Int J Mol Epidemiol Genet 2016;7(2):87-94
www.ijmeg.org /ISSN:1948-1756/IJMEG0028425

Original Article

Association of *PECAM1/CD31* polymorphisms with cerebral malaria

Jun Ohashi[1,2], Izumi Naka[1,2], Hathairad Hananantachai[3], Jintana Patarapotikul[3]

[1]Graduate School of Science, The University of Tokyo, Tokyo, Japan; [2]Faculty of Medicine, University of Tsukuba, Ibaraki, Japan; [3]Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Received March 16, 2016; Accepted April 30, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Platelet/endothelial cell adhesion molecule-1 (PECAM1/CD31), a receptor recognized by *P. falciparum*-infected red blood cells (iRBCs), on the vascular endothelium has been implicated in mediating cytoadherence in patients with *P. falciparum* malaria. To examine associations of *PECAM1* polymorphisms with cerebral malaria, 11 tag single nucleotide polymorphisms (SNPs) of *PECAM1* were analysed for 312 Thai patients with *P. falciparum* malaria (109 with cerebral malaria and 203 with mild malaria). The rs1122800-C allele was significantly associated with protection from cerebral malaria ($P = 0.017$), and the rs9912957-A significantly increased the risk for cerebral malaria ($P = 0.0065$) in malaria patients. Fine-scale mapping using genotyped and imputed SNPs and linkage disequilibrium (LD) analysis revealed that rs1122800 and rs9912957 were located in two distinct LD blocks and were independently associated with cerebral malaria. The rs1122800-C allele was significantly associated with lower expression level of *PECAM1* in EBV-transformed lymphoblastoid cell lines ($P = 0.045$). The present results suggest that PECAM1-mediated cytoadherence of iRBCs to brain endothelium plays a crucial role in the pathogenesis of cerebral malaria.

Keywords: *PECAM1*, cerebral malaria, SNP, association study, candidate gene approach, Thai

Introduction

Cerebral malaria is one of the most severe clinical complications from *Plasmodium falciparum* infection. Although the pathogenesis of cerebral malaria is not yet fully understood, cerebral malaria has been suggested to be caused by cytoadherence of *P. falciparum* infected red blood cells (iRBCs) to brain endothelium. To date, several endothelial receptors, including CD36 [1, 2], intercellular adhesion molecule 1 (ICAM1) [3], chondroitin-4-sulfate (CSA) [4, 5], and platelet/endothelial cell adhesion molecule-1 (PECAM1/CD31) [6, 7], that are recognized by iRBCs have been identified.

Because a part of malaria patients develop cerebral malaria, polymorphisms of human genes or genetic differences among hosts are considered to influence the risk for cerebral malaria. The *PECAM1* (OMIM *173445) gene encoding an endothelial receptor for binding iRBCs is a plausible candidate for cerebral malaria. Two non-synonymous single nucleotide polymorphisms (SNPs) of *PECAM1*, L125V (rs668) and S563N (rs12953), have been shown to be associated with cerebral malaria in Thailand [8], and L125V was associated with severe malaria in India after the data were stratified by disease endemicity [9]. However, no association of L125V with severe malaria was reported in Papua New Guinea and Kenya [10]. Although most previous association studies of *PECAM1* in various diseases have investigated nonsynonymous SNPs such as L125V, S563N, and G670R (rs1131012), there may be *PECAM1* SNPs that can influence the transcription activity or expression level of *PECAM1*. To identify such SNPs, a systematic tagSNP approach is required. In the present study, the possible association of 11 tagSNPs and eight additional SNPs of *PECAM1* with cerebral malaria was examined in Thai patients with *P. falciparum* malaria.

Material and methods

Subjects

A total of 312 adult patients with *P. falciparum* malaria (109 cerebral malaria and 203 mild malaria patients) who lived in northwest

