

Role of CR1 Knops polymorphism in the pathophysiology of malaria: Indian scenario

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Abstract

Background & objectives: *Plasmodium falciparum* is the leading cause of mortality and causes cerebral malaria associated with sequestration caused by cytoadherence of the trophozoite and schizont-infected erythrocytes to the endothelial cells of the deep vascular beds in the brain. Pathophysiology of malaria is complicated by rosetting. Rosetting is a process of binding of uninfected erythrocytes to the erythrocytes infected with mature asexual parasites and is controlled by expression of complement receptor 1 (CR1) on RBC surface. Various polymorphic forms of CR1 are known including molecular weight polymorphism, red blood cell expression levels/density polymorphism and Knops (KN) polymorphism. The Knops blood group includes several allelic pairs; Knops a and b (Kna and Knb), McCoy a and b (McCa, McCb), Swain-Langley (Sla), and Villien (Vil). Knops phenotype SI (a⁻) has been found to rosette less effectively than SI (a⁺) and hence suggested to be more protective. *P. falciparum* cases have not reduced much as compared to the reduction in the total number of malaria cases in the past few years. In addition, *P. falciparum* is the leading cause for all mortality and most of the morbidity in India. We, therefore, investigated the role of CR1 Knops polymorphism in the pathophysiology of malaria in Indian population.

Methods: A case control approach was used for this study. CAPS (Cleaved amplified polymorphic sequence) methodology was adopted. A total of 100 normal individuals (free from any ailment) and 100 individuals suffering from *P. falciparum* infection (uncomplicated malaria) were recruited for this study.

Results: We found that in Indian population (normal individuals and *P. falciparum*-infected individuals), only the wild type allele is present.

Interpretation & conclusion: We concluded that the process of rosetting in the Indian context could be occurring independently of the effect of Knops polymorphism and in part could be controlled by other polymorphisms of the CR1 gene (density and structural polymorphism).

Key words CR1 gene – Knops polymorphism – malaria – *Plasmodium falciparum*

Introduction

Malaria is a disease of poverty and is highly prevalent in underdeveloped countries. This disease claims the life of about 1.5 to 2 million people per year^{1–2}. Malaria has been a problem in India for centuries. The annual incidence of malaria was around 75 million cases in 1953 with about 0.8 million deaths an-

nually. National Malaria Control Programme and National Malaria Eradication Programme (1957 and 1958) were a great success and the incidence dropped to 50,000 cases a year. But malaria staged a dramatic comeback in India in the 1970s after its near eradication³. In recent past (2007 & 2008), malaria positive cases reported annually from India were 1.5 million, with *Plasmodium falciparum* proportion

being 49% in both 2007 and 2008 from the previously reported 44% in 2005⁴. Malaria caused by *P. falciparum* is a malignant variety of malaria. It has been reported that infection with *P. falciparum* is the most deadly form of malaria. The number of deaths due to *P. falciparum* was 963 in 2005 and it further increased to 1311 in 2007, albeit the deaths reported in the provisional data for 2008 was found to be reduced to 935⁵. But clearly the mortality associated with malaria is a big problem.

A key feature of the biology of *P. falciparum* is the ability of the infected red blood cells to adhere to the linings of small blood vessels leading to obstruction of the tissue perfusion. Cerebral malaria is a complication of severe *P. falciparum* malaria associated with cytoadherence of trophozoite and schizont-infected erythrocytes to endothelial cells of cerebral microvasculature. Several receptors including CD36⁶, thrombospondin⁷, intercellular adhesion molecule-1⁸, vascular cell adhesion molecule, and E-selectin⁹ mediate this interaction. The process of binding of uninfected erythrocytes to the erythrocytes infected with mature asexual parasites is called rosetting¹⁰.

