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▣ BRIEF COMMUNICATION

Evolution of Genetic Polymorphisms of *Plasmodium falciparum* Merozoite Surface Protein (*PfMSP*) in Thailand

Jiraporn Kuesap[1], Wanna Chaijaroenkul[1], Kanchanok Ketprathum[1], Puntanat Tattiyapong[1], Kesara Na-Bangchang[2,*]

[1]Faculty of Allied Health Sciences, [2]Chulabhorn International College of Medicine, Thammasat University, Pathumthani, 12121, Thailand

Abstract: *Plasmodium falciparum* malaria is a major public health problem in Thailand due to the emergence of multidrug resistance. The understanding of genetic diversity of malaria parasites is essential for developing effective drugs and vaccines. The genetic diversity of the merozoite surface protein-1 (*PfMSP-1*) and merozoite surface protein-2 (*PfMSP-2*) genes was investigated in a total of 145 *P. falciparum* isolates collected from Mae Sot District, Tak Province, Thailand during 3 different periods (1997-1999, 2005-2007, and 2009-2010). Analysis of genetic polymorphisms was performed to track the evolution of genetic change of *P. falciparum* using PCR. Both individual genes and their combination patterns showed marked genetic diversity during the 3 study periods. The results strongly support that *P. falciparum* isolates in Thailand are markedly diverse and patterns changed with time. These 2 polymorphic genes could be used as molecular markers to detect multiple clone infections and differentiate recrudescence from reinfection in *P. falciparum* isolates in Thailand.

Key words: *Plasmodium falciparum*, merozoite surface protien, genetic polymorphism, Thailand

Malaria remains one of the most important public health problems in several tropical countries. *Plasmodium falciparum* infection causes clinical symptoms ranging from asymptomatic to the rarer complications of severe manifestations. Cerebral malaria (CM) is one of the major pathological complications of *P. falciparum* infection in humans manifesting as coma that can lead to death. The emergence and spread of resistance of *P. falciparum* to antimalarial drugs is an important factor for malaria control in endemic areas [1]. The resistance of *P. falciparum* has occurred to all classes of antimalarial drugs except artemisinin and its derivatives. The understanding of genetic diversity of malaria parasites is essential for developing effective drugs and vaccines. The merozoite surface protein-1 (MSP-1) of *P. falciparum* is a major surface protein with an approximate molecular size of 190 kDa. MSP-1 exerts a key role in erythrocyte invasion by the merozoite [2]. It is a target of human immune responses [3] and a promising candidate for a blood stage subunit vaccine [4]. MSP-2 of *P. falciparum* is another candidate antigen for a subunit malaria vaccine [5]. The objective of this study was to investigate genetic diversity of *PfMSP-1* and *PfMSP-2* genes in blood samples collected from 145 patients with uncomplicated *P. falciparum* malaria in Mae Sot District of Thailand during the 3 different study periods.

A total of 145 blood samples were collected from patients attending the malaria clinic in Mae Sot District, Tak Province during 3 different periods, *i.e.*, 1997-1999 (n=49), 2005-2007 (n=50), and 2009-2010 (n=46). Approval of the study protocol was obtained from the Ethics Committees of Ministry of Public Health, Thailand. Tak Province has been reported as the province with highest malaria incidence with approximately equal ratio of *P. falciparum* and *P. vivax*. Two milliliters of blood samples were collected by venipuncture prior to treatment with standard regimens for *P. falciparum* (a 3-day artesunate-mefloquine combination) and collected into EDTA collecting tubes. Giemsa-stained thin and thick blood smears were prepared and examined microscopically for *P. falciparum*. Parasite genomic DNA was extracted from whole blood using Chelex extraction method and used as the template for PCR amplification.

The amplification of *PfMSP-1* and *PfMSP-2* was carried out using PCR technique [6]. In the reaction, primer pairs corresponding to the conserved sequences spanning the polymorphic regions consisted of forward-5'GAAGATGCAGTATTGACAGG3' and reverse-5'GAGTTCTTTAATAGTGAACAAG3' for MSP-1 and forward-5'GAGTTCTTTAATAGTGAACAAG3' and reverse-

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* Corresponding author (kesaratmu@yahoo.com)

