C-TERMINAL POLYMORPHISM OF *PLASMODIUM FALCIPARIUM* MEROZOITE SURFACE PROTEIN-1 (MSP-1) FROM TAK PROVINCE, THAILAND

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Abstract. This study was undertaken to ascertain the extent of polymorphism in the C-terminal region of *Plasmodium falciparum* merozoite surface protein (MSP-1) from 119 malaria patients in Tak Province on the western border of Thailand, who were admitted to the Bangkok Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. *P. falciparum* infection was confirmed by microscopic examination of peripheral blood smears. Clinical manifestations were categorized into 2 groups: uncomplicated (94 cases) and complicated/severe (25 cases). A 1,040 basepair fragment of *P. falciparum* MSP-1 gene was compared with MSP-1 of reference strains retrieved from GenBank. The consensus sequences of MSP-1 block 16 showed it belonged to MAD20 genotype, which is the major allele of *falciparum* malaria from the western border of Thailand. MSP-1 block 16 amino acid fragment could be separated into 2 groups: similar and dissimilar to reference sequence. Four variations in MSP-1 block 16 were -1494K, D1510G, D1556N, and K1696I. MSP-1 block 16 diversity is not significantly associated with clinical manifestation although MAD 20 genotype is the predominant genotype in this area. The genetic data of MSP1 gene of *faciparum* malaria isolated from western Thai border contribute to the existing genetic database of Thai *P. falciparum* strain.

Keywords: *Plasmodium falciparum*, merzoite surface protein-1, polymorphism

INTRODUCTION

According to World Malaria Report 2011, malaria caused by *Plasmodium* parasites results in 300 to 500 million cases with 0.1% mortality annually worldwide (WHO, 2012). Although the incidence of both morbidity and mortality of malaria is now decreasing but the presence of *falciparum* drug resistance especially to artemisinin along the Thai-Cambodia border is a matter of great concern.

Merozoite surface protein 1 (MSP-1) is one of *falciparum* surface proteins involved in invasion of red blood cell. MSP-1 is synthesized as a 195 kDa protein

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precursor that is cleaved into three fragments, 83 kDa N-terminal, 38 kDa central and 42 kDa C-terminal (Espejo *et al*, 2004, Takala *et al*, 2006). Just before invasion, the 42 kDa C-terminal fragment is further cleaved into 33 and 19 kDa fragments (Ferreira *et al*, 2003), the 33 kDa fragment being shed (Blackman *et al*, 1992) and 19 kDa C-terminal fragment remaining anchored to the merozoite surface at the time of erythrocyte invasion (Ferreira *et al*, 2003). The sequence of this region is conserved with only a few polymorphic sites (Ferreira *et al*, 2003; Ballou *et al*, 2004).

MSP-1 exhibits extensive polymorphism (Tanabe *et al*, 2007a). The protein consists of 17 blocks (N- to C-terminus) according to the degree of amino acid polymorphism among alleles especially in block 2 of the N-terminal and block 17 at the C-terminal regions. (Tanabe *et al*, 1987; Miller *et al*, 1993). Genetic diversity of MSP-1 is defined as unique combination of allelic types, which are named according to parasite strains, *viz* K1, MAD20 and RO33. For instance MSP-1 block 2 genotype in Myanmar is classified into 14 different alleles, 5 for K1 type and 9 for MAD20 type (Kang *et al*, 2010).

In Tanzania, where malaria transmission is intense in endemic area, MSP-1 haplotype diversity shows lower incidence than in less intense transmission areas, such as Solomon Islands, Thailand and Vietnam (Ferreira *et al*, 1998; Tanabe *et al*, 2007b). The number of parasite haplotypes declines from 20 haplotypes to 9 haplotypes during 1993-2003) in Rufiji, eastern coastal Tanzania (Tanabe *et al*, 2007b). In Solomon Islands, diversity of MSP-1 haplotype is greater than the nearby island, Papua New Guinea, which has a higher transmission rate of malaria (Sakihama *et al*, 2006). Parasites isolated from low transmission areas, *eg*, Thailand and Vietnam, express haplotype frequencies higher than Papua New Guinea and Tanzania (Ferreira *et al*, 1998; Sakihama *et al*, 2006).

