Multiple Novel Mutations in *Plasmodium falciparum* Chloroquine Resistance Transporter Gene during Implementation of Artemisinin Combination Therapy in Thailand

Pattakorn Buppan,^{1,2} Sunee Seethamchai,³ Napaporn Kuamsab,¹ Pongchai Harnyuttanakorn,⁴ Chaturong Putaporntip,^{1*} and Somchai Jongwutiwes^{1*}

¹Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand; ²Inter-Department Program of Biomedical Sciences, Faculty of Graduate School, Chulalongkorn University, Bangkok, Thailand; ³Department of Biology, Faculty of Science, Naresuan University, Pitsanulok Province, Thailand; ⁴Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

Abstract. Mutations in the chloroquine resistance transporter gene of *Plasmodium falciparum* (*Pfcr*) are associated with drug susceptibility status of chloroquine and other antimalarials that interfere with heme detoxification process including artemisinin. We aim to investigate whether an increase in duration of artemisinin combination therapy (ACT) in Thailand could affect mutations in *Pfcr*. The complete coding sequences of *Pfcr* and dihydrofolate reductase (*Pfdhfr*), and size polymorphisms of the merozoite surface proteins-1 and 2 (*Pfmsp-1* and *Pfmsp-2*) of 189 *P. falciparum* isolates collected during 1991 and 2016 were analyzed. In total, 12 novel amino acid substitutions and 13 novel PfCRT haplotypes were identified. The most prevalent haplotype belonged to the Dd2 sequence and no wild type was found. A significant positive correlation between the frequency of *Pfcr* mutants and the year of sample collection was observed during nationwide ACT implementation ($r = 0.780$; $P = 0.038$). The number of haplotypes and nucleotide diversity of isolates collected during 3-day ACT (2009–2016) significantly outnumbered those collected before this treatment regimen. Positive Darwinian selection occurred in the transmembrane domains only among isolates collected during 3-day ACT but not among those collected before this period. No remarkable change was observed in the molecular indices for other loci analyzed when similar comparisons were performed. An increase in the duration of artesunate in combination therapy in Thailand could exert selective pressure on the *Pfcr* locus, resulting in emergence of novel variants. The impact of these novel haplotypes on antimalarial susceptibilities requires further study.

INTRODUCTION

A worldwide effort to control malaria has been highly reliant on antimalarial chemotherapy; therefore, drug resistance in malaria parasites can substantially preclude effective therapeutic and preventive interventions. One of the most devastating impacts of this phenomenon has been widely concerned since the emergence and spread of chloroquine resistance in *Plasmodium falciparum*, the most prevalent and malignant malaria species in the tropics. To date, the global dispersal of chloroquine-resistant *P. falciparum* has rendered the drug ineffective in almost all disease-endemic countries.^{1,2}

Plasmodium falciparum chloroquine resistance transporter or chloroquine resistance transporter of *P. falciparum* is a 48.6-kDa transmembrane protein on the digestive vacuolar membrane that confers chloroquine resistance.³ It is encoded by a single copy 13-exon gene, *Pfcr*, comprising 424 codons. A point mutation at codon 76 (K76T) is a key determinant of chloroquine resistance phenotype.^{3–5} However, the hitherto mutation per se may not be the sole predictor of drug resistance, whereas specific substitutions beyond codon 76 could modulate drug susceptibility status or compensate for the fitness costs of chloroquine-resistant parasites.^{3,5,6} To date, at least 30 amino acid substitutions and ~51 haplotypes were identified in PfCRT of natural isolates.^{7,8} In Thailand, all *P. falciparum* isolates collected during the past two decades exhibited chloroquine-resistant phenotypes, and the key mutations at codons 72–76 of the *Pfcr* gene revealed CVIET as the most predominant haplotype.^{9–11}

Antimalarial treatment in Thailand has been relied on parasite-based diagnosis by microscopy, whereas antimalarial drug policies have been implemented and changed in response to therapeutic efficacy based on national surveillance system. Chloroquine was introduced to treat malaria caused by all *Plasmodium* species in 1945 and was implemented nationwide in 1965 despite the first report on drug resistance in *falciparum* malaria patients in 1957.^{12,13} Because of the widespread occurrence of chloroquine resistance in *P. falciparum*, national treatment policy was changed to the combination of sulfadoxine and pyrimethamine (SP) in 1974 and was implemented until 1981 when antifolate resistance deteriorated in most endemic areas of the country.¹⁴ During 1982 and 1985, quinine plus tetracycline were used as a standard treatment regimen and was replaced by the combination of mefloquine and SP because of the problem of drug compliance. Because the lack of evidence for delaying mefloquine resistance in combination therapy and the adverse effects from SP, mefloquine monotherapy was initiated in 1990, fully implemented in 1992, and used until 1994 when severe deterioration of mefloquine efficacy occurred.^{14–16} During 1995 and 2008, artemisinin combination therapy (ACT) with mefloquine as a partner drug was deployed as a 2-day treatment regimen (Figure 1). Because of emergence and increasing tendency in the treatment failure rate of 2-day ACT, treatment with 3-day ACT regimen was initiated in 2008 and completely implemented nationwide in 2009.¹⁷ However, a recent increase in treatment failure of 3-day ACT has prompted the change in treatment regimen to dihydroartemisinin plus piperazine in 2017 and fully implemented in 2018. Nevertheless, chloroquine has been used as the first-line drug to treat vivax and other non-*falciparum* malaria since its first implementation in 1965. Importantly, about 8–10% of vivax malaria patients had

