Infection, Genetics and Evolution 11 (2011) 139–144

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

High CR1 level and related polymorphic variants are associated with cerebral malaria in eastern-India

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ARTICLE INFO

Article history:
Received 9 July 2010
Received in revised form 23 September 2010
Accepted 26 September 2010
Available online 14 October 2010

Keywords:
CR1
Polymorphism
Plasmodium falciparum
Cerebral malaria
Severe malaria anemia

ABSTRACT

The complement receptor 1 (CR1/CD35) protein acts as the major rosetting receptor in *Plasmodium falciparum* infection and several genetic variants of CR1 gene have been shown to be associated with quantitative expression of erythrocyte CR1 (E-CR1) level. However, CR1 level and gene polymorphisms exhibit differences in clinical manifestation of malaria in regions of varying disease endemicity. The result of the present study which analyzed three SNPs (intron 27 HindIIIA>T, exon 22 3650 A>G, and exon 33 5507 C>G) of the CR1 gene in Orissa, a hyperendemic state in eastern-India showed that a significantly increased risk for cerebral malaria (CM) was associated with AA genotype of both intron 27 and exon 22 when compared with mild, severe malaria anemia (SMA) and CM + SMA group respectively. Further, the overall haplotype analysis for all the three loci showed predominantly two major haplotypes 'AAC' coding for higher expression of CR1 and 'TGG' haplotype coding for low expression of CR1 level with the former haplotype being significantly associated with CM (P value < 0.00619 after Bonferroni correction) compared to mild malaria. The 'TGG' haplotype was proportionately more in SMA cases compared to mild malaria though statistically not significant. These findings suggest that the mild malaria group had an intermediate level of E-CR1 and extremely low or high levels of CR1 can cause severity in malaria. Further large scale studies in different endemic regions are needed to explain the epidemiological differences between E-CR1 expression and clinical manifestation of malaria which may contribute to the understanding of malaria pathogenesis.

1. Introduction

The pathogenesis of severe malaria is multifaceted with parasite virulence phenotypes and host genetic factors being two major foci of research. One such *Plasmodium falciparum* virulence phenotype is rosetting, an adhesion property in which parasitized red blood cells (RBCs) bind to unparasitized RBCs to form clumps of cells (Rowe et al., 1997). Although, the mechanisms by which rosetting becomes virulence are still unknown, the possible mechanisms include greater microvascular obstruction to blood flow (Nash et al., 1992), elevated parasite densities in favoring invasion of the merozoites (Rowe et al., 2002a) and immune evasion (Deans and Rowe, 2006). Rosetting is mediated by the binding of parasite ligand *P. falciparum* erythrocyte membrane protein1 (PfEMP1) expressed on the surface of infected RBCs to a variety of uninfected RBC receptors like blood group antigens A and B, serum components, glycosaminoglycans and complement receptor 1 (CR1). Amongst these the CR1, an immunoregulatory membrane glycoprotein expressed on various cell types including RBCs, acts as the major rosetting receptor (Teeranaipong et al., 2008). The susceptibility to severe malaria has been shown to be dependent on the expression level of erythrocytic CR1 with individuals having low-level CR1 are more likely to complement-mediated damage and removal of erythrocytes in the spleen, leading to severe anemia (Stoute et al., 2003). On the contrary, individuals with high levels of erythrocyte CR1 are more likely to form rosettes that contribute to sequestration in brain capillaries and other vital organs leading to cerebral malaria (Rowe et al., 1997). Further, high levels of CR1 also carry more immune complexes (ICs) that can interact with monocytes and endothelial cells to produce pro-inflammatory mediators (Stoute, 2005). However, population survey on CR1 genetic variants that influences the level of CR1 expression and their association with malaria has given varied results.

In humans, the CR1 gene is located at long arm of chromosome 1 and the number of CR1 molecules expressed on erythrocytes is in

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doi:10.1016/j.meegid.2010.09.009

part genetically determined that varies between individuals in the range of 50–1200 molecules per cell (Cockburn et al., 2004). Several genetic polymorphisms of CR1 gene have been shown to be associated with quantitative expression of CR1 in different malaria endemic populations and include SNPs in intron 27 (HindIIIA>T), exon 22 (3650 A>G), exon 33 (5507 C>G) and exon 19 (3093G>T) of the CR1 gene (Xiang et al., 1999). The high expression haplotype (H) is considered to contain $A^{HindIII}$, A^{3650}, C^{5507} and G^{3093} whereas the low expression haplotype (L) allele is considered to contain $T^{HindIII}$, G^{3650}, G^{5507} and T^{3093}. Population survey on the association of CR1 genetic variants with malaria have shown some conflicting findings, with intermediate CR1 level having HL genotype of exon 22 SNP had protective effect against severe malaria in PNG (Cockburn et al., 2004) whereas deficiency of CR1 level having LL genotype of intron 27 was significantly higher in severe malaria (Nagayasu et al., 2001) and CR1 variant associated with higher E-CR1 expression conferred protection against CM (Teeranaipong et al., 2008) in Thailand. However, in African population, the intron 27 and exon 33 variants did not show any significant correlation with erythrocyte CR1 expression level (Rowe et al., 2002b). These differences in association of CR1 genotype with clinical manifestation of the disease might be due to non-categorization of severe malaria group leading to an under or overestimate of the actual CR1 level, age-related changes in CR1 expression on RBC (Waitumbi et al., 2004) and/or regional differences of malaria endemicity (Sinha et al., 2009) or by interactions between CR1 and other genetic effects (Stoute, 2005). Therefore, an attempt has been made to investigate the prevalence of genotype and haplotype frequency of three different CR1 gene polymorphisms and their association with the clinical outcome of severe *P. falciparum* malaria amongst the adult patients in Orissa, a hyperendemic state in the eastern part of the country that contributes almost 50% of malaria associated deaths of the country.