This study was performed in order to determine the polymorphism of MSP-1 C-terminal region of *P. falciparum* in an endemic area where multidrug resistance exist, *ie*, western border of Thailand where malarial infection is endemic (Lopes *et al*, 2002; Chaveepojnkamjorn and Pichainarong, 2004).

MATERIALS AND METHODS

During May to October 2007, 119 subjects between 15 and 65 years of age of either gender, with confirmed mono-infection with *falciparum* malaria parasite by microscopic examination, and who agreed to participate to this study were recruited. Subjects who were younger than 15 and older than 65 years old, pregnant women, or did not agree to participate were excluded. Five ml aliquot of blood was drawn from the patients before treatment with the artemisinin combination regimen. All patients were stratified into 2 categories: uncomplicated and complicated/severe malaria according to WHO (2006) classification. Clinical and laboratory data including blood smear, complete blood count and blood biochemistry were recorded as a routine procedure. Implementation, monitoring procedures and research protocols complied with the regulations of Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

For determination of polymorphism, genomic DNA was extracted from 100 l of whole blood sample using a QIAamp Blood Kit (Qiagen, Valencia, CA) according to manufacturer's instruction. In order to determine polymorphism of PfMSP-1 gene, a 1,040 bp DNA fragment

spanning the variation blocks was amplified by PCR (Ruang-areerate, unpublished data). PCR reaction was carried out in 50 l volume using primers MSP1-up (5′ TATATATTTAAAACCTTTAGCTGGAGTAT 3′) and MSP-1-down (5′ TGGGGTTTAGTCAATTCACAT3′). Thermocycling consisted of 94ºC for 3 minutes, 40 cycles of 94ºC for 30 seconds, 54.3ºC for 1 minute and 72ºC for 1 minute; and final incubation at 72ºC for 7 minutes. The 1,040 bp fragment amplicons were visualized under UV light following 1% agarose gel-electrophoresis.

Amplicons were purified using high pure PCR product purification kit (Roche, Mannheim, Germany) and directly sequenced using an automated ABI prism 377 sequencer (Applied Biosystem, Carlsbad, CA) employing Big Dye Terminator version 3.1 Sequencing Kit (Applied Biosystem, Carlsbad, CA). Analysis of PfMSP-1 C-terminal region of each DNA sequence was performed using Sequencer 4.1.4 software (Gene Codes, Ann Arbor, MI) and subsequently aligned to reference strains of the region, W2 and other strains retrieved from GenBank (www.ncbi.nlm.nih.gov) using DNASTAR (Lasergene, Madison, WI). The MSP1 sequences were submitted to GenBank; accession number JQ664244-JQ664294. PAUP 4.0 was used to construct phylogenetic tree (Swofford, 2002). Protein conformation was predicted using computerized prediction tool, protean of DNASTAR software package (DNASTAR, LaserGene, Madison, WI).

RESULTS

Patients' demography and treatment

Among 119 *falciparum* malaria patients who were admitted to the Bangkok Hospital for Tropical Diseases, Thailand and agreed to participate in the study, 94 (80%) were classified as uncomplicated and 25 (20%) as severe *falciparum* malaria. The average age was 27.2 ± 9.1 (15 - 56) years in the uncomplicated group and 23.1 ± 4.9 (17 - 34) years in the severe group. Seventy-five percent of uncomplicated malaria patients had a history of malaria and infection while only 58% of the severe group had a history of previous infection. Common manifestations of the patients were headache, myalgia, dizziness, anorexia, and vomiting, while some had more additional manifestations including sweating, tiredness, loss of appetite, abdominal pain with liver and spleen enlargement.

Average numbers of white red blood cell were within normal limits in both groups (Table 1). However, the means of hemoglobin, hematorcrit, and platelets were slightly low in both groups (13.3 ± 8.3 *vs* 12.1 ± 2.8 g/dl, 34.7 ± 4.0 *vs* 30.4 ± 6.9%, and 102.7 ± 52.6 *vs* 74.0 ± 42.7 $x10^3/mm^3$, respectively). In addition, the liver enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were higher in the severe group when compare with the uncomplicated group (34.5 ± 24.5 *vs* 54.2 ± 36.7 IU and 33.3 ± 25.0 *vs* 41.6 ± 30.1, respectively).

