcr* K76T, which showed a strong association with chloroquine resistance.⁶ For *pfmdr1*, N86Y mutation was found to be associated with chloroquine resistance *in vitro* in field isolates or laboratory lines.⁷ Amplification of *pfmdr1* has also been proposed as a predictor of treatment failure after

ACT.^{8–11} Apart from *pfcr* 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.^{12,13}

The aim of this study was to investigate the association between polymorphisms (gene mutation and amplification) of *pfcr*, *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.¹⁴ 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* were included into this study. Inclusion criteria for enrollment in the study were according to the World Health Organization protocol for areas with low-to-moderate malaria transmission.¹⁵ Written informed consents were obtained from all patients before study participation.

Patients were treated with the standard regimen of a three-day combination of artesunate and mefloquine in conjunction with the gametocytocidal primaquine.¹⁴ Patients were admitted 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

*Address correspondence to Kesara Na-Bangchang, Thailand Center of Excellence on Drug Discovery and Development, Thammasat University, Rangsit Campus, 99 Moo 18 Paholyothin Road, Klongnueng, Klongluang, Pathumthani 12121, Thailand. E-mail: kesaratmu@yahoo.com

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 *pfprt*, *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 *pfprt*, *pfmdr1*, and *pfatp6* polymorphisms by PCR–restriction fragment length polymorphism. Polymerase chain reaction–restriction fragment length polymorphism was used to detect mutations of *pfprt* (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 μ L with 0.4 μ M of each primer, 2.5 mM MgCl₂, 100 μ M deoxynucleotides (dNTPs), 1 $\times$ PCR buffer (100 mM KCl, 20 mM Tris-HCl, pH 8.0), 0.5 units of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), and 2 μ L 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 *Pf*atp6 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- μ L reaction volume in a 96-well plate containing 1 $\times$ buffer (10 $\times$), 3.5 mM MgCl₂, 200 μ M dNTPs, 1 μ M each of sense and antisense primers, and 12.5 μ L of Platinum™ PCR SuperMix (Invitrogen, Carlsbad, CA).

The 2^{− $\Delta\Delta$ Ct} 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 $\Delta\Delta$ Ct calculation for the relative quantification of targets was $\Delta\Delta$ Ct = (Ct, target gene – Ct, *Pf*- β -actin1)_x – (Ct, target gene – Ct, *Pf*- β -actin1)_y, 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^{− $\Delta\Delta$ Ct}. 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 *pfprt*, *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-signed rank 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.¹⁴ 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 *pfprt*, *pfmdr1*, or *pfatp6*. The mutation of *pfprt* 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 1
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*

Mutation	Treatment outcome, no. (%)			
	Sensitive (n = 89)	Failure (n = 45)	Recrudescence (n = 29)	
			Pre-treatment	Post-treatment
<i>Pfprt</i> K76T	89 (100.0)	45 (100.0)	29 (100.0)	29 (100.0)
<i>Pfprt</i> A220S	88 (98.9)	43 (97.7)	28 (100.0)†	29 (100.0)
<i>Pfprt</i> Q271E	89 (100.0)	45 (100.0)	29 (100.0)	28 (96.6)
<i>Pfprt</i> A326S	89 (100.0)	45 (100.0)	29 (100.0)	29 (100.0)
<i>Pfprt</i> I356T	89 (100.0)	45 (100.0)	29 (100.0)	29 (100.0)
<i>Pfprt</i> R371I	89 (100.0)	45 (100.0)	29 (100.0)	29 (100.0)
<i>Pfmdr1</i> N86Y	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>Pfatp6</i> R37K	ND	0 (0.0)	0 (0.0)	0 (0.0)
<i>Pfatp6</i> G639D	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>Pfatp6</i> S769N	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
<i>Pfatp6</i> I898I	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

* *Pfprt* = *Plasmodium falciparum* chloroquine resistance transporter; *Pfmdr1* = *P. falciparum* multidrug resistance 1; *Pfatp6* = *P. falciparum* ATPase; ND = not determined.

† Analysis was conducted for 28 isolates.

TABLE 2

Prevalence of *pfmdr1* copy number in isolates collected from malaria patients with sensitive, re-infection, and recrudescence (before treatment and at the time of reappearance) responses, Thailand*

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

* *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 ($P = 0.007$).

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 ≥ 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 ≥ 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 ≥ 3 gene copies. No association between *pfatp6* gene copy number and treatment response was found; all isolates carried one gene.

