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Association of *CD40L* gene polymorphism with severe *Plasmodium falciparum* malaria in Indian population

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ABSTRACT

Background & objectives: Many host genetic factors are associated with the disease severity and fatal outcome of falciparum malaria. *CD40L* gene has been found to be one of the most important factors associated with malaria in African countries. This study was aimed to investigate the possible association of *CD40L* gene polymorphism in severe falciparum malaria in Indian adults.

Methods: One hundred fifteen adult cases with severe falciparum malaria were included in the study. Two single-nucleotide polymorphisms (SNPs) of *CD40L* gene, CD40L–726(C/T) and CD40L+220(C/T) were investigated, and the possible association with different clinical sub-phenotypes of severe falciparum malaria were analyzed.

Results: Statistically no significant difference was observed in the incidence of CD40L−726C between the patients and control group. The incidence of CD40L+220C allele was found to be significantly higher (OR, 2.25; $p = 0.03$) in male patients compared to controls but no significant difference was observed in females. Haplotype data showed the susceptibility of –726T/+220C haplotype to severe malaria whereas –726C/+220T was associated with protection against severe malaria. CD40L+220C allele was associated with severe malarial anaemia in males ($\chi^2 = 6.60$; $p = 0.01$).

Interpretation & conclusion: CD40L gene polymorphism was found to be associated with severe falciparum malaria in Indian population especially in severe malarial anaemia. CD40L may be considered as a factor of immunity in understanding the pathophysiology of falciparum malaria.

Key words CD40L; *Plasmodium falciparum;* severe malaria; severe malarial anaemia

INTRODUCTION

Malaria is one of the major public health problem in India which accounted for 0.85 million cases during 2014, including 0.54 million cases of falciparum infection and 316 deaths, as per the National Vector Borne Disease Control Programme (NVBDCP), India[1]. The severity of the falciparum malaria ranges from asymptomatic parasitaemia to the severe form. The spectrum of severe falciparum malaria is also changing across different population groups and age of the patients with varied immunity level[2]. Although, the episodes of fever occur repeatedly during *P. falciparum* infection, only 1% of them develop severe form leading to death[3]. It has been hypothesized that, these differences in the clinical severity of the patients is due to the selective pressures imposed by different host genetic factors. Amongst them the most studied ones were on the influence of HbS, HbC, alpha thalassaemia and G6PD deficiency on the falciparum malaria. Other than these, human innate and adaptive immune response plays an important role against this deadly disease involving various cells and antibodies[4].

Recently, four studies from Africa have emphasized on the association of *CD40L* gene polymorphism and malaria[5-8]. CD40L (CD154) is a ligand gene located on the long arm of human X-chromosome at position q26.3–q27.1, containing five exons spanning about 12 kilo base pairs (*http://www.ncbi.nlm.nih.gov/gene/959*). This gene encodes a 39 kDa cell surface type II membrane glycoprotein, a member of the tumor necrosis factor (TNF) super family. CD40L is expressed by activated $CD4^+$ T-cells and is involved in various immune responses such as B-cell proliferation, antigen presenting cell activation, Ig class switching, formation of germinal centers and prevention of apoptosis of B-cells[5]. Two polymorphisms of *CD40L* gene [CD40L-726 (C/T) (rs3092945) and CD40L+220 (C/T) (rs 1126535)] have been shown to be associated with malaria in African countries[5-8]. Though, there are many studies on the impact of various genetic polymorphism and malaria in India, none of them focused on CD40L. This study aimed to estimate the

alleles frequency of CD40L−726 (C/T) and CD40L+220 (C/T), and the possible association of haplotype with disease severity of falciparum malaria in Indian population.

MATERIAL & METHODS

This case controlled study was undertaken at Sickle Cell Clinic and Molecular Biology Laboratory and the Department of Medicine, Veer Surendra Sai Institute of Medical Sciences and Research (formerly known as Veer Surendra Sai Medical College), Burla, Odisha, India. This tertiary health care centre caters to a population of 12 million residing in western districts of Odisha state and few eastern districts of the state of Chhattisgarh. The state of Odisha contributes to 27% of malaria positive cases, 50% of falciparum cases and 16% of deaths due to malaria in the country as per NVBDCP[9]. In the study area, *P. falciparum* alone accounted for 92% of malarial infections compared to 50% of *P. falciparum* infection in India[10].

