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Episodic positive selection in the Cam734 haplotype and low prevalence of the A144F mutation in *Plasmodium falciparum* chloroquine resistance transporter gene among Thai isolates

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Abstract. Chloroquine resistance transporter of *Plasmodium falciparum* (PfCRT) is a food vacuolar transmembrane protein that mediates susceptibility of the parasite to chloroquine. A mutation at K76T of the *Pfcrt* gene is a key determinant for chloroquine resistance phenotype. In the absence of drug pressure, *in vitro* growth rate of chloroquine-resistance parasites was outcompeted by wild-type parasites unless intragenic compensatory mutations occurred. Chloroquine-resistant *P. falciparum* bearing the Cam734 haplotype known to circulate in endemic areas of Cambodia bordering Thailand contains 9 mutations in *Pfcrt* and exhibits both chloroquine resistance and comparable growth rate to the chloroquine-sensitive 3D7 strain. To analyze the evolution of the Cam734 haplotype, codon-based analysis was performed by using the mixed effects model of evolution (MEME), branch-site random effects likelihood (BR-REL) and other related methods. Results revealed that the Cam734 haplotype has evolved distinctively from other known mutant haplotypes including the most common Dd2 haplotype in Southeast Asia. Evidence of episodic positive selection was detected at codon 144, characterized by c.[430G>T; 431C>T] (p.A144F), known to be indispensable for both chloroquine resistance and restoration of growth rate of the parasites. To survey the prevalence of mutations at codons 76 and 144 in *Pfcrt* among Thai isolates, restriction fragment analysis of 548 *P. falciparum* isolates collected from six endemic provinces of Thailand during 1991 and 2016 was performed. The 144F *Pfcrt* mutant was detected in 7 (1.28%) isolates. All Thai isolates analyzed herein harbored a mutation at codon 76 whilst the wild-type parasite was not found. The low prevalence of isolates bearing the mutation 144F in PfCRT could imply little or lack of survival advantage of this mutant in endemic areas of Thailand where the wild-type parasites seem to be absent or extremely rare.

INTRODUCTION

Chloroquine is a 4-aminoquinoline compound first introduced as an antimalarial agent active against asexual blood stages of malaria parasites in 1945 and was served as the frontline of treatment for all human malaria species for several decades (Wernsdorfer, 1991). It was not until the late 1950s and early 1960s that chloroquine-resistant *Plasmodium falciparum* was reported independently at Thailand-Cambodia border and a few scattered foci in Central America. Recent genetic analysis has suggested at least four distinct geographic origins for the emergence of drug resistant parasites (Awasthi & Das, 2013; Mita *et al.*, 2016). To date, chloroquine-resistance *P. falciparum* occupies the majority of malaria endemic areas.

Chloroquine resistance phenotype of *P. falciparum* is characterized by a higher rate of chloroquine efflux from the parasite's digestive vacuoles than the chloroquine-susceptible one, leading to a suboptimal level of chloroquine remaining inside the acidic vacuoles where heme detoxification takes place (Ecker *et al*., 2012; Summers *et al*., 2012). The leading candidate of protein conferring chloroquine resistance in *P. falciparum* is a 48.6 kDa transmembrane protein located on the digestive vacuolar membrane, designated chloroquine resistance transporter (PfCRT)(Fidock *et al*., 2000). PfCRT consists of 424 amino acids, encoded by a single copy 13-exon gene known as *Pfcrt*. A point mutation at codon 76 (K76T) is considered to be a key determinant of chloroquine resistance that has been ascertained in a number of laboratory and field studies (Ecker *et al*., 2012). Therefore, most of the molecular epidemiological reports on *Pfcrt* genotypes have been confined to sequence variation in exon 2. Importantly, recent evidences have suggested that the hitherto mutation (K76T) *per se* is not the sole predictor of drug resistance while specific substitutions beyond codon 76 could modulate susceptibility status of the parasites to other antimalarials believed to interfere with heme detoxification (Ecker *et al*., 2012).

