Polymorphisms of the pfmdr1 but not the pfnehe-1 gene is associated with in vitro quinine sensitivity in Thai isolates of Plasmodium falciparum

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Abstract

Background: The emergence of Plasmodium falciparum resistance to most currently used anti-malarial drugs is a major problem in malaria control along the Thai-Myanmar and Thai-Cambodia borders. Quinine (QN) with tetracycline/doxycycline has been used as the second-line treatment for uncomplicated falciparum malaria. In addition, QN monotherapy has been the first-line treatment for falciparum malaria in pregnant women. However, reduced in vitro and in vivo responses to QN have been reported. To date, a few genetic markers for QN resistance have been proposed including Plasmodium falciparum chloroquine resistance transporter (pfcrt), P. falciparum multidrug resistance 1 (pfmdr1), and P. falciparum Na+/H+ exchanger (pfnehe-1). This study was to investigate the role of the pfmdr1 and pfnehe-1 gene on in vitro QN sensitivity in Thai isolates of P. falciparum.

Methods: Eighty-five Thai isolates of P. falciparum from the Thai-Myanmar and Thai-Cambodia borders from 2003-2008 were determined for in vitro QN sensitivity using radioisotopic assay. Polymorphisms of the pfmdr1 and pfnehe-1 gene were determined by PCR-RFLP and sequence analysis. Associations between the in vitro QN sensitivity and the polymorphisms of the pfmdr1 and pfnehe-1 gene were evaluated.

Results: The mean QN IC50 was 202.8 nM (range 25.7-654.4 nM). Only four isolates were QN resistant when the IC50 of >500 nM was used as the cut-off point. Significant associations were found between the pfmdr1 mutations at codons N86Y and N1042D and in vitro QN sensitivity. However, no associations with the number of DNNND, DDNNNDNHNDD, and NHNDNHNNDDD repeats in the microsatellite ms4760 of the pfnehe-1 gene were identified.

Conclusion: Data from the present study put doubt regarding the pfnehe-1 gene as to whether it could be used as the suitable marker for QN resistance in Thailand. In contrast, it confirms the influence of the pfmdr1 gene on in vitro QN sensitivity.

Background

The emergence of anti-malarial resistance in Plasmodium falciparum is a major public health threat worldwide, especially in tropical developing countries. The situation of multidrug-resistant falciparum malaria is most serious along the Thai-Myanmar and Thai-Cambodia borders [1]. To handle this situation, WHO recommends artemisinin derivative-based combination treatment (ACT) for the treatment of uncomplicated falciparum malaria [2]. Artesunate-mefloquine combination has been used as the first-line treatment in Thailand for more than 15 years [3]. Quinine (QN)-tetracycline/doxycycline has been used as the second-line treatment for uncomplicated falciparum malaria in Thailand. In addition, QN monotherapy is the first-line treatment for pregnancy [3,4]. Unfortunately reduced in vitro and in vivo response to QN has been reported in Southeast Asia [5,6]. Investigations have been carried out to identify the mechanisms of QN resistance. At least three candidate genes including Plasmodium falciparum chloroquine resistance transporter (pfcrt), Plasmodium falciparum multidrug resistance 1 (pfmdr1),
and *Plasmodium falciparum* Na+/H+ exchanger (*pfnhe-1*) have been linked to reduced QN sensitivity [7-12]. Cooper *et al.* (2002) showed the association between the *pfcr* mutation at codon 76 and QN sensitivity [7]. In addition, recent studies have shown that parasites containing a novel mutation in the *pfcr* gene, Q352K/R, C350R, altered QN sensitivity [8,9]. Concerning the *pfmdr1* gene, it has been shown that both mutations and copy number influenced *in vitro* QN sensitivity [10-12]. Using quantitative trait loci on the genetic cross of HB3 and Dd2 strains, an additional candidate gene, *pfhhe-1*, for QN resistance was identified [13]. This gene encodes a 226 kDa parasite plasma membrane protein containing 12 transmembrane domains, and 3 microsatellite regions, msR1, ms3580 and ms4760. Variations in QN susceptibilities between different parasite strains have also been linked to repeat polymorphisms in the microsatellite locus ms4760 of *pfhhe-1* [13]. A few studies showed that the number of DNNND, DDNDNDNHDD, and NHNDNHNNDDD repeats in the microsatellite ms4760 influenced *in vitro* QN sensitivity [14-19]. However, there is a lack of consensus regarding the specific nature of these associations [20-25]. For instance, while some studies report an association between reduced susceptibility to QN and an increase in the number of ‘DNNND’ repeats in ms4760 [15,18,19], others could not verify this association [22-24], or found that two DNNND repeats was the optimal number for conferring a reduction in QN sensitivity [17]. Moreover, amplification of a second ms4760 repeat ‘NHNDNHNNDDD’ has been linked to increases in the parasite’s susceptibility to QN [15,18,19]. However, the reverse association has also been reported [22], and several studies have failed to confirm either of these findings [23,24]. Considering the situation of multidrug-resistant *P. falciparum* in Thailand, determination of the molecular basis of QN resistance is crucial to be able to monitor parasite resistance. This study was to investigate the influence of the *pfmdr1* and *pfhhe-1* genes on *in vitro* QN sensitivity of Thai isolates of *P. falciparum* from both the Thai-Myanmar and Thai-Cambodia borders.

