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Association Between the *pfmdr1* Gene and *In Vitro* Artemether and Lumefantrine Sensitivity in Thai Isolates of *Plasmodium falciparum*

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Abstract. We evaluated the influence of *pfmdr1* mutations and copy number on *in vitro* artemether and lumefantrine sensitivity in 101 laboratory and adapted Thai isolates of *Plasmodium falciparum*. Approximately one-fourth of these isolates exhibited reduced lumefantrine susceptibility. We found that both mutations and amplification of the *pfmdr1* gene influenced *in vitro* artemether and lumefantrine sensitivity. Using multivariate analysis, 184F or 1042N alleles and a copy number of ≥ 4 were identified as the independent markers for decreased lumefantrine susceptibility. Separate analysis also indicated that parasites from different geographical areas were influenced by different genetic markers.

INTRODUCTION

Drug resistance is a major obstacle to effective treatment and control of potentially life-threatening falciparum malaria. This situation is at its most serious in southeast Asia, particularly along the Thai borders of Myanmar and Cambodia.[1] Resistance to quinolines, including chloroquine and mefloquine, is well-documented in *Plasmodium falciparum* isolates from Thailand. To address the problem of antimalarial drug failure in southeast Asia, artemisinin-based combination treatments (ACTs; e.g., artesunate plus mefloquine) have been introduced. This combination has had a satisfactory cure rate even in the highly multidrug-resistant areas of Thailand.[2,3] Unfortunately, a report of unacceptably low cure rates of this combination has been documented along the Thai–Cambodia border.[4] To date, there is no compelling evidence that failure of ACTs is caused by artemisinin resistance. However, delayed parasite clearance after treatment of an artemisinin derivative in this area has been reported and was indicated to be a genetic basis.[5,6] Artemether–lumefantrine (Coarthem, Novartis, Basal, Switzerland) is the only fixed-dose formulation ACT on the World Health Organization (WHO) essential drug list[7] and is considered a possible replacement for failing mefloquine plus artesunate. Recent studies from Thailand show that the six-dose regimen of artemether–lumefantrine has satisfactory cure rates (> 96%) for the treatment of uncomplicated falciparum malaria.[8,9]

Several studies have shown that single nucleotide polymorphisms (SNPs) and amplification of the *pfmdr1* gene are associated with *in vitro* and *in vivo* response to arylaminoalcohols, especially mefloquine.[10–17] Evidence also exists that the *pfmdr1* gene plays a role in the response to artemisinin derivatives.[18–20] Although the combination of artemether and lumefantrine has been recently introduced, similar molecular mechanisms might influence the response to this combination. Several studies from Africa showed that SNPs, rather than amplification of the *pfmdr1* gene, are involved in the *in vitro* and *in vivo* lumefantrine response.[21–24]

Rationale deployment of the artemether–lumefantrine combination for the treatment of falciparum malaria in Thailand requires greater insight into the potential for resistance evolution. In this study, we have investigated the influence of the mutations and amplification of the *pfmdr1* gene on *in vitro* artemether and lumefantrine sensitivity of Thai isolates of *P. falciparum*. Most *in vitro* studies using Thai isolates and *in vivo* studies conducted in Thailand were limited to single geographical areas (i.e., the Thai–Myanmar border). This may bias the resulting data. We have used adapted isolates from four different areas along the Thai–Myanmar and Thai–Cambodia borders to establish the range of parasite genotypes and drug susceptibilities across a broad geographical area.

MATERIALS AND METHODS

***P. falciparum* strains and cultivation.** One hundred and one isolates of *P. falciparum*, including five standard laboratory isolates (K1, T994, M12, 3D7, and G112) and 96 adapted Thai isolates, were investigated. The recent Thai isolates were collected from patients presenting at four malaria endemic areas including Tak, Kanchanaburi, and Ranong on the Thai–Myanmar border and Chantaburi on the Thai–Cambodia border. All samples were collected between 2003 and 2005. Parasites were maintained in continuous cultures using a modification of the method of Trager and Jensen.[25] Parasites were cultured in human erythrocytes (O^+) and incubated at 37°C in culture flasks containing medium [Roswell Park Memorial Institute (RPMI) 1640 with 23 mM $NaHCO_3$, 25 mM N-(2-hydroxyethyl) piperazine-N-(2-ethanesulphonic acid), and 10% human AB serum]. Cultures were maintained under an atmosphere of 90% N_2, 5% O_2, and 5% CO_2.