DISCUSSION

Polymorphisms of *pfert* 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 *pfert* 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 *pfert* 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 *pfert* 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 *pfert* 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 *pfert* 76T mutation frequency of 96% and a *pfmdr1* 86Y mutation frequency of 8%.³⁴

Copy number	Patients without recrudescence (n, %)						
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
1 (n=84)	84 (100)	84 (100)	84 (100)	83 (98.8)	76 (90.5)	76 (90.5)	72 (85.7)
2 (n=15)	15 (100)	15 (100)	15 (100)	14 (93.3)	13 (86.6)	13 (86.6)	13 (86.6)
≥3 (n=18)	18 (100)	18 (100)	15 (83.3)	13 (72.2)	11 (61.1)	11 (61.1)	11 (61.1)

FIGURE 1. Relationship between *Plasmodium falciparum* multidrug resistance 1 gene copy number (1, 2, and ≥ 3) and time to recrudescence in patients with uncomplicated *P. falciparum* malaria after treatment with a three-day combination of artesunate-mefloquine.

The prevalence and patterns of *pfprt* and *pfmdr1* mutations are also markedly different among malaria-endemic areas of the world. In Saudi Arabia (Asia), *pfprt* 76T and *pfmdr1* 86Y had prevalences of 100% and 40%, respectively.³⁵ In Senegal (Africa), the prevalence of *pfprt* 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.⁸ 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 P 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 Ca^{2+} -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*.¹² However, other studies failed to detect codon 769 polymorphism in the field isolates.^{44,45} 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*, *pfprt*, 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-artesunate 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.

Received April 4, 2011. Accepted for publication June 13, 2011.

Acknowledgment: We would like to thank the staff of Mae Tao Clinic for their support.

Financial support: This study was supported by the Commission on Higher Education, Ministry of Education of Thailand, and the National Research University Project of Thailand, Office of Higher Education Commission. Papichaya Phompradit received a Royal Golden Jubilee Ph.D. scholarship from the Thailand Research Fund.

Authors' addresses: Poonuch Muhamad, Papichaya Phompradit, Wannapa Sornjai, Thunyaluk Maensathian, Wanna Chaijaroenkul, and Kesara Na-Bangchang, Thailand Center of Excellence on Drug Discovery and Development, Thammasat University, Rangsit Campus, 99 Moo 18 Paholyothin Road, Klongnueng, Klongluang, Pathumthani 12121, Thailand, E-mails: nurah_ab@yahoo.com, v_iv_iii_ii_i@hotmail.com, wan_napa_9@hotmail.com, kawaei_sine009@hotmail.com, wn_ap39@yahoo.com, and kesaratmu@yahoo.com. Ronnatrai Rueangweerayut, Mae-Sot General Hospital, Mae-Sot 63110, Tak Province, Thailand, E-mail: ronnatrai@yahoo.com.

REFERENCES

- Chareonviriyaphap T, Bangs MJ, Ratanatham S, 2000. Status of malaria in Thailand. *Southeast Asian J Trop Med Public Health* 31: 225–237.
- Harinasuta T, Suntharasamai P, Viravan C, 1965. Chloroquine resistant falciparum malaria in Thailand. *Lancet* 2: 660–675.
- Na-Bangchang K, Karbwang J, 2009. Current status of malaria chemotherapy and the role of pharmacology in antimalarial drug research and development. *Fundam Clin Pharmacol* 23: 387–409.
- Vijaykadge S, Rojanawatsirivej C, Cholpol S, Phoungmanee D, Nakavej A, Wongsrichanalai C, 2006. *In vivo* sensitivity monitoring of mefloquine monotherapy and artesunate-mefloquine combinations for the treatment of uncomplicated falciparum malaria in Thailand in 2003. *Trop Med Int Health* 11: 211–219.
- Noedl H, Se Y, Schaefer K, Smith BL, Socheat D, Fukuda MM, 2008. Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med* 359: 2619–2620.
- Valderramos SG, Fidock DA, 2006. Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol Sci* 27: 594–601.
- Duraingh MT, Cowman AF, 2005. Contribution of the *pfmdr1* gene to antimalarial drug-resistance. *Acta Trop* 94: 181–190.
- Lim P, Alker AP, Khim N, Shah NK, Incardona S, Doung S, Yi P, Bouth DM, Bouchier C, Puijalon OM, Meshnick SR, Wongsrichanalai C, Fandeur T, Bras JL, Ringwald P, Arie F, 2009. *Pfmdr1* copy number and artemisinin derivatives combination therapy failure in falciparum malaria in Cambodia. *Malar J* 8: 11–17.
- Alker AP, Lim P, Sem R, Shah NK, Yi P, Bouth DM, Tsuyuoka R, Maguire JD, Fandeur T, Arie F, Wongsrichanalai C, Meshnick SR, 2007. *Pfmdr1* and *in vivo* resistance to artesunate-mefloquine in falciparum malaria on the Cambodian-Thai border. *Am J Trop Med Hyg* 76: 641–647.
- Nelson AL, Purfield A, McDaniel P, Uthaimongkol N, Buathong N, Sriwichai S, Miller RS, Wongsrichanalai C, Meshnick SR, 2005. *Pfmdr1* genotyping and *in vivo* mefloquine resistance on the Thai-Myanmar border. *Am J Trop Med Hyg* 72: 586–592.
- Price RN, Uhlemann AC, Brockman A, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ, Nosten F, Krishna S, 2004. Mefloquine resistance in

