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Sequence polymorphism within erythrocyte binding domain of EBA175 in Indian and African field isolates

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Abstract

Invasion of erythrocyte by Plasmodium merozoites is mediated by specific molecular interactions between proteins expressed on merozoite surface and the receptors present on erythrocytes. Erythrocyte binding antigen 175 (EBA175) is one such protein that interacts with the sialic acid residues on glycophorin A present on erythrocytes' surface during invasion. The FII region (PfFII) of EBA175 has been mapped to be critical for binding to erythrocytes. It is reported that antibodies against FII region blocks binding. Polymorphisms in FII region of EBA175 are already reported. The goal of this study was to investigate whether polymorphism in FII region of African P. falciparum field isolates has any effect on erythrocyte binding and also to find whether antibodies raised against FII region from P. falciparum Malayan Camp strain (Camp) can inhibit erythrocyte binding. Genomic DNA of parasites from the blood samples of P. falciparum infected individuals was isolated and PfFII region from these genomic DNA were amplified, cloned and sequenced. Following sequence analysis, we selected three isolates harboring higher PfFII polymorphisms, expressed them on the surface of COS cells as chimeric proteins using secretory signal and transmembrane segments of Herpex simplex virus glycoprotein D (HSVg D) and tested for their erythrocyte binding ability. We further tested the inhibition of erythrocyte binding of these polymorphic FII regions using anti-campPfF2 antibodies. Our results reveal that the polymorphisms in different field isolates included in this study do not have any significant effect on erythrocyte binding and antibodies raised against FII region of camp strain could inhibit erythrocyte binding by all the polymorphic PfFII. This observation strengthens the possibility that PfFII can be a potential candidate vaccine.

Keywords: Diversity, EBA175, PfFII, Field isolate, Polymorphism.

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Introduction

Invasion of erythrocyte by Plasmodium merozoites is mediated by specific molecular interactions between molecules expressed on merozoite surface and the receptors present on erythrocytes [1-3]. Erythrocyte binding antigen 175 (EBA-175) of *Plasmodium falciparum* is one such merozoite surface protein that interacts with the sialic acid residues on glycophorin A present on erythrocytes' surface during invasion [4-6]. EBA-175 is localized in the micronemes at the apical end of the merozoites [7]. The gene encoding EBA-175 consists of an N terminal signal sequence, a long extracellular hydrophilic domain, a transmembrane sequence and a short C terminal cytoplasmic domain [8]. The extracellular domain contains 5' and 3' cysteine rich regions. The 5' cysteine rich region contains two copies named FI and FII [9]. EBA-175 belongs to Duffy binding like erythrocyte binding protein family, because it shares sequence homology with members of this family [10]. The FII region (PfFII) of EBA-175 has been shown to be critical for binding to erythrocytes [11]. The antibodies against FII region block interaction between PfFII and erytrocytes-suggesting that PfFII is a potential vaccine candidate [10]. We investigated polymorphisms in FII region of African *P. falciparum* field isolates by PCR and found that antibodies raised against FII region from the *P. falciparum* Malayan Camp strain can inhibit erythrocyte binding by the field isolates having polymorphism.

Methods

DNA Isolation form filter paper

Genomic DNA of African *P. falciparum* field isolates was extracted from filter paper using the methanol fixation and heat extraction method [12]. Briefly, two-three pieces of filter paper was incubated with 500 µl of methanol for 15 min at room temperature. The methanol was drained off and the papers heated at 95-100°C in 50 µl of sterile distilled water for 15 minutes of incubation with intermittent vortexing. The samples were centrifuged and the final supernatant used as a template for the amplification reaction.

