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High Incidence of Malaria Along the Sino–Burmese Border Is Associated With Polymorphisms of CR1, IL-1A, IL-4R, IL-4, NOS, and TNF, But Not With G6PD Deficiency

Na Ren, MS, Ying-Min Kuang, MD, Qiong-Lin Tang, MS, Long Cheng, MS, Chun-Hua Zhang, MD, Zao-Qing Yang, PhD, Yong-Shu He, MS, and Yue-Chun Zhu, MD

Abstract: Malaria is highly endemic in Yunnan Province, China, with the incidence of malaria being highest along the Sino–Burmese border.

The aim of our study was to determine whether genetic polymorphisms are associated with the prevalence of malaria among Chinese residents of the Sino–Burmese border region. Fourteen otherwise healthy people with glucose-6-phosphate dehydrogenase (G6PD) deficiency, 50 malaria patients, and 67 healthy control subjects were included in our cross-sectional study. We analyzed the frequency of the G3093T and T520C single-nucleotide polymorphisms (SNPs) of *CR1*. Logistic regression was used to calculate the prevalence odds ratio (POR) and 95% confidence interval (CI) of malaria for the T520C SNP of *CR1* and SNPs of *G6PD*, *IL-4*, *IL-4R*, *IL-1A*, *NOS*, *CD40LG*, *TNF*, and *LUC7L*.

The frequency of the 3093T/3093T genotype of *CR1* in the malaria group (0.16) was significantly higher than that in the control group (0.045, $P < 0.05$), and significantly lower than that in the G6PD deficiency group (0.43, $P < 0.01$). The frequency of the 520T/520T genotype of *CR1* was significantly higher in the malaria patients (0.78) than that in the control group (0.67, $P < 0.05$) and G6PD-deficiency group (0.36, $P < 0.05$). The T allele of the T520C variant of *CR1* was significantly associated with the prevalence of malaria (POR: 1.460; 95% CI: 0.703–3.034). Polymorphisms of G6PD did not significantly influence the prevalence malaria ($P > 0.05$). A GTGTGTC haplotype consisting of *IL-1A* (rs17561), *IL-4* (rs2243250), *TNF* (rs1800750), *IL-4R* (rs1805015), *NOS* (rs8078340), *CD40LG* (rs1126535), and *LUC7L* (rs1211375) was significantly associated with the prevalence of malaria (POR: 1.822, 95% CI: 0.998–3.324).

The 3093G/3093G and 520T/520T genotypes are the predominant genetic variants of *CR1* among Chinese residents near the Sino–Burmese border, and the T allele of T520C is associated with the prevalence of malaria in this region. Although G6PD deficiency does not protect against malaria, it may diminish the association between malaria and the *CR1* polymorphisms in this population. The GTGTGTC haplotype is also associated with the prevalence of malaria in this region.

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Abbreviations: CI = confidence interval, G6PD = glucose-6-phosphate dehydrogenase, IL = interleukin, LD = linkage disequilibrium, NOS = nitric oxide synthase, POR = prevalence odds ratio, SNP = single-nucleotide polymorphism, TNF = tumor necrosis factor.

INTRODUCTION

Malaria poses a serious threat to human health in developing regions worldwide, with more than 225 million cases occurring yearly and more than 176 million cases occurring in sub-Saharan Africa alone. Approximately 98% of malaria cases in Africa and 65% of cases elsewhere are caused by the malarial parasite, *Plasmodium falciparum*, which causes high mortality in children. Malaria is endemic throughout greater Southeast Asia and the tropical and subtropical regions of China,[1,2] where severe disease is caused by both *P. falciparum* and *Plasmodium vivax*.[3] In Yunnan Province, which borders Myanmar in southwestern China, the incidence of malaria was >10/10 000 person-years from 2010 to 2012, making it the Chinese province with the highest incidence of malaria.[4–6] The highest incidence of malaria in Yunnan Province occurs along the Sino–Burmese border.[7] A similar pattern in the incidence of malaria in Thailand has been reported for the Thai–Burmese border.[8]

Malaria is considered to be one of the strongest forces of natural selection to have influenced the human genome in recent history. Genetic disorders that diminish the severity of malaria, including sickle-cell disease and thalassemia, demonstrate that mutations causing otherwise deleterious conditions have become fixed in human populations in specific areas as a result of selective pressure related to malaria pathogenesis.[9,10] However, whether such genetic disorders influence susceptibility to malarial parasites has remained unclear.[10,11] Glucose-6-phosphate dehydrogenase (G6PD) deficiency affects more than 400 million people worldwide,[12] primarily in the tropical and subtropical regions of the world, including Southeast Asia, Myanmar, and areas of mainland China.[13] The correlation between the geographic distribution of G6PD deficiency and malaria endemicity suggests that G6PD deficiency might affect malaria susceptibility.[14] However, although studies in Africa have found a correlation between G6PD deficiency and malaria,[15,16] the results of other studies of falciparum and vivax malaria have not.[17,18]

Multiple studies have shown that complement receptor 1 (CR1) functions as the erythrocyte receptor for invasion by *P. falciparum*.[19,20] The CR1 protein functions in the regulation

