CONCISE COMMUNICATION

Identification of a Conserved *Plasmodium falciparum var* Gene Implicated in Malaria in Pregnancy

J. Alexandra Rowe,[1] Sue A. Kyes,[2] Stephen J. Rogerson,[3] Hamza A. Babiker,[1] and Ahmed Raza[1]

[1]Institute of Cell, Animal, and Population Biology, University of Edinburgh, Edinburgh, and [2]Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, United Kingdom; [3]Department of Medicine, University of Melbourne, Melbourne, Australia

The *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) family is a highly polymorphic class of variant surface antigens encoded by *var* genes that play an important role in malaria pathogenesis. This report describes the unexpected finding that 1 of the *var* genes encoding a PfEMP1 variant that binds to the host receptor chondroitin sulfate A (CSA) and is implicated in malaria in pregnancy is well conserved among *P. falciparum* isolates worldwide. The N-terminal domains of this PfEMP1 variant are especially highly conserved, whereas the functional CSA binding domain is more variable. Analysis of *var* gene expression in placental parasites from primigravid women in Malawi did not support a role for this conserved gene in placental infection but identified a second commonly occurring *var* gene. These results indicate the need for reevaluation of previous assumptions of a minimal overlap between *var* gene repertoires from different parasite isolates.

Malaria in pregnancy is a major global health problem. In endemic areas, adults usually develop immunity to clinical malaria; however, women who are pregnant for the first time lose this immunity and become particularly susceptible to infection [1]. This is thought to occur because the placenta provides a niche that allows for survival of a subpopulation of parasites that bind to receptors, such as chondroitin sulfate A (CSA), expressed on syncytiotrophoblasts and leading to sequestration of infected erythrocytes in the placenta [2]. During the course of pregnancy, infected women develop antibodies that recognize the surface of CSA-binding infected erythrocytes and inhibit binding of infected erythrocytes to CSA [3, 4]. Such antibodies may protect women against malaria in subsequent pregnancies and seem to be directed against an antigenically conserved strain-independent parasite ligand [3]. Identification of this antigenically conserved CSA-binding ligand is a priority because it could form the basis of a vaccine to prevent malaria in pregnancy.

Recent work showed that some members of the *Plasmodium falciparum* variant erythrocyte membrane protein 1 (PfEMP1) family, encoded by specific *var* genes, could bind CSA [5, 6]. The *var* gene family is characterized by a high degree of sequence polymorphism [7, 8], and there is thought to be minimal overlap between the *var* gene repertoires of different parasite isolates [7, 9], apart from short blocks of 20–80 aa [8]. Each haploid parasite genome contains ~50 *var* genes, and their expression is controlled so that only 1 PfEMP1 variant is expressed on the surface of each infected red blood cell [10].

Every *var* gene encodes a high-molecular-weight PfEMP1 variant composed of tandemly arranged cysteine-rich domains of ~300 aa known as Duffy binding-like (DBL) domains and the related cysteine-rich interdomain regions (CIDRs). These DBL domains are numbered consecutively from the N-terminus and have been classified into 5 types (α–ϵ) on the basis of their amino acid (aa) sequence [11]. Recent investigations have begun to define the adhesive function of individual DBL domains within PfEMP1: for the 2 CSA-binding PfEMP1 variants described to date, DBLγ has been identified as the binding region [5, 6]. Despite this progress, it is unresolved how PfEMP1, an inherently

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Nucleotide sequence data are available in the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank databases (accession numbers AJ420379–AJ420412).

Reprints or correspondence: Dr. Alex Rowe, Institute of Cell, Animal, and Population Biology, University of Edinburgh, West Mains Rd., Edinburgh EH9 3JT, United Kingdom (Alex.Rowe@ed.ac.uk).

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polymorphic protein, could be the parasite CSA-binding ligand when immunoepidemiologic evidence suggests that the CSA ligand is antigenically conserved between isolates [3]. The aim of this study was to provide a possible explanation for this conundrum by investigating evidence that a particular PfEMP1 variant may be conserved in a range of *P. falciparum* isolates.

Materials and Methods

var *gene cloning and sequencing.* The *TM180var2* and *TM284-var3* genes were identified by reverse-transcriptase (RT) polymerase chain reaction (PCR) with degenerate primers to DBL1α [7] by using RNA from mature pigmented trophozoites of parasite lines TM180 and TM284, respectively. The sequences were extended 5′ and 3′ by use of Vectorette libraries (Sigma Genosys) made from the genomic DNA of each parasite. Sequencing was done with BigDye Terminator chemistry (Applied Biosystems), and sequences were analyzed with Lasergene software (DNAstar).