- Plasmodium falciparum* and increase *pfmdr1* gene copy number. *Lancet* 364: 438–447.
12. Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T, Mercereau-Puijalon O, 2005. Resistance of *Plasmodium falciparum* field isolates to *in vitro* artemether and point mutations of the SERCA-type PfATPase6. *Lancet* 366: 1960–1963.
 13. Uhlemann AC, Cameron A, Eckstein-Ludwig U, Fischbarg J, Iserovich P, Zuniga FA, East M, Lee A, Brady L, Haynes RK, Krishna S, 2005. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nat Struct Mol Biol* 12: 628–629.
 14. Na-Bangchang K, Ruengweeraut R, Mahamad P, Ruengweeraut K, Chaijaroenkul W, 2010. Declining in efficacy of a three-day combination regimen of mefloquine-artesunate in a multi-drug resistance area along the Thai-Myanmar border. *Malar J* 9: 273–280.
 15. World Health Organization, 2003. *Assessment and Monitoring of Antimalarial Drug Efficacy for the Treatment of Uncomplicated falciparum Malaria*. Geneva: World Health Organization.
 16. Wooden J, Gould EE, Paul AT, Sibley CH, 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method for differentiating strains. *Exp Parasitol* 75: 207–212.
 17. Arieu F, Chalvet W, Hommel D, Peneau C, Hulin A, Mercereau-Puijalon O, Duchemin JB, Sarthou JL, Reynes JM, Fandeur T, 1999. *Plasmodium falciparum* parasites in French Guiana: limited genetic diversity and high selfing rate. *Am J Trop Med Hyg* 61: 978–985.
 18. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Welles TE, 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* 6: 861–871.
 19. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Welles TE, Plowe CV, Coulibaly D, 2001. A molecular marker for chloroquine-resistant *falciparum* malaria. *N Engl J Med* 344: 257–263.
 20. Ferreira ID, Martinelli A, Rodrigues LA, do Carmo EL, do Rosário VE, Póvoa MM, Cravo P, 2008. *Plasmodium falciparum* from Para state (Brazil) shows satisfactory *in vitro* response to artemisinin derivatives and absence of the S769N mutation in the SERCA-type PfATPase6. *Trop Med Int Health* 13: 199–207.
 21. Ferreira ID, Rosario VE, Cravo PV, 2006. Real-time quantitative PCR with SYBR Green I detection for estimating copy numbers of nine drug resistance candidate genes in *Plasmodium falciparum*. *Malar J* 5: 1–8.
 22. Lin JT, Julian JJ, Wongsrichanalai C, 2010. Drug-resistant malaria: the era of ACT. *Curr Infect Dis Rep* 12: 165–173.
 23. Sidhu AB, Verdier-Pinard D, Fidock DA, 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfprt* mutations. *Science* 298: 210–213.
 24. Lakshmanan V, Bray PG, Verdier-Pinard D, Johnson DJ, Horrocks P, Muhle RA, Alakpa GE, Hughes RH, Ward SA, Krogstad DJ, Sidhu AB, Fidock DA, 2005. A critical role for PfCRT K76T in *Plasmodium falciparum* verapamil-reversible chloroquine resistance. *EMBO J* 24: 2294–2305.
 25. Hall AP, Segal HE, Pearlman EJ, Phintuyothin P, Kosakal S, 1975. Amodiaquine resistant *falciparum* malaria in Thailand. *Am J Trop Med Hyg* 24: 575–580.
 26. Malikul S, 1988. The current situation of anti-malaria programme in Thailand. *Southeast Asian J Trop Med Public Health* 19: 355–359.
 27. Karbwang J, Harinasuta T, 1992. *Chemotherapy of Malaria in Southeast Asia*. Bangkok: Ruamtassana Co.
 28. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimé AA, Kouriba B, Taylor TE, Plowe CV, 2003. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J Infect Dis* 187: 1870–1875.
 29. Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, Sasi P, Marsh K, Borrmann S, Mackinnon M, Nzila A, 2009. Chloroquine resistance before and after its withdrawal in Kenya. *Malar J* 8: 106–112.
 30. Wongsrichanalai C, Meshnick SR, 2008. Declining artesunate-mefloquine efficacy against *falciparum* malaria on the Cambodia-Thailand border. *Emerg Infect Dis* 14: 716–719.