Table 1. Association *P*-values for all the genotyped SNPs of *PECAM1*

rs #	Allelic status (ancestral/derived)	Position (NCBI B36 coordinates)	Geno-type	Frequency		*P*-value[a]	Odds ratio[b] (95% CI)	Model[c]
				Cerebral malaria (n = 109)	Mild malaria (n = 203)			
rs1867624	C/T	59740823	T/T	58 (0.532)	126 (0.624)	0.12	0.69	Recessive
			T/C	47 (0.431)	71 (0.352)		0.43-1.1	
			C/C	4 (0.037)	5 (0.025)			
rs1122800[d]	G/C	59745097	C/C	52 (0.482)	99 (0.488)	0.017	0.39	Dominant
			C/G	41 (0.380)	92 (0.453)		0.18-0.87	
			G/G	15 (0.139)	12 (0.059)			
rs7213889	A/G	59752300	GG	14 (0.128)	11 (0.054)	0.021	2.6	Recessive
			G/A	40 (0.367)	89 (0.438)		1.1-5.9	
			A/A	55 (0.505)	103 (0.507)			
rs1470453[d]	G/A	59758122	A/A	4 (0.037)	8 (0.040)	0.21	1.3	Dominant
			A/G	49 (0.450)	75 (0.373)		0.84-2.2	
			G/G	56 (0.514)	118 (0.587)			
rs2070783[d]	C/T	59760703	T/T	15 (0.138)	15 (0.074)	0.071	2.0	Recessive
			T/C	44 (0.404)	88 (0.436)		0.93-4.2	
			C/C	50 (0.459)	99 (0.490)			
rs9913080[d]	C/A	59766765	A/A	46 (0.422)	88 (0.434)	0.069	0.50	Dominant
			A/C	48 (0.440)	100 (0.493)		0.23-1.1	
			C/C	15 (0.138)	15 (0.074)			
rs4968723[d]	A/G	59770666	G/G	14 (0.128)	27 (0.133)	0.40	1.2	Dominant
			G/A	61 (0.560)	103 (0.507)		0.75-2.0	
			A/A	34 (0.312)	73 (0.360)			
rs7207019[d]	G/C	59777169	C/C	16 (0.150)	33 (0.167)	0.30	1.3	Dominant
			C/G	60 (0.561)	96 (0.485)		0.79-2.2	
			G/G	31 (0.290)	69 (0.349)			
rs1131012[d] (G670R)	G/A	59781521	A/A	25 (0.232)	53 (0.268)	0.49	0.82	Recessive
			A/G	60 (0.556)	103 (0.520)		0.48-1.4	
			G/G	23 (0.213)	42 (0.212)			
rs11079538[d]	C/T	59806027	T/T	24 (0.220)	46 (0.227)	0.59	1.2	Dominant
			T/C	58 (0.532)	101 (0.498)		0.68-2.0	
			C/C	27 (0.248)	56 (0.276)			
rs11653087	C/T	59810599	T/T	23 (0.211)	45 (0.222)	0.71	1.1	Dominant
			T/C	57 (0.523)	100 (0.493)		0.65-1.7	
			C/C	29 (0.266)	58 (0.286)			
rs9906431[d]	T/C	59814883	C/C	18 (0.167)	17 (0.084)	0.028	2.2	Recessive
			C/T	44 (0.407)	99 (0.488)		1.1-4.4	
			T/T	46 (0.426)	87 (0.429)			
rs59573853	-/AA	59814940	AA/AA	45 (0.421)	87 (0.429)	0.044	0.48	Dominant
			AA/–	45 (0.421)	99 (0.488)		0.24-0.99	
			–/–	17 (0.159)	17 (0.084)			
rs9912957[d]	G/A	59815823	A/A	20 (0.187)	16 (0.081)	0.0065	2.6	Recessive
			A/G	45 (0.421)	93 (0.472)		1.3-5.3	
			G/G	42 (0.393)	88 (0.447)			
rs8065316	T/C	59816347	C/C	43 (0.395)	90 (0.443)	0.0094	0.41	Dominant
			C/T	46 (0.422)	96 (0.473)		0.20-0.81	
			T/T	20 (0.184)	17 (0.084)			
rs12953175	G/A	59820164	A/A	43 (0.395)	93 (0.458)	0.026	0.46	Dominant
			A/G	47 (0.431)	92 (0.453)		0.23-0.92	
			G/G	19 (0.174)	18 (0.089)			
rs12051829[d]	C/T	59831092	T/T	36 (0.330)	79 (0.391)	0.29	0.77	Recessive
			T/C	57 (0.523)	93 (0.460)		0.47-1.3	
			C/C	16 (0.147)	30 (0.149)			
rs6504227	T/C	59831227	C/C	93 (0.853)	181 (0.896)	0.27	0.67	Recessive
			C/T	16 (0.147)	20 (0.099)		0.34-1.4	