**Methods**

**Plasmodium falciparum** strains and cultivation

The 85 isolates of *P. falciparum* used in this study were collected from patients with uncomplicated falciparum malaria, who attended malaria clinics and hospitals in malaria endemic areas along the Thai-Myanmar (Kanchanaburi and Ranong) and Thai-Cambodia (Chantaburi and Srisaket) borders from 2003 to 2008. The research protocol was reviewed and approved by the Ethics Committee of the Royal Thai Army Medical Department. Parasites were maintained in continuous cultures using a modification of the method of Trager and Jensen [26].

**In vitro sensitivity assays**

QN sensitivity of *P. falciparum* isolates was determined by measurement of [3H] hypoxanthine incorporation into parasite nucleic acids as previously described [27]. Drug IC<sub>50</sub> (i.e. concentration of a drug which inhibits parasite growth by 50%) was determined from the log dose/response relationship as fitted by GRAFIT (Erithacus Software, Kent, England).

**Genotypic characterization for pfmdr1 and pfhhe-1 genes**

Parasite DNA was extracted using the Chelex-resin method [28]. Five microliters of DNA preparation was used for a 25 μl PCR reaction. Mutations in the *pfmdr1* gene were determined by the nested PCR and restriction endonuclease digestion method developed by Duraisingham *et al.* for detection of the mutations at codons 86, 184, 1034, 1042 and 1246. K1 and 7G8 strain were used as positive controls [29]. The *pfmdr1* gene copy number was determined by TaqMan real-time PCR (ABI sequence detector 7000; Applied Biosystems) as developed by Price *et al.* [30]. The K1 and Dd2 clone containing 1 and 4 *pfmdr1* copies, respectively was used as the reference DNA sample. The *pfmdr1* and β-tubulin amplification reactions were run in duplicate. Relative *pfmdr1* copy number was assessed as previously described. PCR amplification for the *pfhhe-1* ms4760 microsatellite was performed as previously described [14]. DNA purification and DNA sequencing were conducted by Bioservice Unit, Bangkok, Thailand. Sequences were analyzed for the number of DNNND, DDNDNDNHDD, and NHNDNHNNDDD repeats in the *pfhhe-1* ms4760 microsatellite by BioEdit sequence alignment editor (version 7.0.9.0).

**Statistical analysis**

Data were analysed by SPSS for Windows version 18 (SPSS Inc., Chicago, IL). The SPSS license number is ID5071846. The QN IC<sub>50</sub> of each isolate was the mean IC<sub>50</sub> of three independent experiments. Each experiment was carried out in triplicate. Normally distributed IC<sub>50</sub> data were assessed by the Kolmogorov Smirnov test. Differences among parasites with different genotypes was analysed by Chi square and Fisher’s exact test. Correlations between QN IC<sub>50</sub> and the *pfmdr1* copy number and the number of DNNND, DDNDNDNHDD, and NHNDNHNNDDD repeats in the *pfhhe-1* ms4760 microsatellite were assessed by Pearson’s correlation. Differences of the mean QN IC<sub>50</sub> among parasites from different groups were analyzed by Independent t test or One-way ANOVA. Post Hoc test (Scheffe) for multiple comparisons was used to test for differences among
groups. The level of significance was set at a p value of <0.05.

