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Analysis of *var* genes cloned from a *Plasmodium falciparum* isolate in China

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ABSTRACT

Objective: To analyse the *var* gene repertoire and characterise the chondroitin sulphate A (CSA)-binding activity of the Duffy-binding like (DBL) domains encoded by the *var2csa* gene of a *Plasmodium falciparum* (*P. falciparum*) isolate in Hainan Province, China. **Methods:** The sequences of *var* DBL1 regions were PCR-amplified, sequenced and the sequence characteristics was bioinformatically analysed. Recombinant proteins encoded by the *var2csa* genes were expressed and purified. The binding activities of the recombinant proteins to CSA receptor was detected by ELISA assays. **Results:** Fifty six unique DBL α sequences were obtained, and the sequences represented similar diversity to the *var* genes of the genome parasite 3D7. There are two *var2csa* genes in the *P. falciparum* isolated from Hainan Province. Unlike in other falciparum parasites such as HB3, the two *var2csa* genes are more diverged. The receptor-binding capacity of DBL-5 ε and DBL-6 ε domains of HN var2CSA was studied. **Conclusions:** This work represented the diversity of *var* genes of a *P. falciparum* isolate in China.

1. Introduction

Among the four species of *Plasmodium* parasite, *Plasmodium vivax*, *Plasmodium falciparum* (*P. falciparum*), *Plasmodium malariae*, *Plasmodium ovale* infecting humans, *P. falciparum* is the most prevalent and responsible for the high morbidity and mortality after infection[1]. The virulence of the parasites is associated with their ability of the infected erythrocytes (IE) to adhere host endothelial receptors, which leads to the sequestration of IE in the micro-vasculatures[2-9]. IE adhered to syncytiotrophoblast receptors in the placenta can cause severe malaria during pregnancy, named pregnancy-associated malaria (PAM), which contributes to premature delivery, stillbirth, low birth weight neonates, maternal anaemia, increased maternal mortality[10-12]. The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is a molecule expressed at the IE surface during mature stage of the parasite. PfEMP1 molecules are encoded by a *var* multigene family[13-16]. Each parasite genome has approximately 60 different *var* genes, but only one PfEMP1 is expressed on the surface of an IE at a time[17]. Each PfEMP1 contains several adhesion domains, thus as Duffy binding like (DBL) domain: α, β, γ, δ and ε) and cysteine- rich interdomain region (CIDR) (α, β and γ)[18], a transmembrane domain and an intracellular acidic region. The DBL1 α domains are relatively conserved among different DBL domains, which have been used as a marker to study the diversity of *var*/PfEMP1 gene family[19-24].

The var2 chondroitin sulphate A (CSA) PfEMP1, coded by the *var2csa* gene, is different from other PfEMP1s with a unique domain architecture which is composed of six DBLs (DBL1-X, DBL2-X, DBL3-X, DBL4- ε, DBL5- ε and DBL6- ε) in the extra cellular part of the molecule. Studies indicated that var2CSA PfEMP1 played an important role in PAM[10], which has been suggested as a leading candidate for

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a pregnancy malaria vaccine[25]. *Var2csa* gene homologues are found in all parasite isolates[26] and to be up-regulated in both placental isolates and laboratory parasites that bind to CSA[12,27,28]. One *P. falciparum* genome can have one or several copies of *var2csa* genes[29]. Interestingly, two *var2csa* genes can be expressed simultaneously in the same parasites[30]. In addition, IEs of *P. falciparum* isolates that infect the human placenta were found to bind non-immune IgG which could bridge the IEs to Fc-receptors in the placenta[31]. The DBL2-X, DBL3-X, DBL-5 ε and DBL-6 ε domains of 3D7 var2CSA were able to bind CSA[32].

In this study, we analysed the *var* gene diversity of a *P. falciparum* Hainan (HN) isolate by examining their DBL1 α sequences. We further found that there were two copies of *var2csa* genes in the genome of the parasite and the full-length sequences of the two copies were obtained. The capacity of receptor-binding of the DBL domains was investigated.