Pfcrt has evolved not without fitness cost. In the absence of drug pressure, the chloroquine-resistance parasites were outcompeted in terms of growth rate by the wild-type parasites upon *in vitro* co-cultivation (Rosenthal, 2012). Consistently, withdrawal of chloroquine as a standard treatment regimen for falciparum malaria in endemic areas of Kenya (Kiarie, 2015) and Malawi (Kublin, 2003) has resulted in reemergence and expansion of the remaining chloroquine-sensitive parasites in the containment areas. It is noteworthy that *P. falciparum* bearing different mutant *Pfcrt* haplotypes seem to display differential growth disadvantage in the absence of drug pressure (Sa & Twu, 2010). Interestingly, the Cam734 haplotype, characterized by M74I, N75D, K76T, A144F, L148I, I194T, A220S, Q271E and T333S (Durrand *et al*., 2004), conferred chloroquine resistance status while the growth rate of parasites bearing these mutations was comparable to the wild-type parasites, e.g. the 3D7 strain (Petersen *et al*., 2015).

Meanwhile, the Cam734 haplotype displayed comparatively higher number of nucleotide substitutions than other *Pfcrt* mutants. Whether antimalarial drug pressure on individual mutant codons in this haplotype occurred constantly (pervasive positive selection) or instantaneously and changed over time (episodic or past positive selection) remains to be elucidated. The objectives of this study are (i) to explore the prevalence of mutation at codon 76 in *Pfcrt* among *P. falciparum* isolates collected from diverse malaria endemic areas of Thailand during 1991 and 2016, (ii) to identify specific codons in the Cam734 *Pfcrt* haplotype that have evolved under episodic positive selection and (iii) to determine the prevalence and geographic distribution of *P. falciparum* isolates bearing the Cam734 haplotype in Thailand.

MATERIALS AND METHODS

Ethics Statement

Th ethical issues of this study were reviewed and approved by the Institutional Review Board on Human Research of Faculty of Medicine, Chulalongkorn University (IRB No.257/57). Inform consent was obtained from all participatants or their guardians prior to blood sample collection.

Parasite populations

Blood samples (~1 ml) were obtained from falciparum malaria patients who attended malaria clinics or district hospitals in endemic areas. Diagnosis of *P. falciparum* infections was done by Giemsa-stained thin and thick blood smears. In total, 548 *P. falciparum*-infected blood samples were collected from 5 endemic provinces of Thailand including Tak (n = 200), Ubon Ratchathani (n = 160), Chantaburi (n = 48), Yala (n = 81), Narathiwat (n = 34) and Trat (n = 25) collected during 1991 and 2016. Fresh blood sample from each patient was spotted onto chromatography-grade filter paper

(ET31CHR; Whatman, Madison, UK) and the remaining volume was preserved in EDTA anticoagulant.

DNA extraction and PCR-based detection of Plasmodium species

Genomic DNA was extracted from 200 mL of each blood sample by using QIAamp DNA mini kit (Qiagen, Hilden, Germany) per the manufacturer's recommendation and kept at -40°C until use. *P. falciparum* was reaffirmed by species-specific PCR targeting the mitochondrial cytochrome *b* gene as described previously (Putaporntip *et al.*, 2010). Genomic DNA of known species of human malaria parasites including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* were used as positive control.

