Parasitol Res (2010) 107:539–545
DOI 10.1007/s00436-010-1887-x

ORIGINAL PAPER

The patterns of mutation and amplification of *Plasmodium falciparum pfcrt* and *pfmdr1* genes in Thailand during the year 1988 to 2003

Mathirut Mungthin · Nantana Suwandittakul · Wanna Chaijaroenkul · Kanchana Rungsrihirunrat · Pongchai Harnyuttanakorn · Aree Seugorn · Kesara Na Bangchang

Received: 5 April 2009 / Accepted: 19 April 2010 / Published online: 7 May 2010

Abstract The study investigated the patterns of *pfmdr1* and *pfcrt* genetic polymorphisms in *Plasmodium falciaprum* isolates collected from Thailand during the periods 1988-1993 (35 isolates), and 2003 (21 isolates). *Pfcrt* polymorphisms were almost universal for the mutations at codons K76T, A220S, Q271E, N326S, and R371I. All parasites displayed the chloroquine (CQ)-resistant phenotypes. This data suggested that *pfcrt* gene was sufficient to CQ resistance but did not mediate level of resistance. The prevalence [number of isolates (%)] of *pfmdr1* polymorphisms at codons N86Y, Y184F, S1034C, N1042D and D1246Y were five (9%), 48 (86%), ten (18%), and 15 (27%), respectively. All isolates carried the wild-type nucleotide at position 1246. Results support the role of *pfmdr1* in modulating susceptibilities of the *P. falciparum* to CQ, QN, and MQ. The frequencies of the S1034C and N1042D *pfmdr1* polymorphisms and number of gene copy were significantly different in isolates collected during the two periods, with a trend of increasing prevalence of wild-type genotypes and number of gene copy from 1988 to 2003. The prominent pattern of *pfmdr1* at codons 86/184/1034/1042/1246 was NFSND, with prevalence increasing from 40% to 95% during the 10-year period.

M. Mungthin
Department of Parasitology,
Phramongkutklao College of Medicine,
Ratchathewi,
Bangkok, Thailand

N. Suwandittakul · W. Chaijaroenkul · K. Na Bangchang (✉)
Graduate Program in Biomedical Sciences.
Faculty of Allied Health Sciences, Thammasat University,
Rangsit,
Patumthani 12121, Thailand
e-mail: kesaratmu@yahoo.com

K. Rungsrihirunrat · P. Harnyuttanakorn · A. Seugorn
The College of Public Health Sciences, Chulalongkorn University,
Bangkok 10310, Thailand

Introduction

Malaria is one of the most important public health problems worldwide despite considerable efforts throughout the last century to eradicate or control the disease. Multidrug resistant *Plasmodium falciparum,* including resistance to structurally related antimalarial aminoquinolines such as chloroquine (CQ), quinine (QN), and mefloquine (MQ), is especially prevalent at the border areas of Thailand (Wongsrichanalai et al. 2002). The development and spread of drug resistance involves primarily the parasite, drug, and human host factors (Hastings et al. 2007). Due to the rapid spread of multidrug resistant falciparum malaria, antimalarial treatment policies in Thailand have been revised periodically. CQ was introduced for clinical use during the period 1965-1974 and followed thereafter by Fansidar™ (sulfadoxine/pyrimethamine), QN, and MQ, during the periods 1974-1980, 1980-1986, and 1986-1995, respectively (Wongsrichanalai et al. 2001). At present, the combination therapy of MQ and an artemisinin derivative artesunate is being used as a first-line treatment for uncomplicated falciparum malaria throughout the country (Malaria-Division 1998).

The association between gene mutation and/or amplification and resistance of *P. falciparum* to antimalarials has been controversial and debatable during the past decades, depending on geographical areas reported. CQ resistance has been linked to mutations in the *pfmdr1* and *pfcrt* genes which encode the digestive food vacuole transmembrane

proteins Pgh1 and PfCRT, respectively (Reed et al. 2000; Fidock et al. 2000). Transfection studies with *pfmdr1* indicate that mutation in Pgh1 may modulate the CQ resistance phenotype in vitro; however, in vivo studies showed inconsistent observations (van Es et al. 1994; Zakeri et al. 2008). More recently, a number of point mutations in the *pfcrt* gene have been linked with CQ resistance (Durrand et al. 2004). A mutation at amino acid position 76 (K76T) was present in all resistant isolates and was thus proposed as a molecular marker of CQ-resistant falciparum malaria (Djimde et al. 2001).