2. Materials and methods

2.1. Parasite

The *P. falciparum* HN isolate was collected from a patient living in Hainan Province, China. The clonality of the parasite was determined by PCR. The genomic DNA was obtained from the parasite using AxyPrep Multisource Genomic DNA Miniprep Kit (Invitrogen, CA, USA) according to the protocol of manufacturer.

2.2. PCR amplification of the gene fragment encoding DBL1 α domains

The DBL-1 α fragments of *var* genes of the parasite were amplified with the α AF [5′-gcacg (a/c) agttttgc-3′] and α BR [5′- gcccattc (g/c) tcgaacca-3′] primers as described[33]. The PCR product was cloned into TOPO plasmid (Invitrogen, CA, USA) for sequencing.

To clone the *var2csa* sequences of HN isolate, conserved regions of *var2csa* gene were determined after multiple alignments with all *var2csa* genes available and nine pairs of primers were designed (Table 1) and the corresponding fragments of HN *var2csa* gene were amplified. The rest of the sequences of the two *var2csa* genes was cloned and sequenced by chromosome walking.

The DBL-5 ε and DBL-6 ε domains of the *var2csa* genes of 3D7 and HN parasites were amplified from gDNA using the specific primers (Table 2) according to standard protocols.

2.3. Cloning and sequence analysis

PCR products of *var* DBL-1 α and *var2csa* fragments were cloned into pMD-18-T vector (Takara, Dalian, China). The ligation mixture was transformed into *Escherichia coli* strain DH5 α. The transformed bacteria were selected on LB agar plates containing ampicillin (100 μg/mL). Recombinant plasmids were purified and sequenced using M13 primers. Alignment of the deduced amino acid sequence with the published PfEMP1 sequences in NCBI (www.ncbi.nlm.nih.gov/) for sequences identification. 56 DBL-1 α sequences of HN *var* genes were aligned with that of the 3D7 strain and a phylogenetic tree was constructed using MAGE 4.0.

2.4. Expression of recombinant GST-tagged DBL5-α/DBL6-α fusion proteins

The PCR products of DBL-5 ε and DBL-6 ε were digested with *Bam*H Ⅰ and *Xho* Ⅰ or *Eco*R Ⅰ and *Xho* Ⅰ restriction endonucleases depending on the adaptor sequences at the end of the amplicons. The fragments were ligated with pGEX-4T-1 plasmid treated with corresponding enzymes. The ligation mixtures were transformed in *Escherichia* coli strain BL21. Expression of the four fusion proteins were induced with 0.1 mM isopropy- β -D-thiogalactoside (IPTG) at 25 ℃ for 5 h and the fusion proteins were purified by affinity chromatography on glutathione Sepharose 4B columns (GE Biosystems, Uppsala, Sweden) according to the instructions provided by manufacturer. GST produced by the empty vector was used as control. Proteins purified were verified by SDS-PAGE and Western-blot using the mAb recognizing GST tag as described by Jiang *et al*[34].

2.5. Binding of DBL5- ε and DBL6- ε with CSA in enzyme-linked immunosorbent assay (ELISA)

ELISA plates (Nalge Nunc International, IL, USA) were coated overnight at 4 ℃ with 50 μL of CSA (10 μg/mL) in coating buffer (pH 9.6). Plates were washed three times with phosphate buffered saline with 0.5% Tween 20, blocked with 200 μL of blocking buffer (5% bovine serum albumin) per well for 2 h at 37 ℃. Recombinant proteins diluted from 200 to 6.25 μg/mL were added to the wells and incubated for 1 h at 37 ℃. After washing, an anti-GST mAb diluted 1:4 000 in 0.5% BSA was added in the well and incubated for 1 h at 37 ℃. The reaction was detected by an alkaline phosphatase conjugated goat anti-mouse antibody.