Complement receptor 1: CR1 (CD35, C3b/C4b receptor) is a polymorphic membrane bound glycoprotein, differentially expressed on erythrocytes, eosinophils, monocytes, B-lymphocytes, T-lymphocytes, dendritic cells and kidney podocytes¹¹. The major functions of CR1 are binding to and removal of C4b and C3b bearing immune complexes, to serve as a complement regulatory protein¹².

Various polymorphic forms of CR1, including molecular weight polymorphism, red blood cell expression levels /density polymorphism and Knops (KN) polymorphism are known. CR1 molecular weight differs by approximately 30 kilodaltons (kDa), four different forms are known CR1*1 (220 kDa), CR1*2 (250 kDa), CR1*3 (190 kDa) and CR1*4 (280 kDa), arising by deletion and duplication of the complement control proteins (CCPs), mostly contained in the long homologous repeat-B (LHR-B)¹³. The CR1 structural variants differ in their number of CCPs/

LHRs and the number of C3b binding sites with smallest form having one binding site and the largest form having four sites. In most of the populations studied, the two most frequent alleles are CR1*1 (220 kDa) and CR1*2 (250 kDa)¹⁴. RBC-CR1 copy number can vary as much as 10 fold among normal healthy individuals. RBCs having low levels of CR1 (approximately 10% of normal) have the serologically null phenotype known as the 'Helgeson phenotype'. In Caucasians, the RBC-CR1 copy number is linked to a Hind III restriction fragment length polymorphism (RFLP) called the density polymorphism. It arises by a single base change in the intron of long homologous repeat (LHR-D) region within the CR1 gene¹⁵. The Knops blood group localized to the CR1 includes several presumed allelic pairs; Knops a and b (Kna and Knb), McCoy a and b (McCa, McCb), Swain-Langley (Sla), and Villien (Vil)¹⁶⁻¹⁸.

Single nucleotide polymorphisms occurring in short consensus repeat (SCR) 25, which lead to amino acid substitutions, result in generation of these polymorphic forms¹⁹. These antigens have been localized on the LHR-D segment of CR1. The molecular basis of Kn(a) and Kn(b) polymorphism is the substitution of Valine in Kn (a⁺) to Methionine in Kn(b⁺) at residue 1561. McCa, McCb polymorphism is at 4795 bp, where A to G transition leads to the substitution of Proline to Aspartic acid. Sl(a)/Vil mutation is at 4828 bp where, the A to G transition leads to the substitution of Arginine to Glycine.

The role of Knops polymorphism has been investigated in some disease conditions: leprosy, mycetoma infection (fungal infection) and malaria to name a few. In a study published in 2004, homozygotes for the McCoy b blood group defining variant K1590E in exon 29 of the complement receptor 1 gene appeared to be protected against leprosy susceptibility²⁰. In another study, the allele *Sl2* was more often found in the patients suffering from mycetoma infection compared to the control subjects, whereas the *McCb* allele was more dominant in the control population. This indicated the possibility of *Sl2* and *McCa* being the risk factors for the development of

mycetoma infection. Knops polymorphism has been extensively studied in the context of malaria infection²¹, but only a limited number of studies are available on its role in other inflammatory disorders.

Knops polymorphic forms SI(a⁻) and McC(b⁺) have been found to be present at increased frequency in the malaria endemic regions²². Knops phenotype SI(a⁻) has been found to rosette less effectively than SI(a⁺) and hence suggested to be more protective^{23,24}. This observation led the researchers to hypothesize that the SI(a⁻) allele and, possibly, the McCb⁺ allele evolved in the context of malaria transmission and that in certain combinations probably confer a survival advantage on these populations²⁵. Several studies have indicated that patients with cerebral malaria have higher extent of rosette formation compared to those with mild/uncomplicated malaria²⁶⁻³¹. Given the important role of Knops polymorphic phenotypes of CR1 in rosetting of RBCs and the fact that this polymorphism of CR1 has not been studied till date in the Indian population, we investigated the role of CR1 Knops polymorphism in the pathophysiology of malaria in Indian population. This kind of a study could go a long way in understanding the role of selective gene pressure in the pathophysiology of malaria in the context of Indian population. This type of information could also be helpful in using CR1 as a target for development of antimalarial vaccine.