The role of *pfmdr1* mutation in conferring reduced susceptibility of the parasite to QN has been proposed. In a South America study (Sidhu et al. 2005), CQ-resistant strains carrying *pfmdr1* mutations (N86, C1034, N1042, Y1246) showed a low susceptibility to QN (Mu et al. 2003). In a Gambian study (Zalis et al. 1998), N86Y was weakly associated with decreased QN sensitivity. A molecular study has also implicated the link between *pfmdr1* N86Y variant and increased sensitivity of parasites to artemisinin (Pickard et al. 2003). Investigations on the association between *pfmdr1* gene copy number as well as polymorphisms of the *pfmdr1* gene as possible molecular markers of MQ resistance showed contradictory results (Price et al. 2004). Polymorphisms of *pfmdr1* were found to be associated with MQ resistance in some but not all studies. A higher number of gene copy in MQ-resistant isolates was observed in the two studies in Thailand (Price et al. 2004; Nelson et al. 2005; Alker et al. 2007), South America (Sidhu et al. 2005), and Africa (Basco et al. 1995).

In the present study, we investigated the patterns of mutation and amplification of *pfcrt* and *pfmdr1* genes in *P. falciparum* strains collected from Thai-Cambodian border during two periods, i.e., 1988-1993 and 2003.

Material and methods

Culture system for parasite maintenance

P. falcifarum field isolates were collected from malaria endemic areas of Thailand along Thai-Cambodian border (Trad and Chantaburi Provinces) during the two periods, i.e., 1988-1993 (35 isolates) and 2003 (21 isolates). Finger prick blood samples (100-200 μl) were collected from patients with symptomatic and mono-infection with *P. falciparum* malaria and immediately mixed with 1 ml of transporting medium (RPMI 1640, 5% $NaHCO_3$ and 10 units per miligram heparin; Thaithong et al. 1984). All isolates were continuously cultured using the methods of Trager and Jensen (Trager and Jensen 1976) with modifications. Laboratory strains of 3D7 (CQ sensitive: IC_{50} 11±1.5 nM) and K1 (CQ resistant: IC_{50} 125±15 nM) clones were used as positive control. The study protocol was reviewed and approved by the Ethics Committee of the Ministry of Public Health of Thailand.

In vitro drug susceptibility tests

Susceptibility of *P. falciparum* isolates to CQ, QN, and MQ were investigated based on the incorporation of [^{3}H] hypoxanthine into parasite nucleic acids (Desjardins et al. 1979). Highly synchronous ring stage parasite was used in each assay. An aliquot of parasite inoculum (10 μl) with 1% parasitemia and 20% hematocrit was added into each well of microtiter plate. The final hematocrit for each well was 2%. The 96-well drug plates were dosed with antimalarial drugs at a total of eight final concentrations as follows: CQ (5, 10, 25, 50, 100, 150, 250, 500 nM), QN (10, 25, 50, 100, 150, 250, 500, 1,000 nM), and MQ (1, 5, 10, 25, 50, 100, 150, 200 nM). Each assay was performed in triplicate. IC_{50} values (drug concentrations that inhibit the uptake of [^{3}H] hypoxanthine into parasite by 50%) were obtained from a log dose response curve plotted using the Grafit™ computer program (Erithacus Software Ltd., UK). The IC_{50} value was used to indicate antimalarial potency to allow a direct comparison of the activities of all the tested drugs.

Detection of *pfcrt* and *pfmdr1* polymorphisms by PCR-RFLP

Genomic DNA was extracted using Chelex-resin (Biorad Co. Ltd., USA) according to the method of Wooden and colleague (Wooden et al. 1993). Previously published nested and PCR-RFLP methods were employed to detect *pfmdr1* at the codons 86,184, 1034, 1042, and 1246 (Duraisingh and Cowman 2005; Duraisingh et al. 2000), and *pfcrt* mutations at the codons 76, 220, 271, 326, 356, and 371 (Fidock et al. 2000). The primers and reaction conditions used were according to the previously described methods (Duraisingh and Cowman 2005; Duraisingh et al. 2000; Fidock et al. 2000). PCR was performed in a total volume of 25 μl with the following reaction mixture: 0.1 μM of each primer, 2.5 mM $MgCl_2$, 100 μM deoxynucleotides, 1× PCR buffer (100 mM KCl, 20 mM Tris-HCl pH 8.0), 2 μl of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Promega™). Genomic DNA of K1 and 3D7 clones were included in each experiment as positive (mutation) and negative (wild-type) control.

