Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* malaria in Thailand

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**Summary**

To determine differences in the distribution of drug resistance mutations in the *Plasmodium falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multi-drug resistance 1 (*pfmdr1*) genes of *P. falciparum* isolates in Thailand, a study was conducted using polymerase chain reaction-restriction fragment length polymorphism to detect mutations in *P. falciparum* isolates obtained from three areas with different levels of in vivo mefloquine (MQ) resistance. All isolates carried mutant allele T76 of the *pfcrt* gene and wild-type allele D1246 of the *pfmdr1* gene except for one isolate, which showed the wild-type K76 allele. This isolate was obtained from Chanthaburi Province, an area with high MQ resistance. Relatively low rates of the mutant alleles D1042 and Y86 of the *pfmdr1* gene were found among Thai isolates of *P. falciparum*. However, a statistically significant difference in the distribution was noted. Most of the mutant isolates were found among isolates from areas with moderate or low MQ resistance. Only one isolate with mixed mutant and wild-type N1042 and D1042 and two mutants of Y86 were found among the isolates from areas with high MQ resistance. The findings provide limited support for the hypothesis that mutant alleles of *pfmdr1* may be associated with increased sensitivity to MQ.

**Keywords** *Plasmodium falciparum*, drug resistance marker, mefloquine, *pfmdr1*, *pfcrt*

**Introduction**

In Thailand, malaria is endemic along the border areas, especially the Thai-Myanmar and Thai-Cambodia borders. Multidrug-resistant falciparum malaria has also been a major problem in these areas.

Molecular methods that detect genetic markers of drug resistance in parasites are potentially powerful tools to detect and track drug-resistant malaria (Labbe *et al.* 2003). A number of candidate genes associated with resistance to antimalarial drugs have been identified and studied (Cowman 1997; Plowe *et al.* 1998). Mutation occurring at codon 76 in the *Plasmodium falciparum* chloroquine (CQ) resistance transporter (*pfcrt*) gene is highly correlated with the in vitro response of *P. falciparum* to CQ (Fidock *et al.* 2000). The results from several studies have suggested that amplification of *pfmdr1* may be associated with resistance to CQ and/or arylaminoalcohol drugs (Foote *et al.* 1989; Wilson *et al.* 1989; Peel *et al.* 1994). In addition to gene amplification, the *pfmdr1* gene is known to undergo mutations leading to the substitution of amino acids at four distinct positions: 86, 1034, 1042 and 1246 (Basco & Ringwald 2002). The association between these mutations and the sensitivity of *P. falciparum* to CQ and mefloquine (MQ) are still unclear. Some studies found no association (Cowman 1991; Schneider *et al.* 2002; Pilai *et al.* 2003; Huaman *et al.* 2004) but most recent investigations showed that polymorphisms in *pfmdr1* of *P. falciparum* in some geographical areas were associated with increased in vitro MQ sensitivity (Pickard *et al.* 2003; Price *et al.* 2004). The distribution of these mutations in various geographical areas remains unclear.

In this study, we have, in 2001–2003, investigated proportions and distribution of polymorphisms in the *pfcrt* and *pfmdr1* genes based on blood samples obtained from *P. falciparum*-infected individuals in five areas of Thailand which are endemic for malaria.

**Materials and methods**

*Plasmodium falciparum* field isolates from patients with uncomplicated malaria were obtained before treatment during the years 2001–2003. In total 280 isolates were obtained from three different strata of malarious areas in Thailand, characterized by the levels of MQ resistance, i.e. high MQ resistance (cure rate with MQ 750 mg
<50%; Tak and Chanthaburi Provinces), moderate MQ resistance (cure rate between 50% and 70%; Kanchanaburi Province), and low MQ resistance (cure rate more than 70%; Ranong and Chiangmai Provinces; Figure 1).

Patients were enrolled in the study if they met the following criteria: current fever or history of fever within the past 24 h, monoinfection with *P. falciparum*, parasite density in the range of 1000–100 000 parasites/μl blood, no signs of severe malaria, and no history of recent treatment with antimalarial drugs. Informed consent was obtained from either the patients or a guardian accompanying the patients. One hundred microlitres of blood from a finger prick was collected in a heparinized capillary tube for the *in vitro* MQ sensitivity test. A sample of 10 μl blood was taken on to 3MM filter paper and kept for molecular analysis. Thick and thin blood films were also prepared for the determination of parasite density. The study was approved by the Ethics Committee of the Ministry of Public Health, Thailand.

