Original Article

Lack of Association between *BSG* Polymorphisms and Cerebral Malaria

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SUMMARY: The Ok^a blood group antigen basigin (BSG or CD147) is an erythrocyte receptor for the PfRh5 protein from *Plasmodium falciparum*. A recent study has shown that the PfRh5–BSG interaction is essential for erythrocyte invasion by *P. falciparum*. In this study, 6 SNPs in the *BSG* gene were investigated in 312 adult patients with *P. falciparum* malaria (109 cerebral malaria and 203 mild malaria patients) living in northwest Thailand. To examine the association between *BSG* SNPs and cerebral malaria, the allele and haplotype frequencies were compared in cerebral and mild malaria patients. Non-synonymous SNPs were not assessed in the association analysis. The results showed that common *BSG* polymorphisms and haplotypes were not significantly associated with cerebral malaria. In conclusion, common SNPs in *BSG* do not influence the risk of cerebral malaria in the Thai population.

INTRODUCTION

Basigin (BSG, CD147, EMMPRIN, or M6) is a glycosylated transmembrane protein that belongs to the Ig superfamily (1,2), and it is expressed in a variety of cell types where it plays roles in cell–cell interactions. The BSG protein expressed on erythrocytes is also known as the Ok^a blood group antigen.

Recently, it has been demonstrated that human BSG on erythrocytes is bound by the PfRh5 protein from *Plasmodium falciparum*, and that it acts as a receptor essential for the invasion of erythrocytes by most strains of *P. falciparum* (3). It has also been reported that PfRh5 binds chimpanzee BSG with a significantly lower affinity than human BSG, and that it does not bind gorilla BSG (4). These observations suggest that the PfRh5–BSG interaction plays important roles not only in erythrocyte invasion by *P. falciparum* but also in the host species preferences of *P. falciparum*.

Cerebral malaria, the most severe complication of human infection by *P. falciparum*, is characterized by the sequestration of parasitized erythrocytes in brain microvessels, although the cause of cerebral malaria remains obscure. A previous study found that Ok^a–erythrocytes, which express a BSG variant with the E92K substitution, exhibit a reduced binding affinity for PfRh5 and increased resistance to invasion (3). This reduction in parasitized erythrocytes due to a lower invasion efficiency appears to decrease the risk of cerebral malaria because cerebral malaria is generally associated with hyperparasitemia. A previous study showed that hyperparasitemia led to poor prognosis in cerebral malaria among adult Thai patients (5). Some malaria patients infected with *P. falciparum* develop cerebral malaria; thus, polymorphisms in the *BSG* gene (OMIM *109480) may influence the susceptibility to cerebral

malaria by modifying the PfRh5–BSG interaction due to amino acid replacement in the *BSG* gene or by changing the efficiency of *P. falciparum* invasion due to alterations in the expression level of the *BSG* gene. This study determined whether *BSG* polymorphisms are associated with cerebral malaria in Thai malaria patients.

MATERIALS AND METHODS

Subjects: This study recruited 312 Thai patients infected with *P. falciparum* who lived in northwest Thailand. Malarial infection by *P. falciparum* was confirmed by a positive blood smear for the presence of the asexual form of *P. falciparum*. Clinical manifestations of malaria were classified according to the definitions and associated criteria provided by the World Health Organization. The mild malaria group comprised 203 patients with mild symptoms characterized by fever but without other causes of infection or the following signs of severity or evidence of vital organ dysfunction: high parasitemia (>100,000 parasites/ μ L), hypoglycemia (glucose level <22 mmol/L), severe anemia (hematocrit <20% or hemoglobin level <7 g/dL), and serum creatinine level >3.0 mg/dL. The cerebral malaria group comprised 109 patients who were characterized by an unarousable coma and exclusion of other causes of coma. Patients were aged ≥ 13 years, and the average ages of patients with mild malaria and cerebral malaria were 25.5 and 28.6 years, respectively. All patients underwent treatment at the Hospital for Tropical Disease, Faculty of Tropical Medicine, Mahidol University (Bangkok, Thailand). This study was approved by the Institutional Review Board of the Faculty of Tropical Medicine, Mahidol University, and the Research Ethics Committee of Faculty of Medicine, University of Tsukuba. Written informed consent was obtained from all patients.

SNP selection: We obtained SNP genotype data for Asian subjects (44 Japanese individuals from Tokyo, Japan [JPT] and 45 Han Chinese individuals from Beijing, China [CHB]) from the HapMap database (6,7) to

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select tag SNPs in *BSG*. Five tag SNPs were selected manually from 8 SNPs (from rs4919859 to rs8637) in a 20 kb genomic region that contained the entire 12.2 kb sequence of *BSG*. The 8 *BSG* SNPs with a minor allele frequency >0.05 in JPT + CHB were in linkage disequilibrium (LD) with 1 of 5 tag SNPs, with a pairwise LD parameter, r^2 , of ≥ 0.8 . In addition to 5 tag SNPs, 3 *BSG* polymorphisms (rs55911144 [L90P], rs8259, and rs71724272) were selected for genotyping. The *BSG* variant with the E92K substitution (rs104894669) that results in the Ok^{a-} phenotype is very rare (the Ok^{a-} phenotype has only been identified in 8 Japanese families (8)); thus, E92K was not examined in this study.

