Preliminary Surveillance of *Pfkelch13* Mutations in *Plasmodium falciparum* Isolated from Southern Thailand

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Abstract

The occurrence of artemisinin resistance in the Greater Mekong Subregion (GMS) becomes a significant threat to the global effort to control malaria. In Thailand, the studies of artemisinin resistance have been reported from the area of Thai-Cambodia and Thai-Myanmar border. Little is known about artemisinin resistance in Southern Thailand. This study aimed to identify the molecular markers of artemisinin resistance associated with *Plasmodium falciparum kelch13* mutations (*Pfkelch13*). Seventy-eight dried blood spot samples of *P. falciparum* infection were collected from five provinces in Southern Thailand during 2012 to 2017, two on the Thai-Myanmar border (Chumphon and Ranong), one on Thai-Malaysia border (Yala) and two from non-border provinces (Phang-nga and Suratthani). Seven non-synonymous single nucleotide polymorphism (SNP) including C580Y, P574L, C447Y, H366L, V510G, I590T, and N554S and three synonymous SNP including A582A, F583F, and D547D in *Pfkelch13* were found. The C580Y mutation was the most prevalent type on the Thai-Myanmar border. However, no mutations on *PfKelch13* were detected in Suratthani and Yala. Tracking the spread of artemisinin-resistant parasites is critical to planning for effective treatment. The information of *P. falciparum* artemisinin resistance in this study will provide potent information for malaria public health guideline to optimal treatment strategies in these areas.

Keywords: *Artemisinin resistance, Plasmodium falciparum, Southern Thailand, Molecular marker, PfKelch13 gene, C580Y mutation*

1. Introduction

The first line drug against uncomplicated *P. falciparum* malaria infection, recommended by WHO, is artemisinin-based combination therapies (ACTs) (WHO, 2019). The combination of an artemisinin derivative (dihydroartemisinin, artesunate, and artemether) and their partner drug (lumefantrine, mefloquine, amodiaquine, sulfadoxine/pyrimethamine, and piperaquine) can improve the efficacy of antimalarial agents in malaria-endemic areas (Nguyen et al., 2003). The mechanism of artemisinin compound is to reduce the parasite in blood circulation during the first three days of treatment, while the partner drug plays a role to remove the remaining parasites (Woodrow, Haynes, & Krishna 2005). However, the first reported artemisinin resistance was found in the clinical studies in Western Cambodia since 2008 (Dondorp et al., 2009). Artemisinin resistance is exhibited as slow parasite clearance (Dondorp et al., 2009; Ashley et al., 2014) and reduced the susceptibility of ring stage parasite (Witkowski et al., 2013; Winzeler & Manary, 2014). Molecular studies using whole genome sequencing indicated that artemisinin resistance was associated with mutations in *Kelch13* propeller gene encoded by PF3D7_134700 on *P. falciparum* chromosome 13 (*PfKelch13*) (Ariey et al., 2014). The non-synonymous SNP in *PfKelch13* gene, Y493H, R539T, I543T, and C580Y mutations were common and strongly increased parasite survival rate (Ariey et al., 2014). The *Pfkelch13* mutations associated with artemisinin resistance appear to have arisen in Cambodia, Myanmar, Laos, and Thailand with C580Y as the most frequent allele observed (Ariey et al., 2014; Tun et al., 2015; Imwong et al., 2017; Kobasa et al., 2018).

In Thailand, a regimen of three-day artesunate plus mefloquine (AS/MQ) has been introduced since 2009. Due to high treatment failures of AS/MQ, the national therapy policy replaced it with dihydroartemisinin plus piperaquine (DHA/PPQ) as the first-line drug according to WHO guideline in 2015 (WHO, 2015). Artemisinin resistance gene mutations have been noticed in western, eastern and northern along Thailand borders (Imwong et al., 2015; Talundzic et al., 2015; Phyo et al., 2016). The southern part of Thailand is bordered with two countries, Myanmar in the west and Malaysia in the south where malaria-

endemic areas were influenced by forest environments, expansion of agricultural plantations and cross border migrations. There was limited information about the presence of artemisinin resistance in Southern Thailand. This study aimed to determine the prevalence of *PfKelch13* mutations in Southern Thailand. Knowing the distribution of these polymorphisms in the areas is crucial to monitoring the therapeutic efficacy of antimalarial drugs.