PCR of genomic DNA from diverse parasites. Genomic DNA from *P. falciparum* laboratory lines/clones and field isolates was genotyped by standard methods [12], to ensure that all parasites used were genotypically distinct. Clone FCR3 is genotypically identical to parasite clones derived from the Brazilian IT strain used in many previous publications on *var* genes and PfEMP1 [6, 7]. The *3D7-varT3-2*–specific primers were 5′-CCA AGA TAT CAA ACA AAA TAT AAG G and 5′-GCT TCC AGT GTC ACT AGT GG, which amplify nt 482–2229 of *3D7-varT3-2* (GenBank accession no. AL034559). *FCR3varCSA*-specific primers were 5′-CAC ATA AAG GAA CTT CAG ACG and 5′-TGT TGC ATT ATG GGA TGT TTC, which amplify nt 563–2232 of *FCR3varCSA* (GenBank AJ133811). Amplification conditions for both sets of primers were a hot start, followed by 35 cycles of 94°C for 5 s, 52°C for 15 s, and 65°C for 2 min with 100 ng of genomic DNA, 25 pmol of each primer, 1.5 m*M* $MgCl_2$, and 2 U of Expand High Fidelity Taq/Pwo polymerase (Roche)/50 μL reaction.

RT-PCR of placental isolates. Parasitized erythrocytes were collected by flushing placental tissue with PBS, followed by centrifugation of the collected cells through Percoll (Amersham Biosciences) to enrich for infected cells. Most of the parasites studied were mature pigmented trophozoites and schizonts, which enabled us to detected the predominantly expressed *var* genes, rather than the nonspecific DBL1α transcripts that occur at early ring stage [10]. RNA was extracted by using Trizol (Gibco) and treated with DNAse I (Gibco) to remove contaminating genomic DNA. RT-PCR was done by using unbiased degenerate primers that amplify a region of ~300–400 bp of DBL1α [7]. Controls without RT were carried out for each sample to exclude DNA contamination. The RT-PCR products were cloned (TA cloning kit; Invitrogen), and a number of recombinant plasmids were sequenced, as indicated in table 1. Placental isolates were also genotyped by PCR [12], and we estimated the minimum number of parasite clones per infection.

Panning on CSA. Parasite lines TM180 and TM284 were panned on CSA-expressing CHO cells, as described elsewhere [6, 13]. After 4 rounds of selection, the *var* genes expressed by the selected lines were studied by RT-PCR by using degenerate primers to DBL1α, as described above [7].

Results

While studying *var* genes in 2 genotypically distinct Thai *P. falciparum* lines, TM180 and TM284, we noticed that each parasite expressed a *var* gene encoding a PfEMP1 variant similar to

Table 1. *var* genes expressed by placental isolates from primigravid women in Malawi.

Isolate[a]	P132	P136	P143	P154	CS294
Parasitemia[b]	2/57/32	0/39/54	5/62/31	5/58/19	1/8/11
var genes identified[c]	P132var1 (2)	**P136var1** (6)	PCR 1[d]	**P154var1** (10)	CS294var1 (2)
	P132var2 (2)	P136var2 (4)	P143var1 (5)	P154var2 (2)	**CS294var2** (1)
	P132var3 (1)	P136var3 (2)	P143var2 (3)	P154var3 (1)	CS294var3 (1)
	P132var4 (1)	P136var4 (1)	P143var3 (2)	P154var4 (1)	CS294var4 (1)
	P132var5 (1)	P136var5 (1)	P143var4 (2)	P154var5 (1)	
	P132var6 (1)	P136var6 (1)	PCR 2	P154var6 (1)	
	P132var7 (1)		P143var1 (5)	P154var7 (1)	
	P132var8 (1)		P143var2 (3)		
			P143var3 (2)		
			P143var5 (1)		
			P143var6 (1)		
			P143var7 (1)		
Total sequenced[e]	10	15	25	17	5

NOTE. PCR, polymerase chain reaction.

[a] Six different parasite genotypes were detected in isolate P132; 3 genotypes each were detected in P136, P143, and P154. For CS294, the no. of different genotypes was not determined.

[b] Data are parasitemia (%) after Percoll (Amersham Biosciences) enrichment: rings/pigmented trophozoites/schizonts.

[c] Nos. in parentheses are no. of recombinant plasmids containing that sequence. GenBank accession nos. for these sequences are AJ420379–AJ420410. *var* genes indicated in bold show 97%–100% nucleotide identity to each other and to 3 sequences from GenBank (see text).

[d] Two independent PCRs were done with cDNA from isolate P143 and gave similar results.