Study subjects

Patients aged ≥15 yr of both sexes with suspected severe malarial infections, hospitalized in the Department of Medicine, Veer Surendra Sai Institute of Medical Sciences and Research, from July to December 2014 were screened, and 115 confirmed cases of falciparum malaria were included in the study. During admission in the hospital, 5 ml of venous blood was collected. Two ml of venous blood was used for serum parameter analysis and 3 ml was used for complete blood count and molecular analysis. Age and sex matched 107 healthy controls were also included for the study. Written informed consent was obtained from all the patients and controls. In unconscious patients, the informed consent was obtained either from their father or other family members. This study was approved by the institutional ethical committee (IEC) of Veer Surendra Sai Medical College, Burla, Odisha.

Severity of falciparum malaria was defined as per WHO criteria 2010[11]. The most severe complication, cerebral malaria (CM) was defined as condition of the patients with any of the features like altered sensorium, convulsion or GCS (Glasgow Coma Score) of ≤10. Other complications like severe malarial anaemia (SMA) (haemoglobin <5 g/dl), acute renal failure (ARF) (serum creatinine >3 mg/dl), jaundice (serum bilirubin >3 mg/dl), hepatic dysfunction [alanine transaminase (ALT)/ aspartate transaminase (AST) >3 times of normal range, *i.e*. 120 U/L] and respiratory distress were also considered.

Exclusion criteria

The following cases were excluded from the study: (a) subjects coinfected with other *Plasmodium* species; (b) children <15 yr of age; (c) subjects having chronic disease like tuberculosis, cirrhosis of liver and autoimmune diseases like systemic lupus erythematosus and rheumatoid arthritis; (d) patients with dengue fever; (e) pregnant women; and (f) subjects who refused consent for the study.

Laboratory investigations

Plasmodium falciparum infection was confirmed by single step polymerase chain reaction as described earlier by Patsoula *et al*[12]. A complete blood count was performed on an automated haematology analyzer (Sysmex pocH-100i; Sysmex Corporation, Kobe, Japan). The biochemical parameters such as serum bilirubin, creatinine, urea, alanine transaminase, aspartate transaminase, sodium, potassium and glucose were analysed in a semi auto-analyzer (Erba Chem 7; Erba Diagnostics Mannheim GmbH, Mannheim, Germany) as per the manufacturer's instructions.

Genotyping for CD40L polymorphs

Severe malaria and controls samples were typed for both CD40L−726 (C/T) and CD40L+220 (C/T) polymorphisms using allele specific PCR amplification as described by Sabeti *et al*[5]. Briefly, for CD40L−726 (C/T), allele-specific primers and one reverse consensus complementary primer were used for PCR amplification of 276 bp. For CD40L+220 (C/T) polymorphisms, allele-specific primers and one forward consensus complementary primer were used for PCR amplification of 466 bp. The primer sequences for both the polymorphisms are shown in Table 1. A total of 25 µl of PCR reaction mixture contained 2.5 µl of 10X PCR buffer, 2 µl of $MgCl_2$, 1 µl of 40 mM dNTP, 15 p-mol of each forward and reverse primer, 0.3 U Taq DNA polymerase (QIAGEN India Pvt. Ltd.) and 200 ng of whole genomic DNA. Cycling conditions were 96°C for 1 min, 5 cycles of 96°C for 35 sec, 70°C for 45 sec, and 72°C for 35 sec, then 21 cycles of 96°C for 25 sec, 65°C for 50 sec, and 72°C for 40 sec, then 6 cycles of 96°C for 35 sec and 55°C for 1 min, followed by 72°C for 90 sec. The respective PCR products were electrophoresed in 4% agarose gel and documented by staining with ethidium bromide.

Statistical analysis

Pearson's χ^2-tests were used for initial examination of associations between exposures and each outcome. Unconditional logistic regression was used to estimate

Table 1. Description of primers for amplification of CD40L−726 (C/T) and CD40L+220 (C/T) polymorphism

Name of the primers	5'–3'	Primer specification	Amplification product
	CD40L−726(C/T) polymorphism		
CD40L−726T	CTGAACTGTTACATCAGCAT	Sequence specific for 726T	276 bp
CD40L−726C	CTGAACTGTTACATCAGCAC	Sequence specific for 726C	
CD40L−726R	CTAAACTCAATGAAAGCCTG	Reverse consensus primer	
	CD40L+220(C/T) polymorphism		
CD40L+220T	GTTCATCTTACCTTGTCCAA	Sequence specific for 220T	466 bp
CD40L+220C	GTTCATCTTACCTTGTCCAG	Sequence specific for 220C	
CD40L+220F	GACATTTCAAGGCAAGAATG	Forward consensus primer	

the odds ratios and the associated 95% confidence intervals (CIs). The gene frequency and genotypes were compared between patients and control group by calculating odd ratio with 95% CI. The haplotype frequency in male patients (where both the SNPs were amplified) was compared between the patients and control group with estimation of odd ratio and 95% CI. Hardy-Weinberg equilibrium (HWE) was assessed using a χ^2 statistical test. HWE was not calculated for males because they are hemizygous for the alleles studied. Differences in values were considered significant if *p*-values were less than 0.05. The data were analyzed using the statistical program STATA ver.13.0.