2. Objectives

To determine the prevalence of *PfKelch13* mutations gene in *P. falciparum* isolated from Southern Thailand to provide potent information for malaria treatment strategies.

3. Materials and Methods

3.1 Blood Collection and DNA extraction

The 78 *P. falciparum*-infected blood samples were collected between 2012 – 2017 from malaria clinic under the Office of Disease Prevention and Control 11 and 12 in Chumphon, Ranong, Phang-nga, Suratthani and Yala, Thailand. The blood was spotted on Whatman 3 MM filter paper. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen Inc., Germany) in accordance with the manufacturer's instructions. All samples were confirmed to be *P. falciparum* using PCR amplification based on 18S rRNA as described previously (Snounou et al., 1993).

Ethical approval for the protocol of this study was approved by the Faculty of Medicine, Prince of Songkla University (REC60-096-19-2).

3.2 *PfKelch13* gene amplification

PfKelch13 gene was amplified from the genomic DNA samples using nested PCR (Ariey et al. 2014). The initial primary PCR was amplified to cover region 1-2283 bases. Three nested PCR reactions were performed with amplifying fragment 1 (1-840 bp), fragment 2 (621-1538 bp) and fragment 3 (1344-2129 bp) of the *Pfkelch13* gene. Primer sequences used in this study are shown in Table 1. For the first PCR, the reactions were carried out using a final volume of 20 µl including 125 µM of dNTPs, 125 nM of each primer, 2 mM of $MgCl_2$, 0.5 U of *Platinum* Taq DNA polymerase (Invitrogen, USA), and 5 µl of genomic DNA. The reaction conditions consisted of an initial denaturation at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min. For the second PCR, 3 µl of the first PCR's product was used for the second round, 250 µM of dNTPs, 250 nM of each primer, and 3 mM of $MgCl_2$. The second PCR reaction was completed with 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min, and a final extension at 72°C for 5 min. The PCR products were observed on 1.5% gel electrophoresis, stained with 0.5 µg/ml of ethidium bromide, visualized under UV light, and photographed.

3.3 Sequencing analysis

The *PfKelch13* amplicons were purified using the QIAquick PCR purification kit (QIAGEN Inc., Germany) and sequenced by Macrogen (Macrogen, South Korea). The nucleotide sequences were blasted on the NCBI sequence database to confirm the *P. falciparum Kelch13* gene. Multiple sequences alignment was performed using the Bioedit sequence alignment editor software with *Kelch13* sequence of PF3D7_1343700 retrieving from PlasmoDB as the reference.

Table 1 Primers for detection *P. falciparum Kelch13* mutation

Pfkelch13 **Fragment**	**Primer**	**Sequence (5'>3')**	**Sequencing primer**	**Product Size (bp)**
Nest1	K13_c.1 F K13_c.2283 R	TGGAAGGAGAAAAAGTAAAAACAAAA TGTGCATGAAAATAAATATTAAAGAAG		2283
F1	K13_c.1F K13_c.840R	TGGAAGGAGAAAAAGTAAAAACAAAA TTGTACAATCGTACTCTTTCCATTTC	K13_c.840R	840
F2	K13_c.621F K13_c.1538 R	CGGAATTAAGTGATGCTAGTGA CGATCATACACCTCAGTTTCAA	K13_c.621F	917
F3	K13_c.1344 F K13_c.2129 R	AGGTGGATTTGATGGTGTAGAA GGGCCAAGCTGCCATTCATTTGT	K13_c.1344F	786

