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Research paper

Variation in intronic microsatellites and exon 2 of the *Plasmodium falciparum* chloroquine resistance transporter gene during modification of artemisinin combination therapy in Thailand

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ABSTRACT

The amino acid substitution at residue 76 of the food vacuolar transmembrane protein encoded by the chloroquine resistance transporter gene of *Plasmodium falciparum* (*Pfcrt*) is an important, albeit imperfect, determinant of chloroquine susceptibility status of the parasite. Other mutations in *Pfcrt* can modulate susceptibility of *P. falciparum* to other antimalarials capable of interfering with heme detoxification process, and may exert compensatory effect on parasite growth rate. To address whether nationwide implementation of artemisinin combination therapy (ACT) in Thailand could affect sequence variation in exon 2 and introns of *Pfcrt*, we analyzed 136 *P. falciparum* isolates collected during 1997 and 2016 from endemic areas bordering Myanmar, Cambodia and Malaysia. Results revealed 6 haplotypes in exon 2 of *Pfcrt* with 2 novel substitutions at c.243A > G (p.R81) and c.251A > T (p.N84I). Positive selection was observed at amino acid residues 75, 76 and 97. Four, 3, and 2 alleles of microsatellite (AT/TA) repeats occurred in introns 1, 2 and 4, respectively, resulting in 7 different 3-locus haplotypes. The number of haplotypes and haplotype diversity of exon 2, and introns 1, 2 and 4 were significantly greater among isolates collected during 2009 and 2016 than those collected during 1997 and 2008 when 3-day ACT and 2-day ACT regimens were implemented nationwide, respectively ($p < 0.05$). By contrast, the number of haplotypes and haplotype diversity of the merozoite surface proteins 1 and 2 of these parasite populations did not differ significantly between these periods. Therefore, the *Pfcrt* locus of *P. falciparum* in Thailand continues to evolve and could have been affected by selective pressure from modification of ACT regimen.

1. Introduction

Widespread resistance of *Plasmodium falciparum* to chloroquine has impeded global malaria control program. During its therapeutic efficacy, chloroquine was considered a safe, economical and affordable drug that contributed to remarkable reduction in global malaria morbidity and mortality (Wernsdorfer, 1991). Although chloroquine resistance phenotype of *P. falciparum* has emerged from a complex biological process, an important, albeit imperfect, genetic determinant relies on mutations in the chloroquine resistance transporter gene (*Pfcrt*). PfCRT is a malarial digestive vacuolar transmembrane protein consisting of 424 amino acids encoded by a 13-exon gene located on chromosome 7 (Ecker et al., 2012; Fidock et al., 2000). It has been widely perceived that *Pfcrt* K76T confers chloroquine resistance phenotype while additional nonsynonymous mutations in other codons may exert compensatory adaptation for parasite fitness cost or modulate drug susceptibility status of *P. falciparum* to a number of antimalarials known to interfere with heme detoxification process (Agrawal et al., 2017; Callaghan et al., 2015; Dhingra et al., 2017; Ecker et al., 2012; Gabryszewski et al., 2016; Johnson et al., 2004; Petersen et al., 2015).

The evolution of chloroquine resistance as viewed from variation in amino acid residues 72–76 of PfCRT and its flanking microsatellite loci has suggested at least three distinct geographic origins for the emergence and spread of drug resistant *P. falciparum*. The parental haplotype CVIET originated at the Thai-Cambodia border is believed to spread to Africa whilst the haplotype SVMNT occurred independently in South America and Papua New Guinea that subsequently spread to their

