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Genetic polymorphisms of candidate markers and *in vitro* susceptibility of *Plasmodium falciparum* isolates from Thai-Myanmar border in relation to clinical response to artesunate–mefloquine combination

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ABSTRACT

The genetic polymorphisms of the candidate markers of antimalarial drug resistance *pfcrt*, *pfmdr1*, *pfatp6*, and *pfmrp1* were investigated in relationship with *in vitro* susceptibility of *Plasmodium falciparum* isolates and clinical response following artesunate (AS)–mefloquine (MQ) combination in 21 and 27 samples obtained from patients with recrudescence and adequate clinical response, respectively. MQ (21.0 vs. 49.9 nM) and AS (1.6 vs. 2.8 nM) IC_{50} values (concentrations that inhibit parasite growth by 50%) were significantly higher in isolates collected from patients with recrudescence. Furthermore, a significantly higher MQ IC_{50} was found in isolates from patients with recrudescence that carried *pfmrp1* mutations at amino acid residues 191Y, 437A, and 876V. For AS sensitivity, a significant association was also detected in isolates from patients with recrudescence that carried gene mutations at amino acid residues 437A and 876V. MQ IC_{50} of the isolates with recrudescence which carried ≥4 *pfmdr1* gene copies was significantly higher than that carrying only one gene copy. In addition, a significantly higher proportion of isolates carrying one gene copy was detected in the group with adequate clinical response compared with recrudescence. Results from this limited sample size suggested the potential link between *pfmdr1* gene copy number and *pfmrp1* gene mutation, *in vitro* parasite susceptibility, and AS–MQ treatment response.
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1. Introduction

Artemisinin combination therapy (ACT) is being recommended as first-line treatment for uncomplicated *Plasmodium falciparum* malaria in all malaria endemic areas of the world. In Thailand, the combination of artesunate–mefloquine was adopted as a replacement of mefloquine monotherapy in 1995 to cope with multidrug resistance *P. falciparum*, initially as a 2-day combination regimen (Na-Bangchang and Congpuong, 2007). Recent reports from Cambodia, Myanmar, Vietnam, and Thailand have provided evidence of declining in sensitivity of the parasite to ACT and possibility of artemisinin resistance (Noedl et al., 2008; WHO, 2012). Monitoring the therapeutic efficacy of ACTs is therefore essential for detecting an early change in sensitivity of *P. falciparum* to artemisinins and combination partners and thus, the adjustment of treatment policy (WHO, 2012).

Tracking of gene mutations responsible for resistance of *P. falciparum* to artemisinins is one approach to detect the change in parasite sensitivity to this group of drugs. Several current molecular analyses of *P. falciparum* isolates provide evidence for possible link between the polymorphisms of several candidate genes and artemisinin resistance. These include the gene encoding sarco/endoplasmic reticulum Ca^{2+}-ATPase ortholog of *P. falciparum* (*pfatp6*) (Eckstein-Ludwig et al., 2003; Jambou et al., 2005; Price et al., 2004; Uhlemann and Krishna, 2005), *P. falciparum* multidrug resistance 1 (*pfmdr1*) (Duraisingh et al., 2000; Lim et al., 2009; Sidhu et al., 2005), and *P. falciparum* multidrug resistance protein 1 (*pfmrp1*) (Dahlstrom et al., 2009a; Klokouzas et al., 2004; Mu et al., 2003). Among these, *pfmdr1* gene on chromosome 7 which encodes an ATP-binding cassette (ABC) transporter on the digestive vacuole

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has received the most attention. Several point mutations (at codons N86Y, F184Y, S1034C, N1042D, and D1246Y) or increased in gene copy number have been shown to be associated with the change in sensitivity of *P. falciparum* field isolates to artemisinins as well as to mefloquine chloroquine, quinine, and halofantrine (Cowman et al., 1994; Duraisingh et al., 2000; Foote et al., 1990; Lim et al., 2009; Mungthin et al., 2010a; Price et al., 1999, 2004; Reed et al., 2000; Wilson et al., 1993).

The aim of the present study was to investigate the relationship between *pfcrt, pfmdr1*, *pfatp6*, and *pfmrp1* gene polymorphisms and *in vitro* susceptibility of *P. falciparum* isolates, as well as clinical response following mefloquine (MQ)–artesunate (AS) combination in an area of multidrug resistant *P. falciparum* along the Thai-Myanmar border.

2. Materials and methods

2.1. Blood samples

Investigation of polymorphisms of the candidate molecular markers of antimalarial drug resistance and *in vitro* sensitivity test was performed in a total of 48 *P. falciparum* isolates (culture-adapted) obtained from Burmese patients (24 males and 24 females, aged between 16 and 45 years) with acute uncomplicated *P. falciparum* infection (Na-Bangchang et al., 2010). All patients received treatment with a 3-day combination regimen of 25 mg/kg body weight mefloquine and 12 mg/kg body weight artesunate. The median (95% CI) admission parasitaemia was 6230 (3446–9014) per μl blood (WHO, 2003). Twenty-seven and 21 cases, respectively, had adequate clinical response and PCR-confirmed recrudescence (with genotyping markers *pfmsp1*, *pfmsp2*, and *pfglurp*) during the 42-day follow-up period. A significant prolongation of parasite and fever clearance time (PCT and FCT) was observed in patients with recrudescence compared with adequate clinical response [median (95% CI): 32.0 (29.0–35.0) vs. 26.0 (25.0–27.0) h, and 32.0 (29.0–35.0) vs. 26.0 (25.0–27.0) h, respectively]. The study protocol was approved by the Ethics Committee of Ministry of Public Health of Thailand and written informed consents for study participation were obtained from all patients before study.