3. Results

3.1. Var DBL α domains of P. falciparum HN isolate

The 5′region of *var* genes (350 bp-450 bp) coding for the DBL1 α domain was amplified from gnomic DNA of the HN *P. falciparum* isolate with α AF and α BR primers[33]. The

amplified fragments (350–450 bp) (Figure 1) were cloned and sequenced. 56 different sequences of DBL1 α type (GenBank accession numbers were FJ748609–FJ748664) were obtained from 350 clones. Phylogenetic analysis of all sequences together with the *var* DBL1 α of *P. falciparum* 3D7 strain showed that *var* genes of these two parasites diverged similarly with only two sequences were similar between the two families. This reflected the data obtained with parasites isolated in Africa that the *var* gene family of *P. falcuparum*.

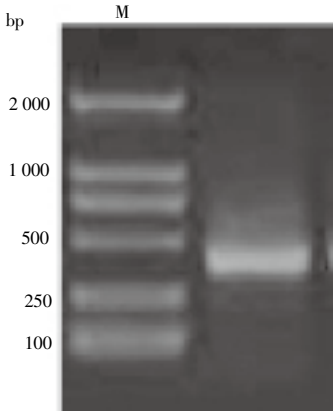

Figure 1. The PCR product of DBL α gene fragments.
The amplified fragments are with molecular sizes between 350 and 500 bp.

3.2. *var2csa* gene of *P. falciparum* HN isolate

The *var2csa* gene of *P. falciparum* HN isolate was also investigated in this study. Two copies (named *var2cas*–HN1 and *var2csa*–HN2) of *var2csa* genes were identified. The length of the two copies was 9 303 and 9 318 bp respectively, with a homology of 87.11% in nucleotide composition. Phylogenetic analysis on the sequences of the two copies with other *var2csa* genes showed that *var2cas*–HN1 and *var2csa*–HN2 were closer to 3D7 and DD2 *var2cas* variants (Figure 2 and Table 3).

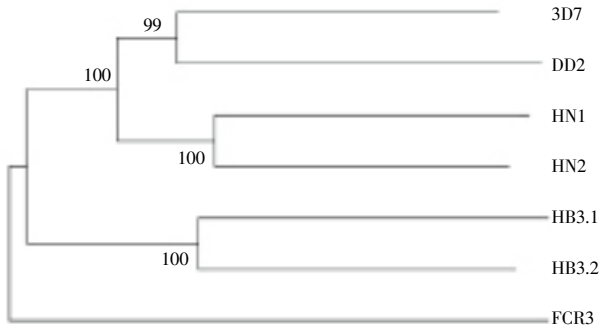

Figure 2. Phylogenetic analysis of the two var2CSA sequences of HN isolates and those of other *P. falciparum* strains.
The two var2CSA sequences were more related than to other homologous members. Numbers indicate distance from the origin.

Table 1
PCR primers for cloning *var2csa* sequences.

Name	Primer sequence
var2csa 1F	5′–tattgctgacaaaattgaagcata–3′
var2csa 1R	5′–gtaataacttcccacacctttt–3′
var2csa 2F	5′–aatagacaaaaggtgtgggaa–3′
var2csa 2R	5′–cattatcccatatacttgtacct–3′
var2csa 3F	5′–aggtacaagtatatgggataatg–3′
var2csa 3R	5′–ttcatatattcttttctatcatca–3′
var2csa 4F	5′–tgatgatagaaaagaatatatgaa–3′
var2csa 4R	5′–ttgctgtacctttatcgtgg–3′
var2csa 5F	5′–ccacgataaaggtacagc–3′
var2csa 5R	5′–gttcataaagacataattgttgcc–3′
var2csa 6F	5′–ggcaacaattatgtctttatg–3′
var2csa 6R	5′–gttcattaaatctagaggtcctt–3′
var2csa 7F	5′–aaggacctctagatttaatgaac–3′
var2csa 7R	5′–cagtccattcttgaaaccatc–3′
var2csa 8F	5′–gatggtttcaagaatggact–3′
var2csa 8R	5′–gtcatctccactagtatctccttcc–3′
var2csa 9F	5′–ggaaggagatactagtggagatgac–3′
var2csa 9R	5′–caacaattgtacgaattttccat–3′

Table 2

PCR primers for cloning the DBL-5 ε and DBL-6 ε domains in the expression vector.