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From the Department of Biochemistry and Molecular Biology (NR, LC, C-HZ, Q-LT, Z-QY, Y-SH, Y-CZ); and First Affiliated Hospital, Kunming Medical University, Kunming, Yunnan, PR China (Y-MK).
Correspondence: Yue-Chun Zhu, Department of Biochemistry and Molecular Biology, Kunming Medical University, No. 1168, Chunrong West Road, Kunming, Yunnan 650500, PR China (e-mail: zhuyue chunbm@126.com).
NR, Y-MK and Q-LT contributed equally to this work.
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of complement activation and the clearance of immune complexes from the bloodstream via erythrocyte recycling.[21] Wilson et al[22,23] found that *Hind*III restriction fragment length polymorphisms (RFLPs) of *CR1* correlated with high-level (H/H), moderate-level (H/L), and low-level (L/L) expression of CR1 in erythrocytes. Although diminished CR1 expression on erythrocytes results in impaired clearance of immune complexes, the persistence of *Hind*III RFLPs that reduce the expression of CR1 in both African and non-African populations, suggesting that low-level CR1 expression might confer a survival benefit. Jallow et al[24] performed a genome-wide investigation of associations between susceptibility to malaria and polymorphisms of IL-4R1, CR1, tumor necrosis factor (TNF), G6PD, interleukin (IL)-10, IL-4, IL-22, nitric oxide synthase (NOS) 2A, CD40, CD54, and CD36 in West Africa. Although notable associations were observed between malaria and SNPs in *CR1*, *TNF*, *CD36*, *IL-22*, *NOS2A*, *CD40*, and *G6PD*, only the G238A SNP in *TNF* was found to be significantly associated with malaria.

We hypothesized that polymorphisms in genes affecting rosette formation, hemoglobin structure, and the innate immune response contribute to variation in malaria susceptibility in the Sino–Burmese border region. Therefore, we examined the frequency of 41 SNPs, including those in *CR1*, *IL-1A*, *TNF*, *IL-4R*, *NOS2*, *CD40LG*, *IL-4*, *G6PD*, and *LUC7L*, in Chinese residents along the Sino–Burmese border to determine whether these polymorphisms are associated with the prevalence of malaria in Yunnan Province. Twenty-nine polymorphisms in *G6PD* were included to examine whether G6PD deficiency influences susceptibility to malarial infection in this geographic area. Polymorphisms in *IL-1A*, *TNF*, *IL-4R*, *NOS2*, *CD40LG*, and *IL-4* were included in our analysis because these genes contribute to the innate immune response,[24,25] and *LUC7L* was included because mutations in *LUC7L* affect the structure of hemoglobin in thalassemia.[26,27]

METHODS

Study Population

Our cross-sectional study was performed in accordance with the Declaration of Helsinki with regard to ethical principles for research involving human subjects, and the protocols for our study were approved by the Ethics Committee of Kunming Medical University (Kunming, Yunnan, China). Signed informed consent was received from all of the subjects before they participated in our study. Fifty malaria patients, 14 otherwise healthy people with G6PD deficiency, and 67 healthy people (controls) were enrolled in our study in 2009. All of the study participants were screened for malaria parasites based on microscopic examination of Giemsa-stained thick blood films, as previously described.[28,29] The malaria patients included 14 cases of falciparum malaria and 36 cases of vivax malaria. All of the G6PD-deficiency cases were confirmed using a previously described colorimetric method for measuring G6PD activity in erythrocytes.[30,31] The healthy control subjects consisted of undergraduate students at our institution who reported no known health problems. All of our study participants had been long-time residents of the Sino–Burmese border region. The mean age and sex distribution of the malaria patients, G6PD-deficient subjects, and healthy controls were 30.92 ± 8.75 years (94% men), 26.07 ± 12.46 years (100% men), and 22.12 ± 3.05 years (68.66% men), respectively.

Genomic DNA Isolation

Three to five milliliters of venous blood was collected from each participant in EDTA anticoagulant tubes, and stored at -20°C. Genomic DNA was extracted from peripheral blood mononuclear cells, and suspended in TE buffer using the AxyPrep Blood Genomic DNA Isolation kit (Baisai Biological Technology, Shanghai, China), according to the manufacturer's instructions. Five microliters of the purified genomic DNA was diluted in 1000 μL of ultrapure water, and the ratios of the optical densities at 260 and 280 nm (OD260/OD280) were measured using a GeneQuant 1300/100 spectrophotometer (GE Healthcare, Waukesha, WI) to determine the concentration and purity of the DNA. The OD260/OD280 of the DNA samples ranged from 1.6 to 1.8.

BstNI RFLP Analysis of the G3093T SNP of CR1

For all of the RFLP analyses performed in our study, the genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The *Bst*NI RFLP was performed as described previously.[32] The F1 and R1 primers (Table 1) were used to PCR amplify cDNA containing the G3093T SNP of CR1 from the genomic DNA template. The 366-bp PCR product was digested using the restriction enzyme, *Bst*NI (Takara Bio, Shiga, Japan), and the restriction fragments were analyzed by agarose gel electrophoresis. Three distinct genotypes can be detected based on the *Bst*NI cleavage pattern. The homozygous wild-type genotype, 3093G/3093G, produces 3 fragments that are 221, 91, and 54 bp in size. The heterozygous genotype, 3093G/3093T, produces 4 fragments that are 312, 221, 91, and 54 bp in size. The homozygous mutant genotype, 3093T/3093T, produces 2 fragments that are 312 and 54 bp in size (Fig. 1).