			T/T	0 (0.000)	1 (0.005)			
rs8080666	G/A	59840612	A/A	39 (0.358)	76 (0.374)	0.71	1.1	Dominant
			A/G	53 (0.486)	92 (0.453)		0.60-2.1	
			G/G	17 (0.156)	35 (0.172)			

[a]The smallest *P*-value among three models (i.e., dominant, recessive, and allelic) is shown. [b]Odds ratio for model showing the smallest *P*-value is presented. [c]Model (i.e., dominant, recessive, or allelic) showing the smallest *P*-value is shown. Model was defined on the basis of a derived allele for a particular SNP. [d]Tag SNP.

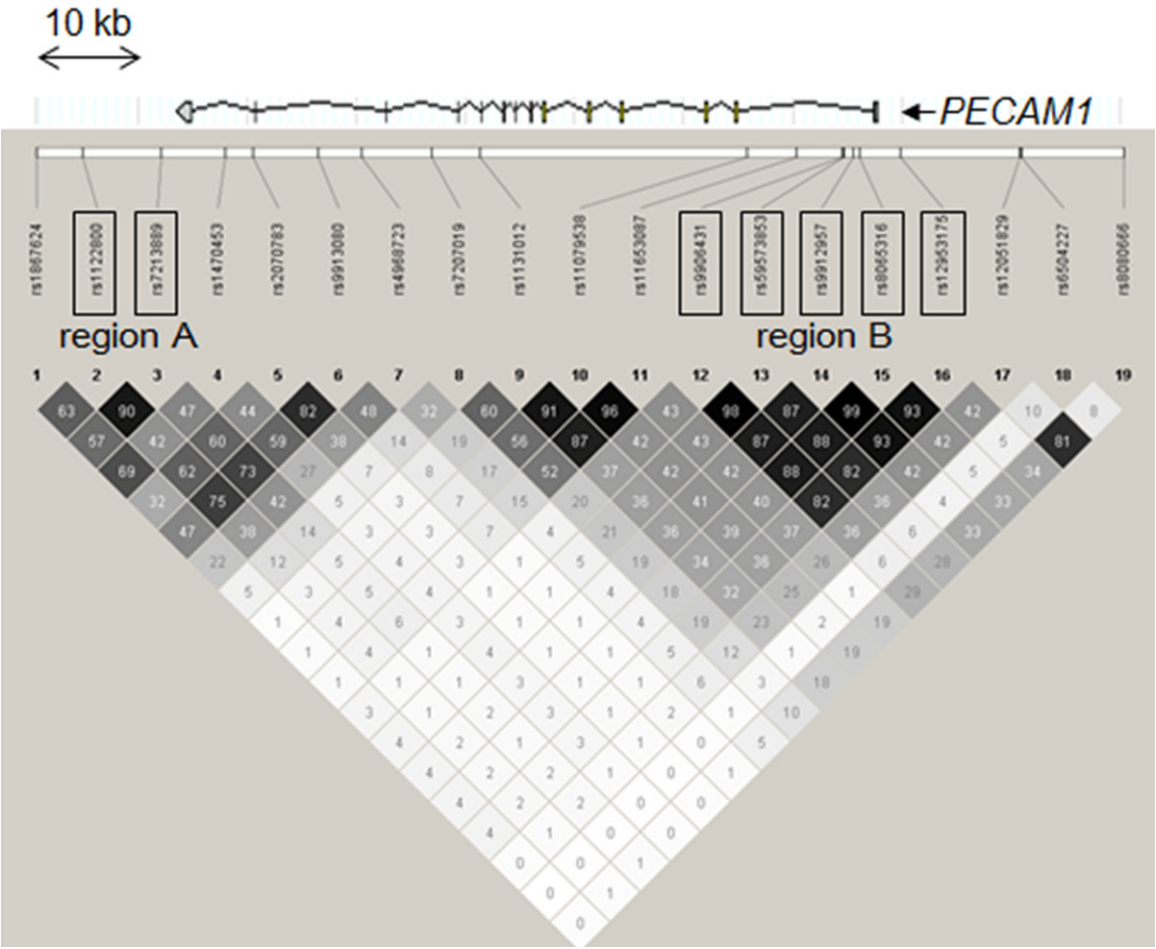

Figure 1. Structure of LD in *PECAM1*. Pairwise LD parameters, r^2, between 19 SNPs were calculated for 312 Thai patients with malaria. White, grey-shaded, and black squares indicate no LD ($r^2 = 0$), intermediate LD ($0 < r^2 < 1$) and strong LD ($r^2 = 1$). The rs# of a SNP showing a significant association with cerebral malaria ($P < 0.05$) is surrounded by a flame.