To observe treatment response, three common parameters were monitored: parasite clearance time (PCT), defined as time in hours that parasites are absolutely cleared from patient's blood after antimalarial drug treatment; fever clearance time (FCT), time in hours when the body temperature returns to normal; and initial parasite count. No significant difference in PCT was observed between the two groups (51.1 ± 24.1 *vs* 57.4 ± 20.3 hours). The uncomplicated group had shorter fever duration compared with the severe group (39.2 ± 36.1 *vs* 54.1 ± 38.2 hours). The initial parasite count was 10 times higher in the severe group.

Table 1
Demography of patients catergorized by clinical presentation.

	Uncomplicated, U (n = 94)	Severe, S (n= 25)
Age (years)		
Mean (SD)	27.2 (9.1)	23.1 (4.9)
Range	15 - 56	17 - 34
No. of patients with		
splenomegaly	6	2
hepatomegaly	7	5
first malaria attack	55	18
geometric mean parasite count (per l)	32,443	230,378
Range	10,360 - 423,750	15,880 - 1,483,520
Laboratory data, mean (SD)		
Packed cell volume (%)	34.7 (4.0)	30.4 (6.9)
WBC count (x 10^3/ l)	5.3 (1.9)	6.7 (2.5)
RBC (x 10^6/ l)	4.6 (0.8)	4.4 (0.9)
Hb (g/dl)	13.3 (8.3)	12.1 (2.8)
Platelet (x10^3/mm^3)	102.7 (52.6)	74.0 (42.7)
Blood glucose (mg/dl)	125.3 (25.1)	133.6 (29.6)
Blood urea (mg/dl)	15.7 (5.1)	23.2 (15.1)
Serum creatinine (mg/dl)	0.8 (0.2)	1.1 (0.3)
Serum AST (IU x 10^3/ l)	34.5 (24.5)	54.2 (36.7)
Serum ALT (IU x 10^3/ l)	33.3 (25.0)	41.6 (30.1)
Alkaline phosphatase (U/l)	95.0 (41.0)	91.7 (34.5)
Sodium (mmol/l)	134.7 (3.3)	130.5 (9.7)
Potassium (mmol/l)	3.6 (0.4)	3.6 (0.5)
Chloride (mmol/l)	100.7 (3.6)	99.2 (4.7)
Carbonate (mmol/l)	25.5 (2.3)	23.0 (2.6)
FCT (hr)	39.2 (36.1)	54.1 (38.2)
PCT (hr)	51.1 (24.1)	57.4 (20.3)

MSP-1 polymorphism

MSP-1 amino acid alignments of MSP-1 block 16 sequences from the patients and 4 reference sequences, comprising 3 clinical Iranian clones (MADCH-2, MADCH-18 and MADCH-19) (Mehrizi *et al*, 2008), and a laboratory clone, 3D7 (Hall *et al*, 2002) retrieved from GenBank (www.ncbi.nlm.nih.gov) were performed (Fig 1). The patients sequences were classified into 2 groups: 12 sequences similar to the reference clones and a distinctive group comprising of 39 sequences containing a missing amino acid resulting in a frame shift. In the latter group, nucleotides "AAG" at position 1492 of MSP-119 corresponding to lysine (K) were missing, and in the former group, there were amino acid variations in position (D1510G), position (D1556N), and position 1574 (K1696I).