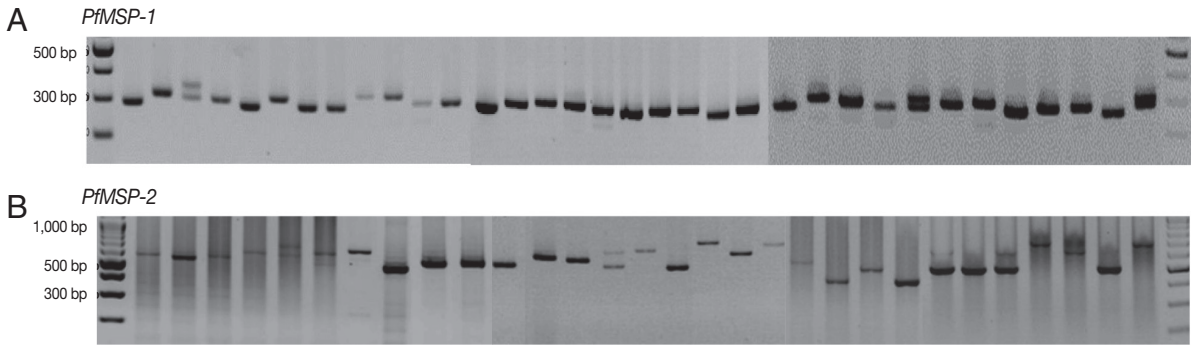

Fig. 1. Genetic polymorphisms of *PfMSP-1* and *PfMSP-2*.

Table 1. Summary of polymorphic sizes of *PfMSP-1* in *Plasmodium falciparum* isolates collected during the 3 different study periods (1997-1999, 2005-2007, and 2009-2010)

Study period	Polymorphic size (bp)		
	1997-1999	2005-2007	2009-2010
	260	280	280
	290	290	285
	300	295	290
	310	300	300
	320	320	310
	300/320	290/300/320	315
	300/330	300/310	320
		300/320	325
		300/320/340	330
		310/320	300/400
			310/360
			315/370
			320/360
			325/360
			330/380
Total no. of polymorphisms	7	10	15

Table 2. Summary of polymorphic sizes of *PfMSP-2* in *Plasmodium falciparum* isolates collected during the 3 different study periods (1997-1999, 2005-2007, and 2009-2010)

Study period	Polymorphic size (bp)		
	1997-1999	2005-2007	2009-2010
	495	450	450
	500	480	480
	550	490	490
	570	500	500
	580	510	510
	590	520	520
	600	530	590
	620	550	600
	630	570	610
	650	580	630
	690	590	650
	700	595	660
	900	600	690
	550/620	610	700
	590/610	690	500/520
	590/690	480/560	520/530
	500/600	500/690	
	500/650		
Total no. of polymorphisms	18	17	16

5'CCTGTACCTTTATTCTCTGG3' for MSP-2 [6]. The reaction volume was 20 µl containing 1 µM of each of primer, 0.5 U of Taq polymerase, 1x of buffer with KCl (Fermentas, Burlington, Canada), 2.5 mM of $MgCl_2$ (Fermentas), 0.5 mM of dNTP and DNA template. PCR was performed under 1 cycle of 5 min at 94℃, then 30 cycles of 1 min at 94℃, 1 min at 50℃, 1 min at 72℃, and final extension at 72℃ for 5 min of amplification condition. PCR products were analyzed on a 2% agarose gel containing ethidium bromide. The variation in size of the amplified products was observed.

The genetic diversity pattern of *PfMSP-1* and *PfMSP-2* were analyzed using GeneTools software (SYNGENE™, Cambridge, UK). This software automatically compensates for smiling or distorted bands and tracks. Molecular weight or base pair values can be calculated using 2 standards for comfirmation. Comparison of difference in gene patterns during 3 different periods of sample collection was performed using the chi-square test (SPSS version 12.0 software, SPSS Inc., Chicago, Illinois, USA). Statistical significance level was set at $P = 0.05$.

The amplification results of 145 samples during the 3 study periods (1997-1999, 2005-2007, and 2009-2010) were successful in 46 (94%), 50 (100%), and 46 (100%) for *PfMSP-1* and 33 (67%), 29 (58%), and 39 (85%) for *PfMSP-2*, respectively. Both *P. falciparum* genes were highly polymorphic (Fig. 1) with different gene patterns in samples collected during the 3 periods (Tables 1-2). The dominant polymorphic sizes of