Analysis of mutations at codons 76 and 144 of *Pfcrt* by PCR-RFLP

The *Pfcrt* gene fragment spanning exons 2 and 3 was amplified by PCR using the forward primer CRT1F (5'-TTGTCGACC TTAACAGATGGCTCAC-3')(Djimde *et al.*, 2001) and the reverse primer CRT0R (5'-TCCGAGATAATTGTATAAGTGATATC-3'). PCR amplification was done in a total volume of 30 µL containing PCR buffer, 200 µM dNTP, 0.2 µM of each primer, nuclease free water, 2 µL of template DNA and 1.25 units of *ExTaq* DNA polymerase (Takara, Seta, Japan). The thermal cycler profiles consisted of a preamplification denaturation at 94°C for 1 min, followed by 35 cycles of 94ºC for 40 s, 56ºC for 30 s, and 72ºC for 1.5 min. Two microlitres of the PCR products were used as template for subsequent PCR amplification of exon 2 using the same amplification conditions except that the primers were replaced with CRT2F (5'-CTTGTCTTGGT AAATGTGCTC-3') and CRT2R (5'-GAACA TAATCATACAAATAAAGT-3') as described previously (Djimde *et al.*, 2001). Likewise, amplification of exon 3 was carried out with primers PFCRTE3-F (5'-TATTTA TTTCTTATGACCTTTTTAGGAACG-3') and PFCRTE3-R (5'-GTAATTTAAAATAGTATA CTTACCTATATC-3') using the same PCR condition. After purification with QIAquick PCR purification kit (Qiagen, Hilden, Germany), the PCR products spanning exon 2 were digested with *Apo* I for detection of the wild-type codon 76 (AAA for lysine) of *Pfcrt*; therefore, indigestible products indicated chloroquine-resistance mutants. Meanwhile, the recognition site of *Hae* III restriction endonuclease (GG|CC) occurred at codon 144 in which digestible PCR products encompassing exon 3 indicated the presence of wild-type codon (GCC for alanine); therefore, uncut products indicated mutation at this codon. The digested PCR products were analyzed by using 1.5% agarose gel electrophoresis.

RNA extraction, RT-PCR amplification and sequencing of Pfcrt

Dry blood spot was excised from the filter paper and cut into small pieces with sterile scissors that had been flamed to eliminate RNase prior to use. Total RNA was extracted from the blood sample using QIAamp RNA blood minikit (Qiagen, Hilden, Germany) following the recommendations provided by the manufacturer. Synthesis of cDNA was obtained from 2 mL of RNA sample using TaKARa RNA PCR (AMV) version 3.0 kit (TaKaRa, Japan) in a total volume of 10 mL. Two microlitres of cDNA product were used as template for nested PCR amplification of the complete coding region of *Pfcrt*. The outer PCR primers were PFCRTF (5'-CATATAACAAAATGAAATTCGC-3') and PFCRTR (5'-TTATTGTGTAATAATTGAAT CGACG-3') and the inner primers were PFCRTF1 (5'-TCAAGCAAAAATGACGA GCG-3') and PFCRTR1 (5'-ACGTTGGTTAA TTCTCCTTC-3'). PCR amplification was performed in a total volume of 30 µL containing PCR buffer, 200 µM dNTP, 0.2 µM of each primer, nuclease free water, 2 µL of template DNA and 1.25 units of ExTaq DNA polymerase (Takara, Seta, Japan). The amplification profiles for both primary and nested PCR were essentially the same, consisting of pre-amplification denaturation at 94ºC, 60 s and 30 cycles of 94ºC, 30 s; 50ºC, 30 s and 72ºC, 2 min; followed by 72ºC, 5 min. DNA amplification was performed by using a GeneAmp 9700 PCR thermal cycler (Applied Biosystems, Foster City, CA). The PCR products were analyzed on 1% agarose gel electrophoresis, stained with

ethidium bromide and visualized under UV transillumination. After purification with QIAquick PCR purification kit (Qiagen, Hilden, Germany), the PCR product was used as template for direct sequencing from both directions using ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems) and sequencing primers.