Detection of gene copy number of *pfmdr1* by TaqMan real-time PCR

Pfmdr1 copy number was assessed by TaqMan real-time PCR (ABI sequence detector 7000; Applied Biosystems,

Warrington, UK). The primers and a FAM-TAMRA (6-carboxyfluorescein-6-carboxytetramethyl rhodamine) probe specific to a conserved region of *pfmdr1* and the primers and a VIC-TAMRA (VIC-6-carboxytetramethyl rhodamine) probe specific to ß-tubulin were designed with Prism 7000 sequence-detection software (Primer Express, Applied Biosystems) according to the method developed by Price (Price et al. 2004). Briefly, the amplification reactions was done as multiplex PCR in MicroAmp 96-well plates (Applied Biosystems) in a 25 µl reaction mixture containing TaqMan buffer pH 8.3 (8% glycerol, 0.625 U DNA polymerase, 5.5 mmol/l $MgCl_2$, 300 µmol/l dNTP, 600 nmol/l passive reference dye 5-carboxy-X-rhodamine), 300 nmol/l of each forward and reverse primer, 100 nmol/l of each probe, and 5 µl of template DNA. The reaction was performed for 50 cycles (95°C for 15 s and at 58°C for 1 min). Fluorescence data was expressed as normalized reporter signal, calculated by dividing the amount of reporter signal by the passive reference signal. The detection threshold was set above the mean baseline value for fluorescence of the first 15 cycles. The threshold cycle (Ct) was when the increase in reporter signal was first detected above baseline. Results were analyzed by a comparative Ct method based on the assumption that the target (*pfmdr1*) and reference (ß tubulin) were amplified with the same efficiency within an appropriate range of DNA concentrations. The comparative ΔΔCt method was used: $\Delta\Delta Ct = C_{tE} - C_{tB}$, where C_{tE} denotes the experimental C_t and C_{tB} the baseline C_t. Every TaqMan run contained two reference DNA samples from both 3D7 and K1 clones which contained only one copy numbers of *pfmdr1*. All reactions were performed in triplicate and results were rejected in case of non-exponential kinetics.

Statistical analysis

Distribution of the data was assessed using Kolmogorov-Smirnov test. Comparison of the prevalence of *pfcrt* and *pfmdr1* gene mutations in *P. falciparum* isolates collected during the two time periods was performed using chi-square test. Association between *pfmdr1* gene copy number and in vitro susceptibility of the parasites to CQ, QN, and MQ was done by student *t* test. Association between *pfmdr1* copy number and allelic polymorphisms at codons 86, 184, 1034, 1042, and 1246 and in vitro susceptibility of *P. falciparum* isolates to CQ, QN, and MQ was assessed using Kruskal Wallis and Mann-Whitney *U* tests. The molecular markers influencing in vitro drug susceptibility level was determined by binary logistic regression. Statistical significance level was set at $\alpha = 0.05$ for all tests.

Results

In vitro drug susceptibility of *P. falciparum* isolates

A total of 56 *P. falciparum* isolates collected from symptomatic malaria patients with mono-infections during the two periods, i.e., 1988-1993 (35 isolates) and 2003 (21 isolates), were investigated for their susceptibility to CQ, QN, and MQ. The criteria for discrimination between the resistant and sensitive isolates to CQ, QN, and MQ followed the previously described criteria (Cerutti et al. 1999; Pickard et al. 2003). CQ susceptibility was categorized into three levels, i.e., sensitive (S: IC_{50}<25 nM), moderately resistant (MR: 25≤IC_{50} < 100 nM), and highly resistant (HR: IC_{50}≥100 nM). QN susceptibility was categorized into two levels, i.e., sensitive (S: IC_{50}< 500 nM), and resistant (R: IC_{50}≥500 nM). MQ susceptibility was categorized into two levels, i.e., S (IC_{50} ≤ 24 nM), and R (IC_{50}>24 nM). The range of IC_{50} values for CQ varied from 27 to 174 nM, with geometric mean (95% CI) value of 88 (79-99) nM. Twenty-eight (50%) isolates were classified as MR and HR. The IC_{50} values for QN ranged from 36 to 483 nM, with geometric mean (95%CI) value of 159 (135-187) nM. All isolates were categorized as QN-sensitive. The IC_{50} values for MQ ranged from 3 to 110 nM, with the mean (95% CI) values of 16 (13-21)nM. Thirty-six (64%), and 20 (36%) isolates were categorized as MQ-sensitive and MQ-resistant, respectively. The drug susceptibility profiles of *P. falciparum* isolates collected during the two periods to all drugs except MQ were similar. MQ susceptibility of parasite isolates collected during the period 1988-1993 was significantly higher (lower IC_{50}) when compared with those collected during the year 2003. (Table 1)