* Address correspondence to Chaturong Putaporntip or Somchai Jongwutiwes, Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand. E-mails: p.chaturong@gmail.com or jongwutiwes@gmail.com

FIGURE 1. (A) Annual numbers of *Plasmodium falciparum*-infected cases in Thailand during 1990 and 2016. Vertical broken demarcation lines indicate the period of nationwide implementation of antimalarial treatment regimens: MSP, mefloquine combined with sulfadoxine and pyrimethamine; M, mefloquine monotherapy; 2-day ACT, artesunate plus mefloquine for 2 days; 3-day ACT, 2-day ACT plus artesunate on day 3. (B) Biannual frequencies of mutant *Plasmodium falciparum* chloroquine resistance transporter (*PfCRT*) haplotypes (spots) relative to the Dd2 sequence (GenBank accession no. AF030694). Correlation between frequencies of mutant haplotypes and years of sample collection (during 1991 and 201 represented in broken line: Pearson's correlation coefficient, $r = 0.382$; $P = 0.350$, and during 2003 and 2016 shown in solid line: $r = 0.780$; $P = 0.038$).

submicroscopic *P. falciparum* infection and were treated with chloroquine.^{18,19} Therefore, it is likely that a small proportion of *P. falciparum* populations in Thailand could have been exposed to chloroquine.

Although mutations in *PfCRT* are not considered to be the key molecular markers for resistance of *P. falciparum* to other antimalarial drugs, transfection-based approaches have shown that certain mutations at this locus conferred alteration in susceptibilities to artemisinin and some partner drugs in ACT, particularly those that interfere with heme detoxification process in the parasite's digestive vacuole such as mefloquine and piperazine.^{6,20-22} Importantly, changing antimalarial treatment regimens to ACT have remarkable impacts on *P. falciparum* drug resistance markers, including *PfCRT* of parasite populations in Uganda and Kenya where chloroquine resistance deteriorated around the turn of the century.²³⁻²⁵ To determine whether nationwide implementation of ACT and its modification could influence mutations in *PfCRT*, we analyzed the complete coding sequences of *PfCRT* and the dihydrofolate reductase gene (*PfDHFR*) along with length polymorphisms in the merozoite surface proteins-1 and 2 (*Pfmsp-1* and *Pfmsp-2*) genes of *P. falciparum* isolates in Thailand. Results revealed multiple novel *PfCRT* haplotypes with greater molecular diversity indices among isolates collected during implementation of the 3-day ACT regimen than those collected before this

period, consistent with ongoing selective pressure on this locus.

METHODS

Parasite populations. Venous blood samples were obtained from 383 *P. falciparum*-infected patients initially diagnosed by microscopy and confirmed by species-specific polymerase chain reaction (PCR) assay.²⁶ Blood samples were collected during 1991, 2003–2006, 2008–2011, and 2013–2016 from seven provinces of Thailand bordering Myanmar, Cambodia, and Malaysia (Supplemental Figure 1). The parasite density determined from thick blood films ranged from 35 to 217,800 parasites/ μ L (geometric mean = 2,761 parasites/ μ L). Each blood sample was aliquoted; one preserved in EDTA anticoagulant and the other in RNA preservative for DNA and RNA extractions, respectively. The annual *P. falciparum* cases and the national treatment regimens for falciparum malaria in Thailand during sample collection period are depicted in Figure 1.

Detection of multiple-clone infections. Clonality of samples was determined by PCR analysis of size polymorphisms in block 2 of the *Pfmsp-1* and the central repeats of the *Pfmsp-2* of *P. falciparum* (Supplemental Material 1).

Sequencing of *PfCRT*. The complete coding region of *PfCRT* was obtained from direct sequencing of the PCR products amplified from cDNA and genomic DNA of each isolate (Supplemental Material 2). All singleton substitutions were re-determined using PCR products from independent amplifications of newly synthesized cDNA template from the same isolate.