DNA extraction and genotyping: Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp Blood Kit (Qiagen, Hilden, Germany). Seven SNPs in the *BSG* gene (rs4919859, rs12611131, rs55911144 [L90P], rs3764938, rs2072309, rs8637, and rs8259) were genotyped using the TaqMan SNP genotyping assay (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. To genotype the rs71724272 ins/del polymorphism, PCR was performed using the primers 5'-GGTTCCAGGCTCCTCTCTCT-3' and 5'-GCCTCCATGTTTCAGGTTCTC-3', followed by electrophoresis on 10% polyacrylamide gels.

Statistical analysis: The deviation of the genotype frequencies from Hardy-Weinberg equilibrium (HWE) were examined using the chi-square test. The pairwise LD parameter, r^2 , was estimated using Haploview (9). The association between each SNP and cerebral malaria was tested using the chi-square test based on a 2×2 allele frequency table. The haplotype frequencies were compared between the cerebral and mild malaria groups using a permutation test implemented in Haploview (9). The genotypes of SNPs spanning a 40 kb genomic region that contained the entire *BSG* gene were retrieved from the HapMap JPT and CHB populations. Using their genotype data as references, the genotypes of Thai malaria patients were imputed using MACH (10). The imputed genotypes of SNPs with r^2 values >0.5 were used for the association test. Thus, 20 imputed SNPs were subjected to single-point association analysis. The nucleotide sequences of the 3 predicted isoforms of *BSG* in humans (ENST00000346916 [isoform 4], ENST00000353555 [isoform 2], and ENST00000333511 [isoform 1]) and the corresponding sequences in *Pan troglodytes* (ENSPTRT00000018631, ENSPTRT00000018632, and ENSPTRT00000018633) were retrieved from the Ensembl site (11). The sequences were aligned using Clustal W (12) for each pair. For each pair of sequences, the number of nonsynonymous substitutions per nonsynonymous site (d_N) and the number of synonymous substitutions per synonymous site (d_S) were estimated using the Nei-Gojobori method with the Jukes-Cantor model (13). The variance in d_N-d_S was calculated using the analytical method. The difference in the number of substitutions, d_N-d_S , was evaluated using the z -test for selection (2-sided test) implemented in MEGA 5.0 (14). $P < 0.05$ was considered statistically significant in this study.

RESULTS

The *BSG* gene encodes long and short splice isoforms (Figure 1(a)). In total, 8 polymorphisms that spanned the entire region of *BSG* were genotyped in this study. Two of these polymorphisms, i.e., rs55911144 (L90P) and rs71724272, were monomorphic (i.e., the 90P allele at rs55911144 or the deletion allele at rs71724272 were not as observed), and they were excluded from the following analyses. Thus, nonsynonymous SNPs were not assessed in the association analysis.

The allele frequencies at each of the 6 polymorphic SNPs (5 tag SNPs and rs8259) in mild and cerebral malaria patients are presented in Table 1. None of the SNPs had significant associations with cerebral malaria. Furthermore, no significant deviation from HWE was observed for any of the SNPs in both mild and cerebral malaria patients.

Fig. 1. (a) Structure of 4 transcript variants encoded by *BSG* and location of SNPs assessed in the association analysis. Since rs55911144 (L90P) and rs71724272 were monomorphic, the positions of these polymorphisms are not shown. (b) Pairwise LD parameter, r^2 , between 6 polymorphic *BSG* SNPs in 312 malaria patients.

Table 1. Allele frequencies at 6 *BSG* polymorphisms in Thai malaria patients

rs #	Allelic status (ancestral/derived)	Derived allele frequency		<i>P</i> -value ¹⁾
		Cerebral malaria (2n = 218)	Mild malaria (2n = 406)	
rs4919859	C/G	0.578	0.586	0.842
rs12611131	C/T	0.280	0.329	0.205
rs3764938	C/T	0.349	0.428	0.055
rs2072309	G/A	0.620	0.653	0.417
rs8637	G/A	0.252	0.219	0.350
rs8259	A/T	0.335	0.321	0.723

¹⁾ *P*-value was calculated by chi-square test with one degree of freedom.