PCR amplification and sequence analysis of FII region of *P. falciparum*

Nested PCR technique was used to amplify gene fragment, which encodes for FII region of *P. falciparum* as described previously [12]. Briefly, for primary PCR reaction, primers EBARIIF (5'ggaagaaatacttcatctaataacg 3') and EBARIIR (5'cgaagtttgttcattatttcttattatag 3') were used and PCR product of primary PCR reaction was used as template for nested PCR with primers EBARIIF2 (5' ttgatttagatgatttttctaaatttg 3') and EBARIIR. The amplified PCR products were resolved on agarose gel (1.0%). PCR product was gel purified, ligated in pGEMT-E vector (Promega) and

transformed in *E. coli* DH10B cells by $CaCl_2$ heat shock method. Two clones of each field isolates, containing inserts were sequenced with T7 promoter, M13R and an internal primer by the cycle sequencing method using Big dye terminator kit (Applied Biosystem) and an ABI prism 310 automated DNA sequencer (Applied Biosystem). Analysis of sequence of FII region of field isolates was done using DNA- Star.

MSP typing of field isolates

PCR typing of these field isolates was done using a highly polymorphism markers, MSP1 and MSP2 as described in [14-15]. The Multiplicity of infection was estimated by the number of PCR fragments per infected individual.

Erythrocyte binding assay

To study the impact of point mutations on binding specificity of region II of P. falciparum field isolates, we chose three field isolates, which harbored more mutations in comparison to Indian field isolates. SE011/2, C032/5, C034/2 have seven, four and seven mutations respectively. These mutants and EBA-175 from CAMP strain were expressed on the surface of COS cells as chimeric proteins using secretory signal and transmembrane segments of Herpex simplex virus glycoprotein D (HSVg D) as described by [7, 16-17]. Surface expression of the chimeric protein on COS cells was determined by immuno-fluorescence using mAb DL6 against proline rich region of HSVgD and anti-mouse Ig-G labeled with FITC (Sigma). Transfected COS cells were incubated with untreated and enzyme (trypsin and neuraminidase) treated erythrocyte (10% hematocrit). Rosettes were scored in 20-25 fields at 40 X magnification.

Erythrocyte binding inhibition assay

Transfected COS cells were incubated with different dilutions (from 1:100 to 1:3200) of anti PfF2 antibodies for 1 hr at 370C and followed by 10% hematocrit. 10 –20 fields were scored for numbers of COS cells with adherent erythrocyte (rosettes) for control and test antibodies against region II of P. falciparum. In presence of different dilutions of anti-PfF2 antibodies, binding pattern was expressed relative to binding in presence of pre immune sera. Pre-immune sera (prior to immunization with PfF2) was used as a control at 1:100 dilution.

Results and Discussion

Diversity in PfFII region of field isolates

We observed 1 Kb amplified product in all field isolates. Region II was present in all field isolates of *P. falciparum*. Analysis of amino acid sequence of FII region from field isolates (Table 1) was showed that more than 98%. Sequence identity. All the cysteines and the positions of these cysteine residues in region II were also conserved. Another interesting observation was

that all field isolates revealed polymorphisms at 15 amino acid positions when compared to the FII amino acid sequence of *P. falciparum* Malayan Camp and Indian field isolates. The present study identified 8 novel polymorphisms. In addition to the 8 novel polymorphisms, sequence diversity at seven positions namely 478, 481, 577, 584, 592, 664 and 716 were commonly present in African and Indian field isolates, which are already reported [18-19]. The non-conservative polymorphisms Asn (N) ↔ Lys (K), Ile (I) ↔ Lys (K), Lys (K) ↔ Gln (Q), Glu (E) ↔ Ala (A), Ser (S) ↔ Arg (R) were found in multiple isolates. And other polymorphisms were present in only one or two field isolates.

Yang et al and colleague did analysis of the genetic diversity of PfEBA 175 from global isolates and the result of study showed a high level of diversity within the parasite population [20].