Sequencing of *PfDHFR*. The complete *PfDHFR* sequence was amplified by PCR using genomic DNA of each isolate (Supplemental Material 3). Sequences were determined directly from purified PCR products.

Ethics. Written informed consent was obtained from all participants or from their parents or guardians before blood sample collection. The ethical aspects 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).

Statistical analysis. The sequences were aligned against the corresponding gene of the 3D7 clone. Haplotype diversity (h) and its sampling variance were calculated using the DnaSP program.²⁷ Nucleotide diversity (π), the rates of synonymous substitutions per synonymous site (d_S), and nonsynonymous substitutions per nonsynonymous site (d_N) and phylogenetic tree were analyzed by using the MEGA 6.0 program.²⁸ Evidence of genetic recombination was determined by various recombination tests implemented in the RDP4 package.²⁹ Tajima's D statistics was used for computing deviation from neutrality.³⁰ Tajima's D test determines the differences between the average number of nucleotide differences and an estimate of θ from the number of segregating sites where $\theta = 4N_e\mu$, in which N_e and μ are the effective population size and the mutation rate, respectively.³⁰ Under an equilibrium model or no influence from population history, significant positive Tajima's D values imply balancing or positive selection, whereas significant negative values suggest purifying or negative selection. Analysis of population genetic structure was performed by using molecular variance approach implemented in the Arlequin 3.5 software.³¹

Novel *Pfcrt* coding sequences.

* Positions found in experimental clones/lines induced by selective drug pressure.²¹ Italicized amino acids denote induced mutations by drug pressures. Dots are identical residues with the 3D7 sequence (GenBank accession no. KM28867). Known isolates/strains carrying haplotypes I–V are shown in bold. Novel residues are highlighted. Representative isolates are in parentheses. Demarcations of exons are indicated by dashes.

TABLE 2
Frequencies of amino acid variants in *Pfcr*t of Thai isolates

Province-year (n)	N75D	K76I	F78L	H97R	H97L	A144F	G147D	L148I	F172L	I174M	I194T	I218F	A220P	A320T	T333S	T333I	I337N	I351V
Trat-1991 (N = 19)	-	-	-	-	15.8%	-	-	-	-	-	-	-	-	-	-	-	-	-
Trat-2003 (N = 13)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranong-2003 (N = 1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trat-2004 (N = 2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranong-2004 (N = 10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trat-2005 (N = 2)	50%	-	-	-	-	50%	-	50%	-	-	50%	-	-	-	-	-	-	-
Tak-2006 (N = 14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ranong-2006 (N = 2)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tak-2008 (N = 14)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ubon-2008 (5)	-	-	-	-	-	-	-	-	-	7.1%	-	-	-	-	-	-	-	-
Yala-2008 (N = 3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Narathiwat-2008 (N = 7)	-	-	-	-	-	-	-	-	-	-	-	-	14.3%	-	-	-	-	-
Tak-2009 (N = 3)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chantaburi-2009 (N = 6)	-	-	-	-	16.7%	-	-	-	-	-	-	-	-	-	-	16.7%	-	-
Tak-2010 (N = 21)	-	-	-	-	-	-	-	-	4.8%	-	-	-	-	-	-	-	4.8%	-
Tak-2011 (N = 22)	-	-	4.5%	-	-	-	-	-	-	-	-	-	-	4.5%	-	-	-	4.5%
Ubon-2012 (N = 1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tak-2013 (N = 10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ubon-2014 (N-10)	20%	-	-	10%	-	20%	-	20%	10%	-	20%	10%	-	-	20%	-	-	-
Ubon-2015 (N = 10)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yala-2015 (N = 8)	-	12.5%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Yala-2016 (N = 5)	20%	-	-	20%	-	20%	-	20%	20%	-	20%	20%	-	-	20%	-	-	-
Ubon-2016 (N = 1)	-	-	-	-	-	-	100%	-	-	-	-	-	-	-	-	-	-	-
Total (N = 189)	2.1%	0.5%	0.5%	1.1%	0.5%	2.1%	0.5%	2.1%	1.6%	0.5%	2.1%	1.1%	0.5%	0.5%	1.6%	0.5%	0.5%	0.5%

*Pfcr*t = *Plasmodium falciparum* chloroquine resistance transporter; Ubon = Ubon Ratchathani. Amino acid variants are those differed from the *Pfcr*t sequences of either the Dd2 or the 3D7 clones (GenBank accession nos. AF030694 and KM28867, respectively).