HindIII RFLP analysis of the T520C SNP of CR1

The *Hind*III RFLP was performed as described previously.[33] The F2 and R2 primers (Table 1) were used to PCR amplify cDNA containing the T520C SNP in intron 27 of CR1,[34] producing a PCR product approximately 1800 bp in size. The PCR product was digested using the restriction enzyme, *Hind*III (New England Biolabs, Ipswich, MA), and the restriction fragments were analyzed by agarose gel electrophoresis. The homozygous wild-type genotype, 520T/520T (H/H allele), produces one 1800-bp restriction fragment. The

TABLE 1. Primers Used for PCR Amplification of the Loci of CR1 Containing the G3093T SNP (F1 and R1) and T520C SNP (F2 and R2) for *Bst*NI and *Hind*III RFLP, Respectively

SNP	Primer	Primer Sequence	Primer (bp)	PCR Product (bp)
G3093T	F1	GCTACATGCACGTTGAGACCTTAC	24	366
	R1	AGCAAGCATACAGATTTTCCCC	22	
T520C	F2	CCTTCAATGGAATGGTGCAT	20	1800
	R2	CCCTTGTAAGGCAAGTCTGG	20	

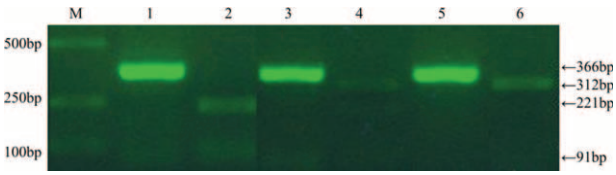

FIGURE 1. *Bst*NI RFLP analysis of the G3093T SNP of CR1. Lanes 1, 3, 5: PCR products produced using primers F1 and R1. Lanes 2, 4, 6: *Bst*NI restriction fragments. Lane 2: homozygous wild-type genotype, 3093G/3093G. Lane 4: heterozygous genotype, 3093G/3093T. Lane 6: homozygous mutant genotype, 3093T/3093T.

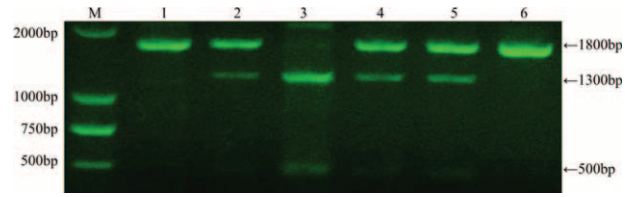

FIGURE 2. *Hind*III RFLP analysis of the T520C SNP of CR1. Lanes 1, 3, 5: PCR products produce using primers F2 and R2. Lanes 2, 4, 6: *Hind*III restriction fragments. Lane 2: homozygous wild-type genotype, 520T/520T. Lane 4: heterozygous genotype, 520T/520C. Lane 6: homozygous mutant genotype, 520C/520C.

heterozygous genotype, 520T/520C, produces 3 fragments that are 1800, 1300, and 500 bp in size. The homozygous mutant genotype, 520C/520TC (L/L allele), produces 2 fragments that are 1300 and 500 bp in size (Fig. 2).

Direct Genotyping of 39 SNP Loci Using SNaPshot Assays

We used the SNaPshot multiplex SNP genotyping assay (Life Technologies, Carlsbad, CA) to determine the genotypes of 39 polymorphic loci (Supplementary Table 1, http://links.lww.com/MD/A450) using gene-specific primers designed to flank both sides of each locus using the Primer Premier 5 program.[35] After the PCR amplification, 3 μL of PCR product was purified using the using the QIAamp DNA Mini Kit to remove the unincorporated primers and dNTPs. The purified PCR product was treated with shrimp alkaline phosphatase (New England Biolabs), and digested with exonuclease I (New England Biolabs). Primer-extension reactions were performed using the SNaPshot Multiplex Reaction Ready Mix, dye-labeled dNTPs, and 1 μL of the PCR product as a template. The extension products were analyzed in an ABI 3730XL sequencer (Life Technologies).

Statistical Analysis

The differences in the continuous data between 2 groups were evaluated using independent sample *t*-tests, and a χ^2 analysis was used to evaluate the intergroup differences involving multiple comparisons. An unconditional logistic regression model was used to calculate the prevalence odds ratios (PORs) and 95% confidence intervals (CIs) of the association between the prevalence of malaria and the various SNPs analyzed.[36] For the SNPs found to be associated with malaria, the Shesis online software was used to perform pair-wise evaluations of linkage disequilibrium (LD) between polymorphic loci and for haplotype construction. We performed a Pearson correlation analysis of the genotype and allele distributions of the SNPs identified in the LD analysis. The Fisher exact test was used to evaluate the statistical significance of the results of the correlational analysis. For all of the aforementioned analyses, the level of statistical significance was set at $P < 0.05$. A Hardy–Weinberg equilibrium test was used to analyze the SNP genotype frequencies within each group, and the distribution was considered to be consistent with Hardy–Weinberg equilibrium when $P > 0.05$.

RESULTS

Genotype and Allele Frequencies of CR1 Based on the BstNI RFLP Analysis of the G3093T SNP

The 3093T genetic variant of *CR1* is associated with the reduced expression of CR1 on erythrocytes due to diminished structural stability of the mutant protein,[34,37] and is therefore described as the L allele, whereas the wild-type variant, 3093G, is described as the H allele. The homozygous 3093G/3093G genotype (H/H) is associated with high-level CR1 expression. The homozygous 3093T/3093T genotype (L/L) is associated with low-level CR1 expression, and the heterozygous 3093G/3093T genotype (H/L) is associated with moderate-level expression due to the codominant H and L alleles of *CR1*.