Results

In vitro QN sensitivity

Characteristics of parasite isolates are presented in Table 1. The mean IC_{50} (± SD) for QN was 202.8 ± 123.3 nM (range 25.7-654.4 nM). QN IC_{50} of isolates were normally distributed. No significant differences were found between QN IC_{50} of parasites isolated from the Thai-Cambodia and Thai-Myanmar borders (p = 0.641, Independent t test). Of 85 isolates, only 4 (4.7%) isolates exhibited QN IC_{50} of >500 nM. Characterization of the four QN-resistant P. falciparum isolates is shown in Table 2.

Characterization of the pfmdr1 and pfhe-1 genes

Characterization of the pfmdr1 gene of 85 isolates is shown in Table 1. Approximately 60% of the parasite isolates contained the pfmdr1 184F allele. Determination of the pfmdr1 gene copy number showed that these isolates contained pfmdr1 copy numbers with a mean of 2.0 (range 0.7-5.6). The pfmdr1 184F allele was more common in the parasites isolated from the Thai-Cambodia border compared with those from the Thai-Myanmar border. In contrast, the parasites isolated from the Thai-Myanmar border had significantly higher copy numbers. A total of 81 isolates were characterized for polymorphisms in the number of DNNND, DDNNNDNHNDD, and NHNDNHNNDDD repeats in the pfhe-1 ms4760 microsatellite (Table 3). The most common number of DNNND, DDNNNDNHNDD, and NHNDNHNNDDD repeats was 2.0 (range 0.7-5.6).

Table 1 In vitro sensitivity to QN and distribution of pfmdr1 polymorphisms of the 85 adapted parasites from Thai-Myanmar and Thai-Cambodia areas

| Area            | No. | Mean QN (nM) ± SD | pfmdr1 mutations | Mean pfmdr1 copy number |
|-----------------|-----|------------------|------------------|------------------------|
| Thai-Myanmar    | 37  | 209.9 ± 117.1     |                  | 2.9 ± 1.4*             |
| Thai-Cambodia   | 48  | 197.2 ± 128.9     |                  | 1.2 ± 0.7              |
| Total           | 85  | 202.8 ± 123.3     |                  | 2.0 ± 1.3              |

* Significant difference between two areas determined by Independent t test (p < 0.001)
** Significant difference between two areas determined by Chi square test (p < 0.001)

Table 2 Characterization of the four QN-resistant isolates of Plasmodium falciparum

| Origin          | SK20 | SK22 | MR2 | KB12 |
|-----------------|------|------|-----|------|
| QN IC_{50} (nM) | 521.8| 503.0| 530.6| 654.4|
| Pfmdr1 mutations|      |      |     |      |
| 86N86           | N    | N    | N   | N    |
| Y184F           | F    | F    | F   | F    |
| S1034C          | S    | S    | S   | C    |
| N1042D          | N    | N    | N   | D    |
| D1246Y          | D    | D    | D   | D    |
| Pfmdr1 copy number | 08  | 0.9 | 1.2 | 2.0 |

Table 3 Comparison of in vitro QN sensitivity among Plasmodium falciparum with different pfmdr1 and pfhe-1 genotypes

| Parasite genotypes | No. (%) | Mean QN IC_{50} (nM) | p value |
|--------------------|---------|----------------------|---------|
| pfmdr1             |         |                      |         |
| 86                 | 70 (82.4)| 216.5 ± 127.5        | 0.025*  |
| 86Y                | 15 (17.6)| 138.3 ± 76.1         | 0.013*  |
| 184                | 32 (37.7)| 160.4 ± 93.6         | 0.144   |
| 184F               | 53 (62.3)| 228.3 ± 132.6        |         |
| 1034               | 71 (83.5)| 194.1 ± 114.0        |         |
| 1034C              | 14 (16.5)| 246.9 ± 160.5        |         |
| 1042               | 68 (80)  | 185.8 ± 113.8        | 0.010** |
| 1042D              | 17 (20)  | 270.3 ± 139.9        |         |
| Copy no.           |         |                      |         |
| <3                 | 69 (81.2)| 195.8 ± 132.2        | 0.284   |
| ≥3                 | 16 (18.8)| 232.7 ± 68.8         |         |
| pfhe-1             |         |                      |         |
| DNNND repeats      | 0      | 1 (1.2)              | 0.958   |
| DNNNDNHNDDNDD repeats | 3      | 4 ± 3 (1.2)         |         |
| DDNNNDNHNND repeats | 1      | 2 ± 3 (1.2)         |         |
| NHNDNHNNDDD repeats | 1      | 1 ± 3 (1.2)         |         |

* Significant difference determined by Independent t test
** Significant difference determined by One-way ANOVA
NHNDNHNNDDD repeats was 4 (48.2%), 2 (79%) and 1 (86.4%). Genotypic characterization of the four QN-resistant isolates is shown in Table 2.