4. Results and Discussion

A total of 78 *P. falciparum* infections, collected from Southern Thailand, as indicated in Figure 1, were evaluated. The *Pfkelch13* gene was successfully amplified, and sequence polymorphism was analyzed. The results showed that 80.8% (63/78) of the samples are *Pfkelch13* wild-type and 19.2% (15/78) are *Pfkelch13* mutation genes. Table 2 indicated the *Pfkelch13* mutation analysis from each province. C580Y mutant was the most predominant allele in this study which accounted for 45.5% (10/22) of the samples from Ranong, 6/9 of these were collected in 2013, and 4/7 were collected in 2015. The mutation at the position P574L was found in one isolate from Chumphon. Two samples of synonymous SNP at codon A582A plus F583F and D547D were detected in Ranong. However, these synonymous SNP had no association with artemisinin resistance (Huang et al., 2015). Seven of non-synonymous SNP were identified in this study. Four novel identified mutations (I590T, C447Y, H366L, and V510G) were observed from the samples collected in 2012 from Ranong and Phang-nga. One I590T plus N554S mutation and one sample combining the mutation of C447Y, H366L and V510G were found in isolates from Ranong and Phang-nga, respectively. Among these mutations, the N554S mutation was found in Kenya, but this position did not responsible for delayed parasite clearance (Isozumi et al., 2015). All parasites isolated from Suratthani (41/41) and Yala (7/7) province carried wild-type *PfKelch13* allele (Figure1).

Overall, this study found that artemisinin resistance associated with *PfKelch13* polymorphism (C580Y and P574L) only spread around in provinces that are bordered by Myanmar (Ranong and Chumphon). The movement of the population from Myanmar crossing the border to Ranong and Chumphon for trading or occupational exposure may contribute to the transmission of artemisinin resistance malaria (Wongsrichanalai et al., 2001; Bhumiratana et al., 2013). On the other hand, the *PfKelch13* mutation was not observed in Yala, the province that is neighboring Malaysia. Several studies have reported the mutations conferring drug resistance from the boundaries between countries such as Thai-Cambodia, Thai-Myanmar and Thai-Laos borders (Ariey et al., 2014; Tun et al., 2015; Imwong et al., 2017). Our findings are consistent with those reported earlier that the C580Y point mutation was the most widespread in the GMS (Ariey et al., 2014; Miotto et al., 2015; Imwong et al., 2017) and Thailand, along the borders with Myanmar and Cambodia but not Malaysia (Putaporntip et al., 2015; Talundzic et al., 2015; Kobasa et al., 2018). *In vivo* and *in vitro* study have confirmed that the C580Y mutation was validated as a marker that linked with high artemisinin-resistant phenotype (Ariey et al., 2014; WHO, 2016). P574L mutant allele was reported to be associated with clinical resistance (WHO, 2016) and was also highly prevalent in Myanmar and China (Tun et al., 2015; Yang et al., 2017).

Table 2 *Pfkelch13* mutation analysis in five provinces in Southern Thailand among 2012-2017

Year	Provinces	number of samples (N)						
		wild-type	C580Y	P574L	C447Y+ H366L V510G	N554S+ I590T	Synonymous SNP	total
2012	Ranong	3	-	-	-	1	2	6
	Phang-nga	1	-	-	1	-	-	2
2013	Ranong	3	6	-	-	-	-	9
	Chumphon	2	-	-	-	-	-	2
2014	Chumphon	1	-	1	-	-	-	2
	Suratthani	16	-	-	-	-	-	16
2015	Ranong	3	4	-	-	-	-	7
	Suratthani	15	-	-	-	-	-	15
	Phang-nga	2	-	-	-	-	-	2
2016	Suratthani	10	-	-	-	-	-	10
2017	Yala	7	-	-	-	-	-	7
	N (%)	63(80.8)	10(12.8)	1 (1.3)	1(1.3)	1(1.3)	2 (2.5)	78