The frequencies of the 3093G/3093G (H/H), 3093G/3093T (H/L), and 3093T/3093T (L/L) genotypes of *CR1* among the 3 study groups were as follows (Table 2): 0.46 (n = 23), 0.38 (n = 19), and 0.16 (n = 8) in malaria group; 0.14 (n = 2), 0.43 (n = 6), and 0.43 (n = 6) in the G6PD-deficient group; and 0.58 (n = 39), 0.37 (n = 25), and 0.045 (n = 3) in the control group, respectively. The frequency of the 3093T/3093T genotype in the malaria patients was significantly lower than that in the

TABLE 2. Genotype Frequencies of the G3093T and T520C SNPs of *CR1* Based on *Bst*NI and *Hind*III RFLP Analyses

	Malaria Group				
Genotype	**Total Malaria (n)**	**Malaria Tertiana (n)**	**Malignant Malaria (n)**	**G6PD-Deficient Group (n)**	**Control Group (n)**
3093G/3093G*	0.46 (23)	0.58 (21)	0.14 (2)	0.14 (2)	0.58 (39)
3093G/3093T	0.38 (19)	0.31 (11)	0.57 (8)	0.43 (6)	0.37 (25)
3093T/3093T	0.16 (8)	0.11 (4)	0.29 (4)	0.43 (6)	0.045 (3)
520T/520T*	0.78 (39)	0.83 (30)	0.64 (9)	0.36 (5)	0.67 (45)
520T/520C	0.18 (9)	0.11 (4)	0.36 (5)	0.50 (7)	0.30 (20)
520C/520C	0.040 (2)	0.055 (2)	0 (0)	0.14 (2)	0.030 (2)

Malaria group, n = 50; G6PD-deficient group, n = 14; control group, n = 67; malaria tertiana subgroup, n = 36; malignant malaria subgroup, n = 14.
* Wild type.

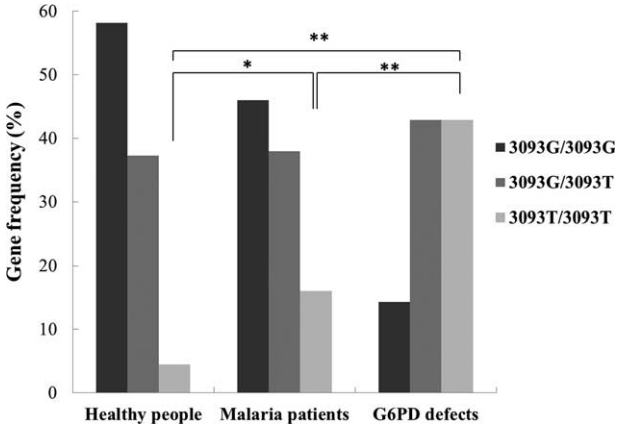

FIGURE 3. Genotype frequencies of the G3093T SNP of CR1 in the study groups (3093G: wild type; 3093T: mutant). The asterisks indicate the comparison of the frequency of the homozygous mutant genotype in each study group ($*P < 0.05$ and $**P < 0.01$).

G6PD-deficient group ($P < 0.05$; Fig. 3). The frequency of the 3093T/3093T genotype in the control group was significantly higher than those in the malaria and G6PD-deficient groups ($P < 0.01$; Fig. 3).

In the subgroup analysis, the frequencies of the 3093G/3093G, 3093G/3093T, and 3093T/3093T genotypes were 0.58 (n = 21), 0.31 (n = 11), and 0.11 (n = 4) in the patients with malaria tertiana and 0.14 (n = 2), 0.57 (n = 8), and 0.29 (n = 4) in the patients with malignant malaria, respectively (Table 2). Based on these data, the frequencies of the H and L alleles in our study groups were as follows: 0.65 and 0.35 in the malaria group, 0.36 and 0.64 in the G6PD-deficient group, and 0.77 and 0.23 in the control group, respectively. In the subgroup analysis of the malaria patients, the H and L allele frequencies were 0.74 and 0.26 in the patients with malaria tertiana and 0.43 and 0.57 in the patients with malignant malaria, respectively (Table 3).

Genotype and Allele Frequencies of CR1 Based on the HindIII RFLP Analysis of the T520C SNP

The 520C genetic variant of *CR1* is associated with the reduced expression of CR1 on erythrocytes through an unknown mechanism,[34,37] and is therefore described as the L allele, whereas the wild-type variant, 520T, is described as the H allele. The homozygous 520T/520T genotype (H/H) is associated with high-level CR1 expression. The homozygous 520C/520C genotype (L/L) is associated with low-level CR1 expression, and the heterozygous 520T/520C genotype (H/L) is associated with moderate-level expression due to the codominant H and L alleles. The genotype frequencies of the 520T/520T, 520T/520C, and 520C/520C genotypes in the 3 study groups were as follows: 0.78 (n = 39), 0.18 (n = 9), and 0.040 (n = 2) in malaria group; 0.36 (n = 5), 0.50 (n = 7), and 0.14 (n = 2) in the G6PD-deficient group; and 0.67 (n = 45), 0.30 (n = 20), and 0.030 (n = 2) in the control group, respectively (Table 2). Based on these data, the H and L allele frequencies were 0.87 and 0.13 in the malaria group, 0.61 and 0.39 in the G6PD-deficient group, and 0.82 and 0.18 in the control group, respectively (Table 3).