The association between in vitro quinine sensitivity and the pfmdr1 and pfne-1 genes

No correlation was found between QN IC50 and the pfmdr1 copy number (r = 0.099, p = 0.367) and the number of DNNND (r = 0.017, p = 0.880), DDNNNDHNDDD (r = -0.76, p = 0.503), and NHNDNHNNDDD (r = -0.420, p = 0.711) repeats in the pfne-1 ms4760 microsatellite. Table 3 shows the in vitro QN sensitivities of these adapted Thai isolates containing different pfmdr1 genotypes and different number of DNNND, DDNNNDHNDDD, and NHNDNHNNDDD repeats in the pfne-1 ms4760 microsatellite. Parasite isolates with pfmdr1 184F and 1042D showed significantly higher QN IC50 than those containing pfmdr1 184Y and 1042N, respectively. In contrast, parasites having pfmdr1 86Y exhibited significantly lower QN IC50 than those having pfmdr1 86N. Parasites containing different pfmdr1 copy number and the number of DNNND and NHNDNHNNDDD repeats in the pfne-1 ms4760 microsatellite showed no significant difference in the mean QN IC50. When the parasites were categorized into subgroups according to their copy number of the pfmdr1 gene, using 3 copies as the cut-off point gave the greatest difference of the QN IC50 between 2 groups compared to other figures. However no significant difference was detected. These parasites were also classified into subgroups with a different number of DNNND repeats. No significant difference were found between QN IC50 of parasites containing ≥2 and less repeats (204.9 ± 121.5 nM & 230.5 ± 204.1 nM, p = 0.692), ≤3 and less repeats (207.7 ± 124.7 nM & 207.7 ± 124.7 nM, p = 0.748) or ≥4 and less repeats (210.4 ± 105.5 nM & 200.5 ± 148.1 nM, p = 0.728). Although analysis by One-way ANOVA showed a significant difference of QN IC50 in the parasites with a different number of DDNNNDHNDDD repeats, multiple comparison showed no significant difference between groups.

According to their pfmdr1 haplotypes, parasites were classified into five groups (Table 4), i.e., the isolates containing the pfmdr1 86Y, 1042D, 184F with copy number <3, 184Y with copy number <3 and 184Y with copy number ≥3. Significant differences of QN IC50 were found among these groups (p = 0.019, One-way ANOVA). Multiple comparison indicated that only parasites containing pfmdr1 1042D were significantly less sensitive to QN than parasites containing pfmdr1 86Y (p = 0.048).

Discussion

In the present study, using the IC50 of >500 nM as the cut-off point for in vitro QN resistance, only 4.7% (4/85) exhibited QN resistance. Although a higher cut-off point at 800 nM has been proposed, no parasite isolate was found showing QN IC50 of >800 nM. This result is similar to those previous reports showing that most Thai isolates of P. falciparum were QN sensitive [6,31,32]. However data from these studies might not be comparable since different methods including schizont maturation inhibition, isotopic and SYBR green 1 based fluorescence assays were used for the determination of QN IC50. In addition, since culture-adapted isolates were used in some studies including the present study, specific phenotypes might be selected during the adaptation process. The situation of QN resistance in Thailand is less serious than those found in mefloquine and chloroquine. This may be due to a lower drug pressure of QN since it has a shorter half life. However, a decline in QN sensitivity of P. falciparum isolated from the Thai-Myanmar border has been indicated in recent study [6].