The prediction of secondary protein structure demonstrated that isoelectric

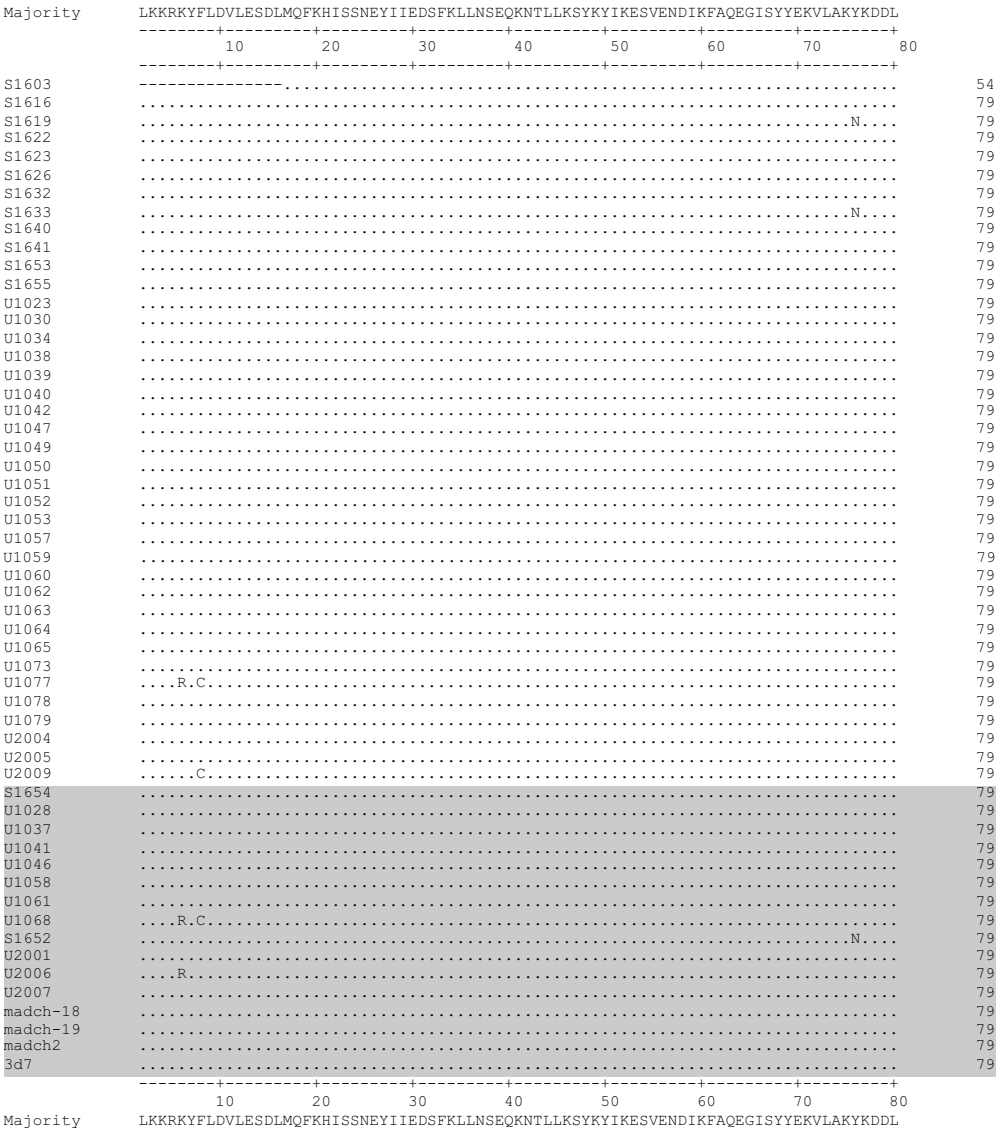

Fig 1–Amino acid alignment of *P. falciparium* MSP-1 isolated from western border region of Thailand. Unshaded label indicates distinct sequence that contains one amino acid deletion causing a frame shift ($n = 39$), and shaded label indicates sequence that is similar to reference sequences. U, uncomplicated malaria; S, severe malaria

point (IEP) and net charge at pH 7.0 between these two groups are different. IEP of 12 sequences (former group) is 6.11 and of 39 sequences (latter group) is 6.74, and net charge at pH 7 is -2.64 and -0.64, respectively.