Table 3. Polymorphic sizes of various combination patterns of *PfMSP-1/PfMSP-2*

Pattern	*PfMSP-1/PfMSP-2* (bp)					
	1997-1999		2005-2007		2009-2010	
1	320	495	290	450	300	450
2	290	500	290	480	310	450
3	300	500	300	480	300	480
4	310	500	320	480	310	480
5	320	500	290/300/320	480/560	300	490
6	310	500/600	320	490	300	500
7	300	500/650	300/320	500	310/360	500
8	300	550	300/310	500/690	315	500
9	310	550	310/320	500/690	320/360	500
10	290	550/620	290	510	320/360	500/520
11	300	570	300/320/340	520	325/360	500/520
12	310	580	290	530	290	510
13	320	580	290	550	300	510
14	310	590	300	570	310	510
15	300/320	590/610	290	580	320/360	510
16	300	590/690	300	580	330/380	520
17	310	600	300	590	310/360	520/530
18	320	600	300	595	290	530
19	290	620	310/320	595	290	590
20	310	620	300	600	300	590
21	290	630	300	610[a]	310	590
22	300	650[a]	300	690	300	600
23	310	690	320	690	310	600
24	290	700			310	610[a]
25	260	900			290	650[a]
26					310	650
27					315	650
28					310	660
29					300	690
30					310	690
31					280	700
32					300	700

[a]Statistically significant difference from other borders (by chi-square test).

PfMSP-1 and *PfMSP-2* detected during 1997-1999, 2005-2007, and 2009-2010 were 300 and 500 bp, 300 and 480 bp, and 310 and 650 bp, respectively. The multiple clone infections were detected by 2 or more PCR fragments. A significant difference in the pattern of *PfMSP-1* was observed between isolates collected during the period 1997-1999 vs 2005-2007 ($P = 0.002$), 2005-2007 vs 2009-2010 ($P < 0.001$), and between 1997-1999 vs 2009-2010 ($P = 0.028$). For the pattern of *PfMSP-2*, significant difference was found between isolates collected during the period 1997-1999 vs 2005-2007 ($P = 0.050$).

The polymorphic sizes of the combined *PfMSP-1*/*PfMSP-2* were more diversed than each individual gene alone; 25, 23, and 32 patterns of *PfMSP-1*/*PfMSP-2* polymorphisms were observed (Table 3). The dominant combination pattern found in samples collected during the period 1997-1999, 2005-2007, and 2009-2010 were 300/500, 300/610, and 310/650, respectively. A total of 5 (15.5%), 3 (17.8%), and 7 (18.4%) samples collected during the 3 periods showed multiple clone infections, respectively.

Several malarial proteins have been proposed as vaccine candidate antigens but MSP-1 is the most promising candidate [7,8]. Results of the phase 1-2b clinical trial of a MSP-2 based vaccine showed that 1 allelic type included in the vaccine may be more effective against malaria parasite [9]. *P. falciparum* MSP-2, apical membrane antigen-1 (AMA-1), and circumsporozoite protein (CSP) are also under investigation as candidate antigens for the development of malaria vaccine [10,11]. The polymorphisms of *PfMSP-1* and *PfMSP-2* have been investigat-

ed in isolates collected from several malaria endemic areas [12-16]. All showed highly polymorphic patterns of these 2 genes. High levels of *PfMSP-1* and *PfMSP-2* polymorphisms and multiple clonal infections were reported in 3 malaria endemic regions of Lao PDR [13]. Similarly, sequence analysis of *PfMSP-1* block 2 in *P. falciparum* isolates collected from Myanmar demonstrated 14 different genotypes (5 for K1 type and 9 for MAD20 type), whereas 22 genotypes (7 for FC27 type and 15 for 3D7 type) were found with *PfMSP-2* block 3 [12]. A recent report from Republic of Congo revealed high polymorphisms and multiple clones of *P. falciparum* isolates [15]. Moreover, isolates collected from Malawi, Tanzania, Uganda, Burkina Faso, and São Tomé exhibited highly polymorphic and low allele frequencies of *PfMSP-1*, *PfMSP-2*, and *glurp*, with a total of 17 *PfMSP-1*, 116 *PfMSP-2*, and 14 *glurp* genotypes [16]. In contrast, relatively low levels of genetic diversity were found in isolates collected from Haiti (9 *PfMSP-1* genotypes) [14].

The results of the present study confirmed the genetic variations of *PfMSP-1* and *PfMSP-2* in isolates collected from Mae Sot District, the endemic area of Thailand with highest malaria incidence. Moreover, the combination of *PfMSP-1*/*PfMSP-2* was relatively more polymorphic, and thus appropriate for application to detect multiple clone infections and differentiate recrudescence from reinfection in *P. falciparum* isolates in Thailand. The low efficacy of vaccine candidate antigens observed in various clinical trials would be due to the highly variable genetic polymorphisms of *PfMSP-1* and *PfMSP-2* in *P. falciparum* isolates.

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CONFLICT OF INTEREST

We have no conflict of interest related with this study.

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