[e] Total recombinant plasmids sequenced per isolate.

the previously described CSA-binding *FCR3varCSA* [5] (figure 1*A*). The N-terminal domains DBL1α to DBL2β are particularly well conserved (89%–96% aa identity), whereas DBL3γ, the CSA-binding domain in FCR3varCSA [5], is the most variable region (figure 1*A*). By comparison, the PfEMP1 variants encoded by 19 different *var* genes [11] in GenBank, compared with FCR3varCSA, show 36%–52% aa identity for DBL1α (usually the most well-conserved PfEMP1 domain [7]) and 16%–40% aa identity for CIDR.

To determine whether *FCR3varCSA* homologues are found in natural *P. falciparum* parasites from diverse geographic locations, we designed oligonucleotide primers specific for the DBL1α/CIDR region of *FCR3varCSA* (figure 1*A, arrows*). For comparison, primers were designed to the same region of 3 unrelated *var* genes, *3D7-varT3-2* (see Materials and Methods), *R29var1* (GenBank Y13402), and a second CSA-binding PfEMP1 variant, *CS2 var* [6]. The primers were used in PCR with genomic DNA from genotypically distinct laboratory clones from Brazil (7G8), Honduras (HB3), and Thailand (T9/96) and randomly chosen field isolates from Sudan, Mali, Kenya, Vietnam, Thailand, and Vanuatu.

As shown in figure 1*B,* with primers to 1 of the controls, *3D7-varT3-2,* a major 1.7-kb product, was detected only in clone 3D7, the parasite from which the *3D7-var-T3* sequence is derived. This result is as expected because *P. falciparum* isolates are thought to have minimally overlapping *var* gene repertoires [7, 9]. Faint PCR products were also seen in 2 field isolates (M58 and TM4C8), possibly indicating the presence of related genes in these isolates. Similarly, primers to *R29var1* and *CS2var* were each only positive in just 2 of 25 isolates tested (data not shown). In contrast, primers to *FCR3varCSA* yielded 1.7-kb PCR products in all parasites, except for the laboratory clone 3D7 and 1 field isolate from Vietnam (figure 1*B*). 3D7 is the parasite clone sequenced in the *P. falciparum* genome project, and a BLAST search of the Malaria Genome Project Database (http://PlasmoDB.org/) confirmed that 3D7 does not have a highly conserved homologue to the 5′ end of *FCR3varCSA*. The closest match was the 3D7 chromosome 5 sequence (chr5_P30000004_glm5, hereafter referred to as *3D7chr5var*), which encodes a putative protein that is 62% identical to FCR3varCSA overall (figure 1*A*). This *3D7chr5var* gene encodes a PfEMP1 variant that is divergent from FCR3varCSA from DBL1α to DBL4ϵ but matches relatively closely from DBL5γ to the end of DBL7ϵ (figure 1*A*). The *3D7chr5var* sequence is truncated at the end of DBL7ϵ and does not contain the expected *var* gene intron and exon II coding sequence, but instead runs into the telomere repeat sequence. It would not therefore be expected to encode a functional protein

