SHORT COMMUNICATION

The genotypes of *GYPA* and *GYPB* carrying the MNSs antigens are not associated with cerebral malaria

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Abstract *Plasmodium falciparum* invades erythrocytes via several routes using different red blood cell receptors that include glycophorin A (GYPA) and glycophorin B (GYPB). *GYPA* has two codominant alleles, i.e., *M* and *N*, that correspond to the M and N antigens, which differ by two amino acids (S1L, G5E); the codominant alleles of *GYPB*, i.e., *S* and *s*, correspond to the S and s antigens, which differ by a single amino acid (T29M). If these antigens influence the efficiency of erythrocyte invasion by malaria parasites, the MNSs phenotype may be associated with the severity of malaria. To examine this, the *GYPA* and *GYPB* genotypes carrying the MNSs antigens were analyzed in 109 and 203 Thai patients with cerebral malaria and mild malaria, respectively. Neither the genotype nor allele frequencies at each locus were statistically different between the cerebral and mild malaria patients. Thus, we conclude that the MNSs antigens do not reveal the difference in susceptibility to cerebral malaria.

Keywords Malaria · MNSs antigens · Glycophorin A · Glycophorin B · Thailand

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Introduction

The malaria parasite *Plasmodium falciparum* invades human erythrocytes and replicates within them. Erythrocyte invasion by merozoites requires interaction between multiple erythrocyte receptors and their corresponding parasite ligands, such as the 175-kD erythrocyte-binding antigen (EBA-175) of *P. falciparum*. It has been demonstrated that the glycophorin A (GYPA) expressed on erythrocytes is a receptor of EBA-175 (Adams et al. 1992; Camus and Hadley 1985; Klotz et al. 1992; Orlandi et al. 1992; Pasvol et al. 1982; Sim et al. 1990), and glycophorin B (GYPB) was suggested to be used as an EBA-175 independent *P. falciparum* receptor (Dolan et al. 1994). These observations indicate that GYPA and GYPB play a crucial role in the process of erythrocyte invasion by merozoites.

The *GYPA* (MIM 111300) and *GYPB* (MIM 111740) genes, located on chromosome 4 (4q28.2-q13.1), code for antigens responsible for the very common MN and Ss blood type polymorphisms, respectively. The M and N antigens differ by two amino acids (S1L and G5E) and the S and s by a single amino acid (M29T). These amino acid sites are located in the extracellular domain that may cause differences in receptor–ligand interaction. If a difference exists, the efficiency of invasion by the malaria parasites in individuals with diverse MNSs antigens is expected to vary. Interestingly, *GYPA* and *GYPB* were reported to be fast-evolving genes, with a high rate of nonsynonymous substitutions between humans and other primates. Thus, it has been hypothesized that the human *GYPA* gene has been evolving rapidly in order to evade malaria parasite invasion (Wang et al. 2003). Further, this hypothesis led us to investigate whether MNSs antigens affect susceptibility to severe malaria. To examine this, we conducted an association study for mild or cerebral malaria in Thai patients.

The deletion of exon 3 in the *GYPC* (MIM 110750) gene, *GYPCΔex3*, causing the Gerbich-negative phenotype has been suggested to confer protection against malaria because the *GYPCΔex3* allele reaches a high frequency in malaria-endemic regions of Papua New Guinea (Booth and McLoughlin 1972; Patel et al. 2001, 2004). In addition, GYPC has been shown to be the receptor for the *P. falciparum* erythrocyte-binding antigen 140 (Maier et al. 2003). The 27-base-pair deletion in the solute carrier family 4, anion exchanger, member 1 (*SLC4A1* [MIM 109270]) gene, *SLC4A1Δ27*, resulting in the Southeast Asian/Melanesian ovalocytosis (SAO), has been reported to be associated with protection from cerebral malaria (Allen et al. 1999; Genton et al. 1995). These observations imply that the *GYPCΔex3* and *SLC4A1Δ27* alleles influence the association between MNSs antigens and cerebral malaria. Thus, the presence of *GYPCΔex3* and *SLC4A1Δ27* was also investigated in this study.