Thailand were investigated in this study. All patients underwent treatment at the Hospital for Tropical Diseases, Faculty of Tropical Medicine, Mahidol University. Malarial infection by *P. falciparum* was confirmed in all patients by a positive blood smear for the asexual form of *P. falciparum*. Clinical manifestations of malaria were classified according to the definitions and associated criteria of the World Health Organization. Cerebral malaria was defined as unrousable coma, a positive result in tests for the presence of the asexual form of *P. falciparum*, and exclusion of other causes of coma. Mild malaria was defined as having a positive blood smear and fever without other causes of infection and no signs indicating severe malaria such as high parasitemia (> 100,000 parasites/L), hypoglycemia (glucose level < 2.2 mmol/L), severe anemia (hematocrit < 20% or hemoglobin level < 7.0 g/

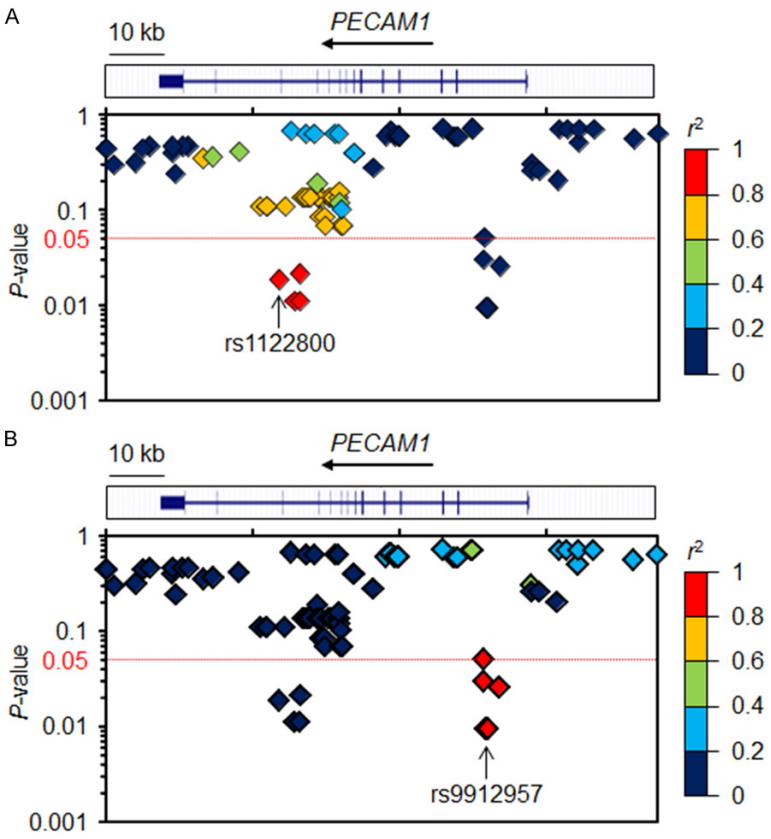

Figure 2. Association plot of SNPs surrounding the *PECAM1* gene. Association plot of 19 genotyped and 63 imputed SNPs. Each SNP is plotted along the chromosomal position. Pairwise r^2 between a particular SNP and (A) rs1122800 or (B) rs9912957 is colored on a scale from low (blue) to high (red).

Table 2. Combined effect of rs1122800 and rs9912957 on risk for cerebral malaria

Genotype		Frequency		*P*-value	OR (95% CI)
rs1122-800	rs9912-957	Cerebral malaria	Mild malaria		
GG	AA	7 (0.066)	2 (0.010)	0.0039	7.6 (1.5-37)
CC+CG	AA	13 (0.123)	14 (0.071)	0.083	2.0 (0.90-4.5)
GG	GG+AG	7 (0.066)	10 (0.051)	0.41	1.5 (0.56-4.1)
CC+CG	GG+AG	79 (0.745)	171 (0.868)	-	1.0

The genotype frequency was compared with that of CC or CG at rs1122800 and AG or GG at rs9912957.

dL) or increased serum creatinine level (> 3.0 mg/dL). All patients were 13 years of age or older. The mean ages of patients with cerebral malaria and mild malaria were 28.6 years and 25.5 years, respectively. This study was approved by the institutional review board of the Faculty of Tropical Medicine, Mahidol University, and the Research Ethics Committee of University of Tsukuba. Written informed consent was obtained from all patients.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using a QIAamp Blood Kit (Qiagen, Hilden, Germany). 11 tagSNPs and eight additional SNPs of *PECAM1* were genotyped using a TaqMan® SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA).