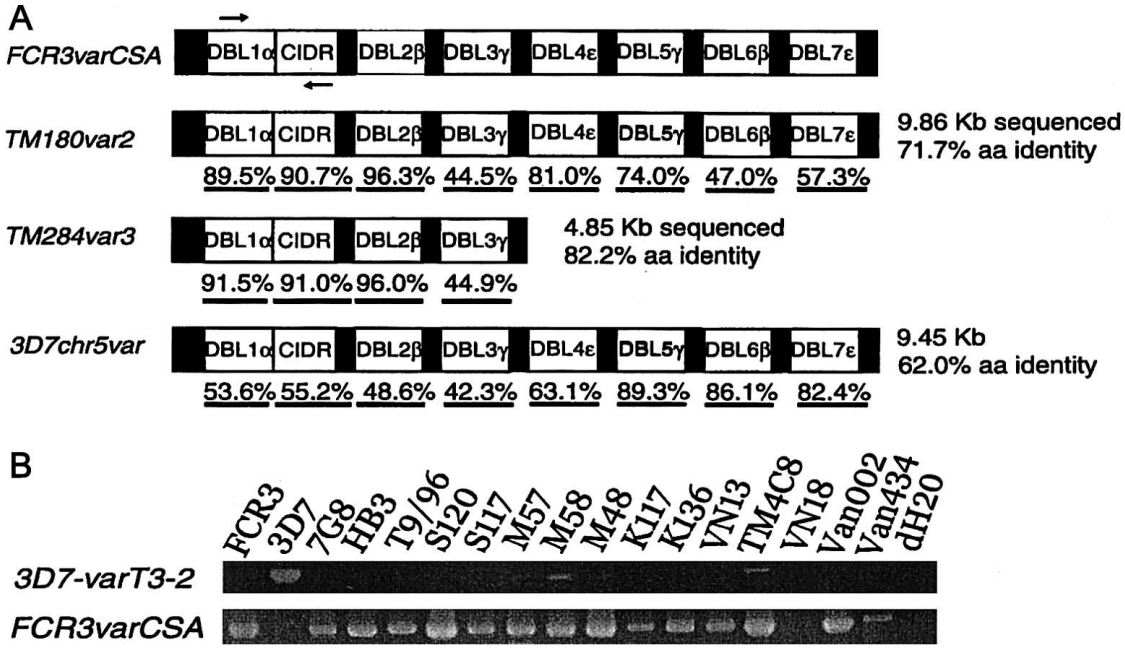

Figure 1. Identification of highly conserved homologues of *FCR3varCSA* in *Plasmodium falciparum* laboratory lines and field isolates. *A,* Comparison of predicted partial amino acid sequence encoded by *TM180var2* (GenBank accession no. AJ4200411) and *TM284var3* (GenBank AJ420412) with FCR3varCSA. Entire sequence of exon 1 of *TM180var2* is shown; *TM284var3* is only partially sequenced. The most similar *var* gene from parasite clone 3D7 sequenced by the Malaria Genome Project Consortium is shown for comparison. Amino acid identity for each domain [11] vs. FCR3varCSA is shown, and the total length of the sequence (in kb) is indicated. Arrows indicate site of oligonucleotide primers used in panel *B. B,* Polymerase chain reaction amplification of genomic DNA from laboratory clones (FCR3, 3D7, 7G8, HB3, and T9/96) and field isolates from around the world (S, Sudan; M, Mali; K, Kenya; VN, Vietnam; TM, Thailand; Van, Vanuatu) with primers to *3D7-varT3-2* and *FCR3varCSA*. CIDR, cysteine-rich interdomain region; DBL, Duffy binding-like region.

in 3D7 parasites. The lack of a 3D7 homologue to the 5′ end of *FCR3varCSA* means that 3D7 provides a useful negative control in figure 1*B* and indicates that the PCR products from other parasites are specific and not due to PCR contamination.

Genomic DNA samples from a further 19 genetically distinct Sudanese field isolates were studied, and 17 gave a PCR product with *FCR3varCSA* primers, whereas none gave a PCR product with *3D7-varT3-2* primers (data not shown). The PCR products from figure 1*B* were sequenced and compared with *FCR3varCSA*. We found 89.7%–99.8% nt sequence identity and 86.0%–99.8% aa sequence identity (the alignment of these sequences is shown in an Appendix published only in the electronic edition of the *Journal* [http://www.journals.uchicago.edu/JID/] or is available from the authors).

To confirm the existence of *FCR3varCSA* homologues in other parasites, genomic DNA from 6 independent laboratory clones/lines (T9/96, FCR3, HB3, TM180, TM284, and 3D7) was analyzed by Southern hybridization with *FCR3varCSA*-specific probes. All parasites apart from 3D7 were positive with probes from the *FCR3varCSA* N-terminal domains (data not shown), whereas 3D7 was positive for DBL5γ, DBL6β, and DBL7ϵ (data not shown), as expected from the existence of a 3D7 *var* gene with high homology to these regions (figure 1*A*).

To determine whether the *FCR3varCSA* homologues have CSA binding functions despite the variability in the DBL3γ domain, we selected TM180 and TM284 parasites for CSA binding by panning [6, 13] and then examined the *var* genes expressed by the selected parasites. The predominant *var* genes expressed by TM180 and TM284 selected on CSA were not *TM180var2* and *TM284var 3,* respectively (data not shown), suggesting that genes with homology to the 5′ end of *FCR3varCSA* are not always functionally linked to the ability to bind CSA.

To determine whether *FCR3varCSA*-like genes play a role in malaria in pregnancy, we studied the *var* genes expressed by placental parasites from primigravid women in Malawi. RT-PCR was done by use of degenerate primers to DBL1α [7], and the placental isolates were genotyped to estimate the minimum number of clones present in each infection [12]. Only DBL1α was studied, because universal, unbiased primers that give a true indication of the diversity of *var* genes being expressed are available for DBL1α only and not for other *var* domains [7]. Table 1 shows that a complex mixture of infecting parasite genotypes was obtained, and a number of different *var* genes were expressed by each isolate. All the *var* genes detected were different apart from those shown in bold, which are discussed below. None of the commonly expressed *var* genes detected by RT-PCR were *FCR3varCSA* homologues, although the *FCR3varCSA* homologues could be detected by PCR of genomic DNA from the placental isolates (data not shown). These results do not support the hypothesis that genes with homology to the 5′ end of *FCR3varCSA* play a role in placental infection.