MSP-1 phylogenetic tree consisted of 4 main clusters: a large group consisting of 25 samples and three smaller groups consisting of 6-12 samples (Fig 2). Therapeutic criteria are added in bracket of the sample name. Most of the patients successfully

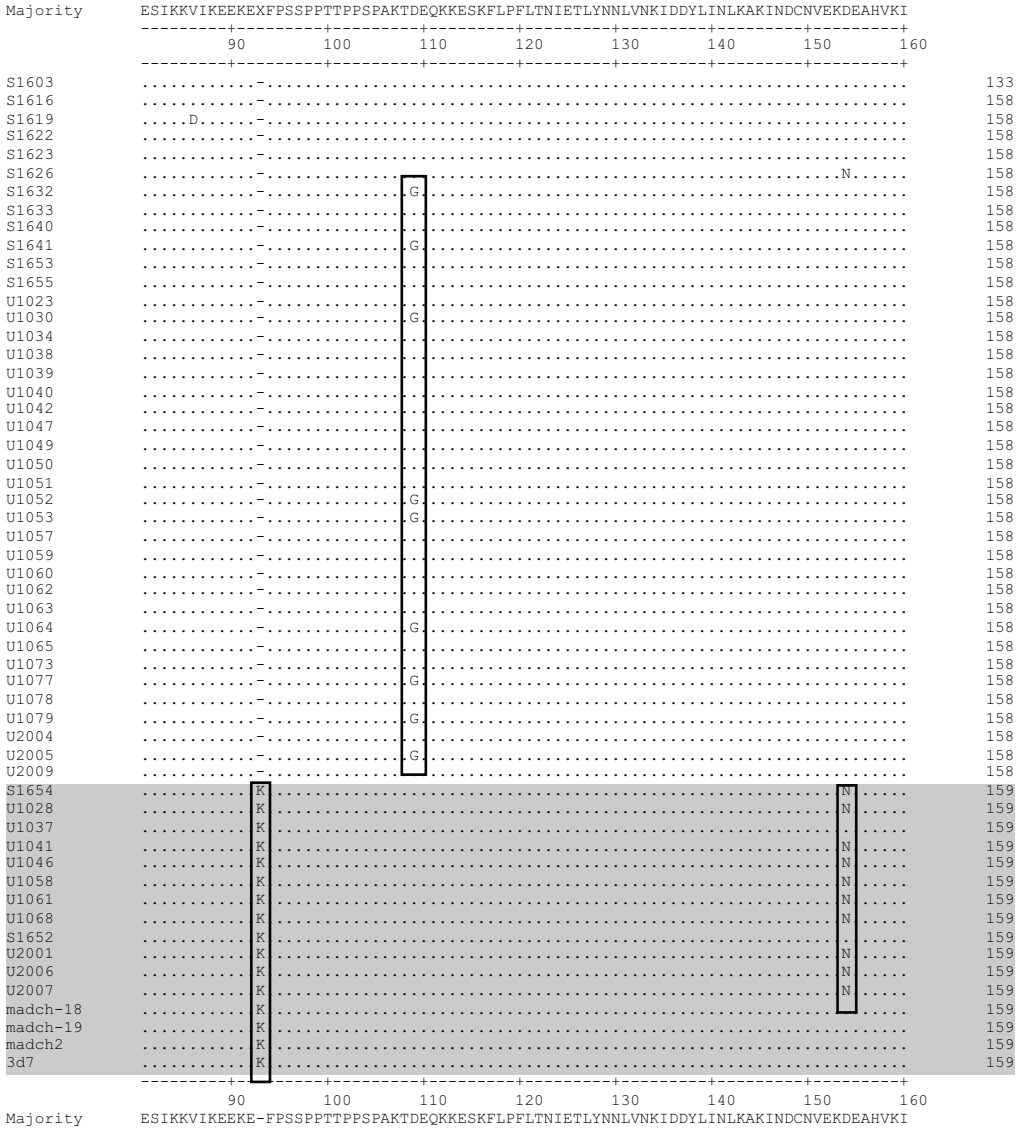

Fig 1–(Continued).

recovered, and a few cases dropped out, as well as late treatment failure. Similar clinical pictures and patients' background were found in all clusters. Hematological and biochemistry parameters revealed no significant difference among the clusters. Biological evolution of MSP-1 gene showed a non-correlated evolution among uncomplicated and severe *P. falciparum* groups. Both uncomplicated and severe *P. falciparum* cases are found in the same clade of different clusters.

