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Pfatp6 polymorphisms of *Plasmodium falciparum* isolates in Thailand and association with *in vitro* sensitivity to artesunate and mefloquine

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Abstract

The sarco/endoplasmic reticulum Ca^{2+}- ATPase of *Plasmodium falciparum* or *pfatp6* has been proposed to be a target for artemisinin and derivatives which are currently used worldwide to combat the emergence of multi-drug resistance *P. falciparum*. Nevertheless, reports of clinical treatment failure with supplemented data on single-nucleotide polymorphisms (SNPs) of *P.falciparum* genes associated with resistance have been increasing in malaria endemic areas including Thailand. In this study, we investigated the association between *Pfatp6* polymorphisms, and *in vitro* sensitivity in a total of 63 *P. falciparum* isolates collected from the Thai-Myanmar border, to artesunate and mefloquine. All isolates were adapted to continuous culture *in vitro* and assessed for their susceptibility to artesunate and mefloquine. Malarial parasite DNA was extracted from blood samples using Chelex-100 assay. Polymorphism of *pfatp6* at codons R37K, G639D, S769N and I898I were analyzed based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Gene copy number of all isolates was analyzed by quantitative real time-polymerase chain reaction (qRT-PCR). Results showed no mutation of *pf*ATP6 gene at any codon investigated. In this limited number of isolates under investigation, no association between SNP and amplification of *pfatp6* gene and *in vitro* sensitivity of *P.falciparum* isolates to artesunate and mefloquine.

Keywords**:** *Plasmodium falciparum*, *pfATPase6*, single-nucleotide polymorphism, gene amplification, artemisinin, drug resistance, PCR-RFLP

Introduction

Multidrug resistance *Plasmodium falciparum* including resistance to structurally related antimalarials such as chloroquine, quinine and mefloquine, is still problematic along the border areas of Thailand, especially Thai-Myanmar border (1). A 3-day artesunate (ARS)-mefloquine (MQ) combination is currently being used as the first-line treatment of uncomplicated falciparum malaria to cope with the situation of multi-drug resistance (1). Recently however, there has been a report of modest increase in resistance of this combination in areas along the Thai-Cambodian and Thai-Myanmar borders (2-5). Apart from assessment of clinical response, *in vitro* sensitivity test together with molecular surveillance system can help target *in vivo* studies to where they are needed the most. Polymorphisms of genes involved in vial process of malaria parasite are suggested as the key factor contributing to drug resistance. Among these, the sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA) PfATP6 is proposed to be an important target for artemisinin and derivatives. Nevertheless, recent observations revealed that polymorphisms (single nucleotide polymorphism: SNP and gene amplification) in this gene were associated with *in vitro* resistance of *P. falciparum* to artemisinin derivatives (6). In addition, amplification of this gene has also been linked with both artesunate and mefloquine resistance (6). In the present study, we investigated the association between *in vitro* sensitivity to artesunate and mefloquine, and the polymorphisms of *pfatp6* gene in in a total of 63 *P. falciparum* isolates collected from an area along the Thai-Myanmar border during 2007-2009.

Methods

Venous blood samples (3 ml) were collected from patients attending malaria clinics in areas along the Thai-Myanmar border after microscopically confirmation of *P.falciparum* mono-infection. Approval of the study protocol was obtained from the Ethics Committee of Ministry of Public Health of Thailand. Written informed consents were obtained from all patients prior to blood collection. *P.falciparum* isolates were adapted to culture *in vitro* (7) for sensitivity testing to artesunate and mefloquine, as well as for investigation of single nucleotide polymorphism (SNP) and amplification of the *pfatp6* gene. *In vitro* sensitivity testing (3 independent experiments, triplicate each) of all isolates to artesunate (concentration range 0.39-50 nM), and mefloquine (concentration range 1.56-200 nM) was performed in a 96-well microtiter pate based on SYBR green I-based Assay (8), in order to obtain the IC_{50} values (concentrations that produce 50% inhibition of parasite growth). SNPs of *pfatp6* gene at codons S769N, R37K, G639D and I898I were examined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method (9). DNA sequencing of *pf*ATP6 and quantitative real time- PCR (10) were performed in 5 isolates with different degree of *in vitro* sensitivity to artesunate.

Results

***In vitro* Sensitivity Testing:** *In vitro* sensitivity test was successful in a total of 50 out of 63 isolates collected. Mean ± SD IC_{50} values for artesunate and mefloquine were 2.21±0.99 and 43.12±26.12 nM, respectively. All were classified as sensitive to artesunate and 68% (34/50) were classified as resistance to mefloquine (IC_{50} >24 nM) (11). There were 9 isolates with marked decline in sensitivity to artesunate (mean $\pm$ SD IC_{50} =3.66$\pm$0.46 nM); mean$\pm$SD IC_{50} of 26 and 15 isolates were between 1.42$\pm$0.27, and 2.50$\pm$0.28 nM, respectively.

SNPs and Amplification of pfatp6: All isolates carried wild-type allele *pfatp6* (Table 1). All of the five isolates selected based on *in vitro* sensitivity to artesunate (IC_{50} =4.41, 3.10, 2.62, 2.46 and 1.22 nM) carried only a single copy of the *pfatp6* gene.

Table 1 Prevalence of SNP in *pfatp6* gene

Gene	***pfatp6* Codon**							
Target allele	**37R**	**37K**	**693G**	**693D**	**769S**	**769N**	**898I**	**898I**
Target SNP	110G	110A	1916G	1916A	2306G	2306A	2694A	2694T
%Prevalence (*N*)	100 (22)	0	100 (63)	0	100 (63)	0	100 (63)	0

Discussion

Our results show the increase in prevalence of mefloquine resistant *P.falciparum* isolates (68%) collected from the multi-drug resistance area of Thailand compared with the isolates collected during 1998-2005 (32%) (10) and 2007 (46%) (11). Sensitivity to artesunate is also gradually declining with about 14.3% (9 isolates) exhibiting IC_{50} of greater than 3 nM. The IC_{50} range observed for artesunate in this study is similar to that reported in Africa (12). Nevertheless, at the molecular level, no genetic changes (SNP and amplification) in the *pfatp6* gene which is proposed to be linked with resistance of artemisinins or mefloquine were found. Continuous monitoring of these genetic changes in parallel with *in vitro* and *in vivo* sensitivity of *P.falciparum* isolates collected from various endemic areas are required to definitely conclude on the involvement of this gene in conferring resistance of the parasite to artemisinins. This would assist in policy making in the malaria control program of the country.

Conclusion

With limited number of isolates (63) under investigation, lack of association between *in vitro* sensitivity of *P.falciparum* isolates to artesunate and mefloquine, and genetic polymorphism (SNP and gene amplification) were observed.

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