All parasite isolates in this study contained chloroquine-resistant haplotype, CVIET of the pfcr. The association between in vitro QN sensitivity and polymorphisms of the pfmdr1 gene, but not the pfne-1 gene, was identified. Although a genetic cross study indicated that QN sensitivity can be modulated by the pfne-1 gene [13], the role of the pfne-1 gene as a molecular marker for QN resistance is still controversial. Some but not all in vitro and in vivo studies identified the association between DDNNND, DDNNNDHNDDD, and NHNDNHNNDDD repeats of the pfne-1 ms4760 microsatellite and QN sensitivity and treatment outcome, respectively [14-19,23-25]. Nearly half of Thai isolates in the present study contained 4 DDNNND repeats while most parasites from other areas in Southeast Asia including Vietnam and the China-Myanmar border contained 3 DDNNND repeats [18,19]. Similar to these 2 studies, most isolates in the present study contained 1 repeat of NHNDNHNNDDD. The studies with positive association usually showed that parasites with 2 or more than 2 DDNNND repeats had a significantly reduced QN sensitivity compared with those with 1 repeat. This association has been found in the studies from Vietnam and the China-Myanmar border as well [18,19]. In contrast, no significant difference of QN IC50 between parasites containing ≥2 and less DDNNND repeats was identified. Inconsistent findings of the association between the response to QN and pfne-1 gene might be due to its interaction with other genes such as pfcr and pfmdr1. This postulation has been provided to explain the result of a knockdown pfne-1 expression resulting in increased QN sensitivity in 2 of 3 parasite lines [16]. In addition, a recent study in Kenya found no significant difference of QN IC50 among parasites with different DDNNND repeats [17]. However parasites containing 2
DNNND repeats with 86Y pfmdr1 showed a decrease in QN sensitivity. When the QN IC$_{50}$ of the parasites containing a similar number of DNNND repeats in the pfnhe-1 ms4760 microsatellite with different 86 alleles in the pfmdr1 gene was compared, no significant difference of QN IC$_{50}$ among these parasites was identified (data not shown). In addition, no significant correlations between the number of DDNNNDNHNDD, and NHNDNHNNDND repeats of the pfnhe-1 ms4760 microsatellite and in vitro QN sensitivity were found. Since there were only four isolates exhibiting reduced QN susceptibility, this may be restrictive for identification or validation of these new markers.

In contrast to the pfnhe-1 gene, in vitro QN sensitivity was significantly associated with the mutations in the pfmdr1 gene in these Thai isolates. The parasites containing the pfmdr1 184F and 1042D allele showed less sensitivity to QN while those with the pfmdr1 86Y exhibited increased QN sensitivity. However, when the parasites were categorized according to their haplotypes of the pfmdr1 gene, the pfmdr1 86Y and 1042D allele influenced QN sensitivity. Compelling evidence for a significant role of N1042D mutation on in vitro QN sensitivity has been shown in a few studies using allelic exchange strategies [10,11]. Reed et al. [10] showed that insertion of the pfmdr1 gene containing the 1034C, 1042D and 1246Y alleles made a QN-sensitive parasite become more resistant to QN [10]. Conversely, a QN-resistant line became more sensitive to QN after these alleles were removed. More recently, a study by Sidhu et al. (2005) identified that a single mutation, the N1042D, could modulate the parasites become less sensitive to QN [11]. Unlike the N1042D mutation, the functional role of the N86Y mutation on QN sensitivity has been explored by expression of the pfmdr1 gene in a heterologous system, Xenopus oocytes [33]. Substituting the asparagines (N) at position 86 for tyrosine (Y) resulted in a loss of QN transport ability. Since the site of QN action is in the food vacuole, [34,35] transport ability of the wild-type Pgh1 reduces drug concentration in the food vacuole, and consequently results in decreased QN susceptibility. This finding is compatible to that found in a few studies using parasites isolated from Southeast Asia, including the present study showing that the parasite isolates containing the pfmdr1 86Y allele showed more sensitive to QN [20,32]. However, a contrary result was shown in the study of parasite isolates from Kenya [17]. The decrease in QN susceptibility was associated with the pfmdr1 86Y allele in parasites harbouring the two DNNND repeats in the pfnhe-1 ms4760 microsatellite. Since reduced susceptibility to QN appear to be governed by a number of proteins whose contributions vary between strains. The contrasting findings might be explained by different variations in genetic background of parasites from different geographical areas. The influence of the pfmdr1 copy number on QN sensitivity has been confirmed by the study of Sidhu et al. using the knockdown strategy [12]. A few studies of parasite isolates from the Thai-Myanmar border also showed the influence of pfmdr1 copy number on in vitro QN sensitivity. However, this association has not been identified in some studies [21,22]. In this study, no association between the pfmdr1 copy number and in vitro QN sensitivity neither in parasites from the Thai-Myanmar nor the Thai-Cambodia border was identified.

In conclusion, the present study confirms the involvement of the pfmdr1 gene in QN sensitivity. Both N86Y and N1042D mutations significantly modulate in vitro response to QN. Although previous studies from Southeast Asia including Vietnam and China-Myanmar border have demonstrated that the pfnhe-1 gene is involved in QN sensitivity, the present study showed no association. This has raised doubt regarding the pfnhe-1 gene as to whether it could be used as the suitable marker for QN resistance in Thailand.

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