**In vitro mefloquine sensitivity**

*In vitro* tests for the measurement of drug sensitivity of *P. falciparum* followed the standard methodology for the assessment of inhibition of schizont maturation (WHO 1990). Sterile, heparinized capillary tubes were used to collect 100 μl of blood from each patient before treatment. Blood was drawn from a finger after a prick with a sterile lancet and immediately placed in 900 μl of RPMI 1640, pre-warmed to body temperature. A thick blood film was also prepared for reading pre-culture parasitaemia. The thick blood film was stained with 10% Giemsa at pH 7.2. WHO standardized MQ pre-dosed test plates were used. The plates were pre-dosed with 0, 2, 4, 8, 16, 32, 64 and 128 pmol MQ (wells B–H). Aliquots of 50 μl of the blood-medium-mixture were placed into each well of the scheduled test line of the plate. The plates were placed in a candle jar and held for up to 30 h in an incubator, maintained at a temperature of 37.5 °C (±0.5 °C). After incubation, the sediments of the wells were harvested, preparing thick films. These were stained with 2% Giemsa solution at pH 6.8 for 30 min. The number of schizonts per 200 asexual forms of the parasites was used to assess schizont maturation in the control and drug wells. Asexual parasites with at least three chromatin bodies were defined as schizonts. Isolates with <10% schizonts in the control well were excluded. All tests were performed in duplicate. The concentration of drug that inhibits schizont maturation by 50% (IC50) and other *in vitro* drug response parameters were calculated using a computer version of log-concentration/response probit analysis (Wernsdorfer & Wernsdorfer 1995) based on the method of Litchfield and Wilcoxon (1949). According to the WHO standard, isolates were considered MQ resistant if the minimum inhibitory concentration was >1280 nm, corresponding to schizont maturation in well G (64 pmol).

**Mutation analysis of pfcrt and pfmdr1 genes**

DNA was extracted from dried blood spots following the method previously described (Wooden *et al.* 1993) with some modifications. Half of the blood spot (corresponding to approximately 5 μl of blood) was cut from the filter paper, transferred to a tube containing 180 μl of 5% Chelex-100 (Bio-Rad Laboratories, Munich, Germany).
and mixed intensively. After incubation in boiling water for 5 min, the tube was vortexed for 30 s and further incubated in boiling water for 10 min. The Chelex was separated by centrifugation (12 000 g for 2 min, repeated once) and the supernatant containing the isolated DNA was transferred to a fresh tube. One microlitre of the DNA solution was used as a template in each 20 μl polymerase chain reaction-restriction fragment length polymorphism matrix as previously described (Lopes et al. 2002). The results were interpreted as follows: pfmdr1 allele 86 (wild-type Asn; mutant Tyr), 1042 (wild-type Asn, mutant Asp), 1246 (wild-type Asp, mutant Tyr) (Foote et al. 1990), pfcrt allele 76 (wild-type Lys, mutant Thr).

Statistical analysis

Association between area-specific degree of MQ resistance and the presence of mutant, wild type and mixed mutant and wild-type alleles was tested by chi-square test. Differences in the in vitro MQ sensitivity among the isolates obtained from areas with different MQ in vivo sensitivity and among isolates with mutant or mixed mutant and wild-type alleles were tested by analysis of variance (ANOVA) after logarithmic transformation of each IC50 value. The IC50 values were expressed as geometric mean and 95% confidence interval. The level of significance was set at P < 0.05.

Results

Distribution of pfcrt and pfmdr1 mutations

A total of 280 P. falciparum field isolates were genotyped for the pfcrt and pfmdr1 genes. The results are shown in Table 1. Of these, 270, 274, 245 and 271 were genotyped for the pfmdr1 polymorphisms at codons 86, 1042, 1246 and for pfcrt polymorphism at position 76 respectively. All isolates carried mutant allele T76 of the pfcrt gene and wild-type allele D1246 of the pfmdr1 gene except for one isolate, which had a wild-type K76 allele. This isolate was obtained from Chanthaburi Province in an area with high MQ resistance. The majority of isolates displayed wild-type pfmdr1 N86 and N1042. A total of 252 of 270 isolates (93.3%) carried the wild-type allele N86, while eight (3%) carried the mutant allele Y86 and 10 (3.7%) had mixed alleles; and 258 of 274 (94.2%) had the wild-type allele N1042, 11 (4%) had the mutant allele D1042, and 5 (1.8%) had mixed alleles. The mutant alleles of pfmdr1 Y86 and D1042 were differently distributed (P = 0.025 and 0.0001 respectively). Six of the mutants of Y86 and 10 isolates with mutant and wild-type alleles were encountered in areas with low or moderate MQ resistance. Only two mutants with Y86 were found in areas with high MQ resistance. Similarly, 11 mutants D1042 and four mixed populations with mutants and wild types were found in areas with low or moderate MQ