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nearby countries (Awasthi and Das, 2013; Gresty et al., 2014; Mita et al., 2009). However, co-existence of other derived haplotypes has been found in these areas. Importantly, genetic hitchhiking or selective sweeps around mutant *Pfcrt* has been observed among chloroquine-resistant parasite populations, characterized by significantly reduced variation in the flanking microsatellite loci (Wootton et al., 2002). The differences in selection intensity seem to be reflected by the width of the selective valley of the flanking microsatellite markers (Nash et al., 2005). Meanwhile, reduced variation in microsatellites immediately surrounding mutant *Pfcrt* has been fixed among *P. falciparum* populations of Southeast Asia, South Asia, South America and Papua New Guinea where choloroquine sensitive parasites have been purged or substantially depopulated from these endemic areas (Chen et al., 2008; Dare et al., 2007; Mallick et al., 2013; Nash et al., 2005). However, analysis of microsatellites in introns of *Pfcrt* has shown a higher level of sequence variation than those flanking this locus. Furthermore, intronic microsatellite variation seems to be more pronounced in high malaria transmission areas, probably from ongoing intragenic recombination in *Pfcrt* (Dare et al., 2007; Mallick et al., 2013).

The national antimalarial treatment regimens in Thailand have been changed or modified promptly in response to the emergence and spread of drug resistance in *P. falciparum*. Chloroquine was introduced as an antimalarial drug in Thailand in 1945 and was implemented as standard treatment for malaria caused by all *Plasmodium* species in 1965 (Malikul, 2000). The emergence of chloroquine-resistant *P. falciparum* occurred in 1957 (Harinasuta et al., 1965) and gradually spread across endemic areas of the country, leading to the necessary change of national treatment regimen to the combination of sulfadoxine and pyrimethamine (SP) in 1974 (Malikul, 1988; Malikul, 2000). It was not until 1981 that antifolate resistance became a substantial therapeutic problem; therefore, SP was replaced by quinine plus tetracycline (QT) during 1982 and 1985 (Thimasarn et al., 1995). Due to the low compliance of QT, the combination of mefloquine, sulfadoxine and pyrimethamine (MSP) was implemented nationwide as the first-line treatment for falciparum malaria during 1986 and 1990. However, mefloquine monotherapy was used during 1990 and 1995 because there was no evidence for SP in delaying mefloquine resistance (Malikul, 2000; Wongsrichanalai et al., 2004). When resistance to mefloquine monotherapy deteriorated, a 2-day course of artesunate plus mefloquine, known as artemisinin-based combination therapy (ACT), was deployed as the national treatment regimen during 1995 and 2008. An increasing treatment failure rate of 2-day ACT has led to a modification to 3-day ACT by the addition of artesunate on the third day of treatment that was implemented nationwide during 2009 and 2016 (World Health Organisation, 2014).

Mutations in *Pfcrt* could alter susceptibility status of *P. falciparum* to both artesunate and mefloquine as evidenced from *in vitro* mutagenesis (Cooper et al., 2002; Dhingra et al., 2017; Gabryszewski et al., 2016; Lakshmanan et al., 2005; Sidhu et al., 2002; Valderramos et al., 2010). For example, a mutation at K76T in *Pfcrt* confers a significant increase in *in vitro* susceptibility to artesunate whilst combinatorial mutations at A220S, N326D and I356L lead to significantly decreased drug susceptibility status (Gabryszewski et al., 2016). Likewise, *P. falciparum* carrying a mutation K76I in *Pfcrt* and those harboring multiple mutations such as the Dd2 haplotype (M74I, N75E, K76T, A220S, Q271E, N326S, I356T and R371I) and the 7G8 haplotype (C72S, K76T, A220S, N326D and I356L) exhibit a remarkable increase in susceptibility to mefloquine (Sidhu et al., 2002). Meanwhile, replacing chloroquine with the combination of artemether and lumefantrine (AL) reportedly led to *in vivo* selection of *P. falciparum* carrying chloroquine-sensitive *Pfcrt* genotype (K76) in Tanzania (Sisowath et al., 2009). Besides the lack of chloroquine selective pressure, chloroquine-resistant parasites carrying the *Pfcrt* K76T mutation are more susceptible to AL and are more likely to be eliminated from the population than the chloroquine-sensitive ones (Sisowath et al., 2009). Therefore, chloroquine-sensitive parasites are more likely to be selected in the population.