Statistical analysis

Despite a number of reports on mutations in PfCRT, complete or near complete nucleotide sequences of this protein were limited in the GenBank database. In this study, we retrieved 13 distinct *Pfcrt* sequences of natural *P. falciparum* isolates and 4 distinct sequences from the Cambodian isolates reported previously (Durrand *et al.*, 2004). The CLUSTAL_X program was deployed for alignment of the complete or near complete *Pfcrt* sequences. Geographic origins, GenBank accession numbers and amino acid substitutions of *Pfcrt* used in this study were listed in Table 1. Phylogenetic tree was constructed by the Maximum Likelihood method based on the Tamura 3-parameter model that gave the lowest Bayesian Information Criterion (BIC) score. The reliability of the tree was determined by the bootstrap method implemented the MEGA 6.0 software (Tamura *et al.*, 2013). Detection of selection on specific codons was performed by estimation of the global ratio of the rate of non-synonymous to synonymous substitutions (dN/dS or ω value) across the *Pfcrt* gene using various models. The fixed effects likelihood (FEL), random effects likelihood (REL), fast unconstrained Bayesian approximation (FUBAR), branch site REL analysis (BR-REL) and mixed effects model of evolution (MEME) methods were used for analysis (Pond & Frost, 2005). FEL model compares the ratio of nonsynonymous to synonymous substitution on a site-by-site basis, without assuming an *a priori*

Table 1. The *Pfcrt* sequences of *P. falciparum* isolates used in this study

Isolate/ strain	Origin	Amino acid substitutions 1111122233333 777792446902723357 245673480450163461	GenBank accession no./references
3D7	Africa?	CMNKHHALLITAQNTSIR	KM288867
Dd2	Thailand	.IET.......SES..TI	AF030694
TM6	Thailand	.IET.R....ASES...I	AF468006
1061	Sudan	.IE........SES...I	AF233065
GM4	Ghana	.IET.......SE....I	HM854027
7G8	Brazil	S..T.......S.D..L.	AF233067
DIV30	Brazil	S..T.......S.D..L.	AF233064
TA6182	Colombia	..ETQ......S..S..I	DQ156109
TA7519	Colombia	..ETQ......S.....T	DQ156107
TU741	Colombia	...T.......S.D.NL.	DQ156108
PH1	Philippines	...T..T.Y....D....	AY254700
FVO	Vietnam	.IET.......SES...I	DQ533840
2300	Indonesia	.IKT.......SES...I	AY651315
Cam734	Cambodia	.IDT..FI.T.SE.S...	Durrand *et al.*, 20
Cam738	Cambodia	.IDT...I.T.SE.S...	Durrand *et al.*, 20
Cam742	Cambodia	.IET.......SE....I	Durrand *et al.*, 20
Cam783	Cambodia	.IET.......SE...TI	Durrand *et al.*, 20

Dots are identical residues.

distribution of rates across sites whereas REL model first fits a distribution of rates across sites and then infers the substitution rate for individual sites. MEME algorithm detects codons under episodic positive selection unmasked by the abundance of purifying selection along the lineages. Significance level settings for FEL, iFEL, REL and MEME were p values < 0.1 and Bayes Factor > 1000 for REL followed the default values available on the Datamonkey Web Server (Pond & Frost, 2005). Searching for natural selection based on the measurement of selective influences on 31 structural and biochemical amino acid properties during cladogenesis was performed by using the TreeSAAP program. Amino acid under positive selection was analyzed by performing goodness-of-fit and categorical statistical tests (Woolley *et al.*, 2003).

RESULTS

Phylogeny and codon-specific selection in Cam734 and other Pfcrt haplotypes

Alignment of 17 *Pfcrt* sequences has revealed 19 nucleotide substitutions, resulting in 18 amino acid changes. Codon-based analysis of departure from neutrality has identified 13 positively selected sites based on positive results by at least one method. Most of these codons (77%) were located in the transmembrane domains (Table 2). Test of positive selection implemented in the TreeSAAP program has detected 10 amino acid substitutions with significant alteration in physicochemical properties (p<0.001). Taken together, 11 codons were under positive selection based on a consensus of concordant results from ≥ 2 tests. Meanwhile, only 9 positively selected codons gave concordant positive results from ≥ 3 tests (Table 2).

The fingerprint of episodic or past positive selection was found in the Cam734 haplotype by both the mixed effects model of evolution (MEME) and the branch-site random effects likelihood (BR-REL) methods in which substitution at codon 144 yielded statistically significant tests (p<0.05). The Cam734 lineage could have experienced a short burst of adaptive evolution, resulting in a mutation A144F (Figure 1).