Genotypes of 39 SNP Loci Based on SNaPshot Assays

The genotypes of 39 SNP loci were analyzed by direct sequencing using SnaPshot assays (Supplementary Table 2, http://links.lww.com/MD/A450). Three malaria patients were excluded from our analysis because the PCR amplification of their samples had failed. The results of genotyping showed that, among the 39 SNP loci analyzed, the following 7 SNPs occurred at a higher frequency in malaria patients: rs17561, rs2243250, rs1800750, rs1805015, rs8078340, rs1126535, and rs1211375 (Supplementary Table 2, http://links.lww.com/MD/A450). Although the A4828G SNP in the 29th exon of *CR1*, which corresponds to a Swain–Langley blood antigen encoded by *CR1* (rs17047661), was higher in malaria patients than the healthy controls, the difference was not statistically significant. Of the 29 SNP loci in *G6PD* that were analyzed, no missense mutations were found in the malaria group or the control group.

SNP Distribution and Prevalence of Malaria

Logistic regression was used to evaluate the relationship between the distribution of the allelic variants of the above-mentioned 7 SNPs and prevalence of malaria, and the results are presented in Table 4. The PORs and 95% CIs for the associations between the polymorphisms and malaria were as follows: T allele of the T520C variant of *CR1* (POR: 1.460; 95% CI: 0.703–3.034); C allele of the T520C variant of *CR1* (POR: 0.685; 95% CI: 0.330–1.423); T allele of *IL-1A* (POR: 0.593; 95% CI: 0.207–1.694); G/T genotype of *IL-1A* (POR: 0.569; 95% CI: 0.191–1.694); T allele of *IL-4* (POR: 0.832; 95% CI: 0.451–1.533); T allele of *IL-4R* (POR: 1.488; 95% CI: 0.637–3.473); T allele of *CD40LG* (POR: 0.41367; 95% CI: 0.131–1.311); and C allele of *LUC7L* (POR: 1.008323; 95% CI: 0.594–1.710). These results suggest that these SNPs are associated with malaria prevalence. Therefore, they were subjected to further analyses to confirm the associations between them and malaria in our study sample.

TABLE 3. Allele Frequencies for the G3093T and T520C SNPs of *CR1* Based on *Bst*NI and *Hind*III RFLP Analyses

	Malaria Group				
Allele (Allelic Variant)	**Total Malaria**	**Malaria Tertiana**	**Malignant Malaria**	**G6PD-Deficient Group**	**Control Group**
H (3093G)*	0.65	0.74	0.43	0.36	0.77
L (3093T)	0.35	0.26	0.57	0.64	0.23
H (520T)*	0.87	0.89	0.82	0.61	0.82
L (520C)	0.13	0.11	0.18	0.39	0.18

* Wild type.

TABLE 4. Associations Between Polymorphisms and Prevalence of Malaria

Gene	Control Group	Malaria Group	POR (95% CI)
CR1[*]			
T	0.821	0.870	1.460 (0.703–3.034)
C	0.179	0.130	0.685 (0.330–1.423)
T/T	0.672	0.780	1.853 (0.802–4.284)
T/C	0.299	0.180	0.516 (0.212–1.258)
C/C	0.030	0.040	1.354 (0.184–9.958)
IL1A			
G	0.948	0.915	0.593 (0.207–1.694)
T	0.052	0.085	1.688 (0.59–4.826)
G/G	0.896	0.83	0.569 (0.191–1.694)
G/T	0.104	0.17	1.758 (0.59–5.238)
IL4			
C	0.269	0.234	0.832 (0.451–1.533)
T	0.731	0.766	1.202 (0.652–2.216)
C/C	0.075	0.043	0.551 (0.102–2.969)
C/T	0.388	0.383	0.979 (0.455–2.106)
T/T	0.537	0.574	1.162 (0.548–2.465)
TNF			
A	0	0.011	2.328E + 9 (0–∞)
G	1.000	0.989	0
A/G	0	0.021	2.353E + 9 (0–∞)
G/G	1.000	0.979	0
IL4R			
C	0.090	0.128	1.488 (0.637–3.473)
T	0.910	0.872	0.672 (0.288–1.569)
C/C	0.015	0	2.253E+9 (0–∞)
C/T	0.149	0.255	1.954 (0.764–4.997)
T/T	0.836	0.745	0.573 (0.228–1.439)
NOS2			
A	0.030	0	0.349 (0.038–3.177)
G	0.070	1	1.168E+9 (0–∞)
A/G	0.060	0	0
G/G	0.940	1	1.205E+9 (0–∞)
CD40LG			
C	0.097	0.043	0.414 (0.131–1.311)
T	0.903	0.957	2.417 (0.763–7.66)
C/C	0.030	0.043	1.444 (0.196–10.636)
C/T	0.134	0	0 (0–∞)
T/T	0.836	0.957	4.420 (0.932–20.967)
LUC7L			
A	0.530	0.532	1.008 (0.594–1.71)
C	0.470	0.468	0.992 (0.585–1.682)
A/A	0.239	0.277	1.219 (0.520–2.854)
A/C	0.582	0.511	0.749 (0.354–1.586)
C/C	0.179	0.213	1.239 (0.485–3.162)

CI = confidence interval, POR = prevalence odds ratio.
[*] T520C variant of CR1.

Pair-Wise LD Analyses of SNPs

Pair-wise LD analyses were performed for the 7 SNPs that were found to be associated with the prevalence of malaria in the logistic regression analysis. The D' values for rs17561–rs1800750, rs17561–rs8078340, rs17561–rs1126535, rs2243250–rs1800750, rs1800750–rs1805015, rs1800750–rs8078340, rs1800750–rs1126535, rs1800750–rs1211375, rs1805015–rs1126535, rs1805015–rs1211375, and rs8078340–rs1126535 indicated LD (Table 5, Fig. 4). The results of the Pearson correlation analysis and Fisher exact test showed that significant LD existed between *TNF*: rs1800750 and *IL-4R*: rs1805015 ($r^2 = 0.037$; $P < 0.05$) and *IL-1A*: rs17561 and *TNF*: rs1800750 ($r^2 = 0.063$; $P < 0.05$), whereas the LD that existed between *IL-4R*: rs1805015 and *LUC7L*: rs1211375 ($r^2 = 0.104$; $P > 0.05$) was not significant (Table 5, Fig. 5).