2. Materials and methods

2.1. Study site and sample collection

The study was conducted from June 2007 to October 2009 in SCB Medical College and Hospital, Cuttack, of Orissa. The state is considered as hyperendemic for malaria and all four species of human malaria are found in Orissa, but >85% of all clinical malaria are due to *P. falciparum* (Ranjit, 2006). Clinically suspected malaria patients were screened for *P. falciparum* infection using both thick and thin film method. Severity of malaria was classified according to the definitions and associated characters published by WHO (2000). The inclusion criteria for the mild malaria cases were axillary temperature >37.5 °C or symptoms of headache, fever and myalgia, no schizontaemia, no intake of antimalarial drugs within the preceding week and no history of hospitalization (to exclude those who already had a severe malarial attack). The inclusion criteria for cerebral malaria cases were unarousable coma for >6 h after severe convulsions, negative for other causes of cerebral involvement and for SMA was Hb levels < 5 g/dl, axillary temperature >37.5 °C. Multi organ dysfunctions (MODS) were considered when there were involvements of two or more organ systems (central nervous systems, respiratory, renal, hepatic, gastrointestinal and hematologic systems). Exclusion criteria were (i) confirmed diagnosis of co-infection with other Plasmodium species, (ii) symptoms of mild or severe malaria with other acute infections including intestinal geohelminthic infections, (iii) chronic diseases like tuberculosis, leprosy and malnutrition, and (iv) pregnant women. Venous blood was collected in EDTA vials after informed consent was obtained from all enrolled (mild as well as severe) patients and stored at −20 °C. Each patient was treated according to local guidelines and care was provided until discharge from the concerned hospital. The Ethical committee of the Regional Medical Research Centre, Bhubaneswar, approved the study.

2.2. DNA isolation and genotyping of CR1 variants

DNA was isolated from 100 μl of blood following the standard protocol (Sambrook and Russel, 2001). In brief, erythrocytes were lysed with lysis buffer (10 mM Tris–HCl pH-8.0, 0.1 M EDTA pH 8.0, RNase 20 μg/ml, 0.5% SDS and proteinase K 100 μg/ml) at 55 °C for 16 h. DNA was obtained by phenol extraction/ethanol precipitation and resuspended in 50 μl of DNase free water. Samples were genotyped for intron 27 HindIIIA>T, exon 22 3650 A>G, and exon 33 5507 C>G of the CR1 gene by PCR-RFLP as described by (Xiang et al., 1999). For intron 27 and exon 22 the RFLP products were separated on 3% Neusive agarose and for exon 33, fragments were resolved in nondenaturing 15% polyacrylamide gels and visualized by ethidium bromide (0.5 μg/ml) staining under trans UV illumination. For confirmation by restriction fragment length polymorphisms, all RFLP experiments were performed for about 10% of the randomly selected samples and three control (known genotype) samples obtained from direct sequencing and the results were observed to be 100% concordant.

2.3. Quantification of erythrocyte CR1 level

The density of CR1 on erythrocytes was measured according to a method described elsewhere (Teeranaipong et al., 2008), with minor modification. In brief, fresh whole blood preserved in heparin was centrifuged at 2000 rpm for 15 min at room temperature, to remove the buffy coat containing the leucocyte. Afterward, 100 μl of RBC pellet was washed 3 times in 1 ml of PBS–RPMI–ABS (i.e., PBS + 4% RPMI + 1% human AB serum). Cells were resuspended in 1 ml of PBS–RPMI–ABS, and 50 μl of this suspension was placed into two tubes for the next step. One tube was incubated with FITC conjugated monoclonal Ab of CR1, E-11 clone (Santa Cruz) and the other tube for isotypic control was incubated with FITC conjugated goat anti-mouse IgG1 (E-Bioscience) in PBS-RPMI at 4 °C for 1 h with occasional agitation. Thereafter, all samples were washed 3 times in PBS–RPMI–ABS and were ready for analysis on the flow cytometer (Beckman Coulter). According to size, the gating strategy was used to define the absolute erythrocyte subset and to gate out platelets and cell debris. The mean fluorescence intensity (MFI) of each sample and isotypic control was measured 3 times in succession. The number of E-CR1 copies for each individual was calculated as the average MFI of each sample minus the average MFI of the isotypic control.

2.4. Statistical analysis

All statistical analysis was performed with Graph pad Prism (4.0). Quantitative data were expressed as the mean SD and were compared by unpaired *t*-test. The association of genotypes and allele frequencies amongst different clinical groups were determined by computing the odds ratio (OR) derived from 2 by 2 contigency table. Statistical comparison also was performed using Fisher's exact test whenever a variable in the contingency table was <5. The χ^2-test was used to test the Hardy–Weinberg equilibrium (HWE). Statistical significance was accepted at $P < 0.05$. Linkage disequilibrium was examined by χ^2-test analysis, and the extent of disequilibrium was determined as follows: $D' = D/D\text{max}$. The SNP Alyze program (version 7.0), which uses the expectation maximization algorithm, was used to estimate the maximum likelihood of haplotype frequencies in each group, and to identify which specific haplotypes were associated with a clinical outcome of malaria, the *P* value was evaluated by