Stable Allele Frequency Distribution of the *Plasmodium falciparum* clag Genes Encoding Components of the High Molecular Weight Rhoptry Protein Complex

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**Abstract:** *Plasmodium falciparum* Clag protein is a candidate component of the plasmodial surface anion channel located on the parasite-infected erythrocyte. This protein is encoded by 5 separated clag genes and forms a RhopH complex with the other components. Previously, a signature of positive diversifying selection was detected on the hypervariable region of clag2 and clag8 by population-based analyses using *P. falciparum* originating from Thailand in 1988–1989. In this study, we obtained the sequence of this region of 3 clag genes (clag2, clag8, and clag9) in 2005 and evaluated the changes over time in the frequency distribution of the polymorphism of these gene products by comparison with the sequences obtained in 1988–1989. We found no difference in the frequency distribution of 18 putatively neutral loci between the 2 groups, evidence that the background of the parasite population structure has remained stable over 14 years. Although the frequency distribution of most of the polymorphic sites in the hypervariable region of Clag2, Clag8, and Clag9 was stable over 14 years, we found that a proportion of the major Clag2 group and one amino acid position of Clag8 changed significantly. This may be a response to a certain type of pressure.

**Key words:** malaria, rhoptry, polymorphism, temporal change

**INTRODUCTION**

*Plasmodium falciparum*, the most deadly causative agent of malaria, is an obligate intra-erythrocytic protozoan parasite in humans. The erythrocyte invasive form, the merozoite, releases a panel of molecules during the erythrocyte invasion and utilizes them to recognize and modulate the host erythrocyte environment for further development. One such molecule is the high molecular mass erythrocyte-binding protein complex, or RhopH complex, secreted from the merozoite rhoptry. Antibodies against this complex have been shown to confer anti-parasite protection to the host [1–4]. However, it remained unclear how antibodies recognize the proteins secreted into the erythrocyte and locate on the erythrocyte cytosol side of the parasitophorous vacuole membrane and the parasite-infected erythrocyte membrane [5, 6]. The RhopH complex is comprised of three distinct proteins: RhopH1, RhopH2 and RhopH3, each encoded by separate genes [7–9]. RhopH1 is encoded by a multigene family, termed the cytoadherence-linked asexual gene

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Abbreviations: aa, amino acid position; Clag, cytoadherence-linked asexual gene; DNA, deoxyribonucleic acid; nt, nucleotide position; PCR, polymerase chain reaction
(rhoph1/clag) family, that consists of at least five paralogous genes (clag2, clag3.1, clag3.2, clag8, and clag9), and each Rhoph complex contains one of the rhoph1/clag gene products [7, 10, 11]. Recently, Nguitragool et al. identified clag3.1 (PFC0120w) gene locus in association with a plasmoidal surface anion channel (PSAC) activity responsible for the nutrient uptake into the infected erythrocyte, by linkage analysis using a genetic cross between HB3 and Dd2 parasite lines, followed by an allelic replacement experiment [12]. They also showed that clag3.1 product could be digested by treatment of intact parasite-infected erythrocytes with Pronase E and proposed that a part of clag3.1 product was exposed on the erythrocyte surface and played a role as a PSAC or interacted with other protein(s) to form functional PSAC [12].

Among seven Rhoph complex-related genes (5 from rhoph1/clag and 1 from each of rhoph2 and rhoph3), clag2, clag3.1, clag3.2 and clag8 were found to be highly polymorphic, especially in a region encompassing amino acid positions 1000–1200 of these gene products [13]. Population-based analyses detected a signature of positive diversifying selection on this region for clag2 and clag8 [13, 14]. Although positive selection was not detected, clag3.1 and clag3.2 showed even higher polymorphism than clag2 and clag8 with insertion/deletions, which makes the proper alignment of these genes impossible [13]. It is noteworthy that Nguitragool et al. proposed that clag3.1 products had 3 atypical transmembrane regions at amino acid positions (aa) 1000–1021, 1038–1062 and 1208–1231 and that the polymorphic region between aa 1062 and 1208 is the region expected three hydrophobic regions (hydrophobic), and the CLAG domain possessing a homology with the rhoptry neck protein 2 (RON2) are indicated. Amino acid positions (aa) predicted for the hydrophobic regions are for P. falciparum 3D7 line Clag3.1 sequence. The representative sequence of each group for Clag2 hypervariable region is shown above the scheme. Dots and bars indicate identical amino acid residues with the 3D7 line sequence and gaps.