P. falciparum populations

Of 548 *P. falciparum*-infected individuals diagnosed by microscopy in this study, 353 (64.4%) were males and 195 (35.6%) females. The population distribution by age ranged from 4 to 67 years old (mean = 25 years). No apparent severe manifestation of malaria was observed during blood sample collection. The duration of self-reported febrile illness prior to seeking medical diagnosis ranged from 1 to 7 days, of which

Table 2. Positive selection on amino acid substitutions in *Pfcrt* of worldwide isolates

Codon	Test method					Consensus	
	FEL	iFEL	REL	FUBAR	TreeSAAP	≥2 tests	≥3 tests
74*			✓				
75*			✓	✓	✓	✓	✓
76*			✓	✓	✓	✓	✓
97*			✓	✓	✓	✓	✓
144*		✓	✓	✓	✓	✓	✓
148			✓		✓	✓	
194*			✓		✓	✓	
220*			✓	✓	✓	✓	✓
271			✓				
326*		✓	✓	✓		✓	✓
333*			✓	✓	✓	✓	✓
356*			✓	✓	✓	✓	✓
371			✓	✓	✓	✓	✓

* Residues in transmembrane domains.

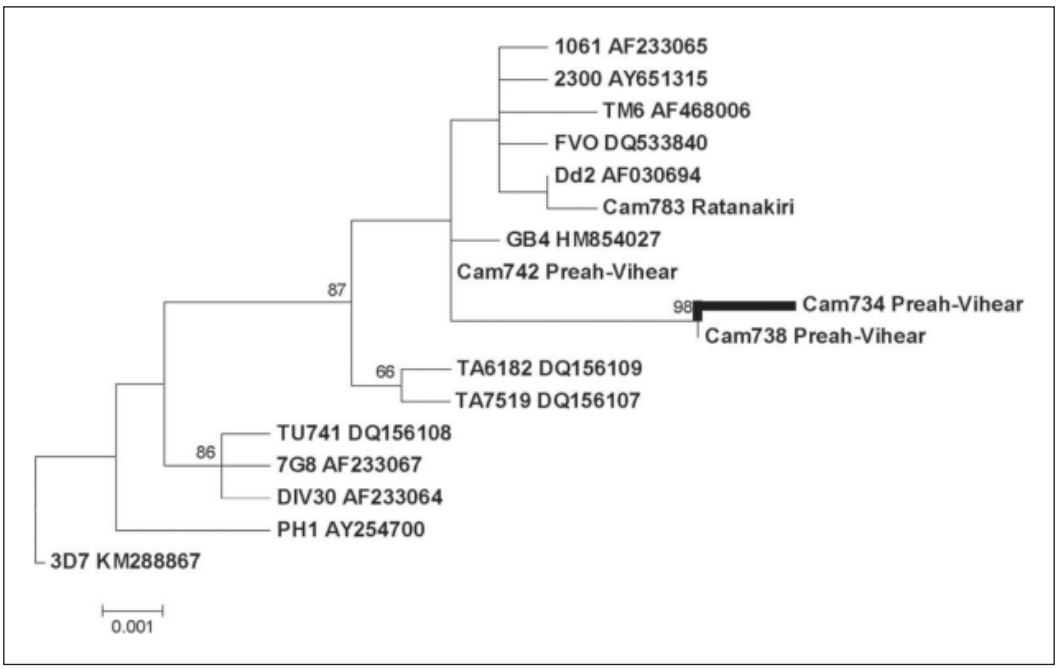

Figure 1. Maximum likelihood tree of the *Pfcrt* sequences among Thai isolates. Thick line indicates branch under episodic positive selection detected by branch-site random effects likelihood (BR-REL) analysis. The tree is rooted by using the 3D7 haplotype. Scale bar represents nucleotide substitutions per site.