DISCUSSION

Merozoite of *P. falciparum* expresses a number of merozoite surface proteins (MSPs) of which MSP-1 is the major

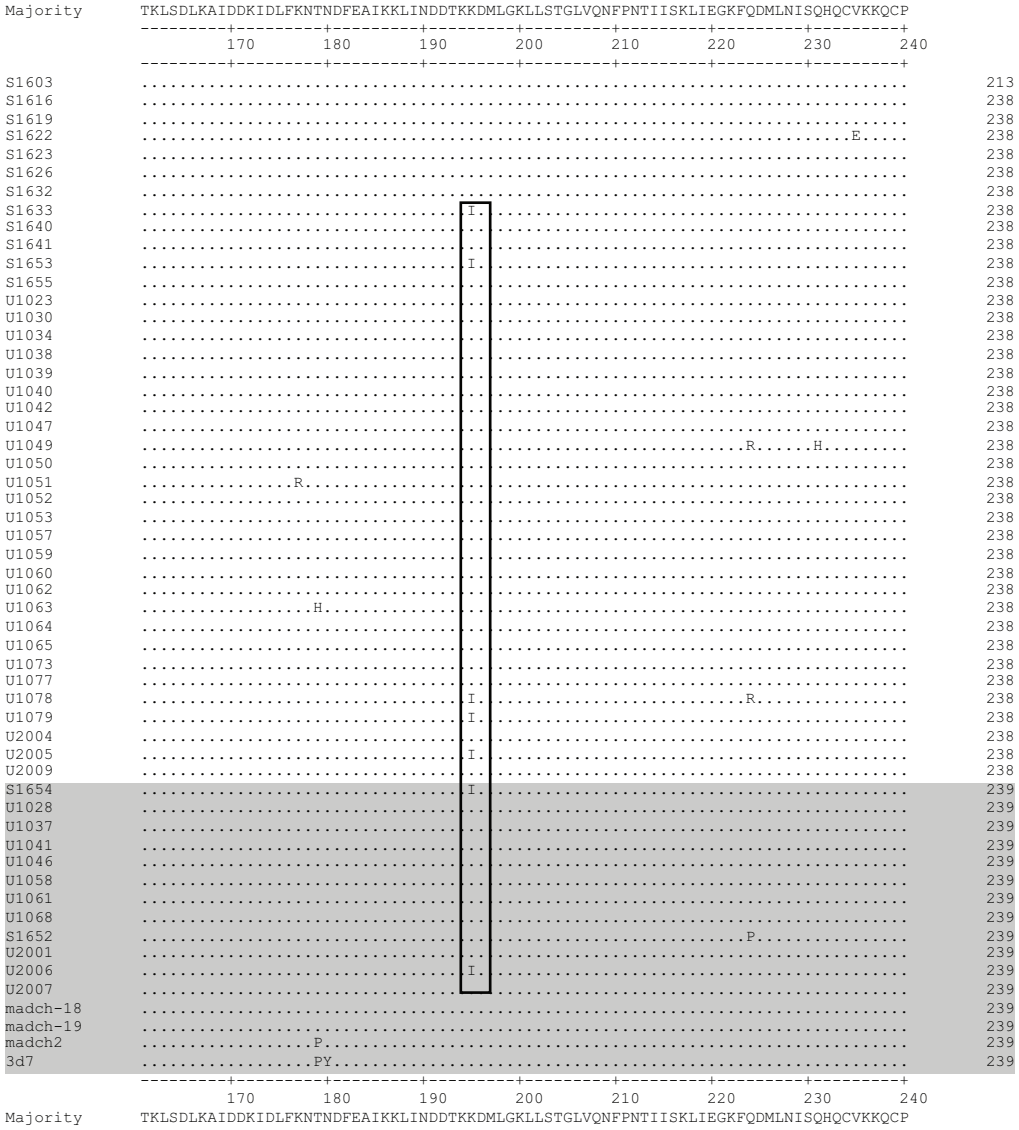

Fig 1–(Continued).