MATERIALS AND METHODS

Parasite DNA, polymerase chain reaction (PCR) amplification and sequencing

P. falciparum genomic DNA were extracted from filter papers containing P. falciparum-infected blood spots collected in Thailand in 2003 (n = 20; sample ID, AA1329, AQ1097, AQ1099, AQ1099, AQ1101, AQ1105, AQ1125, AQ1126, AQ1127, AQ1129, AQ1130, AQ1132, AQ1133, AQ1139, AQ1142, TMPF09, TMPF11, TMPF15, TMPF18 and TMPF44) and 2005 (n = 7; AQ1423, AQ1459, PA009, PA020, PA021, Q2D015 and TMPF338) after approval by the Ethical Committee of Mahidol University as described previously [16]. The known origins in Thailand are: AQ1097, AQ1109, AA1329, AQ1099, PA009, PA020, PA021, Q2D015, TMPF09, TMPF11, TMPF15 and TMPF338 from Tak; AA1329, AQ1098, AQ1126, AQ1129, AQ1132, AQ1133, AQ1139, AQ1142, TMPF09, TMPF11, TMPF15 and TMPF338 from Kanchanaburi; AQ1101 and AQ1125 from Chiangmai; AQ1130 from Chaiyaphumi; and AQ1142 from Saraburi.

DNA fragments were independently PCR-amplified twice with oligonucleotide primers specific for clag2 (resulting in PCR product 702 bp in size), clag8 (750 bp); and clag9 (764 bp) using KOD Plus DNA polymerase (TOYOBO, Japan) under the same conditions described previously [14]. When a single-band product with no back-
ground was observed under UV transillumination after 1.5% agarose gel electrophoresis and ethidium bromide staining. PCR-amplified DNA fragments were directly sequenced following treatment of the PCR mixture with ExoSAP-IT (GE Healthcare, UK). Two independent PCR products were sequenced using a panel of primers described previously [13]; one in the forward direction and the other in the reverse direction, to ensure the accuracy of the obtained sequences. Sequencing reactions were performed using the BigDye® Terminator v1.1 Kit (Applied BioSystems, UK) with an ABI3730 DNA analyzer (Applied BioSystems). Sequences were manually corrected using BioEdit 7.0.0 software [17]. Sequences of 3 P. falciparum clag genes obtained from the samples collected in Thailand from November 1988 to January 1989 were described previously [18, 19].

In addition to the single nucleotide polymorphisms (SNPs) at 4 putatively neutral loci (PFB0200c, PFB0295w, PF10_0363, and PF14_0124) that we selected previously based on the fact that these loci encoded housekeeping proteins [16], SNPs at 10 putatively neutral loci on chromosome (chr) 2 and 3 (PFB0685c, PFB0715w, PFC0180c, PFC0215c, PFC0295c, PFC0350c, PFC0440c, PFC0530w, PFC0745c and PFC0940c), reported by Anderson et al. (2005) were also determined for our samples [16]. In addition to the reported SNPs in these loci, we determined SNPs on chr 2 at nucleotide positions (nt) 619408 and 619410 (PFB0685c), chr 2 nt 649509 (PFB0715w), and chr 3 nt 221411 (PFC0215c), and chr 3 nt 888297 (PFC0940c). We designed oligonucleotide primers surrounding these SNPs (Table 1) and directly sequenced the PCR-amplified product to obtain the SNPs information.

**Data analyses**

Amino acid sequences were aligned using BioEdit 7.0.0 software, and positional amino acid numerical information was obtained from Thai 1988–9 and 2003 group sequences for each clag product. Positional amino acid numerical information was also obtained. The difference in the proportion of the major amino acid residue between 1988-9 and 2003 groups was assessed for each position by Fisher’s exact test (two-tailed).

**RESULTS AND DISCUSSION**

**Stable SNP allele frequency distribution of the putatively neutral loci over 14 years in Thai P. falciparum isolates**

In the previous study, we selected 4 loci encoding housekeeping proteins (thus we considered them as putatively neutral to a positive selection) and evaluated the temporal change in SNP allele frequency distribution of these loci in the Thai P. falciparum population and found that they were stable over a period of 14 years [16]. To confirm this observation, we determined 14 putatively neutral SNPs on chr 2 and 3, which were reported by Anderson et al. (2005) [19], for 26 samples from the 1988-9 group and 16 samples from the 2003 group after excluding MS802, MS803, MS814A1, MS818, MS819, MS820, MS824, MS826, AQ1097, AQ1130, AQ1132 and TMPF44, most of which showed mix-allele infection for the surf4.2 gene locus [16]. With regard to MS820 and MS824, SNPs were obtained only for PF10_0363, PFB0200c and PF14_0124 in the former and only for PFB0295w in the latter. Chr 2 nt 619397 (PFB0685c), chr 2 nt 649509 (PFB0715w), and chr 3 nt 526792 (PFC0530w) showed minor allele only in a mix infection case and so were excluded from the analysis. As a result, we found no significant difference in frequency distribution of any of the 18 putatively neutral SNPs between the 1988-9 and 2003 groups (Fig. 2 and Table 2), suggesting that the parasite population structure did not dramatically change for 14 years from 1988.