***In vitro* sensitivity assays.** Artemether and lumefantrine sensitivity of *P. falciparum* isolates was determined by measuring [^{3}H]hypoxanthine incorporation into parasite nucleic acids as previously described.[26] Drug inhibitory concentration 50% (IC_{50}) (i.e., the concentration of a drug that inhibits parasite growth by 50%) was determined from the log dose/response relationship as described by GRAFIT (Erithacus Software, Kent, England).

Genomic DNA extraction. Parasite DNA was extracted using the Chelex-resin method.[27] A high parasitemia pellet of *P. falciparum* culture collected at the trophozoite stage was lysed by incubation in 1.5 volumes of 0.15% saponin in RPMI at 37°C for 20 minutes. The parasites were then washed in phosphate-buffered saline (PBS; 10 mM phosphate buffered

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saline, pH 7.4, 138 mM NaCl, 2.7 mM KCl). Parasite genomic DNA was extracted by adding 200 μL of the 5% Chelex resin (Bio-Rad, Hercules, CA) and incubated in a boiling water bath for 8 minutes. Chelex was subsequently separated by centrifugation. The supernatant containing the DNA was then collected into a new microcentrifuge tube where 5 μL of DNA preparation was used for a 25-μL PCR reaction.

DNA fingerprinting. Genomic variation between parasite strains was determined by the multiplex polymerase chain reaction (PCR) method using primer pairs specific for three independent genes, merozoite surface protein (MSP)-1, MSP-2, and circumsporozoite protein (CSP). The oligonucleotide primer sequences and procedures were previously described by Wooden and others.[27] The products of PCR were separated by 2% agarose gel electrophoresis and visualized by ultraviolet (UV) transillumination. PCR band pattern of the parasites isolated from the same area during the same period of time was compared with that of K1 and 3D7.

Mutations in the *pfmdr1* gene. Mutations in the *pfmdr1* gene were determined by the nested PCR and restriction endonuclease digestion method developed by Duraisingh and others[18] to detect mutations at codons 86, 184, 1034, 1042, and 1246. K1 and 7G8 strains were used as positive controls.

Estimation of the copy number of *pfmdr1*. The *pfmdr1* gene copy number was determined by TaqMan real-time PCR (ABI sequence detector 7000; Applied Biosystems, Foster City, CA) as developed by Price and others.[15] Primers and fluorescently labeled probes were used to amplify the *pfmdr1* and β-tubulin genes. The PCR and thermal-cycling conditions used were as described in the original publication. The K1 and DD2 clones containing 1 and 4 *pfmdr1* copies, respectively, were used as the reference DNA samples. The *pfmdr1* and β-tubulin amplification reactions were run in duplicate. The relative *pfmdr1* copy number was assessed as described by Price and others.[15]

Statistical analysis. Data were analyzed by SPSS for Windows (SPSS Inc., Chicago, IL). Each IC_{50} value reported represented the mean and standard deviation (SD) of at least three independent experiments. Correlations were assessed by Pearson's correlation. Normally distributed IC_{50} data were assessed by the Kolmogorov–Smirnov test. Quantitative variables among groups were compared by independent *t* test or one-way analysis of variation (ANOVA). The post-hoc test (Scheffe's) for multiple comparisons was used to test for differences among groups. A lumefantrine IC_{50} value of more than 150 nM was considered as decreased lumefantrine susceptibility.[28] Univariate and multivariate analyses were performed to assess the association between *pfmdr1* genotypes and decreased lumefantrine susceptibility of *P. falciparum*.