Polymorphisms of *pfmdr1* and *pfcrt* and *pfmdr1* copy number

Pfcrt polymorphisms were commonly observed in the isolates studied. The distribution of patterns of *pfmdr1* and *pfcrt* polymorphisms was shown in Fig. 1. The prevalence of the mutation at codons K76T, A220S, Q271E, N326S, and R371I were virtually 100%; only one isolate carried wild-type allele at codon 356 (I356). The prevalence of *pfcrt* polymorphism observed during both periods was similar, with TSESTI being the most prevalent haplotype.

The prevalence of *pfmdr1* polymorphisms at codons N86Y, Y184F, S1034C, and N1042D were five (9%), 48 (86%), ten (18%), and 15 (27%), respectively. All isolates carried wild-type allele at codon 1246 (D1246). The *pfmdr1* polymorphisms of the isolates collected during 1988-1993

Table 1 Susceptibility of *P. falciparum* isolates collected during the two periods (1988-1993 and 2003) to CQ, QN, and MQ

		IC_{50} (geometric mean (95% CI) nM)	Classification of susceptibility profiles *n* (%)
CQ	Total (*n*=56)	88 (79-99)	MR 28 (50%), HR 28 (50%)
	1988-1993 (*n*=35)	89 (67-103)	MR 18 (51%), HR 17 (49%)
	2003 (*n*=21)	88 (72-106)	MR 10 (48%), HR 11 (52%))
QN	Total (*n*=56)	159 (135-187)	S 56 (100%)
	1988-1993 (*n*=35)	164 (129-208)	S 35 (100%)
	2003 (*n*=21)	150 (121-186)	S 21 (100%)
MQ[a]	Total (*n*=56)	16 (13-21)	S 36 (64%); R 20 (36%)
	1988-1993 (*n*=35)	13 (9-18)	S 27 (77%); R 8 (23%)
	2003 (*n*=21)	26 (19-34)	S 9 (43%); R 12 (57%)

Data are presented as IC_{50} (geometric mean (95%CI) and range) and number (%) of isolates categorized as S (sensitive), R (resistant), MR (moderately resistant), and HR (highly resistant)

[a] Significantly different in drug susceptibility between parasite isolates collected during 1988-1993 and 2003 (p=0.011; Kruskal Wallis and Mann-Whitney U tests at a significance level of α=0.05)

were more diverse than that of 2003; 6 and 2 haplotypes of *pfmdr1* were found in the isolates collected during 1988-1993 and 2003, respectively. The NFSND was the most prevalent haplotype found in both periods. A total of 19 isolates (34%) carried only one *pfmdr1* gene copy, whereas 37 isolates (66%) carried more than one gene copies.

The associations between antimalarial drug susceptibility, genetic polymorphisms and gene amplification of *P. falciparum*

No association between *pfcrt* polymorphisms and the level of resistance of antimalarial drugs was found. All of the parasite isolates which were either CQ-resistant or CQ-sensitive carried mutant alleles at the codons 76, 220, 271, 326, and 371.

The association between antimalarial drug susceptibility of *P. falciparum* isolates, *pfmdr1* polymorphisms and gene amplification was summarized in Table 2. The mutation of *pfmdr1* at codon 1034 was found to be associated with the level of CQ resistance, whereas the mutations at the codons 184 and 1042 were associated with QN resistance. The mutations at the codons 1034 and 1042 improved susceptibility of the parasite to MQ, while the mutation at codon 184 led to decreased susceptibility to the drug. The parasite isolates containing more than one *pfmdr1* gene copy exhibited decreased susceptibility to MQ comparing to those carrying only one gene copy.