with the 2-day ACT group ($P < 0.05$, randomization test). By contrast, there was no significant difference in the number of haplotypes of *Pfdhfr*, *Pfmsp-1*, and *Pfmsp-2* between these groups (Supplemental Table 3). The distribution of *Pfcr*t haplotypes was more skewed toward few haplotypes in the pre-3-day ACT than in the 3-day ACT groups as viewed from haplotype diversity values of total samples. The haplotype diversity in *Pfdhfr*, *Pfmsp-1*, and *Pfmsp-2* of the corresponding populations was almost comparable (Supplemental Table 3). The number of mutations and nucleotide diversity of *Pfcr*t in the 3-day ACT group exceeded those in the pre-3-day ACT group. Nonsynonymous nucleotide diversity consistently outnumbered synonymous nucleotide diversity for all comparisons shown in Table 3.

Domain-specific selection in *Pfcr*t. A signature of positive selection in *Pfcr*t was evidenced by a significantly greater d_N (0.0036 ± 0.0009) than d_S (0.0010 ± 0.0006) ($P < 0.05$). The transmembrane domains of PfCRT reportedly dictate the transport of chloroquine and other charged molecules across parasite's acid vesicles, thereby affecting drug accumulation and heme detoxification process.³ Hence, we analyzed nucleotide diversity for the putative transmembrane domain-encoding regions containing 199 codons and compared with that for the remainders of the coding regions spanning 225 codons. The mean nucleotide diversity for the transmembrane domains was 7-fold greater than that for the remaining regions and the difference was statistically significant ($P < 0.05$) (Table 4). The nucleotide diversity at nonsynonymous sites (π_N) of 189 isolates significantly outnumbered that at synonymous sites (π_S) for transmembrane domains ($P < 0.05$), but not for the remaining regions, and occurred only in the 3-day ACT group. There was no significant difference in π_S between these groups. Meanwhile, Tajima's D statistics yielded significantly negative values (Table 5). Importantly, significant negative Tajima's D values were observed at nonsynonymous sites but not at synonymous sites. Meanwhile, no evidence of recombination was discernible in *Pfcr*t by all tests analyzed.

DISCUSSION

The complete *Pfcr*t coding sequences of 198 isolates collected over two decades from seven endemic provinces contained 21 distinct genotypes, 13 of which were novel. Together with previous reports, at least 67 naturally occurring *Pfcr*t haplotypes have been documented.^{7,8} Haplotypes I–III in

this study were previously detected among Thai isolates collected over three decades ago, suggesting temporal stability of these haplotypes in this country. Likewise, identical sequences of isolate Cam734 from Cambodia collected a decade ago and haplotype IV, and isolate GB4 from Ghana and haplotype V could imply local and intercontinental spread of chloroquine-resistant *P. falciparum*. Spatiotemporal persistence of particular *Pfcr*t haplotypes has suggested adaptive evolutionary success of parasites carrying these variants in response to antimalarial drug pressure across endemic areas.

Twelve novel amino acid substitutions identified in this study have contributed to a total of 34 naturally occurring amino acid substitution sites in PfCRT so far identified. Most novel amino acid substitutions were found in single isolates and were not confined to particular endemic areas. Therefore, it is likely that mutations in *Pfcr*t could have arisen as independent events, probably as a result of the stochastic nature of a de novo point mutation. The significance of these novel mutations in PfCRT remains to be elucidated. However, a mutation at K76I in *Pfcr*t of two laboratory lines (J3D4 and C5^{K76I}) induced by in vitro chloroquine selective pressure has been observed in isolate YL2823 from Yala collected in 2015.²⁰ Although mutations in *Pfcr*t could have arisen initially from a stochastic mutational process, the shared mutations between a clinical isolate and drug-induced laboratory clones could suggest a common evolutionary pathway exerted by antimalarial selective pressure.

The interplay between antimalarial drug treatment regimens and genetics of malaria parasites has been documented. A timely withdrawal of chloroquine as a standard treatment of falciparum malaria has resulted in reemergence and expansion of chloroquine-sensitive parasites that still remain in the containment areas.³⁷ By contrast, an increase in drug pressure could enhance the emergence of drug resistance genotypes as nationwide modification of ACT from 2-day to 3-day regimens in Thailand gave rise to a remarkable increase in the prevalence of artemisinin resistance genotypes. Several lines of evidence have suggested that mutations in *Pfcr*t can modulate susceptibility of artemisinin and some partner drugs used in ACT such as piperaquine, mefloquine, lumefantrine, and amodiaquine.^{6,21} Importantly, some of these compounds share with chloroquine a quinolone scaffold and affect heme detoxification process of the parasites.³ This in turn suggests that some antimalarials other than chloroquine could mediate selective pressure on *Pfcr*t as evidenced by the emergence of *Pfcr*t mutations induced by stepwise selection with amantadine or halofantrine.²⁰ Therefore, cessation of chloroquine