RESULTS

***In vitro* artemether and lumefantrine sensitivities.** One hundred and one isolates were tested for sensitivity to artemether and lumefantrine. The correlation between the IC_{50}s of artemether and lumefantrine was significant ($P < 0.001$), with a correlation of 0.342. The mean IC_{50}s (±SD) for artemether and lumefantrine were 3.5 ± 2.1 nM (range = 0.7–9.3 nM) and 115.5 ± 96.8 nM (range = 14.0–453.0 nM), respectively. Lumefantrine IC_{50}s in this population of isolates were normally distributed. Table 1 shows the mean artemether and lumefantrine IC_{50}s of the recently adapted isolates by geographical collection site. There were no significant differences of the mean lumefantrine IC_{50}s among parasites isolated from different areas ($P = 0.194$, one-way ANOVA). In contrast, the differences between the mean artemether IC_{50}s were statistically significant at a *P* value of 0.03 (one-way ANOVA). Then, multiple comparisons using Scheffe's test were used to compare between groups and indicated that parasites isolated from Chantaburi had a significantly greater sensitivity to artemether than parasites from Tak ($P = 0.047$).

Characterization of the *pfmdr1* gene. Comparisons of the DNA fingerprints of the parasite isolated from the same area in the same year showed that they were all unrelated based on their genotypic profiles. Only one parasite clone was detected in each isolate. Ninety-six adapted Thai isolates were analyzed for mutations in the *pfmdr1* gene. The profile of *pfmdr1* polymorphisms in these parasite isolates is shown in Table 1. The laboratory isolates K1, T994, and M12 contained the *pfmdr1* 86N allele. Our studies showed that 3D7 contained the *pfmdr1* 184F allele, whereas G112 showed no mutations. The *pfmdr1* 184F and 1042D alleles were more common in the parasites isolated from Chantaburi than those isolated elsewhere (both $P = 0.001$, χ^2 test). None of the isolates contained mutation at codon 1246. Determination of the *pfmdr1* gene copy number showed that these isolates contained a *pfmdr1* copy number with a mean of 2.5 (range = 0.8–5.6). Using one-way ANOVA, the mean *pfmdr1* copy number of the parasites from different areas was significantly different ($P < 0.001$). The mean *pfmdr1* copy number of the parasites isolated from the Thai–Myanmar border (i.e., Tak and Ranong) was significantly higher than those from the Thai–Cambodia border (i.e., Chantaburi; $P = 0.002$ and $P = 0.037$, respectively, Scheffe's test).

The association between *in vitro* sensitivities and the *pfmdr1* gene. Table 2 shows the *in vitro* lumefantrine and artemether sensitivities of *P. falciparum* isolates with different *pfmdr1* genotypes. The parasites containing 86N or 1034S alleles or a *pfmdr1* copy number ≥ 4 exhibited significantly higher lumefantrine $IC_{50.}$ Lower artemether sensitivity was also shown in those parasites containing these genetic

TABLE 1

In vitro sensitivities to artemether and lumefantrine and distribution of *pfmdr1* polymorphisms of the 96 recently adapted parasites from different areas

Area	No. of isolates	Artemether IC_{50} (nM)	Lumefantrine IC_{50} (nM)	*pfmdr1* copy number	*pfmdr1* mutations				
					86Y	184F	1034C	1042D	1246Y
Tak	27	4.3 ± 2.0	147.7 ± 116.4	3.2 ± 1.7	1 (3.7%)	7 (25.9%)	–	–	–
Kanchanaburi	26	3.3 ± 2.0	94.8 ± 72.9	2.4 ± 1.1	5 (19.2%)	9 (34.6%)	2 (7.7%)	2 (7.7%)	–
Ranong	16	4.0 ± 2.4	96.5 ± 65.5	3.0 ± 1.0	1 (6.3%)	6 (37.5%)	2 (12.5%)	2 (12.5%)	–
Chantaburi	27	2.7 ± 2.0	122.2 ± 111.5	1.7 ± 1.1	5 (18.5%)	21 (77.8%)	6 (22.2%)	10 (37.0%)	–
Total	96	3.5 ± 2.1	117 ± 98.3	2.5 ± 1.4	12 (12.5%)	43 (44.8%)	10 (10.4%)	14 (14.6%)	–