The geographical variation of the 10 putatively neutral

| Chr | Position | Gene ID | Forward Primer | Reverse Primer |
|-----|----------|---------|----------------|---------------|
| 2   | 619397   | PFB0685c| ATGTTAATTACAAAAATTTTTTTATGTGTTT | CTTGATATCCGACCCCTCATATG |
| 3   | 649509   | PFB0715w| GTGATAATGACACTGTTATGTATCATC  | CATGTTTTATCCTTCAACCCATAG |
| 3   | 197815   | PFC0180c| GGTATATTTCCCAAAGGATGCTTTCC | CACTACCAAGCCCCTACTGGC |
| 3   | 221432   | PFC0215c| AGCGTCGGACAAAAAGAGCTG  | CTTGTGACCATGAATATATATGTTT |
| 3   | 309640   | PFC0295c| ATGTAGATGTAGGGGAAAGCCTG  | CTGGTCTGACTTTCTCCTAAG |
| 3   | 360148   | PFC0350c| TGTTGTGTTGTCGGCAGGTTG  | CCCCATTCTGCTTTACTTAGATAT |
| 3   | 454628   | PFC0440c| TATAGATGAAAAATGAAGCGGAG  | GAAACATTGTTATACATTTCTTG |
| 3   | 526792   | PFC0530w| AAACTGCTTATCGAGGATACAA  | CATCATGCTATGAGCCCTATAT |
| 3   | 679987   | PFC0745c| TGGGATTGATGAGGAGATGCAA  | GCAGGCTGCTTGTGTTTCTTCC |
| 3   | 888282   | PFC0940c| TATGGAACATATCCATAGAGAGCT  | GATCTATTAGATACCTCATAT |

Table 1. Oligonucleotide primers newly designed to amplify putatively neutral loci
SNPs, some of which were employed in this study, was assessed using parasite populations from Southeast Asia by Anderson et al. (2005) [19]. They found that the *P. falciparum* population in 4 Thai areas (Shoklo, Maela, Ratchaburi and Kanchanaburi) did not significantly differ from each other by evaluating $F_{ST}$ value and speculated that this might indicate an extensive gene flow between parasite populations or rapid population expansion in the region. The stable SNP allele frequency distribution of the putatively neutral loci over 14 years found in this study is in good agreement with this observation, because if *P. falciparum* parasites circulating in the region (i.e., the 4 areas in Thailand mentioned above) form one population, the population structure is likely stable.

Changes in the frequency distribution of *clag2* and *clag8* products over 14 years

Next, we evaluated the changes in allele frequency distribution of the hypervariable region of *clag* gene products over a period of 14 years by assessing the change in the proportion of major amino acid residues at each polymorphic site and that of the major Clag2 group (group 1), which showed 5 distinctive groups at the hypervariable region. Because the sequence of Clag2 is highly diversified and it was impossible to make a proper alignment, we assessed the change in the frequency distribution of Clag2 according to 4 different categories: 1) 5 groups of Clag2 hypervariable region proposed in the previous report (representative sequences for each group are shown in Fig. 1; n = 36 and 18

### Table 2. Frequency distribution of putatively neutral single nucleotide polymorphisms in Thai *P. falciparum* isolates

