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A Cd36 polymorphism associated with eight-times increased susceptibility to cerebral malaria in Central Sudan

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Malaria is one of the biggest known health threats in Africa. Erythrocytes infected with falciparum malaria adhere to a variety of host receptors, including CD36. Cerebral malaria (CM) is a major life-threatening complication of *Plasmodium falciparum* infection. The human protein CD36 is a major receptor for *P. falciparum*-infected erythrocytes and contributes to the pathology of *P. falciparum* malaria. The aim of the present study was to determine the role of the adhesion molecule CD36 in children with CM at Central Sudan. A case-control study included 70 children with cerebral malaria (CM) and 84 controls were enrolled in this study. The method was a mutational analysis for the polymorphism in the CD36-188 T > G using polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) where the distribution of CD36 to 188 T > G genotypes differed significantly between CM patients and controls and children carrying the mutant G allele were associated with eight-times increased relative risk for susceptibility to cerebral malaria (*P-value* = 0.005; odds ratio = 7.962; 95% CI = 1.571 to 29.903).

Key words: *Plasmodium falciparum*, cerebral malaria, erythrocytes, Central Sudan.

INTRODUCTION

Malaria remains a major public health threat to more than 600 million Africans. In sub-Saharan Africa, the disease distribution is closely linked with seasonal patterns of the climate and local environment (Grover-Kopec et al., 2006). According to World Health Organization (WHO) classification of malaria endemic countries, Sudan is categorized in group 4. Sudan represents more than 50% of the total estimated malaria cases in the group (WHO, 2002). In two camps in Khartoum state, Sudan, most of the recorded malaria cases were among children. Risk of malaria attack was significantly associated with tribe, language, education, water supply and food expenditure (Saeed and Ahmed, 2003).

Falciparum malaria is characterized by cytoadherence of host erythrocytes containing mature asexual-stage parasites and the sequestration of these forms in tissue microvasculature (Montgomery et al., 2006). Erythrocytes infected with falciparum malaria adhere to a variety of host receptors, including CD36, which are widely expressed on endothelium, platelets and leucocytes (Combes et al., 2006). The human protein CD36 is a major receptor for *Plasmodium falciparum*-infected

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erythrocytes and contributes to the pathology of *P. falciparum* malaria (Omi et al., 2003). Cerebral malaria (CM) is a major life-threatening complication of *P. falciparum* infection. CM in humans is defined as the presence of unarousable coma with exclusion of other encephalopathies and confirmation of *P. falciparum* infection (Hunt and Grau, 2003). CM is estimated to affect more than 785,000 children who are younger than 9 years in sub-Saharan Africa every year, with 15 to 30% case fatality rate (Murphy and Breman, 2001).

In Gedarif Hospital, Eastern Sudan, the reported CM cases of during three malaria seasons showed that the number of CM patients has increased 6 folds in the year 2003 compared to the year 2001 (Giha et al., 2008). The aim of the present study was to determine the role of the adhesion molecule CD36 in children with CM at Central Sudan, to evaluate the role of CD36 in susceptibility to CM cases and to determine the genetic variations in the CD36 polymorphisms (-188) in CM cases.

MATERIALS AND METHODS

Study area

The study was conducted in three cities: Wad Medani; at the Wad Medani Paediatrics Teaching Hospital, Sinnar; at the Sinnar Teaching Hospital, and Singa; at the Singa Hospital. These cities lie along the bank of the Blue Nile River and are characterized by a prolonged raining period that provides a suitable environment for the breeding of the malaria vector, *Anopheles* mosquitoes. These areas are endemic for *P. falciparum* malaria especially during the autumn season.

Selection of cases and controls

Children admitted to the three hospitals and suspected for cerebral malaria were registered. The mean age of the study subjects was (6.8 ± 3.2) years; the minimum age was 8 months old and the maximum age was 14 years old. Seventy cases were confirmed as having CM by a Pediatrician, according to the criteria of CM (Molyneux, 1990). Children with blood films negative for asexual stages or those with other diseases that may contribute to the coma were excluded from the study (Molyneux, 1990). Eighty-four children were selected as a control group from pupils in schools from the three cities and from other children admitted to the three hospitals with diseases other than CM, and were matched in age, sex and ethnic group.

Collection of blood samples

Samples of 3 ml venous blood were collected in heparinized tubes from the patients and controls. The blood samples were kept frozen at -70°C for later DNA extraction and genetic analyses. DNA was extracted using the salting-out method and was purified by phenol-chloroform method (Sambrook et al., 1989). The quantity of the obtained DNA was measured using an ultra violet (UV) spectrophotometer.

Mutation analysis

PCR-restriction fragment length polymorphism (PCR-RFLP) method was used in the present study. Genotypes for the mutation CD36 – 188 T > G was identified from the restriction enzyme (*Nde*1) digested fragments of PCR amplified products of the exon 10 of CD36 gene with the primer pairs: 5' CTATGCTGTATTTGAATCCGACG 3' and 5' ATGGACTGTGCTACTGAGGTTAT 3', respectively.