TABLE 2
Comparison of lumenfantrine and artemether sensitivity among *P. falciparum* with different *pfmdr1* genotypes

pfmdr1 genotypes		No.	Lumefantrine IC_{50} (nM)	*P* value	Artemether IC_{50} (nM)	*P* value
86	N86	86	125.7 ± 100.4	< 0.001	3.7 ± 2.2	< 0.001
	86Y	15	56.8 ± 36.3		2.1 ± 1.1	
184	Y184	57	102.6 ± 80.5	0.145	3.8 ± 2.3	0.120
	184F	44	132.2 ± 113.3		3.1 ± 1.8	
1034	S1034	91	121.9 ± 99.5	< 0.001	3.7 ± 2.1	< 0.001
	1034C	10	57.3 ± 29.1		1.7 ± 1.1	
1042	N1042	87	120.7 ± 94.6	0.180	3.7 ± 2.1	0.004
	1042D	14	83.2 ± 54.5		2.0 ± 1.9	
Copy no.	< 4	85	101.7 ± 85.6	0.013	3.2 ± 2.0	0.026
	≥ 4	16	188.9 ± 120.7		4.5 ± 2.2	

IC_{50} values are shown as mean values ± standard deviation.

polymorphisms. In addition, parasites with the 1042N allele also showed significantly lower artemether sensitivity.

Using an IC_{50} of 150 nM as the cut-off point, approximately one-fourth (25/101, 24.75%) of the isolates had decreased lumefantrine susceptibility. Table 3 shows the association between *pfmdr1* genotypes and decreased lumefantrine susceptibility using univariate and multivariate analysis. Multivariate analysis of 101 isolates showed that 184F, 1042N and a copy number ≥ 4 were independent factors associated with decreased lumefantrine susceptibility. Parasites isolated from different areas (i.e., the Thai–Myanmar and Thai–Cambodia borders) were also separately examined. Only 1042N was significantly associated with decreased lumefantrine susceptibility in the parasites isolated from the Thai–Cambodia border (odds ratio [OR] = 12.1, 95% confidence interval [CI] = 1.1–139.4, P = 0.045). In contrast, the parasites from the Thai–Myanmar border containing ≥ 4 copies of *pfmdr1* gene were more likely to have decreased lumefantrine susceptibility (OR = 5.9, 95% CI = 1.5–22.7, P = 0.011).

DISCUSSION

Although the artemether–lumefantrine combination has not been used for the treatment of uncomplicated falciparum malaria in Thailand, approximately one-fourth of Thai isolates in this study exhibited decreased lumefantrine susceptibility. This may be because of a cross-resistance among arylaminoalcohols. From our data, mefloquine and lumefantrine IC_{50} of 96 Thai isolates showed a significant correlation of 0.333 (P = 0.001). These parasites were isolated at the same time that the reduction of artesunate–mefloquine cure rate was noticed along the Thai–Cambodia border. However, according to our data, artemether was still active against these Thai isolates.

The majority of these Thai isolates contained 184F allele (44.8%) and an increased copy number (80.2%) of the *pfmdr1* gene. Our data showed geographical differences in the *pfmdr1* haplotype pattern between the Thai–Myanmar and Thai–Cambodia borders. The parasites isolated from Thai–Cambodia areas exhibited significantly lower copy numbers but a higher prevalence of the 184F allele of the *pfmdr1* gene compared with those isolated from the Thai–Myanmar border. This finding indicates the diversity throughout these two different geographical areas. The selection of particular *pfmdr1* haplotypes in specific geographical areas was identified in some previous studies.[29,30]