Subjects and methods

Patients with malaria

A total of 312 adult patients with *P. falciparum* malaria (109 cerebral malaria and 203 mild malaria patients) living in northwest Thailand were recruited for this study. All of them underwent treatment at the Hospital for Tropical Diseases of Mahidol University. Clinical manifestations of malaria were as follows. Cerebral malaria was characterized by unrousable coma, positive blood smear for the asexual form of *P. falciparum*, and exclusion of other causes of coma. Mild malaria was characterized by a positive blood smear, fever without other causes of infection, and no manifestation of the following symptoms: high parasitemia (>100,000 parasites/ml), hypoglycemia (glucose <22 nmol/l), severe anemia (hematocrit <20% or hemoglobin <7.0 g/dl), and increased serum creatinine levels (>3.0 mg/dl). All individuals were 13 years or older, and means of age in the cerebral malaria and mild malaria patients were 28.6 ± 9.6 (mean ± SD) and 25.5 ± 10.3 (mean ± SD) years, respectively. This study was approved by the institutional review board of the Faculty of Tropical Medicine, Mahidol University, and the Faculty of Medicine, The University of Tokyo. Written informed consent was obtained from all patients.

Genotyping

Genomic DNA was extracted from the peripheral blood leukocytes using a QIAamp Blood Kit (Qiagen, Hilden, Germany). The *GYPA* genotyping was performed by polymerase chain reaction (PCR) direct sequencing.

A 256-bp fragment containing three single nucleotide polymorphisms (SNPs) (59C > T, 71G > A, 72G > T) was amplified by PCR using previously reported primer sets (Sasaki et al. 2000), i.e., a 5′ primer GYPA-F (5′-GAG-GGAATTTGTCTTTTGCA-3′) and 3′ primer GYPA-R (5′-GGTCCCCTAAAATGGGTTA-3′); this fragment determined the *M* and *N* alleles. PCR was performed using GeneAmp reagents and AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The amplification process comprised initial denaturation at 96°C for 10 min, followed by 40 cycles of denaturation at 96°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s using a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems). The PCR products were subjected to direct sequencing using an ABI PRISM™ 3100 Genetic Analyzer (Perkin-Elmer Applied Biosystems). The genotyping for *S* and *s* alleles of *GYPB* (rs7683365) was performed using the TaqMan technology (Assay-on-Demand) on an ABI 7500 system (Applied Biosystems). The genotyping for *GYPCΔex3* was performed by using previously reported primer sets (Patel et al. 2001). The presence of exons 2 and 3 was examined by a primer set (GPCup 5′-CAGATTCTTGTCCTCTGT-TCACAG-3′ and GPCdn 5′-TCAAAACCACCTCTGA-GGGAGAG-3′) annealing to conserved sequence around exons 2 and 3. The amplification process comprised initial denaturation at 96°C for 10 min, followed by 40 cycles of denaturation at 96°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s using a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems). The PCR products were subjected to electrophoresis on 10% acrylamide gel. A 264-bp band (exon 2) is detected in normal/normal, normal/*GYPCΔex3*, and *GYPCΔex3*/*GYPCΔex3*, and a 240-bp band (exon 3) is detected in only normal/normal and normal/*GYPCΔex3*. To identify the normal allele, a primer set (GPC349up 5′-GGAAACTGCCGTG-ACTTCAGA-3′ and 1,679dn 5′-CATGGTCCTTGCAAC-AGTTGC-3′) was used to amplify a 1,376-bp product. To identify the *GYPCΔex3* allele and distinguish normal/*GYPCΔex3* from normal/normal, a primer set (GPC349up and GPC1680dn 5′-AGGTTAGAATCATACCCCAGG-3′) was used to amplify a 1,377-bp product. The amplification process specific for normal or *GYPCΔex3* allele comprised initial denaturation at 94°C for 60 s, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 75 s using a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems). The PCR products were subjected to electrophoresis on 10% acrylamide gel. In the genotyping, a Melanesian individual heterozygous for *GYPCΔex3* was analyzed as a positive control for PCR, together with Thai malaria patients. A PCR for *SLC4A1Δ27* was performed using a previously reported primer set (Kimura et al. 2003) (5′-GGGCCCA-

GATGACCCTCTGC-3′) and (5′-GCCGAAGGTGATGG CGGGTG-3′) spanning the 27-bp deletion. The amplification process comprised initial denaturation at 96°C for 10 min, followed by 40 cycles of denaturation at 96°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s using a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems). The PCR products were separated on 10% acrylamide gel to identify the presence of 175-bp (normal) and 148-bp (SLC4A1Δ27) fragments.