Of interest, 4 of the 5 placental isolates expressed a gene *(P132var5, P136var1, P154var1,* and *CS294var2)* that has also been reported in GenBank from 3 independent isolates (clone 3D7 sequence AFBR4, accession no. AF133860; clone 1776 type b, accession no. AF084578; and Kenyan field isolate 2 type 2B5, accession no. AF221823). The 3D7 AFBR4 sequence was cross-referenced against the Malaria Genome Project Database to derive the full-length sequence, and this gene was found to be *3D7chr5var,* which, as noted, although different from *FCR3varCSA* at the 5′ end has close homology at the 3′ end of the gene encoding DBL5γ to DBL7ϵ (figure 1*A*). Therefore, although *var* genes with homology to the 5′ end of *FCR3varCSA* are not commonly expressed in placental isolates, it appears that a number of related genes, as typified by the *3D7chr5var* sequence, may be common in placental parasites.

Discussion

The above data show that well-conserved homologues of the gene *FCR3varCSA* occur in the majority of *P. falciparum* isolates worldwide and suggest that a second gene homologous to *3D7chr5var* also occurs in many isolates. These 2 genes are related because, although their 5′ ends are distinct, they share sequence homology further downstream. Previous work has emphasized the extensive diversity in *var*/PfEMP1 [7–9, 11, 14]. We believe that the work described here is the first to illustrate the occurrence of sequence conservation in the PfEMP1 family.

The existence of PfEMP1 variants that are relatively conserved in different *P. falciparum* isolates may explain how it is possible for the parasite CSA-binding ligand implicated in malaria in pregnancy to be antigenically conserved yet also to be encoded by a *var* gene. Our results do not, however, prove the existence of a conserved *var* gene expressed by parasites infecting the placenta but merely provide a first glimpse at the range of *var* genes expressed by placental isolates.

Previous work on immune responses to variant surface antigens has demonstrated the existence of rare and prevalent PfEMP1 variants in field isolates, with the commonly recognized variants being associated with severe disease [14]. It seems possible that the proteins encoded by *FCR3varCSA* and its homologues, because they occur in many different parasite isolates, could be examples of commonly recognized PfEMP1 variants, although this cannot be confirmed without further experimental work.

It remains unclear why some *var* genes should be well conserved when most are thought to undergo extensive recombination and rearrangement [8, 9]. One consideration is gene location. Many *var* genes are subtelomeric, whereas some occur centrally on chromosomes 4, 7, and 12 [15]. It has been suggested that the subtelomeric location encourages recombination and the generation of diversity [9, 15], and it might therefore be expected that the central *var* genes would undergo less recombination and be more highly conserved than the subtelomeric ones. *FCR3varCSA* is a subtelomeric gene on chromosome 10 [13], a finding we confirmed by pulsed-field gel electrophoresis and Southern hybridization (data not shown). The location of *FCR3varCSA* does

not therefore explain its high degree of sequence conservation. Another possibility concerns the orientation of the gene. Preliminary mapping studies show that most subtelomeric *var* genes point toward the centromere; however, one of us (S.A.K., unpublished data) found an example of a *var* gene pointing in the opposite orientation toward the telomere. Any such inverted *var* gene would be unlikely to undergo ectopic recombination with the majority of *var* genes that point toward the centromere, and, therefore, such a gene might be relatively well conserved. The orientation of the *FCR3varCSA* gene is unknown at present, but the truncated *3D7chr5var* points toward the telomere. Further possibilities to explain the well-conserved nature of the *FCR3varCSA* homologues are that they have an essential function unrelated to malaria in pregnancy that is important for parasite survival or transmission or that they are rarely expressed in the human host and are therefore under less immune selection for diversity than other *var* genes.

Our findings are based on a relatively small number of isolates and are therefore preliminary. Clearly, further investigations are required to determine the exact function of the *FCR3varCSA* and *3D7chr5var* homologues and their relationship to placental malaria. We can conclude from this work, however, that initial assumptions of a minimal overlap between *var* gene repertoires from different parasite isolates are probably an oversimplification and that some degree of overlap occurs. This has major implications for the development of PfEMP1-based vaccines against malaria; however, detailed studies of *var* gene sequence and function in diverse parasite isolates will be essential before rational progress toward a vaccine can be made.

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