Selection of tag SNPs

SNP genotype data for Asian subjects, 45 Japanese in Tokyo, Japan (JPT) and 45 Han Chinese in Beijing, China (CHB), were obtained from the HapMap database [11, 12] to select tag SNPs for *PECAM1*. A total of 11 tag SNPs were manually selected among 52 SNPs (from rs4968720 to rs12051829) in a 100-kb genomic region that included the entire *PECAM1* gene (the size of *PECAM1* is 64.2 kb). The 52 SNPs with minor allele frequency (MAF) of ≥0.05 in HapMap JPT+CHB were in linkage disequilibrium (LD) with one of the 11 tag SNPs with a pairwise LD parameter r^2 of ≥0.8.

PECAM1 mRNA expression

To evaluate the effect of a *PECAM1* SNP on *PECAM1* mRNA expression, normalized mRNA data from Epstein-Barr virus-transformed lymphoblastoid cell lines derived from 45 JPT and 45 CHB HapMap subjects and their genotype data were obtained from the databases of the Gene Expression Variation (GENEVAR) project [13] and the HapMap database [11, 12], respectively. The mRNA expression was measured by using the GI_21314616-S probe for *PECAM1* in the GENEVAR database.

Table 3. Haplotype association test

Haplotype		Estimated frequency		Permutation *P*-value	OR
rs1122800	rs9912957	Cerebral malaria	Mild malaria		
G	A	0.1863	0.1025	0.021	2.0
C	A	0.2146	0.2147	1.0	1.0
G	G	0.1344	0.1894	0.15	0.66
C	G	0.4646	0.4934	0.51	0.89

The haplotype frequency was compared with that of the other haplotypes.

Statistical analysis

Deviation of genotype frequencies from Hardy-Weinberg equilibrium in each malaria group was examined by chi-squared test. Pairwise LD parameter r^2 was estimated using the Haploview software [11]. The association between each SNP and cerebral malaria was assessed by a chi-squared test based on a 2 × 2 table using three models: dominant, recessive, and allelic. The smallest *P*-value among three models was considered representative for each SNP. The haplotype frequencies were compared between cerebral malaria and mild malaria patients by a permutation test implemented in the SNPAlyze software, ver. 7.0 (Dynacom Co., Ltd., Yokohama, Japan). The genotypes of 178 SNPs with MAF of ≥0.05 spanning a 198-kb genomic region that included the entire *PECAM1* gene were retrieved from the HapMap JPT and CHB populations [11, 12], after which the genotypes of Thai malaria patients were imputed using the MACH software [14]. The 63 imputed SNPs that had Rsq values ≥0.3 and 19 genotyped SNPs were used for fine-scale mapping. The association between each of *PECAM1* SNPs and the mRNA expression level was evaluated using a simple linear regression analysis with the number of derived alleles for a subject as the independent variable (i.e., 0, 1, or 2). A *P*-value of ≤0.05 was considered statistically significant in this study.

Results

Association tests for genotyped PECAM1 SNPs

To evaluate possible associations of *PECAM1* polymorphisms with cerebral malaria, 11 tag SNPs, rs1122800, rs1470453, rs2070783, rs9913080, rs4968723, rs7207019, rs1131012, rs11079538, rs9906431, rs9912957, and rs12051829 were investigated in 312 Thai patients. No SNPs significantly deviated from HWE either in cerebral malaria or mild malaria patients (data not shown). The genotype or allele frequencies were compared between cerebral and mild malaria patients (**Table 1**). The smallest *P*-value among three models (i.e., dominant, recessive, and allelic) was considered representative for each SNP. Among 11 tag SNPs, three SNPs, rs1122800, rs9906431, and rs9912957, were significantly associated with cerebral malaria (**Table 1**). The rs1122800-C was associated with protection from cerebral malaria in the dominant model ($P = 0.017$, OR = 0.39, 95% CI = 0.18-0.87). The rs9906431-C and rs9912957-A increased the risk for cerebral malaria in the recessive model ($P = 0.028$, OR = 2.2, 95% CI = 1.1-4.4 for rs9906431-C; $P = 0.0065$, OR = 2.6, 95% CI = 1.3-5.3 for rs9912957-A). It should be noted that the direction of association (i.e., OR) was defined on the basis of a derived allele for a particular SNP in this study. A derived allele was determined by comparing the human sequence with the chimpanzee sequence (i.e., the same allele found in chimpanzee was defined as ancestral, and the other allele was defined as derived).