Table 3. Chronological and geographic distribution of *P. falciparum*-infected blood samples

Province	Year of sample collection (n)	Total
Tak	1995 (13), 1996 (26), 1997 (8), 2003 (14), 2004 (11), 2005 (5), 2006 (14), 2008 (13), 2009 (3), 2010 (20), 2011 (21), 2012 (16), 2013 (36)	200
Ubon Ratchathani	2008 (10), 2014 (123), 2015 (27)	160
Chanthaburi	2007 (24), 2009 (21), 2010 (3)	48
Yala	2007 (19), 2008 (26), 2015 (17), 2016 (19)	81
Narathiwat	2008 (34)	34
Trat	1991 (25)	25
Total	1991-2016	548

308 (56.2%) patients had fever for 1 to 3 days. No previous self-administration of antimalarial drugs was reported among the study populations. The chronological distribution of samples by geographic origins is listed in Table 3. PCR analysis reaffirmed that all blood samples were infected with *P. falciparum*. However, co-infections of *P. falciparum* and *P. vivax* were detected in 15 patients (2.74%).

Analysis of mutation at codon 76 in *Pfcrt*

The *Pfcrt* fragment encompassing exon 2 could be amplified from genomic DNA of all isolates examined. The resulting PCR products contained 200 bp without size variation among isolates. For *Apo* I restriction endonuclease analysis, the recognition site is 5'-R|AATT|Y-3' where R represents A or G and Y can be either C or T. The wild-type codon 76 coding for lysine

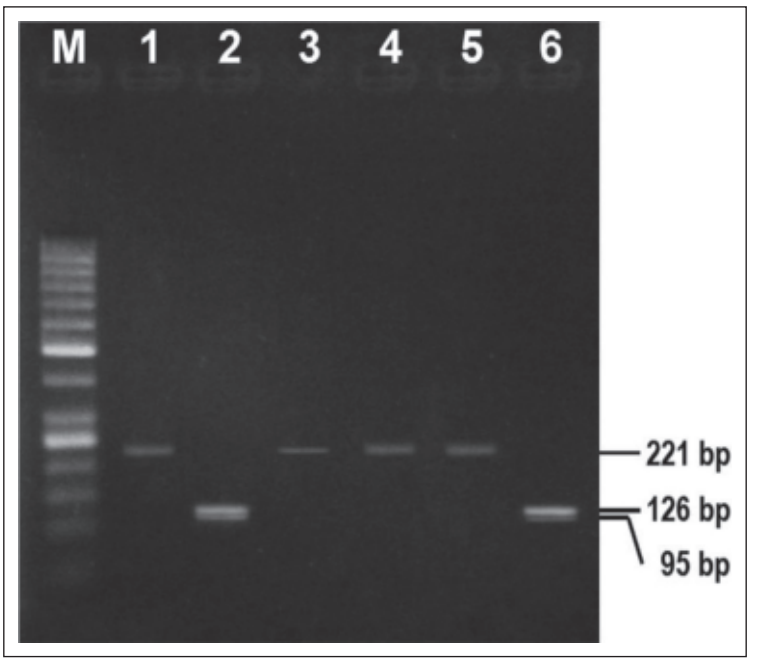

Figure 2. Agarose gel electrophoresis of representative PCR products spanning exon 3 of *Pfcrt* digested with *Hae* III. M denotes 50-bp ladder marker. Sample numbers 2 and 6 contained *Hae* III restriction site.

(codon AAA) and its adjacent codons 75 and 77 provide a restriction site for this enzyme. Therefore, the PCR amplified fragments that were digestible with *Apo* I, producing 2 DNA fragments of 112 and 88 bp, indicate the presence of codon for lysine at position 76, the key residue for chloroquine-sensitive parasites. On the other hand, if mutation(s) occurs at codon 76, the restriction site for *Apo* I would be disrupted; thereby the PCR products remained uncut with this restriction enzyme. Although the intact PCR fragments after *Apo* I digestion indicate nonspecific nucleotide substitution(s) at codon 76, previous sequencing analysis has shown that almost all nucleotide substitutions at this codon were due to A → C change, resulting in a change in amino acid from lysine to threonine. The PCR products from all 548 isolates in this study were indigestible by *Apo* I. Hence, no evidence of chloroquine-sensitive parasites was detected in the study populations.