TABLE 3

Nucleotide diversity in *Plasmodium falciparum* chloroquine resistance transporter of *Plasmodium falciparum* populations in relation to sample collection period

Population bordering	Year	<i>n</i>	<i>M</i>	<i>h</i>	$\pi \pm SE (\times 10^{-3})$	$\pi_S \pm SE (\times 10^{-3})$	$\pi_N \pm SE (\times 10^{-3})$
Myanmar	1991–2008	41	10	2	0.038 $\pm$ 0.037	0.000 $\pm$ 0.000	0.049 $\pm$ 0.045
	2009–2016	56	15	7	0.197 $\pm$ 0.074	0.131 $\pm$ 0.127	0.215 $\pm$ 0.084
Cambodia	1991–2008	41	15	4	0.340 $\pm$ 0.140	0.179 $\pm$ 0.166	0.384 $\pm$ 0.180
	2009–2016	28	20	9	1.488 $\pm$ 0.392##	0.000 $\pm$ 0.000	1.896 $\pm$ 0.571*
Malaysia	1991–2008	10	10	2	0.157 $\pm$ 0.154	0.000 $\pm$ 0.000	0.200 $\pm$ 0.190
	2009–2016	13	19	5	1.701 $\pm$ 0.409###	0.565 $\pm$ 0.537	2.013 $\pm$ 0.572***
Total	1991–2008	92	17	6	0.187 $\pm$ 0.071	0.080 $\pm$ 0.078	0.217 $\pm$ 0.087
	2009–2016	97	27	17¶	0.780 $\pm$ 0.228#	0.151 $\pm$ 0.106	0.951 $\pm$ 0.255**

h = number of haplotypes; *M* = number of mutations relative to the 3D7 coding regions; π = nucleotide diversity; π_S = nucleotide diversity at synonymous sites; π_N = nucleotide diversity at nonsynonymous sites. Test of significant difference between π for population during 1991 and 2008 and those during 2009 and 2016: # $P < 0.05$; ## $P < 0.01$; ### $P < 0.005$. Test of significant difference between π_N for population during 1991 and 2008 and those during 2009 and 2016: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. Randomization test of the hypothesis that the number of haplotypes for the population during 1991 and 2008 equals those during 2009 and 2016: ¶ $P < 0.05$.

TABLE 4

Nucleotide diversity (π) at synonymous sites (π_S) and nonsynonymous sites (π_N) of the *Pfcr*t coding sequences of Thai isolates collected during 1991 and 2016

Collection period/ <i>Pfcr</i> t domain	π ($\times 10^{-3}$)	π_S ($\times 10^{-3}$)	π_N ($\times 10^{-3}$)
1991–2008 (<i>N</i> = 92)			
All coding region	0.187 $\pm$ 0.071	0.080 $\pm$ 0.078	0.217 $\pm$ 0.084
Transmembrane domains	0.363 $\pm$ 0.154♦	0.162 $\pm$ 0.163	0.422 $\pm$ 0.187
Remaining regions	0.032 $\pm$ 0.032	0.000 $\pm$ 0.000	0.041 $\pm$ 0.039
2009–2016 (<i>N</i> = 97)			
All coding region	0.780 $\pm$ 0.228♦	0.151 $\pm$ 0.102	0.951 $\pm$ 0.245###*
Transmembrane domains	1.427 $\pm$ 0.431♦	0.307 $\pm$ 0.210	1.753 $\pm$ 0.523#*
Remaining regions	0.211 $\pm$ 0.126	0.000 $\pm$ 0.000	0.265 $\pm$ 0.159
All			
All coding region	0.494 $\pm$ 0.128	0.117 $\pm$ 0.067	0.597 $\pm$ 0.160#
Transmembrane domains	0.915 $\pm$ 0.253♦	0.236 $\pm$ 0.134	1.113 $\pm$ 0.330#
Remaining regions	0.124 $\pm$ 0.076	0.000 $\pm$ 0.000	0.156 $\pm$ 0.094

*Pfcr*t = *Plasmodium falciparum* chloroquine resistance transporter. Test of the hypothesis that mean π for transmembrane domains equals that for the remaining regions: ♦*P* < 0.05. Test of the hypothesis that mean π for all coding regions of population during 1991 and 2008 equals the corresponding value of population during 2009 and 2016: ♦*P* < 0.05. Test of the hypothesis that mean π_S equals the corresponding mean π_N : #*P* < 0.05; ##*P* < 0.01. Test of the hypothesis that mean π_N for population during 1991 and 2008 equals mean π_N for population during 2009 and 2016: **P* < 0.05; ***P* < 0.01.