To identify other polymorphisms possibly associated with cerebral malaria, eight SNPs (rs1867624, rs7213889, rs11653087, rs59573853, rs8065316, rs12953175, rs6504227 and rs8080666) closely located to rs1122800, rs9906431, and rs9912957 were additionally genotyped. Of these eight SNPs, four SNPs, rs7213889, rs59573853, rs8065316, and rs12953175, were significantly associated with cerebral malaria (**Table 1**).

Linkage disequilibrium analysis

The structure of pairwise LD between 19 genotyped SNPs in Thai malaria patients revealed that associated SNPs were located in two distinct LD blocks (**Figure 1**). These two blocks are tentatively defined "region A", including rs1122800 and rs7213889 and "region B", including rs9906431, rs59573853, rs9912957, rs8065316, and rs12953175. The rs1122800 and rs9912957 had the smallest *P*-values in region A and region B, respectively. No strong LD was found between rs1122800 and rs9912957 ($r^2 = 0.02$).

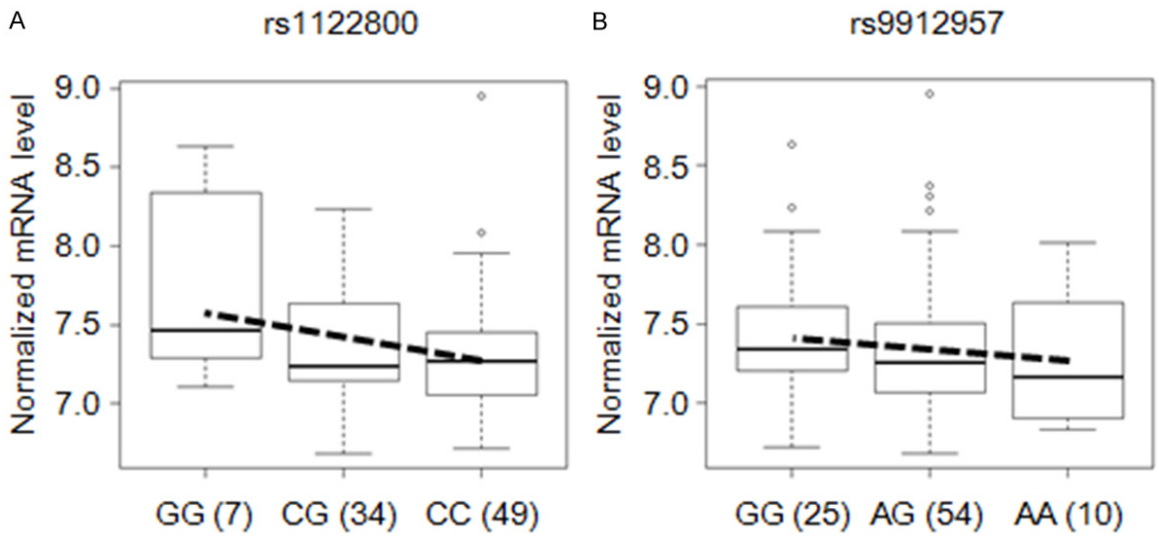

Figure 3. mRNA expression level of *PECAM1* and SNP genotype. A. mRNA expression level of *PECAM1* in each genotype of rs1122800. The rs1122800-C allele was associated with lower mRNA expression level of *PECAM1* (*P*-value = 0.045 and regression coefficient = -0.15). B. mRNA expression level of *PECAM1* in each genotype of rs9912957. The rs9912957-A allele was not associated with mRNA expression level of *PECAM1* (*P*-value = 0.36 and regression coefficient = -0.071). A regression line is shown as a dashed line. The number of samples for each genotype is shown in parentheses.