Clinical studies in Africa have shown that parasites carrying the *pfmdr1* N86 and 184F allele were selected after exposure to artemether–lumefantrine.[21,22,24] Amplification of the *pfmdr1* gene was not observed in these studies. These findings indicate that parasites containing these alleles are more tolerant to lumefantrine. Indeed, an *in vitro* study of African isolates confirmed that parasites containing the 86N allele were less sensitive to lumefantrine.[23] In contrast, lumefantrine sensitivity of parasite isolates from the Thai–Myanmar border was influenced by the amplification of the *pfmdr1* gene but not the N86 allele.[9] The influence of the *pfmdr1* copy number on lumefantrine sensitivity has been confirmed by the study of Sidhu and others[31] using knockdown strategy. In the present study, parasites containing 86N or 1034S alleles or ≥ 4 copies of the *pfmdr1* gene had significantly higher lumefantrine $IC_{50.}$ However, when we considered those isolates exhibiting lumefantrine IC_{50} of more than 150 nM as decreased lumefantrine susceptibility, 184F or 1042N alleles and copy number of ≥ 4 were identified as the independent markers for decreased lumefantrine susceptibility. These genetic markers play an important role among isolates from different

TABLE 3
Univariate and multivariate analysis of the association between *pfmdr1* genotypes and decreased lumefantrine susceptibility

Genotypes	No. (%)	Prevalence of decreased lumefantrine susceptibility (%)	Crude odds ratio (95% CI)	*P* value	Adjusted odds ratio (95% CI)	*P* value
86Y	15 (14.9)	0 (0)	1	0.019		
N86	86 (85.1)	25 (29.1)	1.2 (1.1–1.4)			
Y184	57 (56.4)	11 (19.3)	1	0.169	1	0.009
184F	44 (43.6)	14 (31.8)	1.4 (0.9–2.2)		4.2 (1.4–12.5)	
1034C	10 (9.9)	0 (0)	1	0.063		
S1034	91 (90.1)	25 (27.5)	1.2 (1.1–1.3)			
1042D	14 (13.9)	1 (7.1)	1	0.179	1	0.044
N1042	87 (86.1)	24 (27.6)	1.2 (1.0–1.3)		9.7 (1.18–8.1)	
Copy no. < 4	85 (84.2)	17 (20)	1	0.023	1	0.006
Copy no. ≥ 4	16 (15.8)	8 (50)	3.0 (1.3–7.3)		5.6 (1.6–19.6)	

geographical areas. The 1042N allele and the amplification of the *pfmdr1* gene were identified as the markers for decreased lumefantrine susceptibility in parasites isolated from the Thai–Cambodia and Thai–Myanmar borders, respectively. Different molecular markers of decreased lumefantrine susceptibility were identified in each area that should be a result of the different distribution of the *pfmdr1* haplotypes. In the past few years, different drug regimens were used for the treatment of uncomplicated falciparum malaria in different areas. The combination of artesunate and mefloquine has been used along the Thai–Cambodia area for more than 15 years, whereas along some areas of Thai–Myanmar border, artesunate has been added in the past few years.[4] Our drug policy in the past might contribute to the different *pfmdr1* haplotypes in each area.

The influence of the *pfmdr1* gene on the response to artemisinin derivative has been shown in several studies.[18,19,30] Reduced artemether sensitivity has been observed in parasites containing 86N, 1034S, or 1042N alleles or ≥ 4 copies of the *pfmdr1* gene. Because artemether and lumefantrine sensitivity is influenced by the alterations in common genes (i.e., *pfmdr1*), it is probable that these combinations may select parasites with a common resistance mechanism, especially because for most of the dosing interval, these drugs persist in the patient as monotherapy. This contention is supported by data from clinical trials for artemether–lumefantrine treatment of falciparum malaria in Africa.[21,22]

In conclusion, our results confirm the involvement of the *pfmdr1* gene in both artemether and lumefantrine sensitivity. Molecular mechanisms modulating their sensitivities are similar to the combination currently used for the treatment of uncomplicated falciparum malaria in Thailand, artesunate and mefloquine. Thus, this combination should be carefully evaluated before it is considered as an alternative treatment in Thailand. The association between these molecular markers and *in vivo* response of this combination should be investigated. Although it is frequently found that *in vitro* and *in vivo* resistance to antimalarial drugs might not be associated, monitoring of *in vitro* drug susceptibility should be performed to detect early warning signals.

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