usage against falciparum malaria may not prevent ongoing selection in the *Pfcr*t locus because ACT may influence the evolution of this gene. A significant increase in the number of haplotypes and nonsynonymous mutations in *Pfcr*t across malaria-endemic areas in Thailand after nationwide change in ACT to 3-day regimen could be exerted by an increase in intensity of drug pressure from either increased duration of artesunate administration or long-term use and cumulative effect of mefloquine. This phenomenon occurred neither by chance nor by population process because no significant change in the number of haplotypes was observed in other unrelated genetic loci (*Pfdhfr*, *Pfmsp-1*, and *Pfmsp-2*), whereas gene flow was limited across endemic areas. Furthermore, intra-genic recombination seems not to play an important role in generating sequence variation in *Pfcr*t among samples analyzed.

Natural selection exerted by antimalarial drugs could have influenced sequence variation in genes conferring drug resistance.^{12,38} Our study has shown that positive Darwinian selection favoring amino acid variants in *Pfcr*t was evidenced in the 3-day ACT group but not in isolates collected previously, suggesting that selective pressure has been triggered by an increase in duration of treatment regimen. Evidence of positive selection occurred exclusively in the transmembrane domains of *Pfcr*t for the 3-day ACT group (Table 3). Tajima's *D* test for nonsynonymous sites in *Pfcr*t yielded significant deviation from zero in negative direction, whereas a nonsignificant *D* value was found at synonymous sites (Table 4). The absence of recombination event along with the low level of gene (haplotype) diversity in the *Pfcr*t locus and the excess of

low-frequency variants at nonsynonymous sites (strong negative Tajima's *D* value) are consistent with the presence of slightly deleterious mutations in coding regions of this gene. In fact, variation in the growth rate of *Pfcr*t mutants has been documented among different haplotypes.⁵ Therefore, slightly deleterious mutations in *Pfcr*t will be subject to ongoing purifying selection which will either eliminate the change from the population or drive them to low frequencies.³⁸ On the other hand, some compensatory mutations seem to be required to restore the fitness of some mutants. For example, the Cam734 haplotype (haplotype IV in this study) reportedly acquired the mutations A144F, I148L, and S333T with dual chloroquine resistance phenotype and compensatory restoration of fitness.⁶ A prolonged bottleneck effect or selective sweeps on the *Pfcr*t locus could occur during several decades of chloroquine selective pressure in Thailand as evidenced by analysis of flanking microsatellite loci.³⁹ Several lines of evidence have supported the prediction that slightly deleterious variants will accumulate in a species that has undergone a severe bottleneck or in cases where recombination is reduced or absent.^{40,41} A number of singleton substitutions and the presence of synonymous polymorphism in *Pfcr*t of Thai isolates could have arisen from genetic drift, whereas the emergence of slightly deleterious mutations is a transient feature of evolution and these nonsynonymous mutations are subject to be eliminated by ongoing purifying selection. Importantly, integrated malaria control strategies in Thailand other than antimalarial treatment policy during the sample collection period were unchanged. Taken together, it is likely that an increase in duration of artesunate as a 3-day ACT regimen could provoke

TABLE 5

Genetic diversity in *Plasmodium falciparum* chloroquine resistance transporter of *Plasmodium falciparum* populations in Thailand

Population	Sampling period		Endemic areas bordering			Total
	1991–2008	2009–2016	Myanmar	Cambodia	Malaysia	
<i>N</i>	92	97	97	69	23	189
No. haplotypes	6	17	8	11	6	21
No. polymorphic sites	9	22	8	16	15	25
No. segregating sites	9	20	8	14	15	23
Tajima's <i>D</i>	−2.187###	−2.264###	−2.178###	−2.026#	−2.423###	−2.376###
<i>D</i> (synonymous sites)	−1.037	−1.382	−1.031	−1.069	−1.161	−1.516
<i>D</i> (nonsynonymous sites)	−2.119#	−2.198###	−2.105#	−1.972#	−2.403###	−2.302###

#, ##, and ### denote significant values at *P* < 0.05, *P* < 0.02, and *P* < 0.01, respectively.

further selective pressure on the *Pfcr*t locus among *P. falciparum* isolates in Thailand. The impact of these novel haplotypes on parasite growth rate and drug susceptibility status would require further elucidation such as by transfection-based approaches.

Received May 11, 2018. Accepted for publication July 5, 2018.

Published online August 20, 2018.

Note: Supplemental materials, figures, and tables appear at www.ajtmh.org.