PCR reactions were performed in a reaction volume of 30 µl containing 3 µl from 10 × PCR buffer, 3 µl of 200 µM dNTPs, 1.5 µl $MgCl_2$, 200 ng genomic DNA, 2 µl of 10 picomoles from each primer, 0.2 µl Taq DNA polymerase and completed to the final volume with distilled water. The denaturation temperature was 90°C for 1 min, annealing temperature 55°C for 2 min and prolongation temperature 72°C for 1 min. This reaction was run for 40 cycles. In 2% agarose gel, the PCR product was stained in 1 µg/ml Ethidium bromide for 10 to 15 min and visualized under Ultra Violet (UV) light in a Gel Documentation System (GDS) to check for the presence or absence of the DNA bands. The mutation of thymine for guanine at position (-188) appolishes a restriction site for *Nde*1. Digestion with this enzyme was used for typing this polymorphism. In a total volume of 15 µl, 2 µl PCR product was digested overnight at 65°C with 2.5 U *Nde*1.1.5 µl 10× NE Buffer 1 [100 mM Bis Tris Propane-HCl, 100 mM magnesium chloride and 10 mM dithiothreitol (pH 7.0)] and deionized water. Digestion reactions were loaded on 8% non-denaturaning polyacrylamide gel (30% Protogel (Acrylamide/Bis-acrylamide), 37.5:1) (FMC BioProducts) and electrophoresed at 140 V for one hour. The gel was stained in 1 µg/ml ethidium bromide solution for 10 to 15 min and visualized under UV light in a Gel Documentation System (GDS).

Statistical analysis

Statistical analysis was done using statistical package for the social sciences (SPSS) programme and Chi-square test.

RESULTS

70 cerebral malaria (CM) cases and 84 controls were included in the study. 39 (55.7%) were males and 31 (44.3%) were females. The highest incidence of the disease was among the age group 4 < 6 years. There were 11 different tribal stocks included in the study subjects. Juhaina Arab group had the higher incidence of CM among the study subjects (22.9%). The mutant allele remains uncut with *Nde*1, it is a characteristic of the homozygous mutant type (-188 GG) appears as single 213 base pair band. Complete cleavage into the 148 and 65 bp fragments is a characteristic of the homozygous wild type (-188 TT) which appears as two bands. Incomplete cleavage into 213, 148 and 65 bp is a characteristic of the heterozygous (–188 TG) and appears as three bands. In this study the allele frequency for CD36-188 T > G in CM cases was 88% wild type T allele and 12% mutant type G allele. The allele frequency in controls was 99% T allele and 1% G allele. The distribution of CD36-188 T > G genotypes differs significantly between CM patients and controls and those carrying the mutant G allele were associated with eight times increased relative risk for susceptibility to CM (P-value = 0.003; odds ratio = 7.636; 95% CI = 1.048 to 13.630) (Table 1).

Table 1. The frequency of CD36 -188 T>G alleles in CM patients and controls.

Study subjects	Genotype	
	T T	GG+TG
CM Patients	59 (84.3%)	11 (15.7%)
Controls	82 (97.6%)	2 (2.4%)
(P-value = 0.003; odds ratio = 7.636; 95% CI = 1.048 – 13.630)		

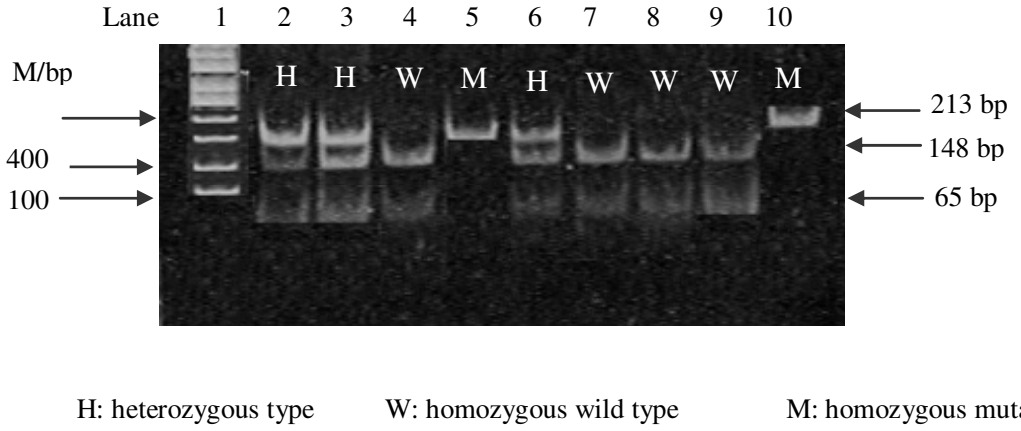

Figure 1. Pattern of the CD36-188 T>G alleles in the CM cases using PCR-RFLP. Lane 1: 100 bp DNA marker, lanes 2, 3 and 6 are heterozygous type (TG) for CD36 -188, lane 4, 7, 8 and 9 are homozygous wild type (TT), lane 5 and 10 are homozygous mutant type (GG).