 31. Cooper RA, Hartwig CL, Ferdig MT, 2005. *Pfprt* is more than the *Plasmodium falciparum* chloroquine resistance gene: a functional and evolutionary perspective. *Acta Trop* 94: 170–180.
 32. Munghin M, Suwandittakul N, Chaijaroenkul W, Rungsirhirunrat K, Harnyuttanakorn P, Seugorn A, Na Bangchang K, 2010. The patterns of mutation and amplification of *Plasmodium falciparum pfprt* and *pfmdr1* genes in Thailand during the year 1988 to 2003. *Parasitol Res* 107: 539–545.
 33. Rungsirhirunrat K, Chaijaroenkul W, Seugorn A, Na-Bangchang K, Thaithong S, 2009. Association between chloroquine resistance phenotypes and point mutations in *pfprt* and *pfmdr1* in *Plasmodium falciparum* isolates from Thailand. *Acta Trop* 109: 37–40.
 34. Chaijaroenkul W, Wisedpanichkij R, Na-Bangchang K, 2010. Monitoring of *in vitro* susceptibilities and molecular markers of resistance of *Plasmodium falciparum* isolates from Thai-Myanmar border to chloroquine, quinine, mefloquine and artesunate. *Acta Trop* 113: 190–194.
 35. Bin Dajem SM, Al-Qahtani A, 2010. Analysis of gene mutations involved in chloroquine resistance in *Plasmodium falciparum* parasites isolated from patients in the southwest of Saudi Arabia. *Ann Saudi Med* 30: 187–192.
 36. Daily JP, Roberts C, Thomas SM, Ndir O, Dieng T, Mboup S, Wirth DF, 2003. Prevalence of *Plasmodium falciparum pfprt* polymorphisms and *in vitro* chloroquine sensitivity in Senegal. *Parasitology* 126: 401–405.
 37. Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, Nash D, Singhasivanon P, Anderson TJ, Krishna S, White NJ, Nosten F, 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. *Clin Infect Dis* 42: 1570–1577.
 38. Rodrigues LA, Henriques G, Borges ST, Hunt P, Sanchez CP, Martinelli A, Cravo P, 2010. Experimental evolution of resistance to artemisinin combination therapy results in amplification of the *mdr1* gene in a rodent malaria parasite. *PLoS ONE* 5: e11593.
 39. Ghanchi NK, Ursing J, Beg MA, Veiga MI, Jafri S, Mårtensson A, 2011. Prevalence of resistance associated polymorphisms in *Plasmodium falciparum* field isolates from southern Pakistan. *Malar J* 10: 18–23.
 40. Griffing S, Syphard L, Sridaran S, McCollum AM, Mixson-Hayden T, Vinayak S, Villegas L, Barnwell JW, Escalante AA, Udhayakumar V, 2010. *Pfmdr1* amplification and fixation of *pfprt* chloroquine resistance alleles in *Plasmodium falciparum* in Venezuela. *Antimicrob Agents Chemother* 54: 1572–1579.
 41. Witkowski B, Iriart X, Njomnang Soh P, Menard S, Alvarez M, Naneix-Laroche V, Marchou B, Magnaval JF, Benoit-Vical F, Berry A, 2010. *Pfmdr1* amplification associated with clinical resistance to mefloquine in West Africa: implication in artemisinin combination therapies efficacy. *J Clin Microbiol* 48: 3797–3799.
 42. Vinayak S, Alam MT, Sem R, Shah NK, Susanti AI, Lim P, Muth S, Maguire JD, Rogers WO, Fandeur T, Barnwell JW, Escalante AA, Wongsrichanalai C, Arieu F, Meshnick SR, Udhayakumar V, 2010. Multiple genetic backgrounds of the amplified *Plasmodium falciparum* multidrug resistance (*pfmdr1*) gene and selective sweep of 184F mutation in Cambodia. *J Infect Dis* 20: 1551–1560.
 43. Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S, 2003. Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature* 424: 957–961.
 44. Mugittu K, Genton B, Mshinda H, Beck HP, 2006. Molecular monitoring of *Plasmodium falciparum* resistance to artemisinin in Tanzania. *Malar J* 5: 126.
 45. Zhang GQ, Guan YY, Zheng B, Wu S, Tang LH, 2008. No PfATPase6 S769N mutation found in *Plasmodium falciparum* isolates from China. *Malar J* 7: 122.
 46. Imwong M, Dondorp AM, Nosten F, Yi P, Munghin M, Hanchana S, Das D, Phyo AP, Lwin KM, Pukrittayakamee S, Lee SJ, Saisung S, Koecharoen K, Nguon C, Day NP, Socheat D, White NJ, 2010. Exploring the contribution of candidate genes to artemisinin resistance in *Plasmodium falciparum*. *Antimicrob Agents Chemother* 54: 2886–2892.