Acknowledgments: We are grateful to all patients who donated their blood samples for this study. We thank the staff of the Bureau of Vector Borne Disease, Department of Disease Control, Ministry of Public Health, Thailand, for assistance in field work.

Financial support: The Thai Government Research Budgets (GRB-APS-12593011 and GBA-600093004) and Chulalongkorn University Research Grants (Fiscal Years 2006–2008) to S. J., C. P., and S. S.; The Thailand Research Fund (RSA5980054) to C. P.; and Ratchadapiseksompetch Fund, Faculty of Medicine, Chulalongkorn University (RA60/109) to C. P., S. J., and P. B.

Authors' addresses: Pattakorn Buppan, Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand and Inter-Department Program of Biomedical Sciences, Faculty of Graduate School, Chulalongkorn University, Bangkok, Thailand, E-mail: pattakorn.b@gmail.com. Napaporn Kuamsab, Chaturong Putaporn, and Somchai Jongwutiwes, Molecular Biology of Malaria and Opportunistic Parasites Research Unit, Department of Parasitology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, E-mails: kuamsab@gmail.com, p.chaturong@gmail.com, and jongwutiwes@gmail.com. Suneer Seethamchai, Department of Biology, Faculty of Science, Naresuan University, Pitsanulok Province, Thailand, E-mail: sunoat@gmail.com. Pongchai Harnyuttanakorn, Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, E-mail: hponchai@gmail.com.

REFERENCES

- Wellems TE, Plowe CV, 2001. Chloroquine-resistant malaria. *J Infect Dis* 184: 770–776.
- Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR, 2002. Epidemiology of drug-resistant malaria. *Lancet Infect Dis* 2: 209–218.
- Fidock DA et al., 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* 6: 861–871.
- Ecker A, Lehane AM, Clain J, Fidock DA, 2012. PfCRT and its role in antimalarial drug resistance. *Trends Parasitol* 28: 504–514.
- Petersen I et al., 2015. Balancing drug resistance and growth rates via compensatory mutations in the *Plasmodium falciparum* chloroquine resistance transporter. *Mol Microbiol* 97: 381–395.
- Gabrysiewicz SJ et al., 2016. Evolution of fitness cost-neutral mutant PfCRT conferring *P. falciparum* 4-aminoquinoline drug resistance is accompanied by altered parasite metabolism and digestive vacuole physiology. *PLoS Pathog* 12: e1005976.
- Callaghan PS, Hassett MR, Roepe PD, 2015. Functional comparison of 45 naturally occurring isoforms of the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT). *Biochemistry* 54: 5083–5094.
- Agrawal S et al., 2017. Association of a novel mutation in the *Plasmodium falciparum* chloroquine resistance transporter with decreased piperaquine sensitivity. *J Infect Dis* 216: 468–476.
- Parker D, Lerdprom R, Srisatjarak W, Yan G, Sattabongkot J, Wood J, Sirichaisinthop J, Cui L, 1994. Longitudinal in vitro surveillance of *Plasmodium falciparum* sensitivity to common antimalarials in Thailand between 1994 and 2010. *Malar J* 11: 290.
- Chaijaroenkul W, Ward SA, Mungthin M, Johnson D, Owen A, Bray PG, Na-Bangchang K, 2011. Sequence and gene expression of chloroquine resistance transporter (*Pfcr*t) in the association of in vitro drugs resistance of *Plasmodium falciparum*. *Malar J* 10: 42.
- Takahashi N et al., 2012. Large-scale survey for novel genotypes of *Plasmodium falciparum* chloroquine-resistance gene *Pfcr*t. *Malar J* 11: 92.
- Harinasuta T, Suntharasamai P, Viravan C, 1965. Chloroquine-resistant falciparum malaria in Thailand. *Lancet* 2: 657–660.
- Malikul S, 2000. *Malariaology 1999 in Commemoration of 50 Years of Malaria Control in Thailand*. Bangkok, Thailand: The Agricultural Co-operative Federation of Thailand Press (in Thai).
- Thimasam K, Sirichaisinthop J, Vijaykudga S, Tansopalaks S, Yamokgul P, Laomiphol A, Palanant C, Thamewat U, Thaitong S, Rooney W, 1995. In vivo study of the response of *Plasmodium falciparum* to standard mefloquine/sulfadoxine/pyrimethamine (MSP) treatment among gem miners returning from Cambodia. *Southeast Asian J Trop Med Public Health* 26: 204–212.
- Wongsrichanalai C, Prajakwong S, Meshnick SR, Shanks GD, Thimasam K, 2004. Mefloquine-its 20 years in the Thai malaria control program. *Southeast Asian J Trop Med Public Health* 35: 300–308.
- Zhou G, Sirichaisinthop J, Sattabongkot J, Jones J, Bjørnstad ON, Yan G, Cui L, 2005. Spatio-temporal distribution of *Plasmodium falciparum* and *P. vivax* malaria in Thailand. *Am J Trop Med Hyg* 72: 256–262.
- World Health Organisation, 2014. *Status Report on Artemisinin Resistance: September 2014*. Geneva, Switzerland: WHO.
- Putaporn C, Kuamsab N, Kosuvin R, Tantiwattanasub W, Vejakama P, Sueblinvong T, Seethamchai S, Jongwutiwes S, Hughes AL, 2016. Natural selection of K13 mutants of *Plasmodium falciparum* in response to artemisinin combination therapies in Thailand. *Clin Microbiol Infect* 285: e1–e8.
- Jongwutiwes S, Buppan P, Kosuvin R, Seethamchai S, Pattanawong U, Sirichaisinthop J, Putaporn C, 2011. *Plasmodium knowlesi* malaria in humans and macaques, Thailand. *Emerg Infect Dis* 17: 1799–1806.
- Sidhu AB, Verdier-Pinard D, Fidock DA, 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by PfCRT mutations. *Science* 298: 210–213.
- 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.
- Dhingra SK et al., 2017. A variant PfCRT isoform can contribute to *Plasmodium falciparum* resistance to the first-line partner drug piperaquine. *MBio* 8: e00303–e00317.
- Conrad MD et al., 2014. Comparative impacts over 5 years of artemisinin-based combination therapies on *Plasmodium falciparum* polymorphisms that modulate drug sensitivity in Ugandan children. *J Infect Dis* 210: 344–353.
- Hemming-Schroeder E et al., 2018. Impacts of antimalarial drugs on *Plasmodium falciparum* drug resistance markers, western Kenya, 2003–2015. *Am J Trop Med Hyg* 98: 692–699.
- Tumwebaze P et al., 2017. Changing antimalarial drug resistance patterns identified by surveillance at three sites in Uganda. *J Infect Dis* 215: 631–635.
- Putaporn C, Buppan P, Jongwutiwes S, 2011. Improved performance with saliva and urine as alternative DNA sources for malaria diagnosis by mitochondrial DNA-based PCR assays. *Clin Microbiol Infect* 17: 1484–1491.
- Librado P, Rozas J, 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25: 1451–1452.
- Tamura K, Stecher G, Peterson D, Filipski A, Kumar S, 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30: 2725–2729.
- Martin DP, Murrell B, Khoosal A, Muhire B, 2017. Detecting and analyzing genetic recombination using RDP4. *Methods Mol Biol* 1525: 433–460.
- Tajima F, 1983. Evolutionary relationship of DNA sequences in finite populations. *Genetics* 105: 437–460.
- Excoffier L, Lischer HE, 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour* 10: 564–567.
- Looareesuwan S, Viravan C, Webster HK, Kyle DE, Hutchinson DB, Canfield CJ, 1996. Clinical studies of atovaquone, alone or in combination with other antimalarial drugs, for treatment of

