Tropical Medicine and Health Vol. 40 No. 3, 2012, pp. 71-77
doi:10.2149/tmh.2012-13



**Original article**

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

Jean Semé Fils Alexandre[1,2,¶], Phonepadith Xangsayarath[1,3,¶], Morakot Kaewthamasorn[1,4], Kazuhide Yahata[1], Jetsumon Sattabongkot[5], Rachanee Udomsangpetch[6] and Osamu Kaneko[1]*

Received 8 May, 2012 Accepted 13 May, 2012 Published online 11 August, 2012

**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

[1] Department of Protozoology, Institute of Tropical Medicine (NEKKEN) and the Global COE Program, Nagasaki University, Sakamoto, Nagasaki 852-8523, Japan
[2] Centro Nacional de Control de Enfermedades Tropicales, Santo Domingo, República Dominicana
[3] National Institute of Public Health, Vientiane, Lao PDR
[4] Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[5] Mahidol Vivax Research Center, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
[6] Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
*Corresponding author:
Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Sakamoto, Nagasaki 852-8523, Japan
Tel: (+81) 95 819 7838
Fax: (+81) 95 819 7805
E-mail: okaneko@nagasaki-u.ac.jp
¶ These authors contributed equally to this work.
Sequence data reported in this article are available in the GenBank™/EMBL/DDBJ databases under the accession numbers AB710386–AB710454.
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 plasmodial 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 exposed on the erythrocyte surface [12] (Fig. 1). Thus, the polymorphism of *clag* products appears to be evolved under host immune pressure targeting the region exposed to the host immune system throughout the asexual blood stage. This model might answer the question why rhoptry body proteins such as the RhopH complex mainly secreted into the erythrocytes exhibit higher polymorphism than microneme proteins such as apical membrane antigen 1 (AMA1), which is released before the secretion of the rhoptry body contents during invasion, briefly exposed to the host immune system, and believed to undergo positive diversifying selection [15].

To design an intervention strategy targeting the polymorphic region of *clag* products, it is important to understand the dynamics of the antigenic polymorphism in the parasite population. In this study, we determined the sequences of the polymorphic region of *clag2*, *clag8*, and *clag9* in Thai *P. falciparum* DNA samples collected in 2003 and 2005 and assessed the changes in frequency distribution of the polymorphic sites over time by comparing them with those obtained from the parasites collected in 1988–1989 [14].

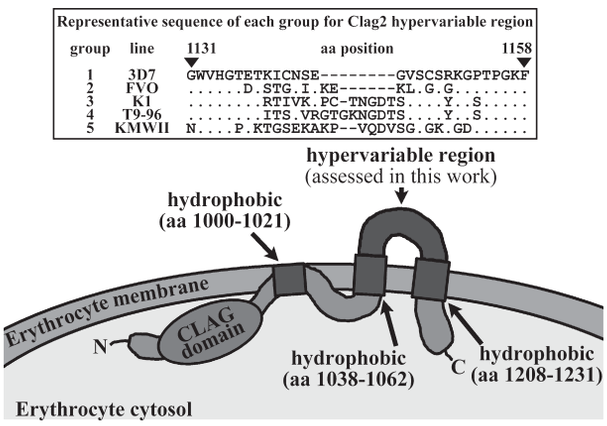


Fig. 1. Schematic topology of the *Plasmodium falciparum clag* product based on that proposed for Clag3.1 by Nguitragool *et al.* (2011) [12]. The hypervariable region evaluated in this study expected three hydrophobic regions (hydrophobic), and the CLAG domain possessing a homology with the rhoptry 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, AQ1098, 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, AQ1099, AQ1105, PA009, PA020, PA021, Q2D015, TMPF09, TMPF11, TMPF15 and TMPF338 from Tak; AA1329, AQ1098, AQ1126, AQ1129, AQ1132, AQ1133, AQ1139, AQ1423, AQ1459 and TMPF44 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 in chr 2 at nucleotide positions (nt) 619408 and 619410 (PFB0685c), 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 $surf_{4.2}$ 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 (PFC530w) 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