Table 2. Permutation P -values for *BSG* haplotypes

Haplotype ¹⁾	SNP ²⁾						Haplotype frequency		P -value ³⁾
	1	2	3	4	5	6	Cerebral malaria	Mild malaria	
H1	C	C	T	G	G	T	0.046	0.088	0.366
H2	G	T	T	G	G	A	0.273	0.322	0.739
H3	C	C	C	G	A	T	0.030	0.017	0.955
H4	G	C	C	G	A	T	0.213	0.187	0.968
H5	G	C	C	A	G	A	0.059	0.047	0.969
H6	C	C	C	A	G	A	0.322	0.293	1.000

¹⁾: Haplotypes with estimated frequency of more than 0.02 in 312 malaria patients are shown.

²⁾: SNP1, SNP2, SNP3, SNP4, SNP5, and SNP6 indicate rs4919859, rs12611131, rs3764938, rs2072309, rs8637, and rs8259, respectively.

³⁾: P -value was calculated by permutation test only for haplotype frequency data.

Fig. 2. Regional association plot. The genotypes of SNPs around the *BSG* gene in Thai malaria patients were imputed by the MACH software by using the genotype data of HapMap JPT and CHB samples as the reference panel. The association P -values for 6 genotyped and 20 imputed SNPs are plotted as shaded diamonds and open diamonds, respectively.

A plot of the pairwise r^2 values among the 6 *BSG* SNPs is shown in Figure 1(b). The structure of LD in the Thai malaria patients was similar to that in HapMap JPT + CHB (data not shown); thus, the genotyped SNPs were considered to be suitable tag SNPs in Thai subjects. To evaluate whether any *BSG* haplotypes with the 6 *BSG* SNPs exhibited significant associations with cerebral malaria, permutation P -values were calculated for the single SNPs and haplotypes to compare the mild and cerebral malaria groups. No haplotypes exhibited significant differences in terms of the estimated frequency between the cerebral and mild malaria groups (Table 2).

To determine whether the other common SNPs in the genomic region of *BSG* are associated with cerebral malaria, a multiple regression analysis was performed for 20 SNPs imputed by MACH (10) using the genotype data from the HapMap JPT + CHB populations as the

reference panel. The imputed SNPs and the genotyped SNPs had no significant association with cerebral malaria (Figure 2).

To explore the possibility of positive or negative (purifying) selection acting on the *BSG* gene in the human lineage, the rate of nonsynonymous substitutions was compared with that of synonymous substitutions based on the human and chimpanzee sequences, where 3 predicted isoforms of *BSG* in humans, i.e., ENST00000346916 (isoform 4), ENST00000353555 (isoform 2), and ENST00000333511 (isoform 1), were compared with the corresponding sequences in *P. troglodytes*, i.e., ENSPTRT00000018631, ENSPTRT00000018632, and ENSPTRT00000018633, respectively. None of the pairs differed significantly in their nonsynonymous and synonymous substitution rates ($z = -1.539$ and 2-sided $P = 0.126$ for ENST00000346916 [isoform 4] vs ENSPTRT00000018631; $z = -1.461$ and 2-sided $P = 0.147$ for ENST00000353555 [isoform 2] vs ENSPTRT00000018632; and $z = -1.829$ and 2-sided $P = 0.070$ for ENST00000333511 [isoform 1] vs ENSPTRT00000018633).

DISCUSSION

In this study, *BSG* polymorphisms were investigated in Thai malaria patients, and we determined the possible associations between these polymorphisms and cerebral malaria. Our results showed that common *BSG* SNPs and common haplotypes were not significantly associated with cerebral malaria.

It is unknown whether rs8259 can affect the expression level of *BSG* in erythrocytes, as found in peripheral blood mononuclear cells (15), but the lack of an association between rs8259 and cerebral malaria suggests that a change in the expression level of *BSG* might not affect the efficiency of *P. falciparum* invading erythrocytes. We can not exclude the possibility that rare *BSG* variants not considered in this study might have statistically significant associations with cerebral malaria if sufficient subjects are investigated, but we conclude that the risk of cerebral malaria in Thai adults is not affected greatly by common SNPs in *BSG*.

Amino acid substitutions in the *BSG* gene may reduce the binding affinity between PfRh5 and *BSG*. Because *BSG* is a crucial receptor for the PfRh5 protein from *P. falciparum*, the number of nonsynonymous substitutions per nonsynonymous site (d_N) is expected to exceed the number of synonymous substitutions per synonymous site (d_S) in species that can be infected with *P. falciparum*. Thus, the *BSG* gene has probably been subjected to positive selection because amino acid substitutions in *BSG* due to the reduced binding affinity between PfRh5 and *BSG* are likely to have been fixed in humans. However, the value of $d_N - d_S$ was negative according to the comparison of each *BSG* isoform, thereby implying that there may be strong selective constraint on *BSG* because of its crucial role in reproduction, including spermatogenesis (16,17).

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Conflict of interest None to declare.

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