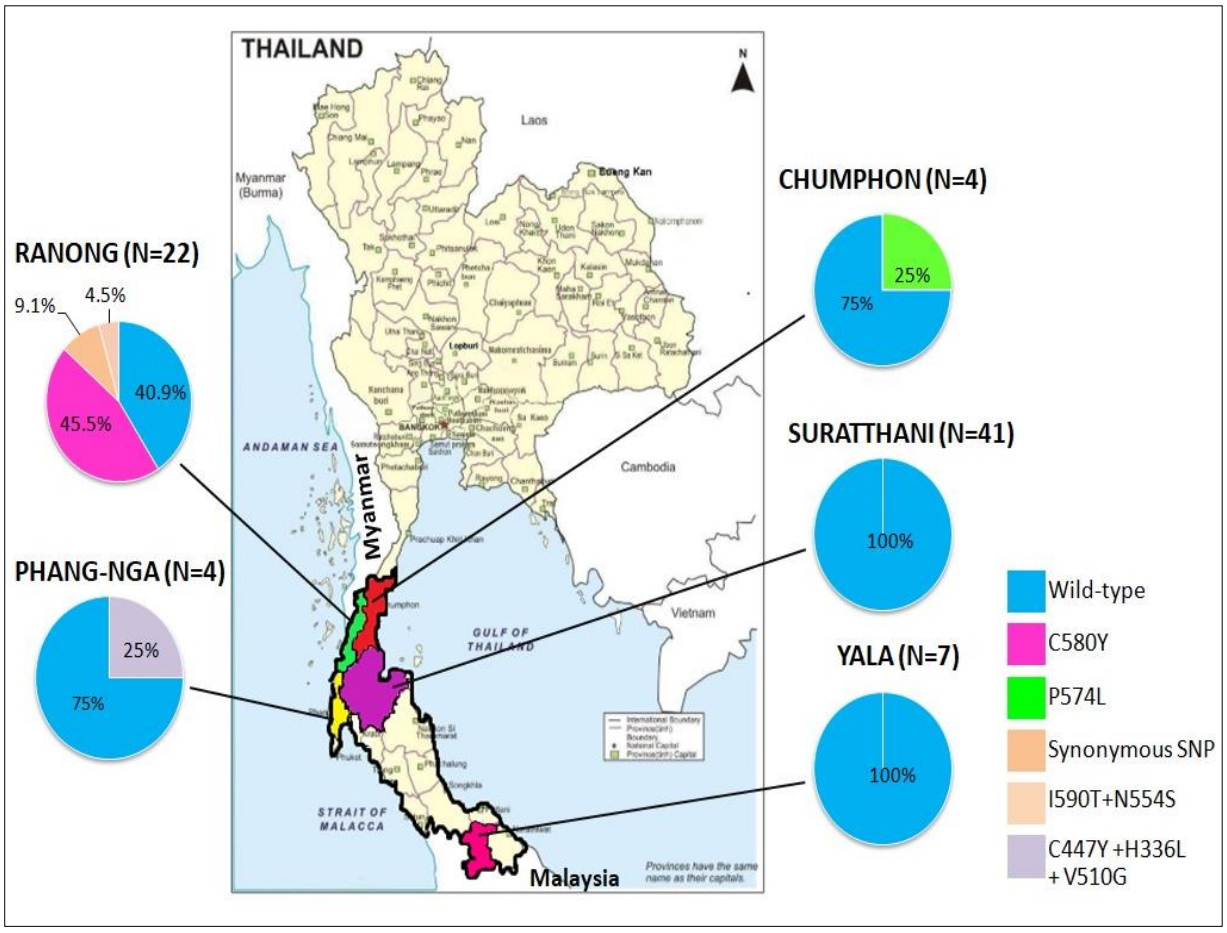

Figure 1 The distribution of *PfKelch13* mutations collected in five provinces of Southern Thailand

5. Conclusion

In conclusion, this study presents an overview of the prevalence of the *Pfkelch13* mutations from some provinces in Southern Thailand. The data indicated that the artemisinin resistance still limited to the west border of Southern Thailand. Thus, continuous drug resistance assessment will facilitate the tracking of the artemisinin resistance. Despite the delayed response to artemisinin that has appeared in various areas of the GMS, ACTs treatment remains the most useful drug for uncomplicated falciparum malaria. A number of patients that manifest as delayed parasite clearance are treated so long as the partner drug remains effective (WHO, 2018). Investigating these molecular markers is critical to enhancing the understanding of the artemisinin resistance and predicting its spread.

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