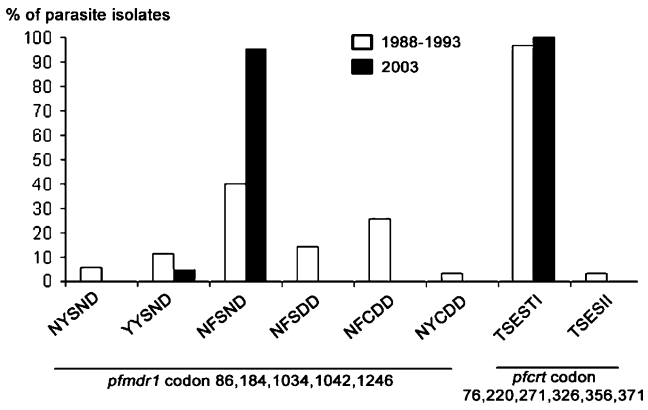

Fig. 1 The distribution of *pfmdr1* and *pfcrt* haplotypes during the periods 1988-1993 and 2003

Pfcrt and *pfmdr1* polymorphism/amplification patterns among parasite isolates collected during the two periods are summarized in Table 3. The mutation of *pfcrt* in *P. falciparum* isolates was stable with low prevalence during the observed 10-year period. The prevalence of N86Y, S1034C, and N1042D, but Y184F mutations were lower during the year 1988-2003 compared with 2003. Significant association between *pfmdr1* polymorphisms and time periods was found only with the two codons, with the prevalence of the wild-type S1034C and N1042D polymorphisms significantly higher in isolates collected during 2003 compared with 1988-1993. In addition, isolates collected in 2003 with more than one *pfmdr1* gene copy were found to be more prevalent compared with the period 1988-1993.

Discussion

The present study investigated in vitro susceptibility profiles in association with patterns of molecular markers of resistance of *P. falciparum* isolates collected from the Thai-Cambodian border during the two periods (1988-1993 and 2003) to CQ, MQ, and QN. Our data showed no significant change in the pattern of mutations of *pfcrt* gene which is the key determinant of CQ resistance. The observation of high prevalence of mutations of *pfcrt* in almost all isolates at the codons A220S, Q271E, N326S,

Table 2 The association between *pfmdr1* polymorphisms, gene copy number and susceptibility of the parasite isolates to CQ, QN, and MQ

Pfmdr 1		Geometric mean IC_{50} (95% CI) nM		
		CQ[a]	QN[B]	MQ[C]
Codon 86	Wild type (n=51)	90 (80-101)	167 (141-197)	18 (14-23)
	Mutation (n=5)	71 (34-148)	97 (40-236)	8 (4-18)
Codon 184	Wild type(n=8)	73 (45-119)	80 (48-133)	8 (5-12)
	Mutation (n=48)	91 (81-102)	178 (152-208)	19 (14-24)
Codon 1034	Wild type (n=46)	83 (73-95)	149 (124-179)	21 (16-27)
	Mutation (n=10)	116 (99-137)	211 (140-318)	6 (4-8)
Codon 1042	Wild type (n=41)	84 (73-97)	141 (116-171)	23 (18-30)
	Mutation (n=15)	101 (82-123)	219 (164-293)	7 (5-9)
Codon 1246	Wild type (n=56)	88 (79-99)	159 (135-187)	16 (13-21)
Copy number	1 (n=37)	87 (75-102)	159 (128-196)	14 (10-19)
	>1 (n=19)	90 (76-106)	159 (119-211)	24 (17-34)

Data are presented as IC_{50} (geometric mean (95%CI)) and number of isolates (n); the statistical test was performed by Kruskal Wallis and Mann-Whitney U tests at a statistical significance level of a<0.05

[a] Statistically significant difference in susceptibility of the parasite isolates with pfmdr1 polymorphisms at codon 1034 (p=0.031)

[b] Statistically significant difference in susceptibility of the parasite isolates with pfmdr1 polymorphisms at codon 184 (p=0.005) and 1042 (p=0.010)

[c] Statistically significant difference in susceptibility of the parasite isolates with pfmdr1 polymorphisms at codon 184 (p=0.010), 1034 (p<0.001), 1042 (p<0.001), and copy number (p=0.026)

and R371I, was in line with the in vitro susceptibility data found in the present study as well as other previous studies (Chen et al. 2001, 2003; Fidock et al. 2000). The previous study reported virtually 100% mutation at codon T76 in all, except one isolate obtained from Chantaburi Province close to Thai-Cambodian border (Congpuong et al. 2005). This might suggest that, *pfcrt* polymorphism was sufficient to CQ-resistance but apart from *pfcrt*, other gene(s) may also play role in mediate level of CQ resistance.