Haplotypes of SNPs Correlating With the Prevalence of Malaria

Haplotypes were constructed based on an exclusion analysis of the 7 SNPs that had been shown to be associated with malaria using the Shesis software with all frequencies <0.03 (Table 6). The results suggested that the GTGTGTC haplotype significantly correlated with the prevalence of malaria in our study population (POR: 1.822; 95% CI: 0.998–3.324, $P < 0.05$), whereas the GCGTGTA haplotype (POR: 1.096; 95% CI: 0.356–3.374); GCGTGTC haplotype (POR: 0.879; 95% CI: 0.394–1.962); GCGTGTC haplotype (POR: 1.960; 95% CI: 0.647–5.936); GCGTGTC haplotype (POR: 1.342; 95% CI: 0.328–5.489); and GCGTGTC haplotype (POR: 0.578; 95% CI: 0.319–1.046) did not correlate significantly with the prevalence of malaria ($P > 0.05$).

DISCUSSION

We examined the frequency of various SNPs in Chinese residents of the Sino–Burmese border in Yunnan Province to determine whether genetic variants in this population contribute to the increased incidence of malaria in this region, compared with that in other provinces in China. We first focused our investigation on genetic variants of CR1 because it serves as the receptor for erythrocyte rosette formation by *P. falciparum*. To evaluate whether SNPs associated with G6PD deficiency contribute to malaria susceptibility in this region, we included malaria patients, otherwise healthy people with G6PD deficiency, and healthy control subjects in our study.

In a study in Papua New Guinea, a malaria endemic area, Cockburn et al[38] found that a polymorphism that caused a reduction in the number of CR1 molecules on erythrocytes conferred protection against severe malaria. Thomas et al found that the frequencies of polymorphisms that caused low-level expression of CR1 on erythrocytes, including the L/L allelic variant and the G3093T (Q981H) single-nucleotide polymorphism (SNP), were highest in populations in malaria-endemic regions in Asia, compared with frequencies in populations originating from Africa, North America, and Europe. Another SNP of CR1, A4828G (R1601G), is highly prevalent in malaria-endemic areas of Africa, but a study in Gambia showed that it was not associated with severe malaria.[39] However, the findings of a similar study of various European populations supported the role of malaria in the positive selection of CR1 on the Mediterranean island of Sardinia.[40]

We found that the frequency of the 3093T/3093T mutant genotype, which is associated with reduced expression of CR1 on erythrocytes, was significantly lower in the malaria (0.16) and control (0.04) groups than that in the malaria-free (0.43) G6PD-deficient group ($P < 0.01$; Table 2, Fig. 3). However, the frequency of the 3093T/3093T genotype in the malaria group was significantly higher than that in the control group ($P < 0.05$; Fig. 3). A similar trend in the frequencies of the 520C/520C mutant genotype was also observed. In addition, the frequency of the wild-type 520T/520T genotype was significantly higher in the malaria group (0.78) than that in the control group (0.67; Table 4), and the frequency of the wild-type 3093G/3093G

TABLE 5. *D'*, Pearson's r^2, and Fisher's *P* Values for the Pair-Wise Analyses of Linkage Disequilibrium Among SNPs Associated With Prevalence of Malaria

D' Values	rs2243250	rs1800750	rs1805015	rs8078340	rs1126535	rs1211375
rs17561	0.161	1	0.168	1	1	0.531
rs2243250	–	1	0.241	0.037	0.114	0.29
rs1800750	–	–	1	1	1	1
rs1805015	–	–	–	0.049	0.996	0.999
rs8078340	–	–	–	–	1	0.113
rs1126535	–	–	–	–	–	0.088
Pearson's r^2	rs2243250	rs1800750	rs1805015	rs8078340	rs1126535	rs1211375
rs17561	0.001	0.063	0.017	0.001	0.006	0.018
rs2243250	–	0.002	0.002	0	0	0.033
rs1800750	–	–	0.037	0	0	0.004
rs1805015	–	–	–	0	0.009	0.104
rs8078340	–	–	–	–	0.001	0
rs1126535	–	–	–	–	–	0.001
Fisher *P*	rs2243250	rs1800750	rs1805015	rs8078340	rs1126535	rs1211375
rs17561	0.706574	0.000161	0.050404	0.592354	0.255436	0.045275
rs2243250	–	0.558311	0.465287	0.837940	0.774824	0.006496
rs1800750	–	–	0.003498	0.893467	0.776061	0.346011
rs1805015	–	–	–	0.421086	0.253203	0.000002
rs8078340	–	–	–	–	0.566847	0.807618
rs1126535	–	–	–	–	–	0.723268

genotype was higher in the patients with malaria tertiana than that in the patients with malignant malaria (Table 2). These data suggest that the wild-type 520T/520T and mutant 3093T/3093T genotypes are associated with the prevalence of malaria. Therefore, the high frequency of these genetic variants of *CR1* in the population residing along the Sino–Burmese border might contribute to the high incidence of malaria in this region, compared with other malaria endemic regions in China.