protein that recognizes red cell surface receptor (Holder, 1994). The precursor protein of MSP-1 (195 kDa) is cleaved into a number of fragments, namely, 83 kDa (MSP-183), 28-30 kDa (MSP-128), 38 kDa (MSP-138) and 42 kDa (MSP-142). MSP-142 fragment is further processed into 33 kDa (MSP-133) and 19 kDa (MSP-119). The 19 kDa piece is a membrane protein that is carried along with the parasite when the merozoite invades the red cell (Holder *et al*, 1992). Interestingly, MSP-119 appears to play essential roles both in parasite invasion and as a immunodominant epitope of T and B cells (Jongwutiwes *et al*, 1992; Hui *et al*, 1996; Hirunpetcharat *et al*, 1997; Tian *et al*, 1998; Galamo *et al*, 2009; Holder, 2009).

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Majority      ENSGCFRHLDEREECKCLLNYKQEGDKCVENPNPTCNE
              ---------+---------+---------+--------
                       250       260       270
              ---------+---------+---------+--------
S1603         ....................................... 251
S1616         ....................................... 276
S1619         ....................................... 276
S1622         ....................................... 276
S1623         ....................................... 276
S1626         .....................................Y. 276
S1632         ....................................... 276
S1633         ....................................... 276
S1640         ....................................... 276
S1641         ....................................... 276
S1653         ....................................... 276
S1655         ....................................... 276
U1023         .....................................D 276
U1030         ....................................... 276
U1034         ....................................... 276
U1038         ....................................... 276
U1039         ....................................... 276
U1040         ....................................... 276
U1042         ....................................... 276
U1047         ....................................... 276
U1049         ....................................... 276
U1050         ....................................... 276
U1051         ....................................... 276
U1052         ....................................... 276
U1053         ....................................... 276
U1057         ....................................... 276
U1059         ....................................... 276
U1060         ....................................... 276
U1062         ....................................... 276
U1063         Q...................................... 276
U1064         ....................................... 276
U1065         ....................................... 276
U1073         ....................................... 276
U1077         ....................................... 276
U1078         ....................................... 276
U1079         ....................................... 276
U2004         ....................................... 276
U2005         ....................................... 276
U2009         ....................................... 276
S1654         ....................................... 277
U1028         ....................................... 277
U1037         Q...................................... 277
U1041         ....................................... 277
U1046         ....................................... 277
U1058         ....................................... 277
U1061         ....................................... 277
U1068         ....................................... 277
S1652         Q...................................... 277
U2001         ....................................... 277
U2006         ....................................... 277
U2007         ....................................... 277
madch-18      ....................................... 277
madch-19      ....................................... 277
madch2        ....................................... 277
3d7           ....................................... 277
              ---------+---------+---------+--------
                       250       260       270
              ---------+---------+---------+--------
Majority      ENSGCFRHLDEREECKCLLNYKQEGDKCVENPNPTCNE
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Fig 1–(Continued).

In terms of allelic determinants, MSP-1 block 2 near the N-terminus is classified into 3 allelic types based on 3 reference isolates, namely MAD20, K1 and RO33 (Certa *et al*, 1987; Tanabe *et al*, 1987). A study of MSP-1 polymorphism in isolates from Pakistan showed that MAD20 is the outstanding allelic form and the other 2 allelic types are found in other geographical areas (Ghanchi *et al*, 2010). A survey of Thai isolates has revealed that MAD20 is the major allelic form with five different variant sizes of between 160-220 base pairs (Snounou *et al*, 1999). Block 12 and 17 are highly conserved among isolates of MAD20, whereas block 16 is a variable