Table 1. Oligonucleotide primers newly designed to amplify putatively neutral loci

| Chr | Position | Gene ID | Forward Primer | Reverse Primer |
|---|---|---|---|---|
| 2 | 619397 | PFB0685c | ATGTTAATTTACAATTTTTTTATTGTGTT | CTTGTATTCCTGTACCTTCATATGTA |
| 2 | 649509 | PFB0715w | GTGTAATAGATACTGTTATGTTATCATC | CATGTATTTTATCTTCAACCATATG |
| 3 | 197185 | PFC0180c | GGTATATTCCCAAGGATGCTTCC | CACATACAAGACCCACTAAGGCTA |
| 3 | 221432 | PFC0215c | AGCGTCGACCCAAAAGTATGG | CTTGTTACACATGAATAATTATGTTTT |
| 3 | 309640 | PFC0295c | ATGTAGTAGTTGAGGAAAAGGCTG | CTGCTGAGTCCTCTCCAAAATCA |
| 3 | 360148 | PFC0350c | TGTTGTTGTTGTGGCAGGTG | CCCATTCAGCTTGTACTATAGAAT |
| 3 | 454628 | PFC0440c | TATCAGGATAAATATAAGAACGGAG | GAACATTGTTTACATTATCTTCTGTT |
| 3 | 526792 | PFC0530w | AATCAGCTTGTATCAGAGGATAAC | CATCTGCTATGATGCCAAATATTAA |
| 3 | 679987 | PFC0745c | TCAGGATTTGATGGAGATGCAAG | GCACGCTCCGTTTGGTTCAATA |
| 3 | 888282 | PFC0940 | TATGTAACATATCCATAGCAGAGTC | GTACTTATAGGATTATCATTCCTATG |

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] |
| | 2 | 199252 | Nsyn | A/T | 29/4 | 15/5 | 0.27 | [16] |
| PFB0295w | 2 | 271860 | Syn | A/G | 18/16 | 13/7 | 0.41 | [16] |
| | 2 | 272601 | Syn | 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 | Nsyn | A/C | 4/22 | 1/15 | 0.3 | 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.

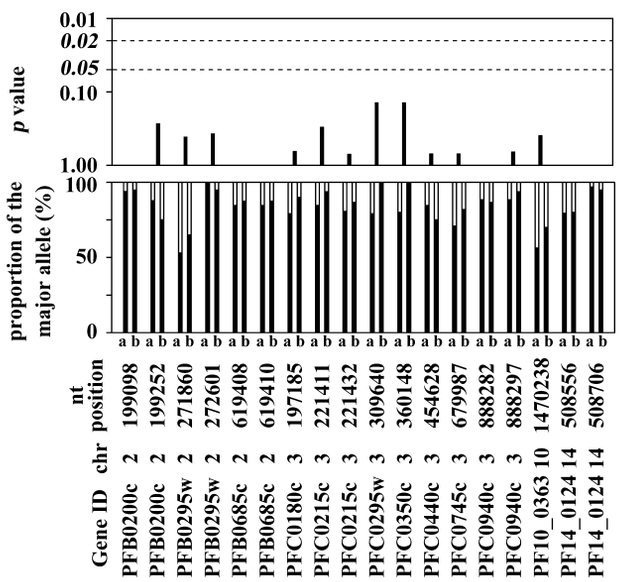


Fig. 2. Frequency distribution of putatively neutral single nucleotide polymorphisms (SNPs). Bottom panel indicates the proportion of the major nucleotide allele (black box) for each nucleotide (nt) position (a, 1988-9 group; b, 2003 group). *p* values were obtained by Fisher's exact test (two-tail) and shown in the top panel with the Y-axis shown as an inverted and logarithmic scale. 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. Details are summarized in Table 2.