Statistical analysis

Fisher's exact test was performed to compare the genotype and allele frequency distributions observed for the cerebral and mild malaria patients. The haplotype frequencies were estimated using the EH program (Xie and Ott 1993) based on the expectation–maximization (EM) algorithm. The differences in the haplotype frequency distribution between the cerebral and mild malaria patients were assessed by a likelihood ratio test. In the EM algorithm for estimating haplotype frequency, the logarithm of the likelihood of the sample $\ln L$ was maximized in each subject group (i.e., $\ln L_c$ for cerebral malaria subjects, $\ln L_m$ for mild malaria subjects, and $\ln L_{all}$ for both sets of subjects). The likelihood ratio statistic was defined by $LR = 2 [\ln L_c + \ln L_m - \ln L_{all}]$, which had an approximate chi-square distribution with three degrees of freedom under the null hypothesis of no difference in haplotype frequency distribution. Linkage disequilibrium parameters between GYPA and GYPB alleles, D' (Lewontin 1964) and r^2, were calculated based on the estimated haplotype frequencies for all subjects.

Results and discussion

The major alleles at the GYPA and GYPB loci observed in the Thai patients were M and s, respectively (Table 1). Neither the genotype nor the allele frequencies were significantly different between the cerebral and mild malaria patients (Table 1). In addition, the likelihood-ratio test detected no differences in the estimated haplotype frequency between cerebral and mild malaria patients (Table 2). Although GYPA and GYPB are closely located on human chromosome 4, statistically significant linkage disequilibrium was not observed between them in the Thai population ($|D'|$ and r^2 values for all the subjects were 0.328 and 0.004, respectively). Neither GYPCΔex3 nor SLC4A1Δ27 was detected in 24 cerebral and 24 mild malaria patients. Thus, we decided not to analyze the rest of samples and conclude that the presence of GYPCΔex3 and SLC4A1Δ27 does not affect the lack of association of the MNSs antigens with cerebral malaria observed in this study. As these deletions may be associated with protection

Table 1 Genotype and allele frequencies of glycophorin A (GYPA) and glycophorin B (GYPB) in patients with malaria

Gene	Cerebral malaria ($n = 109$)	Mild malaria ($n = 203$)	P value*
GYPA			
Genotype			
MM	58 (55.2%)	118 (59.3%)	0.351
MN	42 (40.0%)	65 (32.7%)	
NN	5 (4.8%)	16 (8.0%)	
Allele:			
M	158 (75.2%)	301 (75.6%)	0.921
N	52 (24.8%)	97 (24.4%)	
GYPB			
Genotype:			
SS	1 (0.9%)	1 (0.5%)	1
Ss	19 (17.4%)	36 (17.9%)	
ss	89 (81.7%)	164 (81.6%)	
Allele:			
S	21 (9.6%)	38 (9.5%)	0.942
s	197 (90.4%)	364 (90.5%)	

* P values were obtained by Fisher's exact test based on 3×2 table in genotype frequency comparison and based on 2×2 table in allele frequency comparison

Table 2 Estimated frequencies of glycophorin A (GYPA) and glycophorin B (GYPB) haplotypes in patients with malaria

Haplotype	Cerebral malaria	Mild malaria	P value*
M–S	0.098336	0.077834	0.830
M–s	0.654045	0.682267	
N–S	0.001664	0.018126	
N–s	0.245955	0.221773	

*P value for the comparison of haplotype frequencies between cerebral and mild malaria groups was obtained by log-likelihood ratio test

Estimated $|D'|$ and r^2 values for all the subjects were 0.328 and 0.004, respectively

against malaria infection rather than severe malaria, a further study of malaria-free individuals needs to be conducted in Thailand.

The present results led us to conclude that the MNSs antigens do not reveal a difference in susceptibility to cerebral malaria. The polymorphisms at the following amino acid residues may not influence the receptor–ligand interaction: the 1st and 5th of GYPA (S1L and G5E) and the 29th of GYPB (M29T). Although it has been suggested that the GYPA and GYPB genes undergo positive selection (Wang et al. 2003), the nonsynonymous polymorphisms investigated in this study may not be subjected to selection by P. falciparum malaria.

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