Material & Methods

Study subjects: A case control study was undertaken on two groups of study subjects (100 normal individuals free from any ailment at the time of collection of blood sample and 100 individuals suffering from uncomplicated *P. falciparum* infection). The mean age of the normal individuals ranged from 29.14±8.7 yr. Patients with *P. falciparum* infection were from the regions of Assam, Gujarat, Madhya Pradesh, Chennai and Delhi (NCR), where *P. falciparum* infection is prevalent. The mean age of the patients ranges from 30.5±3.2 yr that was comparable to those of the normal individuals. Informed and free consent was taken from normal individuals

and the patients before collection of the blood samples. The protocol for sample collection was examined and certified by Biosafety and Ethics Committee of the participating institutes.

Genomic DNA isolation: DNA from normal healthy Indian subjects was isolated using the standardized protocol using EDTA as an anticoagulant. Peripheral blood was collected by venipuncture. The plasma part was separated from the cells by centrifuging at 3500 rpm for 10 min. The cells were immediately stored at -80°C for isolation of DNA. DNA from patients infected with *P. falciparum* was isolated from the dried blood spots using QIAamp DNA blood Mini Kit (Qiagen).

Polymerase chain reaction for the amplification of CR1 gene: For the identification of the Knops genotype of the individuals, DNA from the patients and normal individuals were subjected to amplification using the primers and the conditions standardized previously²² with a slight modification in the conditions. PCR was set up in a total of 50 µl volume with 250 ng of genomic DNA, 5 µl of 10X *Taq* polymerase buffer, 0.75 mM of MgCl₂ (25 mM), 0.1 mM each of dNTPs (1 µl of dNTP mix-10 mM each), 0.25 µl of each primer, and 1.25 Units of *Taq* polymerase. The amplification was carried out using the following conditions: initial denaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 59°C for 1 min, extension at 72°C for 1 min for 30 cycles, and a final extension at 72°C for 10 min in an MJ, Thermocycler (Perkin Elmer). The product obtained after PCR was 305 bp long.

Restriction digestion: The Knops polymorphic forms were detected using three enzymes, viz. NdeI, BsmI and MunI. Twenty units each of NdeI, BsmI, MunI were used for the detection of Kna/Knb, McCa/McCb and Sla/SIb detection respectively. The expected size of the bands produced after digestion of the PCR amplified fragment are 261, 44; 166, 139; and 161, 144, respectively for NdeI, BsmI and MunI. Reaction mixture for the detection of each polymorphism was made with 10 µl of PCR product, 20 U of the

respective enzymes and 10X buffer in different cocktails. The reaction mixtures were incubated at 37°C overnight. Digestion patterns were analyzed on 2% agarose gel containing ethidium bromide. The sizes of the restricted fragments were compared with that of the marker DNA (Phi-X DNA marker or 100 bp DNA ladder).

Results

Knops genotyping using polymerase chain reaction and restriction digestion: DNA samples from 100 normal healthy individuals and 100 patients suffering from *P. falciparum* infection were analyzed in