Name	Primer sequence
3D7-DBL5F	5′-ggatccggagatgctataggatgtaaac-3′(*Bam*H Ⅰ)
3D7-DBL5R	5′-ctcgagtccacacggacatttagaacaat-3′(*Xho* Ⅰ)
3D7-DBL6F	5′-ggatccataaacgttaatat-3′(*Bam*H Ⅰ)
3D7-DBL6R	5′-ctcgaggcacatacaattatt-3′(*Xho* Ⅰ)
HN2-DBL5F	5′-ggatccggagatgctataggatgtaaac-3′(*Bam*H Ⅰ)
HN2-DBL5R	5′-ctcgagtccacacggacatttagaacaat-3′(*Xho* Ⅰ)
HN2-DBL6F	5′-gaattcttatattctcgtatgcaacataat -3′(*Eco*R Ⅰ)
HN2-DBL6R	5′-ctcgaggcaatcacatttacctttaag-3′(*Xho* Ⅰ)

Table 3

Sequence similarities in both nucleotides and amino acids (bracketed) between *var2csa*-HN1, var2csa-HN2 and other *var2csa* variants.

	HN1	HN2	3D7	FCR3	HB3-1	HB3-2	DD2
HN1		87.11(76.83)	75.67(76.22)	64.74(67.24)	59.72(66.54)	66.26(67.59)	60.83(63.84)
HN2			64.64(65.88)	58.50(65.16)	60.30(62.20)	59.48(53.27)	59.22(58.02)

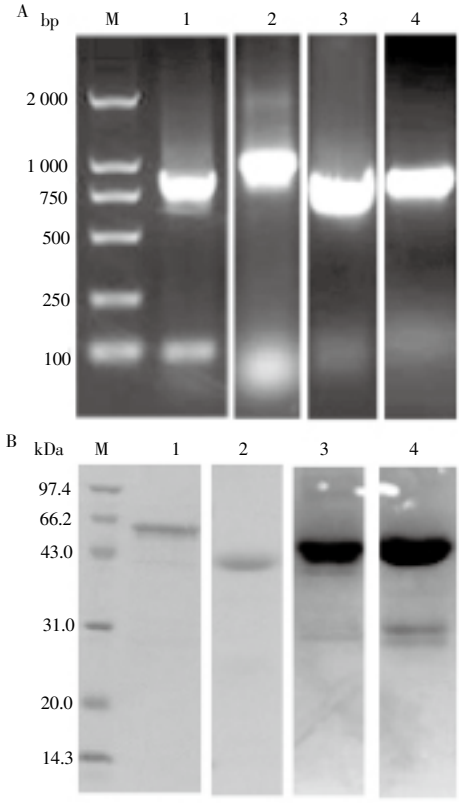

Figure 3. Cloning and expression of the DBL-5 ε and DBL-6 ε domains.

A. PCR amplification of DBL-5 ε and DBL-6 ε domains. 1 and 3 are DBL-5 ε of 3D7 strain and HN isolate; 2 and 4 are DBL-6 ε of 3D7 strain and HN isolate. B. SDS-PAGE analysis of the GST-DBL fusion proteins purified on glutathione sepharose. 1 and 3 are GST-3D7-DBL-5 ε and GST-HN2-DBL5- ε . 2 and 4 are GST-3D7-DBL-6 ε and GST-HN2-DBL-6 ε .