Analysis of mutation at codon 144 in Pfcrt

The 221-bp PCR product spanning exon 3 of *Pfcrt* could be amplified from all 548 isolates. The wild-type codon 144 could be digestible with *Hae* III generating 95 bp and 126 bp fragments (Figure 2). Analysis of mutation at codon 144 has revealed that 7 of 548 (1.28%) isolates were indigestible with *Hae* III, indicating the absence of GGCC at nucleotides 429-431. These isolates were collected from Trat province in 1991 (n = 1), Tak province in 1995 (n = 2), Chanthaburi province in 2007 (n = 1) and Ubon Ratchathani province in 2014 (n = 3). The complete *Pfcrt* sequences of these isolates lacking *Hae*III resistriction site revealed perfect sequence identity with the Cam734 haplotype, characterized by mutations at c.222G>T (p.M74I), c.223A>G (p.N75D), c.227A>C (K76T), c430G>T;431C>T (A144F), c.442C>A (p.L148I), c.581T>C (p.I194T), c.658G>T(p.A220S), c.811C>G (p.Q271E) and c.998C>G (p.T333S).

DISCUSSION

In Thailand, *P. falciparum* isolates collected during the past two decades exhibited chloroquine resistance phenotype while previous analysis of the key mutation at codon 76 of the *Pfcrt* gene failed to detect the wild-type codon (Chaicharoenkul *et al.*, 2011; Parker *et al.*, 2012; Takahashi *et al.*, 2012). Our analysis of mutation at codon 76 of *Pfcrt* among 548 isolates collected during 1991 and 2016 from six endemic provinces of the country was in line with these previous studies. Therefore, nationwide withdrawal of chloroquine for the treatment of falciparum malaria during the past 4 decades has no discernible impact on drug resistance status of *P. falciparum* in this country (Wongsrichanalai *et al.*, 2002). It is likely that *P. falciparum* bearing drug resistance phenotypes, which could have arisen as a new allelic variant or which could have circulated in the population at low prevalence, became populated and eventually reached fixation because of survival advantage following substantial chloroquine pressure. Intriguingly, about 10.8% of microscopically diagnosed *P. vivax* infections were co-infected with submicroscopic *P. falciparum* based on PCR detection and were treated with chloroquine (Jongwutiwes *et al.*, 2011). Therefore, selective pressure from chloroquine on subpopulations of *P. falciparum* could remain in Thailand, especially along Myanmar border where mixed species infections were prevalent (~23% to ~24%)(Putaporntip *et al.*, 2009). Importantly, coexistence of *P. falciparum* and *P. vivax* has been identified in all malaria endemic areas of Thailand. Therefore, it is likely that *P. falciparum* in infected mosquito vectors could have the possibility of exposure to chloroquine upon taking blood meals from individuals infected with *P. vivax* who were treated with chloroquine. Furthermore, the long half-life of chloroquine and its metabolites, lasting for 45 to 55 days and 59 to 67 days, respectively (Gustafsson *et al.*, 1987), in the circulation of chloroquine-treated patients would exert selective pressure on *P. falciparum* upon subsequent infections.

The emergence of chloroquine resistance phenotype in *P. falciparum* occurred with associated fitness cost as exemplified by the inferior growth rate of parasites bearing the Dd2 *Pfcrt* haplotype in comparison with the wild-type strains. On the other hand, intragenic compensatory mutations in the Cam734 haplotype has enhanced growth rate of *P. falciparum* carrying these mutations. Importantly, gene editing approach has shown that mutations at N75D, A144F, L148I and T333S in the Cam734 haplotype contributed directly to both chloroquine resistance and restoration of parasite growth rate whereas a mutation at I194T enhanced parasite growth without conferring drug resistance (Gabryszewski *et al.*, 2016). Although the Cam734 haplotype contained 9 amino acid substitutions in comparison with the chloroquine-sensitive haplotype in the 3D7 strain, reversal of A144F to F144A by gene editing approach has led to complete to near-complete sensitivity to chloroquine albeit the presence of K76T and other associated mutations in this parasite. Therefore, mutation at codon 144 seems to be crucial for chloroquine resistance in the Cam734 haplotype (Gabryszewski *et al.*, 2016).