Allelic diversity in MSP typing

The population diversity was found in field isolates of *P. falciparum* namely C003, C032, C034, C044 and SE011. Three different patterns of genotype were observed (Table 2). K1 allele of MSP1 gene, 3D7 and FC27 alleles of MSP2 gene were observed in C032 and C034. C044 had K1, MAD20 and RO33 alleles of MSP1 and 3D7 allele of MSP2 gene. C003 and SE011 had K1, MAD20 and RO33 alleles of MSP1 gene and 3D7 and FC27 alleles of MSP2 gene. This suggested that the sampled patients had mixed infection.

Erythrocyte binding of filed isolates

COS cells expressing region II bound normal human erythrocytes but not to enzyme (trypsin and neuraminidase) treated human erythrocytes (Table 3). The specificity of binding of normal and enzyme treated erythrocyte to region II expressed on COS cells was identical in native EBA-175 and *P. falciparum field* isolates [21]. From these experiments we conclude that region II of EBA-175 derived from *P. falciparum* field isolates binds erythrocyte with the same specificity as native EBA-175 despite the presence of polymorphic sequences.

To evaluate the relative binding efficiency of variants, we measured inhibition of erythrocytes binding to region II expressed on COS cells with anti PfF2-antibodies. 70% inhibition was observed in all variants by anti-PfFII antibodies (figure 1). The result showed that antibodies against region II of *P. falciparum* inhibit invasion of erythrocytes in vitro and thus provides strong support for developing human vaccine based on region II of *P.falciparum*.

Table 1: Comparison of sequences of F2 region of EBA-175 derived from different *P. falciparum* isolates.

AA no	466	469	478	481	498	507	526	555	567	577	582	584	589	592	614	620	622	625	631	637	650	655	657	663	664	669	716	722
Camp	S	T	N	I	L	K	I	G	K	K	N	K	F	E	V	I	N	Q	S	Y	V	K	C	K	S	Y	E	N
IND			i/k	k	F			e	i	n	d	q/e	S	a	f	I	h		i/r	h			r	r	r	f	k	
SE10			K	K						N		Q		A														
C010			K	K						N		Q													R			
C077			K	K						N		Q													R			
C044-12			K	R			V					Q						N							R			
C044-13			K	K			I					Q													R			
SE015			K	K						N		Q													R			
SE005			K	K						N		E		A														
SE019			K	K						N		Q																
SE011-2			K	K						N		Q						R				R			R			
SE011-5			K							N															R			
C034-2	P		K			Q																						D
C034-3			K																									
C032-2			K	K						N		E		A													K	
C032-5		S	K	K						N		E		A													K	
C003-15			K	K						N		Q		E											R			
C003-18			K	K						N		E		A							A							
SE013																												
CO33-7												Q													R			
CO33-9			K	K								Q																
SE021			K	K								Q													R			

* Red color indicates field isolates which harboring higher mutations and used for further studies.

Table 2: Population diversity in field isolates of P. falciparum.

ALLELE \ ISOLATES		C003	C032	C034	C044	SE011
	K1	+	+	+	+	+
MSP1	MAD20	+	-	-	+	+
	R033	+	-	-	+	+
MSP2	3D7	+	+	+	+	+
	FC27	+	+	+	-	+

(+) indicates: Presence of allele and (-): absent of allele

Table 3: Binding of region F2 derived from EBA-175 from P. falciparum field isolates to normal and enzyme-treated human erythrocytes.

Isolates	AA polymorphism[1]	No Treatment	Enzyme Treatment[2] Neuraminidase	Trypsin
Camp	NA	+	-	-
SE011/2	7	+	-	-
C032/5	7	+	-	-
C034/2	4	+	-	-

[1]Number of differences in amino acid sequence of region F2 from *P. falciparum* field isolates compared to amino acid sequence of region F2 of EBA-175 derived from *P. falciparum* Camp strain.

[2]COS 7 cells transfected to express region F2 from diverse *P. falciparum* isolates were tested for binding to normal and enzyme-treated human erythrocytes. The number of COS 7 cells covered with rosettes of adherent erythrocytes was scored in 15 random fields at 40X magnification. +, denotes that adherent rosettes were observed. -, indicates that no rosettes were seen in the entire well used for transfection and binding assays.