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

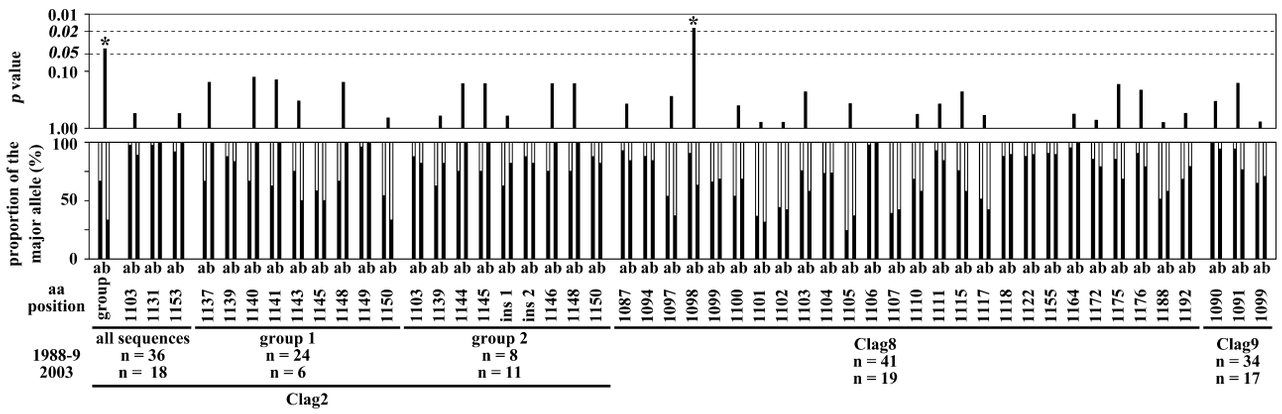


Fig. 3. Frequency distribution of Clag2 group and amino acid at each polymorphic site of *clag* gene products. Bottom panel indicates the proportion of the major amino acid allele (black box) for each amino acid (aa) position (a, 1988-9 group; b, 2003 group). The word "group" indicates the proportion of Clag2 group 1; ins 1 and 2 indicate first and second position of the insertion found in the group 2 Clag2 sequence between aa 1145 and 1146 of group 1 sequence. *p* values were obtained by Fisher's exact test (two-tail) and shown in the top panel with the Y-axis shown as an inverted and logarithmic scale. Asterisks indicate significant differences between the 1988-9 and 2003 groups ($p<0.05$ or $p<0.02$).

Table 3. Temporal change of allele-frequency distribution of *clag* gene loci

| Gene ID | product | chr | amino acid position | allele | 1988–9 | 2003 | *p* value [a] |
|---|---|---|---|---|---|---|---|
| PFB0935w | Clag2 | 2 | aa 1143-6 | group 1/not group 1 | 24/12 | 6/12 | 0.041 |
| MAL7P1.229 | Clag8 | 8 | aa 1098 | S/not S | 37/4 | 12/7 | 0.018 |

[a] *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.

## ACKNOWLEDGEMENTS

We are grateful to I. Sekine, head of the Nagasaki Red Cross Blood Center for human erythrocytes and plasma. This work was supported in part by Grants-in-Aids for Scientific Research 19590428, 22390079 and 24406012 (to OK) and the Global COE Program, Nagasaki University (to OK) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The nucleotide sequence data reported in this paper are available in the GenBank™/EMBL/DDBJ databases under the accession numbers: AB710386–AB710454. Part of this work was conducted during a Master of Tropical Medicine course at Nagasaki University supported by the Japanese International Cooperation Agency (JICA). J.A. is a recipient of a MEXT Ph.D. scholarship, Japan. M.K. and P.X. are recipients of a Nagasaki University Ph.D. research scholarship and Yeh Kuo Shii scholarship, Nagasaki University, Japan.