Table 1 Distribution of the pfcrt and pfmdr1 polymorphisms of Plasmodium falciparum in three different MQ-resistant areas of Thailand

<table>
<thead>
<tr>
<th>Genes/areas of different level of MQ resistance</th>
<th>n</th>
<th>% Mutant</th>
<th>% Wild type</th>
<th>% Mixed of mutant and wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfmdr1 N86Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>100</td>
<td>2 (2)</td>
<td>98 (98)</td>
<td>0</td>
</tr>
<tr>
<td>Medium</td>
<td>52</td>
<td>5.8 (3)</td>
<td>88.5 (46)</td>
<td>5.8 (3)</td>
</tr>
<tr>
<td>Low</td>
<td>118</td>
<td>2.5 (3)</td>
<td>91.5 (108)</td>
<td>5.9 (7)</td>
</tr>
<tr>
<td>Total</td>
<td>270</td>
<td>3 (8)</td>
<td>93.3 (252)</td>
<td>3.7 (10)</td>
</tr>
<tr>
<td>pfmdr1 N1042D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>103</td>
<td>0</td>
<td>99 (102)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Medium</td>
<td>51</td>
<td>7.8 (4)</td>
<td>84.3 (43)</td>
<td>7.8 (4)</td>
</tr>
<tr>
<td>Low</td>
<td>120</td>
<td>5.8 (7)</td>
<td>94.2 (113)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>274</td>
<td>4 (11)</td>
<td>94.2 (258)</td>
<td>1.8 (5)</td>
</tr>
<tr>
<td>pfmdr1 D1246Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>93</td>
<td>0</td>
<td>100 (93)</td>
<td>0</td>
</tr>
<tr>
<td>Medium</td>
<td>46</td>
<td>0</td>
<td>100 (46)</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>106</td>
<td>0</td>
<td>100 (106)</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>245</td>
<td>0</td>
<td>100 (245)</td>
<td>0</td>
</tr>
<tr>
<td>pfcrt K76T</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>100</td>
<td>99 (99)</td>
<td>1 (1)</td>
<td>0</td>
</tr>
<tr>
<td>Medium</td>
<td>52</td>
<td>100 (52)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>119</td>
<td>100 (119)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>271</td>
<td>(271)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values in parenthesis are number of isolates.
Table 2  In vitro mefloquine sensitivity of Plasmodium falciparum and the distribution of pfcr t and pfmdr1 polymorphism from samples obtained from three different MQ-sensitive areas of Thailand

<table>
<thead>
<tr>
<th>Areas of different level of MQ resistance</th>
<th>N</th>
<th>IC50 of MQ (nm)</th>
<th>pfcr t 76</th>
<th>pfmdr1 86</th>
<th>pfmdr1 1042</th>
<th>pfmdr1 1246</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>K</td>
<td>T</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>High</td>
<td>60</td>
<td>90 (77–105)</td>
<td>–</td>
<td>60</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Medium</td>
<td>20</td>
<td>84 (69–101)</td>
<td>–</td>
<td>20</td>
<td>18</td>
<td>2</td>
</tr>
<tr>
<td>Low</td>
<td>65</td>
<td>78 (68–90)</td>
<td>–</td>
<td>65</td>
<td>61</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>145</td>
<td>84 (76–92)</td>
<td>–</td>
<td>145</td>
<td>139</td>
<td>3</td>
</tr>
</tbody>
</table>

T = Mutant allele Thr-76 of pfcr t gene; K = Wild-type allele Lys-76 of pfcr t gene; N = Wild-type allele Asn-86 of pfmdr1 gene; Y = Mutant allele Tyr-86 of pfmdr1 gene; N = Wild-type allele Asn-1042 of pfmdr1 gene; D = Mutant allele Asp-1042 of pfmdr1 gene; D = Wild-type allele Asp-1246 of pfmdr1 gene; Y = Mutant allele Tyr-1246 of pfmdr1 gene. Values are geometric mean IC50 and 95% confidence interval in parenthesis.