The frequencies of the L allele of the G3093T SNP observed in the malaria (0.35) and control groups (0.23) are consistent with those previously reported in Chinese/Taiwanese (0.29) subjects, and were lower than those reported in Cambodians (0.47) and Papuans (0.54), for which the relatively high frequency of the mutant allele correlated with malaria endemicity[41] in regions with higher incidences of vivax malaria, compared with that in malaria endemic regions of Africa.[3] Based on a *Hind*III RFLP analysis of intron 27 of *CR1*, Nagayasu et al[33] found that the frequency of the 520T/520T genotype (LL) in patients with severe malaria in Thailand was significantly higher (34.5%) than that in Thai patients with uncomplicated malaria (23.33%, $P < 0.05$), and that the heterozygous (HL) and LL genotypes were more prevalent among

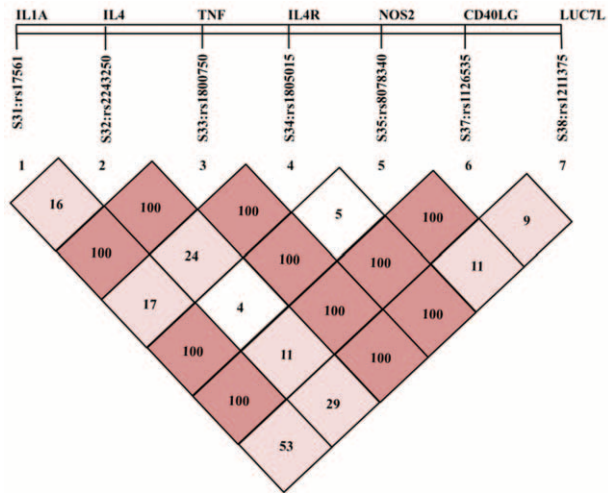

FIGURE 4. Pair-wise linkage disequilibrium analyses of the 7 SNPs found to be associated with the prevalence of malaria in the logistic regression analysis. The numbers in the squares are the D' value for each pair-wise analysis.

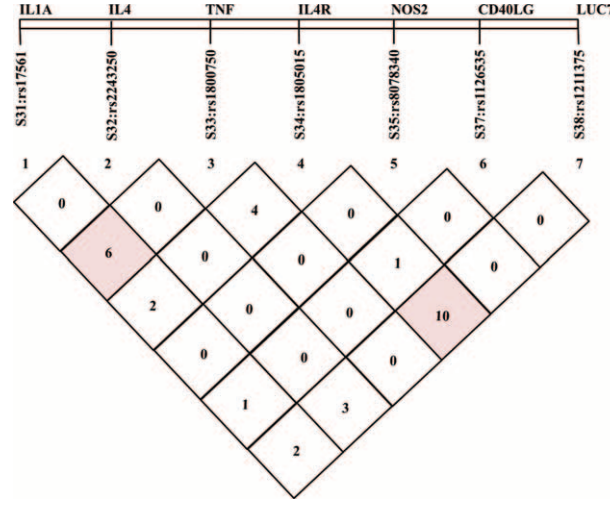

FIGURE 5. Pearson's correlation analysis of linkage disequilibrium between SNPs found to be associated with malaria in the logistic regression analysis. The numbers in the squares are the r^2 values for each of the pair-wise analyses of linkage disequilibrium.

TABLE 6. Associations Between the Prevalence of Malaria and Haplotypes Based on Single-Nucleotide Polymorphisms in 7 Different Genes

N	Haplotype	Malaria (Frequency)	Control (Frequency)	χ^2	Fisher *P* Value	Pearson's *P* Value	POR (95% CI)
1	GCGCATC	0.00 (0.000)	0.82 (0.006)	–	–	–	–
2	GCGCATC	0.00 (0.000)	3.86 (0.029)	–	–	–	–
3	GCGTATA	0.00 (0.000)	1.00 (0.007)	–	–	–	–
4	GCGTGCC	0.00 (0.000)	4.79 (0.036)	3.638	0.056518	0.056475	–
5	GCGTGTA	5.87 (0.062)	7.26 (0.054)	0.026	0.873034	0.873009	1.096 (0.356–3.374)
6	GCGTGTC	11.46 (0.122)	17.27 (0.129)	0.099	0.75338	0.753373	0.879 (0.394–1.962)
7	GTGCGCC	0.00 (0.000)	0.56 (0.004)	–	–	–	–
8	GTGCGTA	8.01 (0.085)	5.76 (0.043)	1.462	0.226657	0.226579	1.960 (0.647–5.936)
9	GTGTATC	0.00 (0.000)	2.18 (0.016)	–	–	–	–
10	GTGTGCA	4.00 (0.043)	4.06 (0.030)	0.169	0.681183	0.681184	1.342 (0.328–5.489)
11	GTGTGCC	0.00 (0.000)	3.58 (0.027)	–	–	–	–
12	GTGTGTA	24.15 (0.257)	47.04 (0.351)	3.31	0.068922	0.068871	0.578 (0.319–1.046)
13	GTGTGTC	32.51 (0.346)	28.81 (0.215)	3.864	0.049393	0.049354	1.822 (0.998–3.324)
14	TCGTGTA	1.65 (0.018)	1.00 (0.007)	–	–	–	–
15	TTGCGTA	0.00 (0.000)	1.00 (0.007)	–	–	–	–
16	TTGTGTA	2.33 (0.025)	0.02 (0.000)	–	–	–	–
17	TTGTGTC	0.00 (0.000)	4.99 (0.037)	3.79	0.051622	0.051581	–
18	TCGCGTA	2.99 (0.032)	0.00 (0.000)	4.091	0.04317	0.043135	–
19	TCGTGTC	0.03 (0.000)	0.00 (0.000)	–	–	–	–
20	TTACGTA	1.00 (0.011)	0.00 (0.000)	–	–	–	–

CI = confidence interval, POR = prevalence odds ratio.

their malaria patients than the homozygous wild-type genotype (HH).