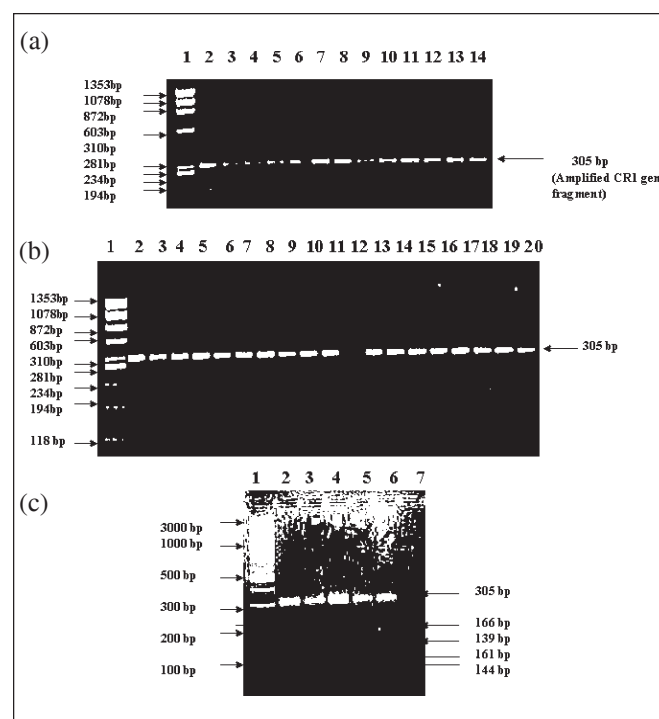


Fig. 1: (a) The PCR amplified fragments of patients and normal samples [Lane 1: Marker DNA, lanes 2–14: amplified products of CR1 gene in Indian subjects]; (b) Digested fragments of the PCR amplified products of Indian population [Lane 1: Marker DNA, lanes 1–20: Restriction digests of PCR amplified products of Indian subjects]; and (c) PCR amplified products from African individuals [Lane 1: Marker DNA, lanes 2 and 3: Kna/Kna pattern produced by NdeI digestion, lanes 4 and 5: McCa/McCb pattern produced by BsmI digestion, lane 6: Observed Sla/SIb pattern and lane 7: observed SIb/SIb pattern (African subjects)]

the present study. A single sharp band of length 305 bp was obtained in all the samples for CR1 gene (Fig. 1a). Upon digestion with NdeI, BsmI and MunI, the homozygous wild type alleles for Kna, McCa and Sla were obtained in all the samples (Fig. 1b).

Restriction digestion of Lambda DNA and DNA from African individuals: To check for the effects of PCR components on the activity of enzymes, the digestion pattern of pure Lambda DNA (taken as positive control of digestion) was analyzed with respect to Lambda DNA spiked with PCR amplicon in three different forms: (a) untreated PCR amplicon spiked with Lambda DNA; (b) salt precipitated PCR amplicon spiked with Lambda DNA; and (c) column precipitated PCR amplicon spiked with Lambda DNA. The reaction mixtures were digested with 20 units of NdeI and the fragments obtained thereof were analyzed on agarose gel (Fig. 2). Lambda DNA produced the desired number of fragments in all the tubes whereas for the samples, even after using the salt precipitated and column precipitated amplicons, only the homozygous form of Knops polymorphism

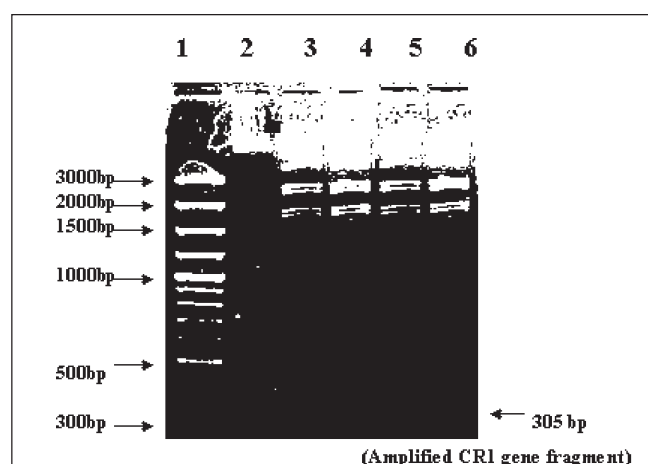


Fig. 2: Digestion of PCR amplicon spiked with Lambda DNA [Lane 1: Marker DNA, lane 2: undigested lambda DNA, lane 3: digested lambda DNA, Digestion of untreated PCR amplicon spiked with Lambda DNA is seen in lane 4, lane 5 contains the salt precipitated PCR amplicon spiked with Lambda DNA and the pattern of digestion of column precipitated PCR amplicon spiked with Lambda DNA is obtained in lane 6]