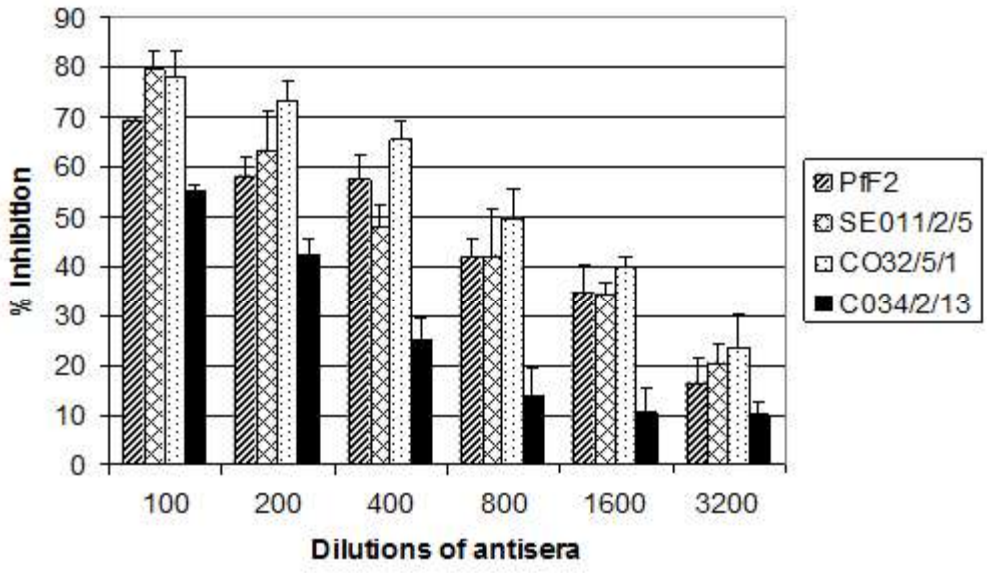

Fig. 1: Inhibition of binding of erythrocytes to region F2 of EBA-175 from diverse P. falciparum field isolates using rabbit sera raised against region F2 of EBA-175 from P. falciparum Camp strain. COS 7 cells transfected to express region F2 of EBA-175 derived from P. falciparum Camp strain and diverse *P. falciparum* field isolates (SE011/2, C034/2 and C032/5) were tested for binding to human erythrocytes in presence of rabbit sera raised against recombinant region F2 of EBA-175 from *P. falciparum* Camp strain. Binding in presence of pre-immune serum (1:100 dilution) was used as control. Number of rosettes of adherent erythrocytes was scored in 20 fields at 400× magnification. Number of rosettes in presence of anti-F2 sera is shown relative to number of rosettes of adherent erythrocytes in presence of pre-immune sera.

Conclusion

Erythrocyte binding antigen 175 (EBA-175) of Plasmodium falciparum belongs to Duffy binding like erythrocyte binding protein family. It contains three cysteine-rich regions F1, F2 and C in which F1 and F2 at the N-terminus are responsible for the glycophorin A binding on the erythrocyte membrane. The FII region (PfFII) of EBA-175 has been shown as a potential vaccine candidate. We investigated polymorphisms in FII region of African P. falciparum field isolates by PCR and found that antibodies raised against FII region from the P. falciparum Malayan Camp strain can inhibit erythrocyte binding by the field isolates having polymorphism. Our findings suggest that antibodies to region II of EBA-175 are largely unaffected by polymorphism in EBA-175 and is a good ligand-blocking malaria vaccine.

Authors' contributions

Conceived and designed experiments: MS, SS. Performed the experiments: MS, and SS. Analyzed the data: MS, SS. Wrote the paper: MS, SS. Involved in all the experiments: MS and SS.

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