In our cohort of residents of the Sino–Burmese border region, we observed no significant differences between the frequencies of the H and L alleles of the T520C SNP in our malaria patients and those in our healthy controls. However, the frequency of the L allele of the G3093T SNP was significantly higher in the patients with falciparum malaria than that of the patients with vivax malaria, whereas the frequency of the H allele of G3093T was significantly higher in vivax malaria patients than that in the falciparum malaria patients. These data suggest that low CR1 expression might be a risk factor for falciparum malaria. In addition, the 3093G/3093G genotype might be associated with vivax malaria. Although *P. vivax* does not utilize CR1 to invade erythrocytes, high-level CR1 expression might reduce the risk of severe anemia in vivax malaria patients,[42] which could improve survival and create selective evolutionary pressure that maintains the H allele in populations in malaria endemic regions in Asia where *P. vivax* is highly prevalent.

In our direct genotyping analysis, we examined SNPs in a number of genes involved in the inflammatory response. The logistic regression analysis showed that polymorphisms of *IL-1A*, *IL-4*, *IL-4R*, *TNF*, *NOS*, *CD40LG*, and *LUC7L* were associated with the prevalence of malaria in our cohort ($P < 0.05$ for all). Significant linkage disequilibrium ($P < 0.05$) was observed between *IL-1A* (rs17561) and *TNF* (rs1800750) and between *TNF* (rs1800750) and *IL-4R* (rs1805015). Based on these data, we constructed a GTGTGTC haplotype model consisting of *IL-1A* (rs17561), *IL-4* (rs2243250), *TNF* (rs1800750), *IL-4R* (rs1805015), *NOS* (rs8078340), *CD40LG* (rs1126535), and *LUC7L* (rs1211375) that significantly correlated with the prevalence of malaria (POR: 1.822, 95% CI: 0.998–3.324, $P < 0.05$). The *IL-1A* gene encodes proinflammatory cytokines, including IL-1A and IL-1D, and is considered a key regulatory gene of the inflammatory response,[25] which supports the importance of *IL-1A* in the response to immune challenge by *P. falciparum* and *P. vivax*. Our findings are also supported to some extent by those of Eid et al,[43] who examined SNPs of candidate malaria susceptibility genes in an East African cohort, and found significant differences between the frequencies of SNPs of *IL-4R1* and *IL-4* in malaria patients and those in control subjects.

Although Eid et al[43] reported no significant genetic variation among East Africans in exon 29 of *CR1*, which encodes Knops blood group antigens, Li et al[44] reported significant variation in the A4870G and A4646G SNPs of exon 29 between 5 Chinese ethnic groups. Our Chinese cohort consisted primarily of Han Chinese subjects, but also included a small number of people from the Dai ($n = 3$), Jingpo ($n = 2$), Lisu ($n = 1$), and Deang ($n = 1$) ethnic groups. We observed no variation in sequences encoding KnopS blood group antigens. In our direct genotyping analysis, we also investigated the frequencies of 29 SNPs of *G6PD* in male malaria patients, and no exonic missense mutations were found. Because G6PD deficiency is a relatively common genetic disorder, this absence of G6PD dysfunction in malaria patients suggests that G6PD deficiency confers resistance against malaria. However, our logistic regression analysis did not show that these loci correlated with the prevalence of malaria. Nonetheless, the H allele of T520C and L allele of G3093T were predominant in the G6PD-deficient subjects, both of which were associated with malaria. Therefore, it is possible that, although G6PD deficiency does not protect against malaria, it may influence the role of CR1 in malaria susceptibility.

Our findings are subject to certain limitations. Malaria is endemic year round in rural areas of Yunnan Province,[45] and all of our control subjects reported being long-time residents of the Sino–Burmese border region. Although our statistical analysis showed that certain SNPs were associated with the prevalence of malaria, it is possible that other environmental factors or

demographic factors have also exerted an influence over the frequency of these SNPs in the general population along the Sino–Burmese border. In addition, the extent to which socio-economic factors influence the transmission of malaria in this region is largely unclear. Therefore, the enrollment of only university students as control subjects for our study might also have influenced our findings as a result selection bias. Furthermore, our findings might also have been influenced by the relatively small size our study sample and the cross-sectional design of our study. Future longitudinal studies of associations between the 3093G/3093G and 520T/520T genotypes and the GTGTGTC haplotype and the incidence of malaria in larger samples are warranted to confirm our findings. Future biochemical studies are also warranted to determine the collective effects of these polymorphisms on the pathophysiology of malaria.

In conclusion, the 3093G/3093G and 520T/520T genotypes are the predominant genetic variants of *CR1* among Chinese residents living near the Sino–Burmese border, and significantly correlate with the prevalence of malaria in this region. Although G6PD deficiency does not protect against malaria, it may diminish the association between malaria and the *CR1* polymorphisms in this population. Polymorphisms of *IL-1A*, *IL-4*, *IL-4R*, *TNF*, *NOS*, *CD40LG*, and *LUC7L* are associated with the prevalence of malaria, and the GTGTGTC haplotype significantly correlates with the prevalence of malaria in this region.

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