RESULTS

In this case-controlled study, 115 adults with severe falciparum malaria and 107 healthy controls were included. The mean age of the patients and controls were 36.9 ± 13.9 yr (range, 15–64) and 35.1 ± 9.9 yr (range, 17–58), respectively. The male to female ratios were 1.88:1 in patients and 1.89:1 in controls. The CD40L + 220 (C/T) was genotyped successfully in 102 patients and 96 controls. Similarly, CD40L−726 (C/T) was genotyped successfully in 93 patients and 92 controls. The detailed genotypes of both SNPs have been illustrated in Table 2. No significant difference was observed in the incidence of variant allele 'C' for CD40L–726 (C/T) in males or females. The incidence of 'C' allele for CD40L+220 (C/T) was found to be significantly higher [Odd ratio = 2.25; 95% CI (1.09–4.68); $p = 0.03$] in male patients with severe malaria compared to control but no difference was seen in females. Similarly, the 'T' allele for CD40L+220 (C/T) was found to be significantly higher in male controls compared to patients [Odd ratio, 0.44; 95% CI (0.21–0.92); $p = 0.03$]. In females, the alleles were in accordance with HWE, both in patients and control group for both SNPs. In males, both CD40L–726(T/C) and CD40L+220 (T/C) alleles were amplified in 55 patients with severe malaria and 49 healthy controls respectively. The comparison in the frequency of haplotype data in male patients and control group showed that the –726T/+220C haplotype was significantly associated with severe malaria, where-

Table 2. Genotype and allele frequencies of the *CD40L* gene polymorphisms in severe *P. falciparum* malaria and non-malarial individuals (Controls)

	CD40L+220 (C/T) (rs1126535)					CD40L−726 (C/T) (rs3092945)				
	Severe malaria (n = 102)	Control (n = 96)	OR	95% CI	*p*-value	Severe malaria (n = 93)	Control (n = 92)	OR	95% CI	*p*-value
Age (yr)	37.6 ± 13.6	34.8 ± 9.6			0.57	36.6 ± 13.9	35.1 ± 9.6			0.81
Male	69 (67.6%)	64 (66.7%)				62 (66.7%)	61 (66.3%)			
C	0.45 (31/69)	0.26 (17/64)	2.25	1.09–4.68	0.03	0.05 (3/62)	0.1 (6/61)	0.44	0.11–1.95	0.32
T	0.55 (38/69)	0.73 (47/64)	0.44	0.21–0.92	0.03	0.95 (59/62)	0.9 (55/61)	2.14	0.51–9	0.32
Female	33 (32.4%)	32 (33.3%)				31 (33.3%)	31 (33.7%)			
C/C	0.21 (7/33)	0.13 (4/32)	1.88	0.49–7.19	0.51	0.03 (1/31)	0.06 (2/31)	0.48	0.04–5.62	1
C/T	0.33 (11/33)	0.25 (8/32)	1.5	0.50–4.41	0.58	0.2 (6/31)	0.23 (7/31)	0.79	0.24–2.69	0.76
T/T	0.46 (15/33)	0.63 (20/32)	0.5	0.18–1.34	0.18	0.77 (24/31)	0.71 (22/31)	0.71	0.22–2.24	0.77
C	0.38 (25/66)	0.25 (16/64)	1.83	0.86–3.88	0.13	0.13 (8/62)	0.19 (12/62)	0.61	0.23–1.63	0.46
T	0.62 (41/66)	0.75 (48/64)	0.54	0.25–1.16	0.13	0.87 (54/62)	0.81 (50/62)	1.62	0.61–4.29	0.46
HW	0.0937	0.0595				0.4397	0.2075			

Genotypes data of the *CD40L* gene in males are not presented because men are hemizygous for the gene; OR—Odd ratio; CI—Confidence interval; *HW*—Hardy-Weinberg equilibrium.

as –726C/+220T was higher in controls group. The frequency of four haplotypes in male patients is illustrated in Table 3.