With the complex interplay between ACT and mutations in *Pfcrt*, we hypothesized that modification of ACT from 2-day to 3-day regimens in Thailand could affect the evolution of *Pfcrt*. Exon 2 of *Pfcrt* encodes key residues involved in drug susceptibility status and served as a target marker for tracing the origin of drug resistance mutants (Awasthi and Das, 2013) while introns 1, 2 and 4 are considered to evolve independently from drug pressure and exhibit extensive allelic variation among isolates (Dare et al., 2007; Mallick et al., 2013; Vinayak et al., 2006). The objective of this study is to analyse sequence variation in exon 2 and microsatellite diversity in these introns in the *Pfcrt* locus among Thai isolates collected during implementation of ACT. Results revealed an increase in molecular diversity indices among isolates collected after modification of ACT policy, consistent with ongoing evolution in the *Pfcrt* locus, probably in response to drug pressure.

2. Materials and methods

2.1. Human ethics statement

Research protocol was approved by the Institutional Review Board in Human Research of Faculty of Medicine, Chulalongkorn University (IRB Nos. 019/2006 and 257/57). Informed consent was obtained from all participants or from their parents or guardians prior to blood sample collection.

2.2. Parasite populations

Blood samples were obtained from individuals attending malaria clinics or local district hospitals in malaria endemic areas by venepuncture (~1 mL) under standard aseptic precaution. Initial diagnosis of *P. falciparum* was done by microscopic examination of Giemsa-stained thin and thick blood films. Blood samples were collected from Tak, Ubon Ratchathani, Chantaburi, Yala and Narathiwat provinces during 1997 and 2016. All patients presented with uncomplicated malaria by the time of blood sample collection.

2.3. DNA preparation and species confirmation

DNA was purified from 200 μL of EDTA-preserved blood samples using QIAamp DNA purification kit (Qiagen, Hilden, Germany) following the manufacturer's recommendation and stored at −40 °C until use. All *P. falciparum* positive samples were reaffirmed by species-specific PCR as described previously (Putaporntip et al., 2011).

2.4. Detection of multiple-clone infections

Clonality in each *P. falciparum* isolate was assessed by size variation in the 5′ portion of the merozoite surface protein 1 locus (*Pfmsp-1*) and in the central region of the merozoite surface protein 2 (*Pfmsp-2*). For *Pfmsp-1*, PCR was done by using 2 μL of DNA sample with outer primers FMSP1B1F (5′-CACAATGTGTAACACATGAAAG-3′) and FMSP1B5R (5′-CAAGTGGATCAGTAAATAAACTATC-3′) that generated ~1.1–1.2 kb PCR fragment. Nested PCR was performed to distinguish the three parental types of block 2 in 3 separate reactions containing the following forward primers: FK1F (5′GAAATTACTACAAAGGTGCAA GTG-3′), FMAD20F (5′-GAACAGCTGTTACAACTAGTACAC-3′) and FRO33F (5′-GGAGCAAATACTCAAGTTGTTGC-3′) for the parental K1, MAD20 and RO33 allelic types, respectively, and a reverse conserved primer from block 3 of *Pfmsp-1* (FMSP1B3R: 5′-CCATCAATTAAATAT TTGAAACC-3′)(Jongwutiwes et al., 1992). The thermal cycle profile for primary PCR contained preamplification denaturation at 94 °C, 1 min, followed by 35 cycles of 94 °C, 20 s; 55 °C, 30 s and 72 °C, 1 min 30 s, and a final extension at 72 °C, 5 min. For nested PCR, the same thermal cycling profile was used except the replacement of 35 cycles of amplification with 25 cycles. The PCR products were analyzed in 2% agarose gel electrophoresis. Size variation in *Pfmsp-2* was determined by PCR