- acute uncomplicated malaria in Thailand. *Am J Trop Med Hyg* 54: 62–66.
33. Thaithong S, Beale GH, 1981. Resistance of ten Thai isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by in vitro tests. *Trans R Soc Trop Med Hyg* 75: 271–273.
 34. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF, 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature* 345: 255–258.
 35. 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 (*Pfcr*) and their relationship to chloroquine resistance in vitro. *Mol Biochem Parasitol* 136: 273–285.
 36. Jongwutiwes S, Putaporntip C, Hughes AL, 2010. Bottleneck effects on vaccine-candidate antigen diversity of malaria parasites in Thailand. *Vaccine* 28: 3112–3117.
 37. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimdé AA, Kouriba B, Taylor TE, Plowe CV, 2003. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. *J Infect Dis* 187: 1870–1875.
 38. Hughes AL, 2008. Near neutrality: leading edge of the neutral theory of molecular evolution. *Ann NY Acad Sci* 1133: 162–179.
 39. Nash D, Nair S, Mayxay M, Newton PN, Guthmann JP, Nosten F, Anderson TJ, 2005. Selection strength and hitchhiking around two anti-malarial resistance genes. *Proc Biol Sci* 272: 1153–1161.
 40. Hughes AL, Packer B, Welch R, Bergen AW, Chanock SJ, Yeager M, 2003. Widespread purifying selection at polymorphic sites in human protein-coding loci. *Proc Natl Acad Sci USA* 100: 15754–15757.
 41. Price MN, Arkin AP, 2015. Weakly deleterious mutations and low rates of recombination limit the impact of natural selection on bacterial genomes. *MBio* 6: e01302–e01315.