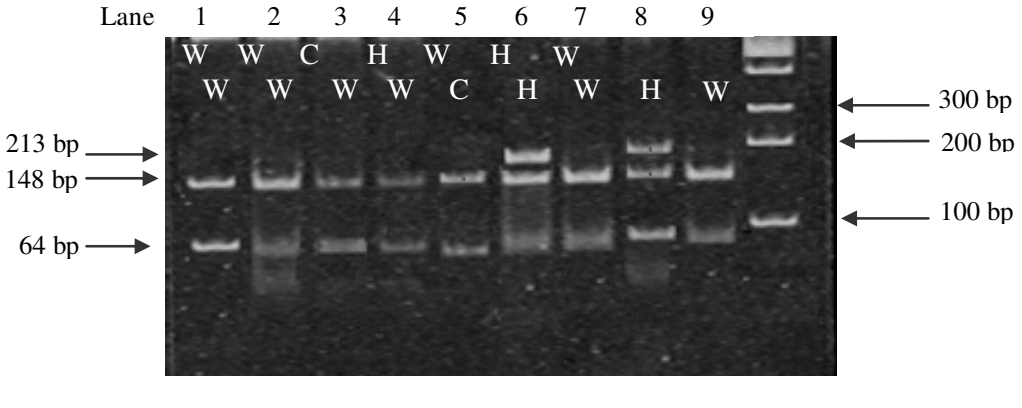

Figure 2. Pattern of CD36 –188 T>G alleles in the controls using PCR-RFLP. Lanes 1, 2, 3, 4, 5, 7 and 9 were homozygous wild type (TT), lane 6 and 8 were heterozygous mutant for CD36-188.

Genetic analysis

CD36-188 T > G genotypes in CM and controls

CD36 -188 T > G polymorphism was screened using the PCR-RFLP method both for CM cases and controls (Figures 1 and 2). The gene frequency for CD36-188 T > G in CM cases was 84.3% homozygous wild type (TT), 7.1% homozygous mutant type (GG) and 8.6% heterozygous mutant type (TG). The gene frequency in controls was 97.6% homozygous wild type (TT), 2.4% homozygous mutant type (GG) and 0.0% heterozygous mutant type (TG). The distribution of CD36-188 T > G genotypes differs significantly between CM patients and

Table 2. The allele frequency of CD36 -188T>G in CM patients and controls.

CD36 -188T>G	Genotypes			Alleles	
Group	T T	G G	T G	T	G
CM patients	59 (84.3%)	5 (7.1%)	6 (8.6%)	0.88	0.12
Control	82 (97.6%)	2 (2.4%)	0 (0.0%)	0.99	0.01

(χ^2 = 9.846, df = 2, *P-value* = 0.007).

controls (*P* = 0.007) (Table 2).

DISCUSSION

Clustering of CM in certain tribes such as Juhaina Arab stocks may be due to more representation in the population in the study area, and some families and tribes may be genetically susceptible to CM. This result shows that those carrying the mutant G allele were more likely to produce the adhesion molecule CD36 in the endothelial microvaculature in response to malaria infection. The production of the adhesion molecule CD36 leads to massive sequestration of the infected red blood corpuscles (RBCs) leading to the known complications of CM. The association of CD36-188 mutant G allele; with increased susceptibility to CM; showed that there is clustering of certain alleles, not only in Sudanese populations, but also in African populations as shown by Pain et al. (2001) in a study in Kilifi, Kenya. This study was consistent with a study done by Aitman et al. (2000) in children from East and West Africa, that showed a significant association of CD36 mutations with susceptibility to severe malaria in general, and to CM in particular. *In vitro* studies indicate that sequestration of parasitized red blood corpuscles (PRBCs) in the microvessels is mediated by the attachment of knobs on PRBCs to receptors on the endothelial cell surface such as CD36, TSP and ICAM-1 (Aikawa et al., 1992). In mice infected with malaria, erythrocytes infected with malaria parasite adhere *in vitro* to purified CD36, a critical endothelium receptor for binding *P. falciparum*-infected erythrocytes (Mota et al., 2000).

Conclusion

The G allele in the mutation T188G is associated with eight-times increased relative risk for susceptibility to CM in children in the Central region of Sudan. This study recommend the establishment of studies exploring the genetic factors related to CM in children in Central Sudan where clustering of CM in certain tribes and families points to the possibility of involvement of a genetic factor in the control of susceptibility to CM. Other mutations in CD36 gene should be studied.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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