In vitro sensitivity to MQ

A total of 145 isolates were characterized for in vitro sensitivity to MQ. The geometric mean IC50 was 84 nm [95% confidence interval (CI) = 76–92 nm]. The isolates obtained from areas with different degrees of in vivo MQ resistance had different geometric mean IC50 values (Table 2). The highest value was found in the areas with high resistance, followed by those with moderate and low resistance respectively. However, the difference failed to reach statistical significance (P = 0.394).

The majority of isolates displayed wild-type alleles N86 and N1042. A total of 139 of 145 (95.8%) carried the wild-type allele N86, while three (2.1%) carried the mutant allele Y86 and three (2.1%) had mixed alleles; 140 of 145 (96.6%) had the wild-type allele N1042, two (1.4%) had the mutant allele D1042 and three (2%) had mixed alleles. All the mutant and mixed mutant/wild-type isolates were MQ sensitive. Although lower IC50 values for MQ were observed among the isolates containing mutant or mixed mutant and wild-type alleles of pfmdr1 Y86 and D1042, there was no statistically significant association between the mutant allele of the pfmdr1 Y86 and D1042 and the MQ in vitro sensitivity. The geometric mean IC50 values were 61 nm (95% CI = 10–370) in parasites with the Y86 allele (n = 3), 85 (77–93), in those with the N86 allele (n = 137) and 68 (42–109) in those with mixed alleles (n = 6) (P > 0.05). The geometric mean IC50 values were 63 nm (95% CI = 8–476) in parasites with the D1042 allele (n = 2), 82 (76–92) in those with the N1042 allele (n = 141) and 69 (21–228) in those with mixed alleles (n = 3) (P > 0.05).

Discussion

In this study the areas were classified according to the in vivo MQ responses. Chiangmai and Ranong Provinces were classified as areas with low MQ resistance, Kanchanaburi Province as an area with moderate MQ resistance, and Tak and Chanthaburi Provinces as areas with high MQ resistance. The in vitro MQ sensitivity is consistent with the current criteria for MQ-resistant areas in this study. Plasmodium falciparum isolates from Chiangmai and Ranong Provinces had the lowest geometric mean IC50 values for MQ (76 and 81 nm). Tak and Chanthaburi Provinces had the highest values (107 and 89 nm). The value for the isolates from Kanchanaburi was 84 nm.

We have observed a predominance of wild-type N86, N1042 and D1246 of the pfmdr1 gene and of the mutant T76 of the pfcr t gene. The predominance of wild-type N86 allele among the Thai P. falciparum isolates was quite distinct from the African isolates which mostly displayed the mutant Y86 alleles (Basco & Ringwald 2002). There were statistically significant differences in the distribution of the mutant Y86 alleles and D1042 isolates. Sixteen of 18 isolates with mutant Y86 or with mixed Y86 and N86 alleles and 15 of 16 isolates with mutant D1042 alleles or mixed D1042 and Y1042 alleles were found in the areas with low or moderate MQ resistance. All these isolates displayed an MQ-sensitive phenotype. These findings may be supportive evidence to previous studies which indicated that mutant alleles of the pfmdr1 gene may be associated with increased sensitivity to MQ (Mungthin et al. 1999; Price et al. 1999; Duraisingh et al. 2000; Basco & Ringwald 2002).

The role of an amplification of the pfmdr1 gene in the expression of resistance to CQ or MQ in P. falciparum has been unclear for considerable time (Cowman 1991; Basco
et al. 1995; Lim et al. 1996; Zalis et al. 1998). However, a recent study in Thai isolates of P. falciparum obtained along the Thai-Myanmar border produced strong evidence that amplification of pfmdr1 is the main cause of resistance to MQ (Price et al. 2004). Additional data from other areas of Thailand are certainly needed to clarify the association of the amplification of pfmdr1 and clinico-parasitological and in vitro response to MQ.

The prevalence of mutant allele T76 of the pfcrt gene in Malawi was substantially lower after discontinuing the use of CQ that had originally been held responsible for the selection of the pfcrt K76T mutation (Mita et al. 2003). Quite in contrast, in Thailand where CQ was not used anymore for treating falciparum malaria since 1973, the present study has shown that all P. falciparum isolates, except one, still displayed the mutant allele T76 of the pfcrt gene.

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