## REFERENCES

1. Siddiqui WA, Tam LQ, Kramer KJ, Hui GS, Case SE, Yamaga KM, Chang SP, Chan EB, Kan SC. Merozoite surface coat precursor protein completely protects Aotus monkeys against *Plasmodium falciparum* malaria. Proc Natl Acad Sci U S A 1987; 84: 3014–3018.
2. Cooper JA, Ingram LT, Bushell GR, Fardoulys CA, Stenzel D, Schofield L, Saul AJ. The 140/130/105 kilodalton protein complex in the rhoptries of *Plasmodium falciparum* consists of discrete polypeptides. Mol Biochem Parasitol 1988; 29: 251–260.
3. Doury JC, Bonnefoy S, Roger N, Dubremetz JF, Mercereau-Puijalon O. Analysis of the high molecular weight rhoptry complex of *Plasmodium falciparum* using monoclonal antibodies. Parasitology 1994; 108: 269–280.
4. Rungruang T, Kaneko O, Murakami Y, Tsuboi T, Hamamoto H, Akimitsu N, Sekimizu K, Kinoshita T, Torii M. Erythrocyte surface glycosyl–phosphatidyl inositol anchored receptor for the malaria parasite. Mol Biochem Parasitol 2005; 140: 13–21.
5. Hiller NL, Akompong T, Morrow JS, Holder AA, Haldar K. Identification of a stomatin orthologue in vacuoles induced in human erythrocytes by malaria parasites. A role for microbial raft proteins in apicomplexan vacuole biogenesis. J Biol Chem 2003; 278: 48413–48421.
6. Goel S, Valiyaveettil M, Achur RN, Goyal A, Mattei D, Salanti A, Trenholme KR, Gardiner DL, Gowda DC. Dual stage synthesis and crucial role of cytoadherence-linked asexual gene 9 in the surface expression of malaria parasite *var* proteins. Proc Natl Acad Sci U S A 2010; 107: 16643–16648.
7. Kaneko O, Tsuboi T, Ling IT, Howell S, Shirano M, Tachibana M, Cao YM, Holder AA, Torii M. The high molecular mass rhoptry protein, RhopH1, is encoded by members of the *clag* multigene family in *Plasmodium falciparum* and *Plasmodium yoelii*. Mol Biochem Parasitol 2001; 118: 223–231.
8. Ling IT, Kaneko O, Narum DL, Tsuboi T, Howell S, Taylor HM, Scott-Finnigan TJ, Torii M, Holder AA. Characterization of the *rhoph2* gene of *Plasmodium falciparum* and *Plasmodium yoelii*. Mol Biochem Parasitol 2003; 127: 47–57.
9. Shirano M, Tsuboi T, Kaneko O, Tachibana M, Adams JH, Torii M. Conserved regions of the *Plasmodium yoelii* rhoptry protein RhopH3 revealed by comparison with the *P. falciparum* homologue. Mol Biochem Parasitol 2001; 112: 297–299.
10. Holt DC, Gardiner DL, Thomas EA, Mayo M, Bourke PF, Sutherland CJ, Carter R, Myers G, Kemp DJ, Trenholme KR. The cytoadherence linked asexual gene family of *Plasmodium falciparum*: are there roles other than cytoadherence? Int J Parasitol 1999; 29: 939–944.
11. Kaneko O, Yim Lim BY, Iriko H, Ling IT, Otsuki H, Grainger M, Tsuboi T, Adams JH, Mattei D, Holder AA, Torii M. Apical expression of three RhopH1/Clag proteins as components of the *Plasmodium falciparum* RhopH complex. Mol Biochem Parasitol 2005; 143: 20–28.
12. Nguitragool W, Bokhari AA, Pillai AD, Rayavara K, Sharma P, Turpin B, Aravind L, Desai SA. Malaria parasite *clag3* genes determine channel-mediated nutrient uptake by infected red blood cells. Cell 2011; 145: 665–677.
13. Iriko H, Kaneko O, Otsuki H, Tsuboi T, Su XZ, Tanabe K, Torii M. Diversity and evolution of the *rhoph1*/*clag* multigene family of *Plasmodium falciparum*. Mol Biochem Parasitol 2008; 158: 11–21.
14. Alexandre JS, Kaewthamasorn M, Yahata K, Nakazawa S, Kaneko O. Positive selection on the *Plasmodium falciparum clag2* gene encoding a component of the erythrocyte-binding rhoptry protein complex. Trop Med Health 2011; 39: 77–82.
15. Crewther PE, Matthew ML, Flegg RH, Anders RF. Protective immune responses to apical membrane antigen 1 of *Plasmodium chabaudi* involve recognition of strain-specific epitopes. Infect Immun 1996; 64: 3310–3317.
16. Kaewthamasorn M, Yahata K, Alexandre JS, Xangsayarath P, Nakazawa S, Torii M, Sattabongkot J, Udomsangpetch R, Kaneko O. Stable allele frequency distribution of the polymorphic region of $SURFIN_{4.2}$ in *Plasmodium falciparum* isolates from Thailand. Parasitol Int 2012; 61: 317–323.
17. Hall TA. BioEdit: a user–friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 1999; 41: 95–98.
18. Nakazawa S, Culleton R, Maeno Y. In vivo and in vitro gametocyte production of *Plasmodium falciparum* isolates from Northern Thailand. Int J Parasitol 2011; 41: 317–323.
19. Anderson TJC, Nair S, Sudimack D, Williams JT, Mayxay

M, Newton PN, Guthmann JP, Smithuis FM, Tran TH, van den Broek IV, White NJ, Nosten F. Geographical distribution of selected and putatively neutral SNPs in Southeast Asian malaria parasites. Mol Biol Evol 2005; 22: 2362–2374.
20. Lipsitch M, O'Hagan JJ. Patterns of antigenic diversity and the mechanisms that maintain them. J R Soc Interface 2007; 4: 787–802.
21. Smith DL, McKenzie FE, Snow RW, Hay SI. Revisiting the basic reproductive number for malaria and its implications for malaria control. PLoS Biol 2007; 5: e42.