2.2. Culture system and in vitro susceptibility assay

All parasite isolates were adapted to continuous culture according to the method of Trager and Jensen (1976) with modification. The laboratory-adapted 3D7 (chloroquine sensitive) and K1 (chloroquine resistant), *P. falciparum* were used as control parasite clones. Susceptibility of *P. falciparum* isolates to MQ and AS were investigated using SYBR Green I assay (Bennett et al., 2004; Smilkstein et al., 2004). Highly synchronous ring stage parasites were used in each assay. An aliquot of parasite inoculum (50 μl) with 2% parasitemia and 1% hematocrit was added into each well of the 96-well microtiter plate. The plate was pre-dosed with each antimalarial drug at a total of eight final concentrations as follows: MQ (3.13, 6.25, 12.5, 25, 50, 100, 200, and 400 nM); AS (0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 nM). AS and MQ hydrochloride were purchased from Sigma-Aldrich, Co. (St.Louis, MO, USA). All compounds were stored at −20 °C as 10 mM stock solutions in 50% ethanol. The experiments were repeated three times, triplicate for each experiment. IC_{50} value was used as an indicator for antimalarial susceptibility and was determined from a log-dose response analysis using the CalcuSyn™ computer program (Biosoft, Cambridge, UK). Susceptibility of the parasite isolates to MQ was categorized into two levels, *i.e.*, "sensitive" ($IC_{50} \leq 24$ nM) and "resistant" ($IC_{50} > 24$ nM) (Chaijaroenkul et al., 2010). For AS, the susceptibility was classified as "sensitive" ($IC_{50} \leq$ upper limit of 95% CI of the median IC_{50}) and "declined sensitivity" (IC_{50} > upper limit of 95% confidence interval (CI) of the median IC_{50}) (Phompradit et al., 2011).

2.3. Investigation of pfcrt, pfmdr1, pfatp6, and pfmrp1 gene mutations using PCR-RFLP

Genomic DNA was extracted from all samples (culture-adapted or dried blood spot) using chelex resin modified technique (Wooden et al., 1993). Prior to using as a DNA template, concentration of the malaria genomic DNA was determined by spectrophotometry (Nanodrop™, Thermo fisher Scientific, Massachusetts, USA).

Previously published nested and PCR-RFLP methods were employed to detect the polymorphisms of *pfcrt* gene at amino acid residues 76, 220, 271, 326, 356, and 371 (Fidock et al., 2000); *pfmdr1* gene atresidues 86,184,1034, 1042, and 1246 (Duraisingh and Cowman, 2005; Duraisingh et al., 2000), *pfatp6* gene at codons 37, 693, 769, and 898 (Ferreira et al., 2008); *pfmrp1* gene at codons 191 and 437 (Holmgren et al., 2006), and *pfmrp1* gene at codons 876, 1390, and 1466 (Pirahmadi et al., 2013).

2.4. Detection of pfatp6 and pfmdr1 gene copy number by SYBR Green I real-time PCR

Pfatp6 and pfmdr1 gene copy number was determined by SYBR Green I real-time PCR (iCycler™, Bio-Rad, California, USA) using the default thermocycler program: 10 min of pre-incubation at 95 °C, followed by 40 cycles for 15 s at 95 °C and 1 min at 60 °C. The oligonucleotide primers used were those previously designed by Ferreira et al. (2006) with modification. Individual real-time PCR reaction was carried out in a 25 μl reaction volume in a 96-well plate containing 1 μM each of sense and antisense primer and 12.5 μl of Platinum™ PCR SuperMix (Invitrogen, California, USA).

The $2^{-\Delta\Delta Ct}$ method of relative quantification was adapted to estimate copy number in *P. falciparum* genes. The genomic DNA extracted from *P. falciparum* 3D7 clone known to harbor a single copy of each gene was used as a calibrator, while Pf-β-actin 1 served as the house-keeping gene in all experiments. Dd2 genomic DNA carrying four copies of *pfmdr1* was used as a second calibrator. The threshold cycle (Ct) was determined as the increase in reporter signal which was first detected above baseline. Results were analyzed by a comparative Ct method based on the assumption that the target (*pfatp6* and *pfmdr1*) and reference (pf-β-actin 1) genes were amplified with the same efficiency within an appropriate range of DNA concentrations.

The ΔΔCt calculation for the relative quantification of target was as followed: ΔΔCt = (Ct, target gene − Ct, Pf-β- actin1)$_x$ − (Ct, target gene − Ct, Pf-β-actin1)$_y$, where x represents unknown sample and y represents *P. falciparum* 3D7 clone. Results for each sample was expressed as an *N*-fold change in χ target gene copies, normalized to Pf-β-actin-1 relative to the copy number of the target gene in *P. falciparum* 3D7 clone, according to the following equation: amount of target = $2^{-\Delta\Delta Ct}$. A minimum of two experiments were carried out for each gene and each sample. In each experiment, each individual sample was analyzed in duplicate wells and the Ct of each well was recorded at the end of the reaction.

2.5. Statistical analysis

The association between *in vitro* susceptibility of *P. falciparum* isolates to MQ and AS (IC_{50}) in patients with adequate clinical response and recrudescence following treatment with AS–MQ combination was performed using Chi-square test. The association between *pfcrt, pfmdr1*, *pfmrp1*, and *pfatp6* polymorphisms and *in vitro* parasite susceptibility to MQ and AS and between gene

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