Fine-scale mapping

Genotype imputation enables evaluating the possible associations of SNPs that are not directly genotyped [9]. To further clarify the genomic region in which SNPs associated with cerebral malaria were located, the genotypes of 63 non-genotyped SNPs in malaria patients were imputed using the MACH software [14]. In addition, the genotypes for 19 SNPs of malaria patients that could not be determined by direct genotyping were also imputed. Association plots for a total of 82 SNPs revealed two genomic regions in which SNPs associated with cerebral malaria were located (**Figure 2**). Regions A and B contained four and six SNPs with $P < 0.05$ and spanned 7.2 kb and 5.3 kb, respectively. In region A (**Figure 2A**), the most significant result was found for rs12941698, which was not directly genotyped, and in region B, rs9912957 had the smallest *P*-value (**Figure 2B**); however, it was difficult to specify a SNP that was primarily associated with cerebral malaria in each region due to the strong LD ($r^2 \geq 0.9$). Because rs1122800 and rs9912957 were actually genotyped in this study, rs1122800 and rs9912957 were regarded as representatives of region A and region B, respectively, and were focused in the subsequent analyses.

Combined effect of two PECAM1 SNPs on susceptibility to cerebral malaria

The rs1122800-C carriers (rs1122800-CC and rs1122800-CG) were associated with protection from cerebral malaria (**Table 1**). In other words, rs1122800-GG was associated with a risk for cerebral malaria. To examine the combined effect of rs1122800 and rs9912957 on susceptibility to cerebral malaria, the odds ratios of risk genotypes were estimated (**Table 2**). As expected, the rs1122800-GG and rs9912957-AA genotype significantly increased the risk for cerebral malaria compared with the rs1122800-CC or CG genotype and rs9912957-GG or AG genotype ($P = 0.0039$, OR = 7.6, 95% CI = 1.5-37).

Haplotype association test

Next, the associations of haplotypes consisting of rs1122800 and rs9912957 were evaluated (**Table 3**). The haplotype of rs1122800-G and rs9912957-A was significantly associated with a risk for cerebral malaria (permutation $P = 0.021$).

Association of PECAM1 SNP with mRNA expression

The association of rs1122800 or rs9912957 with mRNA level of *PECAM1* in EBV-transformed

lymphoblastoid cell lines was assessed to evaluate the functional significance of those SNPs that showed the significant associations with cerebral malaria. The rs1122800-C allele was significantly associated with lower expression level of *PECAM1* (P = 0.045, regression coefficient = -0.15), whereas the rs9912957-A allele was not (**Figure 3**).

Discussion

In this study, two genomic regions of the *PECAM1* gene were found to be independently associated with cerebral malaria in a Thai population. The functional significance of rs9912957 in region B remains unclear, whereas rs1122800-C in region A was associated with lower mRNA level of *PECAM1* (**Figure 3A**). A reduction in cytoadherence caused by lower expression of PECAM1 on vascular endothelium may decrease the risk for cerebral malaria. Although it is unknown if rs1122800 itself is a causative SNP that directly affects the expression level of *PECAM1*, the present results suggest that the PECAM1-mediated cytoadherence of iRBCs to vascular endothelium plays a crucial role in the pathogenesis of cerebral malaria. The differences in cytoadherence of iRBC to PECAM1 on vascular endothelium between different *PECAM1* genotypes may explain why a part of malaria patients develop cerebral malaria.

The results from previous studies on the association of nonsynonymous SNPs, L125V, S563N, and G670R, with severe malaria are inconsistent [8-10]. These three SNPs are in strong LD (r^2 of ≥0.8 in JPT+CHB) with each other in Asian populations. In this present study, G670R, which can act as a tag SNP of L125V and S563N, was not associated with cerebral malaria (**Table 1**). Thus, we conclude that L125V, S563N, and G670R were not associated with cerebral malaria in Thai patients, although false negative results may have occurred due to small sample size of the present study.

Acknowledgements

We sincerely thank all patients who kindly participated in this study. This work was partly supported by KAKENHI (25650125) Grant-in-Aid for Challenging Exploratory Research of the Ministry of Education, Science, Sports and Culture (MEXT) and the Takeda Science Foundation.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Ohashi, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel: +81-3-5841-8395; E-mail: juno-tky@umin.ac.jp

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