Analysis of departure from neutrality has shown that all mutations in the Cam734 haplotype have evolved under positive selection as determined by codon-based methods and alteration in physicochemical properties of amino acid substitutions. Importantly, both MEME and BR-REL methods gave a concordant result for episodic positive selection at codon A144F in the Cam734 haplotype, suggesting a short burst of selective pressure on this codon. Phylogenetic analysis has suggested that the Cam734 haplotype could have arisen from step-wise mutation from the Dd2 lineage. The Cam734 haplotype was closely related with the Cam738 haplotype whose sequence was almost identical except the absence of mutation at codon 144 in the latter haplotype (Durrand *et al.*, 2004). Interestingly, mutations in the Cam738 haplotype did not confer growth advantage in the absence of drug pressure when compared with the Cam734 haplotype (Gabryszewski *et al.*,

2016). A strikingly lower prevalence of the Cam738 haplotype (2.5%) in comparison with the Cam734 haplotype (22.5%) in Cambodia in 2004 (Durrand *et al.*, 2004) and the absence of this haplotype in a more recent study (Gabryszewski *et al.*, 2016) could suggest survival advantage of additional mutation at codon 144 in the Cam734 haplotype. Although our screening of the Cam734 haplotype was performed by analysis of a mutation at codon 144, subsequent sequence analysis of the complete coding region of *Pfcrt* of isolates carrying the mutant codon at this position has revealed identical sequences with the Cam734 haplotype. It is noteworthy that the Cam734 haplotype could be detected almost across the sampling period and circulated at low frequency (1.28%) in diverse endemic provinces of Thailand bordering Myanmar and Cambodia.

Despite evidence for growth advantage while maintaining chloroquine resistance status, the Cam734 haplotype was less abundant than the Dd2 haplotype in Southeast Asia (Durrand *et al.*, 2004; Gabryszewski *et al.*, 2016). A recent genome-wide survey revealed a comparable prevalence of this haplotype in Laos and Vietnam, accounting for 34.12% and 32.99%, respectively, while a lower prevalence was observed in Cambodia, ranging from 18.42% to 22.50% (Durrand *et al.*, 2004; Gabryszewski *et al.*, 2016). The low prevalence of the Cam734 haplotype in Thailand in this study was in line with the previous survey in which only one of 148 (0.68%) isolates was found to carry this haplotype (Gabryszewski *et al.*, 2016). Differential prevalence of the Cam734 haplotype in this region could stem from differences in national antimalarial drug policies of these countries whilst specific mutations in *Pfcrt* could mediate alteration in susceptibility of *P. falciparum* to other antimalarials such as artemisinin, piperquine, mefloquine and amodiaquine. Intriguingly, other antimalarial treatment regimens that target heme detoxification process could affect evolution of the *Pfcrt* locus (Conrad *et al.*, 2014; Agrawal *et al.*, 2017). Meanwhile, chloroquine resistance associated with intragenic compensatory mutations *per se* may not be sufficient for *P. falciparum* to outcompete other variants because additional selective forces from parasite genetic background, mosquito-human transmission and selection within anopheline mosquito vector could have influenced the survival and competitiveness of the parasites (Rosenthal, 2013).

CONCLUSION

The low prevalence of *P. falciparum* populations bearing the mutant 144F in *Pfcrt* known to confer compensatory mutation in terms of growth rate may suggest little or lack of survival advantage of these mutants in the absence of wild-type parasites circulating in Thailand.

COMPETEING INTEREST

The authors declare no conflicts of interest.

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