Comparison was also made between the incidence of different clinical severity with presence of 'C' allele in males and the presence of either CC or CT genotypes in females for both the SNPs. The association of different clinical severity with CD40L–726C and CD40L+220C alleles has been illustrated in Tables 4 and 5 respectively. There were no significant associations of 'C' allele in males or 'CC/CT' genotypes in females on the incidence of various clinical manifestations except severe malarial anaemia in males for CD40L+220C allele ($\chi^2 = 6.60$; $p = 0.01$). All the five male patients with severe malaria with anaemia had CD40L+220C allele.

Table 3. CD40L−726/+220 haplotype frequencies in male patients and controls

Haplotype	Haplotype frequency (Individuals with specified haplotype)		OR (95% CI)	
	Severe malaria patients (n = 55)	Control (n = 49)		
–726T/+220T	0.60 (33)	0.69 (34)	1.51	(0.67–3.40)
–726T/+220C	0.36 (20)	0.06 (3)	0.11	(0.03–0.41)*
–726C/+220T	0.02 (1)	0.20 (10)	13.8	(1.70–112.7)*
–726C/+220C	0.02 (1)	0.04 (2)	2.29	(0.20–26.2)

*Indicates a significant Odds ratio (OR) with $p < 0.05$; –726T/+220T represent the wild type haplotype; CI—Confidence interval

DISCUSSION

For decades, many national and international agencies have focused on malaria control strategies in the country, but till date this parasitic disease remains a major public health burden and is responsible for significant fatal outcome in India[2]. Various scientific communities have reported on different genome level association on the phenotypic expression of falciparum malaria[3, 8]. In India, the population is very diverse and it becomes more important when the studies are undertaken in hospitalized patients,

Table 4. Association of *CD40L−726 (C/T) (rs3092945)* gene polymorphism on different clinical spectrum of *P. falciparum* malaria

Clinical parameters	Male (n = 62)				Female (n = 31)			
	Incidence number	Hemizygotes (C) number	χ^2	*p*–value	Incidence number	Heterozygotes (CT) and homozygotes (CC) number	χ^2	*p*–value
ARF	14 (22.6)	1 (7.1)	0.20	0.648	10 (32.3)	3 (30)	0.46	0.498
CM	25 (40.3)	0 (0)	2.13	0.144	10 (32.3)	4 (40)	2.32	0.127
HD	20 (32.3)	1 (5)	0.001	0.967	8 (25.8)	2 (25)	0.62	0.429
Jaundice	7 (11.3)	1 (14.3)	1.52	0.216	6 (19.4)	2 (33)	0.49	0.483
RD	4 (6.5)	0 (0)	0.21	0.641	6 (19.4)	2 (33)	0.49	0.483
SMA	3 (4.8)	0 (0)	0.16	0.689	5 (16.1)	1 (20)	0.02	0.880

ARF—Acute renal failure; CM—Cerebral malaria; HD—Hepatic dysfunction; RD—Respiratory distress; SMA—Severe malaria anaemia; Figures in parentheses indicate percentages.

Table 5. Association of CD40L+220 (C/T) (rs1126535) gene polymorphism on different clinical spectrum of *P. falciparum* malaria

Clinical parameters	Male (n = 69)				Female (n = 33)			
	Incidence number	Hemizygotes (C) number	χ^2	*p*–value	Incidence number	Heterozygotes (CT) and homozygotes (CC) number	χ^2	*p*–value
ARF	18 (26)	9 (50)	0.25	0.615	11 (33.3)	7 (63.6)	0.55	0.458
CM	28 (40.6)	10 (35.7)	1.61	0.20	12 (36.4)	7 (58.3)	0.10	0.741
HD	22 (31.9)	11 (50)	0.33	0.562	6 (18.2)	3 (50)	0.06	0.80
Jaundice	7 (10.1)	4 (57.1)	0.47	0.493	6 (18.2)	4 (66.7)	0.43	0.510
RD	7 (10.1)	2 (28.6)	0.84	0.359	5 (15.2)	4 (80)	1.54	0.225
SMA	5 (7.2)	5 (10)	6.60	0.010	5 (15.2)	3 (60)	0.07	0.790

ARF—Acute renal failure; CM—Cerebral malaria; HD—Hepatic dysfunction; RD—Respiratory distress; SMA—Severe malaria anaemia; Figures in parentheses indicate percentages.

because subjects of severe malaria admitted in a hospital are those with the lowest level of protection which tends to enrich the sample for strong genetic effect[3].