Table 3 Distribution of *pfmdr1* polymorphisms of parasite isolates collected from different time periods

Pfmdr 1		1988-1993 (n=35)	2003 (n=21)
Codon 86	Wild type	31 (88.6)	20 (95.2)
	Mutation	4 (11.4)	1 (4.8)
Codon 184	Wild type	7 (20)	1 (4.8)
	Mutation	28 (80)	20 (95.2)
Codon 1034[a]	Wild type	25 (71.4)	21 (100)
	Mutation	10 (28.6)	0
Codon 1042[b]	Wild type	20 (57.1)	21 (100)
	Mutation	15 (42.9)	0
Codon 1246	Wild type	35 (100)	21 (100)
	Mutation	0	0
Copy number[c]	1	29 (82.9)	8 (38.1)
	>1	6 (17.1)	13 (61.9)

Data are presented as number of cases (n) and percentage (%) values

[a] Significant difference between 1988-1993 and 2003 (p=0.007; chi-square test at a significance level of α=0.05)

[b] Significant difference between 1988-1989 and 2003 (p<0.001; chi-square test at a significance level of α=0.05)

[c] Significant difference between 1988-1989 and 2003 (p=0.001; chi-square test at a significance level of α=0.05)

Numerous in vitro studies have found the association between both copy number and polymorphism of the *pfmdr1* gene and in vitro antimalarial drug susceptibility (Price et al. 1999; Chaiyaroj et al. 1999; Duraisingh and Cowman 2005; Pickard et al. 2003; Price et al. 2004). The current results support the role of *pfmdr1* in modulating the degree of CQ, MQ, and QN susceptibility. *Pfmdr1* SNPs have been proposed as mediating CQ resistance (corresponding to substitutions N86Y, Y184F, S1034C, N1042D, and D1246Y). In the present study, all these *pfmdr1* polymorphisms were identified in *P. falciparum* isolates from Thailand collected during the period 1988-1993 and 2003. The majority of the isolates (91%, 83%, 74%, and 100%) carried wild-type (N86, S1034, N1042, D1246) at the codons 86, 1034, 1042, and 1246, respectively. The percentage of isolates carried mutant (Y184F) at codon 184 was 86%. All isolates were identified as wild-type (D1246) at codon 1246, which is in agreement with that previously reported in isolates from Thailand (Chaiyaroj et al. 1999; Congpuong et al. 2005; Price et al. 1999). These polymorphisms were found to be generally associated with reduced parasite susceptibility to CQ, QN, and MQ, except for the mutation at codons 1034 and 1042 which were associated with improved sensitivity to MQ.

Gene amplification alone was found to be associated with reduced susceptibility of the parasite isolates to MQ. It has been suggested that the increase in *pfmdr1* copy number might cause resistance to both artesunate and mefloquine (Price et al. 2004; Alker et al. 2007). In one study, *pfmdr1* copy number was found to be associated with treatment failure following the combination regimen of mefloquine-artesunate but not with mefloquine-artemether (Price et al. 2006).

The prevalence of *pfmdr1* polymorphisms including gene copy number of the wild types S1034C, N1042D were significantly different during the two observation periods. There was a trend of increasing prevalence of isolates with wild-type genotypes at these codons including that with more than one gene copy from the year 1988 towards 2003, and this appeared correlated with the decline in MQ susceptibility. In the absence of CQ pressure during the 10-year observation period, the prevalence of the dominant pattern of the wild-type *pfmdr1* polymorphism NFSND (codon 86/184/1034/1042/1246) was significantly increased.

Our results showed that *pfmdr1* polymorphisms influenced CQ susceptibility level, but did not resume the parasite susceptibility to sensitive level. Except for S1034C mutation, no association between *pfmdr1* polymorphisms (N86Y, Y184F, N1042D, and D1246Y) and CQ susceptibility was found. All these results signify the possibility of the hypothesis that *pfcrt* mutation is the key determinant of CQ susceptibility but also requires involvement of the interactions with other genes. Conclusion on the association between *pfmdr1* allele polymorphisms and CQ resistance had been contradictory among various studies, with either positive association (Basco et al. 1995), or lack of association (Adagu et al. 1995; Chaiyaroj et al. 1999; Wilson et al. 1993). The study by Reed et al. (2000) was later on, definitively confirmed its involvement in high level CQ resistance in such a way that mutation of *pfmdr1* could modify CQ sensitivity only when present in a parasite expressing other resistance genes such as *pfcrt* (Reed et al. 2000). In addition, Sidhu et al. (2005) found that the introduction of *pfmdr1* three mutations into the CQ-resistant 3BA6 line did not noticeably alter the degree of CQ resistance. These data suggest that the contribution of *pfmdr1*to CQ-resistant may only apply to a limited subset of genetic backgrounds (Sidhu et al. 2005).