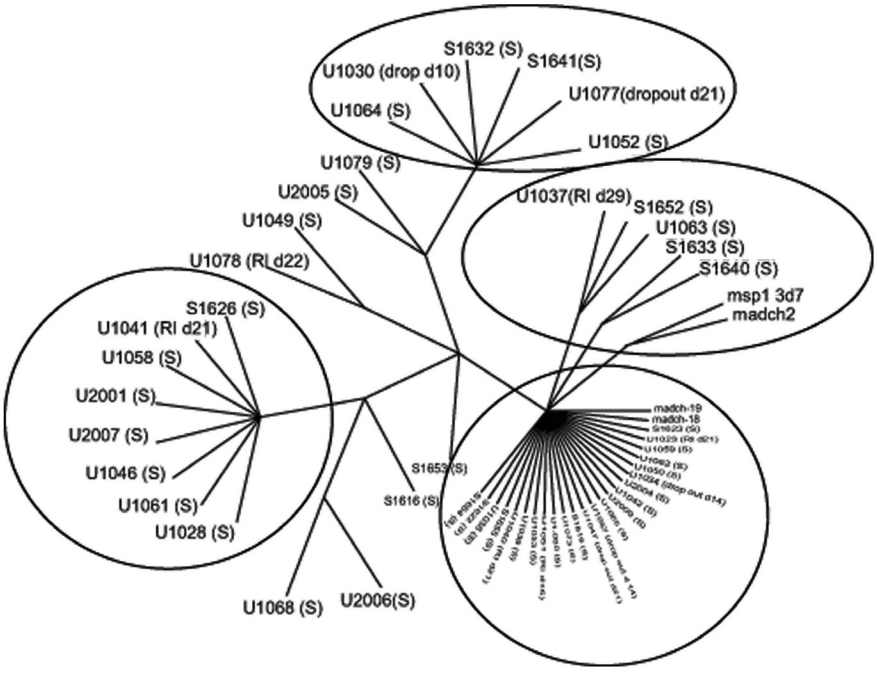

Fig 2–Phylogenetic tree based on *P. falciparum* MSP-1 gene. S, drug sensitive; R, drug resistant

block (Ferreira *et al*, 1998).

In this study, malaria patients were classified into 2 clinical distinctive groups: uncomplicated and severe. MSP-119 amino acid sequence alignment and phylogenetic tree of block 16 of these 2 groups revealed no correlation with clinical appearance. Phylogenetic tree analysis demonstrated that the uncomplicated and severe groups belonged to a similar clade, indicating that MSP-1 block 16 is not directly related with disease pathogenesis. An investigation of the influence of genetic diversity of *P. falciparum* and clinical malaria presentation in rural Bolifamba, Cameroon revealed that 3 allelic variants of MSP-1 block 2 (K1 and RO33) are significantly associated with age and various categories of anemia, while K1/RO33 allele is associated with fever and high level of parasitemia (Anong *et al*, 2010). Cerebral malaria and severe anemia in children living in Sundegath, a malaria-endemic area in India, are associated with MSP-1 RO33 subtype, 3D7 subtype of MSP-2 and K76T mutation of pfcrt protein (Sahu *et al*, 2008).

The sequences of MSP-1 block 16 of *P. falciparum* from western border of Thailand belonged to MAD20 genotype in agreement with a previous study (Paul *et al*, 1998). It is still unclear why the MAD20 genotype is not associated with clinical expression as it is the predominant genotype in this area (Tolle *et al*, 1995; Lalitha *et al*, 1999; Ghanchi *et al*, 2010).

Based on amino acid sequence diversity, MSP-1 block 16 fragment was able to be divided into 2 groups, namely reference sequence group and non-reference sequence group, the later having four variations: -1494K, D1510G, D1556N and K1696I. Changes in amino acid sequences produced different net charges. At pH 7.0, the reference group has a net charge

of -2.64 whereas the net charge of the non-reference group is -0.64. Normally, net charge of C-terminal region of *P. falciparum* MSP-119 is -2.0 while that of *P. knowlesi* and *P. cynomogi* is -7.0 and -4.0, respectively (Garman *et al*, 2003). The negative charge is located in 69-74 loop of domain 2 of MSP-119 (Garman *et al*, 2003). This loop has been suggested as being responsible for host specificity among *Plasmodium* species (Garman *et al*, 2003).

In summary, the polymorphism of merozoite surface protein (MSP-1) C-terminal region isolated from western border of Thailand revealed that this region was highly conserved among the parasite population in this area. It belonged to MAD20 genotype. There is no association between clinical manifestations and genetic diversity of MSP-1. Nevertheless, the data generated from this study contribute to existing genetic database of *P. falciparum* in Thailand.

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