3.3. Cloning, expression and purification of recombinant DBL domains

In order to characterize the receptor-binding property of the two VAR2CSA proteins of HN isolate, gene fragments encoding the DBL-5 ε and DBL-6 ε domains were cloned in expression vectors and recombinant proteins were generated with that of 3D7 VAR2CSA domains as a positive control (Figure 3A, B). The DBL domains of VAR2CSA-HN2 were relatively easy to be generated than that of 3D7 (Figure 3B). The recombinant proteins of VAR2CSA HN1 were insoluble, which were not further analyzed (data not shown).

3.4. VAR2CSA DBL-5 ε of VAR2CSA-HN2 adhered to CSA

In this study, the binding of DBL-5 ε and DBL-6 ε domains of the VAR2CSAs of 3D7 strain and HN isolate with CSA was investigated. The results showed that both DBL-5 ε and DBL-6 ε domains of the two VAR2CSA variants could adhere CSA receptor (Figure 4).

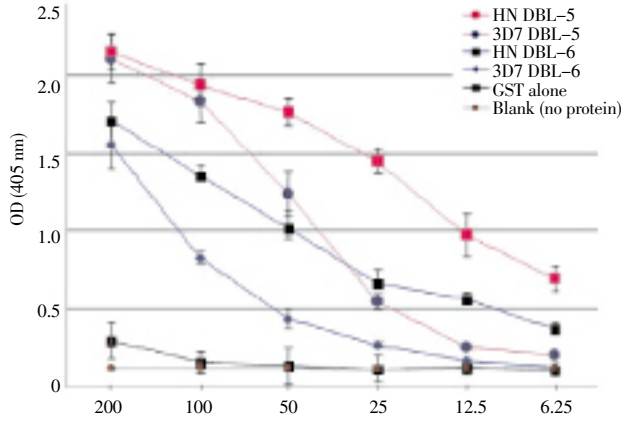

Figure 4. Binding of DBL-5 ε and DBL-6 ε to CSA in ELISA.

Recombinant GST fusion proteins of DBL-5 ε and DBL-6 ε domains from 3D7 strain and HN isolate were tested for their binding activity to CSA. The DBL-5 ε domains of both 3D7 and HN parasite showed higher affinity to CSA than the DBL-6 ε domains from the two parasites. GST did not show any binding to CSA.

4. Discussion

The diversity of the *var* gene family coding for PfEMP1 features the parasite with a capability of unlimited antigenic variation. In this study, we invested the *var* repertoire in a parasite isolated in Hainan Province. The result indicated that the isolate has a similar *var* repertoire as other *P. faciparum*. Interestingly, the parasite contains two copies of *var2csa* genes which has been diverged quite extensively compared to that of the HB3 parasite[30]. Further, analysis of the *var2csa* sequences found that the sequence variation was predominantly located in DBL-2X and DBL-6 ε domains (data not shown), indicating the two domains were under more immuno-pressure than other domain. Recent studies suggested that immuno-recognition of VAR2CSA DBL-5 ε domain was associated with protection of PAM[35]. It has been generally believed that VAR2CSA protein mediated adhesion to the CSA receptor on the syncytiotrophoblast in placenta[10,12,36-43]. Studies earlier have demonstrated that several DBL domains of VAR2CSA potentially mediated adhesion to CSA receptor[27]. Available data pointed to the DBL-5 ε domain which might play a critical role in binding to CSA receptor[44,45]. But the affinity of DBL-5 ε domains to CSA was significantly higher than that of DBL-6 ε domains. The fact that two DBL-5 ε variants showed higher affinity to the same receptor argued the importance of this domain in parasite sequestration in placenta and the domain-specific immunity in resistance against PAM[35,45]. In conclusion, the *var* gene repertoire of a HN isolate in China has been investigated in this study. The data indicated that the *var* gene family of the parasite had similar features as parasites isolated in other malaria endemic areas. The *var2csa* genes were sequenced in full-length and the binding capacity to CSA receptor was investigated. Results suggested that the DBL-5 ε domain may have high affinity to CSA receptor and play important role in parasite sequestration in placenta.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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