This study has investigated the role of two SNPs of *CD40L* gene (CD40L–726 (C/T) and CD40L+220 (C/T), in severe falciparum malaria in hospitalized patients and compared the results with the age and gender matched control. The incidence of CD40L–726C allele both in female patients (both in heterozygous and homozygous state) and male patients (hemizygous state) were comparable to controls; however, there was significant difference (p <0.03) in the incidence of CD40L+220C allele in male patients (0.45, 31/69) compared to controls (0.26, 17/64). This observation was different from the study undertaken in Gambian population[5]. They reported the CD40L–726C allele in male hemizygotes to be higher in controls compared to patients with severe malaria. In another study involving Malian population, the incidence of CD40L–726C allele was low in female patients compared to controls, whereas the incidence of CD40L+220C allele was higher in female patients compared to controls. They did not find any difference in the incidence of above said two alleles in male patients compared to controls[6]. These differences might be due to the inclusion of diverse ethnic groups in various studies. The diverse allele distribution in different study areas was again supported by a recent study undertaken by Malaria Genomic Epidemiology Network[8]. The comparison of haplotype data in male patients and control in this study revealed the susceptibility of –726T/+220C haplotype to severe falciparum malaria, whereas –726C/+220T haplotype was associated with protection against severe malaria.

In the Gambian males, CD40L–726C allele was associated with protection against both cerebral malaria and severe malarial anaemia[5]. In a multicentre study of Malaria Genomic Epidemiology Network, CD40L–726C was associated with reduced risk of severe malaria when the data were aggregated for all the study sites. However, when the sites were analyzed individually, homozygotes for the derived allele showed significantly reduced risk of severe malaria in the Gambia (Odd ratio = 0.54; $p = 2.3 \times 10^{-22}$) but significantly increased risk in Kenya (OR = 1.42; $p = 7.8 \times 10^{-6}$)[8]. CD40L–726C was associated with an increased risk of respiratory distress whereas CD40L+220C was associated with decreased risk of respiratory distress in females in Tanzanian population[7]. In this study, CD40L+220C allele was associated with severe malarial anaemia in male patients ($\chi^2 = 6.60$; $p = 0.01$). This difference might be due to two reasons; first, inclusion of varied ethnic groups in different studies[8]; second, clearance of antimalarial drug resistant *P. falciparum* parasite as suggested by an African study[13]. This African study showed the association of one SNPs of CD40L (CD40L+220C) in the significant clearance of resistant *P. falciparum* species and hence, influences the pathophysiology of the disease. For comparison purpose, the association of two SNPs of *CD40L* gene with severe malaria in different studies has been illustrated in Table 6.

CONCLUSION

In conclusion, this is the first study in India reflecting the significant association of CD40L polymorphism in severe falciparum malaria. *CD40L* gene polymorphism may be considered as a prognostic factor with regard to the severity of falciparum malaria for those who are interested in the study related to the influence of different host genetic polymorphisms on the severity of falciparum

Table 6. Comparison of five different studies describing the possible association of two SNPs [(CD40L–726 (C/T) and CD40L+220 (C/T)] of *CD40L* genes with severe *P. falciparum* malaria

Reference	Place of the study	Age of the study subjects	Association with severe malaria
Sabeti *et al*[5]	Gambia	Both paediatric and adults	CD40L–726C protects against severe malaria, cerebral and severe malarial anaemia in male hemizygous.
Toure *et al*[6]	Mali	Paediatric	CD40L–726C protects against severe malaria, whereas CD40L+220C increases susceptibility to severe malaria in females.
Manjurano *et al*[7]	Tanzania	Paediatric	CD40L–726C increases the risk and CD40L+220C decrease the risk of respiratory distress in females.
Rockett *et al*[8]	12 countries in Malaria Genomic Epidemiology Network	Both paediatric and adults	CD40L–726C protects against severe malaria; Homozygous for CD40L–726C showed significantly reduced risk of severe malaria in the Gambian females and significantly increased the risk of severe malaria in Kenyan females.
Present study	India	Adults	CD40L+220C increases susceptibility to severe malaria specially severe malarial anaemia in male hemizygous; CD40L–726C has no influence on severity of malaria.

malaria. This study again helped for understanding the role of CD40L as an important factor of immunity in the malarial pathophysiology. Inclusion of more number of cases in a longitudinal study can provide a more conclusive result on the association of this genetic marker with different degree of severity in falciparum malaria in the Indian subcontinent.

Conflict of interest

The authors declare no conflict of interest.

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