The influence of *pfmdr1* gene amplification on antimalarial susceptibility was observed with MQ in this study. Reduced susceptibility to MQ was found in the isolates carrying more than one *pfmdr1* gene copies compared with those carrying only one copy. A few experiments selecting for resistance to MQ in vitro have demonstrated the influence of *pfmdr1* gene amplification and over-expression on reduced sensitivity of the parasite to MQ (Sidhu et al. 2006).

Similar to CQ, susceptibility of the parasite to QN could also be influenced by multiple genes. We found stable pattern of QN susceptibility in parasite isolates collected from Thailand during the 10-year period. QN susceptibility does not seem to be linked with the *pfmdr1* gene amplification in several studies of field isolates (Anderson et al. 2005; Pickard et al. 2003; Price et al. 2004). However, Sidhu et al (Sidhu et al. 2006) highlighted the importance of *pfmdr1* copy number in determining *P. falciparum* susceptibility to QN using knockdown technique. Moreover, there was also evidence showing that QN sensitivity was modulated by other genes (Ferdig et al. 2004; Mu et al. 2003). Cooper et al (Cooper et al. 2007) selected parasite lines to become more resistant to QN and found that QN resistance was associated with *pfcrt* mutation that resulted in amino acid changes in PfCRT. Furthermore, SNPs in nine of 49 transporter gene sequences of *P. falciparum* were identified found to be related to QN susceptibility (Mu et al. 2003). However, there was no association between any of those genes and QN response in a cohort of samples from near the Thai-Myanmar border (Anderson et al. 2005)

Acknowledgements The study was supported by Pramongkutklao Medical College and The Commission on Higher Education, Ministry of Education of Thailand. We are grateful to Malaria Research Unit, Chulalongkorn University, Thailand for parasites isolates.

References

Adagu IS, Warhurst DC, Carucci D, Duraisingh MT (1995) Antimalarial drug response of *Plasmodium falciparum* from Zaria, Nigeria. Trans R Soc Trop Med Hyg 89:442–445

Alker AP, Lim P, Sem R, Shah NK, Yi P, Bouth DM, Tsuyuoka R, Maguire JD, Fandeur T, Ariey F, Wongsrichanalai C (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

Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F (2005) Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr1*) associated with antimalarial drug resistance? Antimicrob Agents Chemother 49:2180–2188

Basco LK, Le Bras J, Rhoades Z, Wilson CM (1995) Analysis of *pfmdr1* and drug susceptibility in fresh isolates of *Plasmodium falciparum* from sub-Saharan Africa. Mol Biochem Parasitol 74:157–166

Cerutti C Jr, Marques AC, de Alencar FEC, Durlacher RR, Alween A, Segurado AAC, Pang L, Zalis MG (1999) Antimalarial drug susceptibility testing of *Plasmodium falciparum* in Brazil using a radioisotope method. Mem Inst Oswaldo Cruz 94:803–809

Chaiyaroj SC, Buranakiti A, Angkasekwinai P, Looressuwan S, Cowman AF (1999) Analysis of mefloquine resistance and amplification of *pfmdr1* in multidrug-resistant *Plasmodium falciparum* isolates from Thailand. Am J Trop Med Hyg 61:780–783

Chen N, Russell B, Staley J, Kotecka B, Nasveld P, Cheng Q (2001) Sequence polymorphisms in *pfcrt* are strongly associated with

chloroquine resistance in *Plasmodium falciparum*. J Infect Dis 183:1543–1545

Chen N, Kyle DE, Pasay C, Fowler EV, Baker J, Peters JM, Cheng Q (2003) *Pfcrt* Allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. Antimicrob Agents Chemother 47:3500–3505

Congpuong K, Na Bangchang K, Mungthin M, Bualombai P, Wernsdorfer WH (2005) Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* malaria in Thailand. Trop Med Int Health 10:717–722

Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Wellems TE (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. Mol Pharmacol 61:35–42

Cooper RA, Lane KD, Deng B, Mu J, Patel JJ, Wellems TE, Su X, Ferdig MT (2007) Mutations in transmembrane domains 1, 4 and 9 of the *Plasmodium falciparum* chloroquine resistance transporter alter susceptibility to chloroquine, quinine and quinidine. Mol Microbiol 63:270–280