| Gene ID | chr | position | type | allele | 1988–9$^a$ | 2003$^a$ | p value$^b$ | reference |
|---------|-----|----------|------|--------|------------|----------|------------|-----------|
| PFB0200c | 2   | 199098   | Syn  | A/C    | 31/2       | 19/1     | 1.00       | [16]      |
| PFB0295w | 2   | 199252   | Nsyn | A/T    | 29/4       | 15/5     | 0.27       | [16]      |
| PFB0685c | 2   | 271860   | Syn  | A/G    | 18/16      | 13/7     | 0.41       | [16]      |
|         | 2   | 272601   | Nsyn | A/G    | 34/0       | 19/1     | 0.37       | [16]      |
| PFB0685c | 2   | 619408   | Nsyn | T/C    | 22/4       | 14/2     | 1.00       | this study |
|         | 2   | 619410   | Syn  | A/T    | 22/4       | 14/2     | 1.00       | this study |
| PFC0180c | 3   | 197185   | Nsyn | T/G    | 19/5       | 9/1      | 0.64       | [19]      |
| PFC0215c | 3   | 221411   | Syn  | A/C    | 4/22       | 1/15     | 0.30       | this study |
|         | 3   | 221432   | Nsyn | C/G    | 5/21       | 2/13     | 0.70       | [19]      |
| PFC0295w | 3   | 309640   | Syn  | T/G    | 19/5       | 14/0     | 0.14       | [19]      |
| PFC0350c | 3   | 360148   | Syn  | A/G    | 5/20       | 0/13     | 0.14       | [19]      |
| PFC0440c | 3   | 454628   | Nsyn | A/T    | 4/22       | 4/12     | 0.69       | [19]      |
| PFC0745c | 3   | 679987   | Syn  | A/G    | 7/17       | 2/9      | 0.69       | [19]      |
| PFC0940c | 3   | 888282   | Nsyn | A/G    | 23/3       | 13/2     | 1.00       | [19]      |
|         | 3   | 888297   | Nsyn | A/C    | 3/23       | 1/15     | 0.65       | this study |
| PF10_0363 | 10  | 1470238  | Syn  | A/T    | 14/18      | 6/14     | 0.39       | [16]      |
| PF14_0124 | 14  | 508556   | Syn  | A/G    | 7/27       | 4/16     | 1.00       | [16]      |
|         | 14  | 508706   | Syn  | A/C    | 1/33       | 1/19     | 1.00       | [16]      |

$^a$ Sites showing mix peak were excluded from the analysis. chr 2 nt 619397 (PFB0685c), chr 2 nt 649509 (PFB0715w), and chr 3 nt 526792 (PFC530w) showed minor allele only in a mixed-allele case and so were excluded from the analysis. $^b$ p-values are obtained by two-tailed Fisher’s exact test.
for the 1988–9 and 2003 group, respectively), 2) Clag2 group 1 sequence only (n = 24 and 6; 9 sites), 3) Clag2 group 2 sequence only (n = 8 and 11; 9 sites), and 4) the region conserved for all Clag2 sequences (n = 36 and 18; 3 sites) [14]. Clag2 groups 3 to 5 were not assessed because the sequence number was too low (only 3, 1, and 1 in all 1988–9 and 2003 group sequences). We found that the proportion of the Clag2 group 1 was significantly reduced in the 2003 group as compared to the 1988–9 group (p < 0.05) based on category 1, but no significant difference was observed for the other assessed polymorphic sites (Fig. 3 and Table 3) based on categories 2 to 3. With regard to Clag8 (n = 41 and 19), among the 26 polymorphic amino acid sites assessed, Ser residue at aa 1098 (number is after 3D7 line amino acid sequence) showed a significant reduction in the 2003 group as compared to the 1988–9 group (p < 0.02), but no significant difference was observed for the other polymorphic sites. No significant difference was observed for the frequency distribution of 3 polymorphic amino acid sites of Clag9 (n = 34 and 17). The SNP allele frequency distribution of 3 clag products in the 2005 group was not significantly different from that in either the 1988–9 or 2003 group, a finding consistent with the above observation that the frequency distribution of most of the polymorphic sites did not differ between the 1988–9 and 2003 groups, although the background of the parasite population structure was not assessed for the 2005 group.

The polymorphic sites of the antigenic protein, such as clag products, are considered to be exposed to and selected by the host immune system, which provides an allele-specific protection to the human host. Thus, the parasite population structure circulating in one patient would change at the individual level. Such allele-specific immunity would be able to change the pathogen’s population structure quickly if the pathogen has a short generation time and spreads quickly in the host population, which is exemplified by the antigenic drift of the influenza virus with an estimated generation time of about 10 days [20]. However, the time needed for the malaria parasite to complete one generation is much longer, estimated to be more than 200 days [21], and the change in the parasite population at the individual level may not (or not efficiently if any) be reflected on the whole parasite population structure. Although we detected significant changes in the proportion of Clag2 polymorphic region and one site in the hypervariable region of Clag8, most of the sites were stable. Thus, it remains unclear at this point whether these changes are truly due to a response against human immunity at the population level.
Further studies are required to determine how *P. falciparum* Clag polymorphism changes over time in natural circumstances.

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