was obtained. This result confirmed that none of the components of PCR was hindering the enzyme activity. To further confirm our results and rule out any experimental error, the samples obtained from African individuals (gifted by Dr J.M. Moulds, Shreveport, U.S.A.) were subjected to amplification and restriction digestion with NdeI, BsmI and MunI. When these samples were analyzed for the Knops polymorphism, the wild type homozygous (Kna/Kna), homozygous mutant (McCb/McCb) and heterozygous (McCa/McCb and Sla/SIb) forms of the Knops polymorphism were obtained (Fig. 1c). From the above results, it was concluded that only the homozygous wild type alleles for Kna, McCa and Sla are present in the Indian population and it reflects the true picture of allelic distribution at the Knops locus.

Discussion

In the present study, a total of 100 normal healthy individuals and 100 patients were genotyped for Knops polymorphism. All the individuals contained only the homozygous wild type alleles and the homozygous mutant type, and the heterozygous forms of Knops polymorphism were completely absent from the Indian population. It has already been reported that the SIb gene is almost completely absent in the Caucasian and Asian populations³². In Caucasians, the frequencies of Kna and Knb; McCa and McCb; and Sla and SIb are 0.99 and 0.01; 0.99 and <0.01; and 0.99 and <0.01, respectively, thus are in agreement with our observations.

Previously, in a study conducted on Brazilian population, the heterozygous Kna/Knb, Sla/SIb and McCa/McCb individuals were identified only in populations of European and African descent³³. In another study, the frequencies of Kna and Knb, McCa and McCb and Sla and SIb were 0.98 and 0.02, 0.78 and 0.22, 0.48 and 0.52, respectively in African-American persons. The frequency of SIb was found to be highest (0.77) in the Bambaran region of Africa. The allele frequencies of Kna and Knb in the Bandiagara population were 0.90 for the Kna allele and 0.10 for Knb allele²².

From the results obtained in the present study, we have concluded that only the homozygous wild type form of the Knops polymorphism is prevalent in the Indian population and that the process of rosetting in the Indian context could be occurring independently of the effect of Knops polymorphism loci of CR1. In other words, the rosetting could be mediated by other polymorphisms of the CR1 gene including the density and the structural polymorphism. These two polymorphisms could have a role in increasing the susceptibility to malaria or in providing resistance against the severity of malaria. It can also be suggested that the genes for Knb, McCb and SIb may be under selective pressure in India. It has been reported previously that false negative results can be obtained when the RBCs have low CR1 copy number either owing to an inherited or acquired deficiency after prolonged storage of the blood samples³⁴. Since the CR1 levels of the patients or the density polymorphism were not determined in the present study, it could be possible that the density polymorphism is linked somehow to the absence of Knops polymorphism in Indian population. The density and structural polymorphisms of CR1 gene need to be investigated in the context of the pathophysiology of malaria. There is also a need to explore the role of these polymorphic forms in controlling the process of rosetting and the severity of malaria. This information could be useful for designing the vaccines against cerebral malaria.

Acknowledgement

We are very thankful to Dr J.M. Moulds, Director, Clinical Immunogenetics, Life Share Blood Centers, Shreveport, U.S.A. for providing the DNA samples from the African individuals. We also thank all the blood donors who have donated their blood for this study. The help and grants provided by the University School of Biotechnology, Guru Gobind Singh Indraprastha University and DST, Delhi are also duly acknowledged. We would also like to thank technical staff members of National Institute of Malaria Research, HQs and the field units for their support and cooperation during the study.

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Received: 27 July 2009

Accepted in revised form: 5 October 2009