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD (1979) Quantitative assessment of antimalarial activity in vitro by a semi-automated microdilution technique. Antimicrob Agents Chemother 16:710–718

Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulibaly D (2001) A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 344:257–263

Duraisingh MT, Cowman AF (2005) Contribution of the *pfmdr 1* gene to antimalarial drug-resistance. Acta Trop 94:181–190

Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC (2000) The tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisisnin. Mol Biochem Parasitol 108:13–23

Durrand V, Berry A, Sem R, Glaziou P, Beaudou J, Fandeur T (2004) Variations in the sequence and expression of the *Plasmodium falciparum* chloroquine resistance transporter (Pfcrt) and their relationship to chloroquine resistance in vitro. Mol Biochem Parasitol 136:273–285

Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE (2004) Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol 52:985–997

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, Wellems 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

Hastings I, Korenromp E, Bloland P (2007) The anatomy of a malaria disaster: drug policy choice and mortality in African children. Lancet Infect Dis 7:739–748

Johnson DJ, Fidock DA, Mungthin M, Lakshmanan V, Sidhu AB, Bray PG, Ward SA (2004) Evidence for a central role for PfCRT in conferring *Plasmodium falciparum* resistance to diverse antimalarial agents. Mol Cell 15:867–877

Malaria Division (1998) Malaria Control Programme in Thailand. In.: Malaria Division, Department of Communicable Disease Control (CDC) of the Ministry of Public Health, Thailand

Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC, Xiong M, Su XZ (2003) Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol 49:977–989

Nelson AL, Purfield A, McDaniel P, Uthaimongkol N, Buathong N, Sriwichai S, Miller RS, Wongsrichanalai C, Meshnick SR (2005) *Pfmdr 1* genotyping and in vivo mefloquine resistance on the Thai-Myanmar border. Am J Trop Med Hyg 72:586–592

Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR (2003) Resistance to antimalarials in Southeast Asia and genetic polymorphisms in *pfmdr 1*. Antimicrob Agents Chemother 47:2418–2423

Price RN, Cassar C, Brockman A, Duraisingh M, Van Vugt M, White NJ, Nosten F, Krishna S (1999) The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. Antimicrob Agents Chemother 43:2943–2949

Price RN, Uhlemann AC, 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 increased *pfmdr1* gene copy number. Lancet 364:438–447

Price RN, Uhlemann AC, Wan 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

Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403:906–909

Sidhu AB, Valderramos SG, Fidock DA (2005) *Pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol Microbiol 57:913–926

Sidhu AB, Uhlemann AC, Valderramos SG, Valderramos JC, Krishna S, Fidock DA (2006) Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. J Infect Dis 194:528–535

Thaithong S, Beale GH, Fenton B, McBride J, Rosario V, Walker A, Walliker D (1984) Clonal diversity in a single isolate of the malaria parasite *Plasmodium falciparum*. Trans R Soc Trop Med Hyg 78(2):242–245

Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. Science 193:673–675

Van Es HH, Renkema H, Aerts H, Schurr E (1994) Enhanced lysosomal acidification leads to increased chloroquine accumulation in CHO cells expressing the *pfmdr1* gene. Mol Biochem Parasitol 68:209–219

Wilson CM, Volkman SK, Thaithong S, Martin SK, Kyle DE, Milhous WK, Wirth DF (1993) Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. Mol Biochem Parasitol 57:151–160

Wongsrichanalai C, Sirichaisinthop J, Karwacki JJ, Congpuong K, Miller RS, Pang L, Thimasarn K (2001) Drug resistant malaria on the Thai-Myanmar and Thai-Cambodian borders. Southeast Asian J Trop Med Public Health 32:41–49

Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR (2002) Epidemiology of drug-resistant malaria. Lancet Infect Dis 2:209–218

Wooden J, Kyes S, Sibley CH (1993) PCR and strain identification in *Plasmodium falciparum*. Parasitol Today 9:303–305

Zakeri S, Afsharpad M, Kazemzadeh T, Mehdizadeh K, Shabani A, Djadid ND (2008) Association of *pfcrt* but not *pfmdr1* alleles with chloroquine resistance in Iranian isolates of *Plasmodium falciparum*. Am J Trop Med Hyg 78:633–640

Zalis MG, Pang L, Silveira MS, Milhous WK, Wirth DF (1998) Characterization of *Plasmodium falciparum* isolated from the Amazon region of Brazil: evidence for quinine resistance. Am J Trop Med Hyg 58:630–637