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Transcribed var Genes Associated with Placental Malaria in Malawian Women†

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Malaria is more common in pregnant than nonpregnant women and predisposes those infected to maternal and infant death (37). The placenta provides a unique site for infected erythrocytes (IE) to be sequestered through adhesion to receptors, including chondroitin sulfate A (CSA) and hyaluronic acid (3, 14). IE from nonpregnant individuals rarely adhere to CSA and hyaluronic acid (3). The restricted adhesion phenotype of parasites isolated from placentas is associated with the expression of a restricted repertoire of parasite variant surface antigens (VSAs). Consequently, with increasing parity, women acquire antibodies that bind to parasites isolated from their own and other placentas (1, 16). These antibodies are associated with protection (11, 16, 36), and some are capable of blocking IE adhesion to CSA (2, 16, 39). There has been a need for candidate CSA binding parasite ligands to be identified. A more promising candidate CSA binding parasite ligand is the VAR2CSA PfEMP1 identified by Salanti et al. (36). The most abundant var2csa transcript in multiple allogeneic CSA-adherent parasites is VAR2CSA var2csa (9, 12, 35), these parasites are antigenically cross-reactive (12), and evidence of parity-dependent antibody binding to defined, CSA-adherent laboratory isolates is currently restricted to parasites that express var2csa (2, 35). However, the identity of the conserved, CSA-binding parasite antigen remains unresolved because parity-dependent antibodies bind multiple recombinant proteins derived from both domains of VAR2CSA (33) and the DBLγ domain of at least one other PfEMP1 (5, 7). Furthermore, DBLγ domains present in multiple PfEMP1 molecules and multiple domains of VAR2CSA all bind to CSA (6, 18, 30).

Previous nonquantitative studies of the var genes expressed by placental isolates have either used degenerate primers designed to amplify multiple DBLγ domains or specific primers to amplify DBL domains from some of the three var genes identified as candidate CSA adhesion ligands (15, 22, 23, 32). The amplification of multiple sequences and lack of quantita-
tive data prevented the detection of a clear association between a specific var gene and placental sequestration. These studies also did not detect var2csa because it lacks the DBLα and DBLγ domains for which the degenerate primers were designed. Quantitative analyses of var transcription in placental isolates have been restricted to comparisons of var2csa and FCR3.varCSA (35, 40). These studies were suggestive of a major role for var2csa in malaria during pregnancy and excluded a similar role for FCR3.varCSA but did not address the potential roles of other var genes possessing DBLγ domains that may also have been transcribed at high levels in placental isolates. We used degenerate primers capable of amplifying both DBLγ domains and var2csa DBL3x domains to amplify a range of transcribed var genes from parasites isolated from placentas and from the peripheral blood of children. We then used quantitative reverse transcription (Q-RT-PCR) on cDNAs from the parasite isolates to determine the levels of multiple var gene transcripts, including DBLγ sequences, that had been amplified from the cognate cDNAs.

MATERIALS AND METHODS

Parasites. Informed consent was obtained from all patients (or for children) their parents or guardians for participation in the study. The study was approved by the College of Medicine Research and Ethics Committee, University of Malawi, and the Royal Melbourne Hospital Clinical Research Ethics Committee. P. falciparum-infected blood was obtained from placental biopsies from 19 women, as previously described (3), and from peripheral veins of six children admitted with severe malaria at the Queen Elizabeth Central Hospital in Blantyre, Malawi (31). Placental and peripheral blood samples were washed, and the pellets were solubilized at a 1-in-10 dilution in Trizol (Invitrogen, Carlsbad, CA) and stored frozen in liquid nitrogen. The Mplc series of placental isolates was collected from 1997 to 1999 (32), while the Mpc placental samples and the Mch children’s samples were collected from 1999 to 2001 (31).

DNA and total RNA preparation. Genomic DNA was isolated from 100 μl of packed erythrocytes by using a QIAmp DNA mini blood kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. For RT-PCR, RNAs were extracted from infected erythrocyte pellets solubilized in Trizol as described previously (9). RNAs were treated at 37°C with DNase I (4 U; Ambion, Austin, TX) in the presence of total RNA carrier (Sigma, Sydney, Australia) and with 40 U of RNasin RNase inhibitor (Promega, Madison, WI).

PCR and RT-PCR assays. Reverse transcription was performed as previously described (9). Reverse transcriptase negative controls were used for each sample. DBLγ and var2csa DBL3x sequences of approximately 600 bp were amplified from cDNAs and genomic DNA (gDNA) from children’s and Mplc series placental samples by PCRs using the degenerate oligonucleotides D3F (CCATCKIARAAATTGIGGTYT) and D3R2 (CCATTCGGTTAGCCATCGTAAA) (forward, AGGAATCACGAGCAACAAC; and reverse, TTCATATCATCAATCGTTGTAATCTCTTCTTTT). The use of a semi-nested PCR master mix (PE Biosystems) in a 10-μl reaction mix that was amplified using the ABI Prism 7900HT sequence detection system. PCR master mixes were incubated at 95°C for 10 min and then subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. A final incubation of 95°C for 2 min was followed by a dissociation step at 60°C for 2 min, with a 2% ramp rate to 95°C for 2 min. The specificity of each primer pair was determined by dissociation curve analysis per the manufacturer’s instructions. The primers used to amplify var2csa DBL3x (forward, TGTCATG CGTTCAGAAAGGTTTATT; and reverse, TGGTACCCAAAATCATATTAT CATTTATACT) were designed using an alignment of the 3’D and 17g var2csa sequences and the 24 var2csa sequences amplified in this study. Nucleotide 16 of the DBL3x reverse primer had a C-for-T mismatch with Mplc22.5, and nucleotide 2 had a T-for-G mismatch with Rplc132c, Rplc132l, Rplc143d, and Rplc143e. However, var2csa DBL3x sequences with 100% identity to the var2csa DBL3x forward and reverse primers were also amplified from the same isolates. Nucleotide 11 of the DBL3x forward primer had a C-for-T mismatch with the only var2csa sequence amplified from Mpfcl78. Consequently, for quantitation of var2csa in Mpfcl78 cDNA, the DBL3x forward primer was replaced with TGGTACCCAAAATCATATTAT CATTTATACT. The quantities in each cDNA of the skeleton binding protein 1 gene (SBP) (PFE_005w) (forward, TTGGAGGAATTTGCCAAACG; and reverse, TTGGGTTGTCTCTGGTAC TGCA) and a hypothetical protein gene (PF11_0050) (forward, TCTGAAAGCCAAAACACA; and reverse, AGGGCTTGGAAAACACCTT) were determined by Q-RT-PCR, using standard curves of diluted 3D7 isolate gDNA. SBP and PF11_0050 levels were used to normalize the quantitative var2csa gDNA data. Other primers used are shown in Table 1.

Sequence analysis. Sequence analysis was performed using BioEdit (21). Clustal W multiple sequence alignments with 100 bootstrap replications were created to use protein distance matrices and phylogenetic trees by the neighbor-joining method (13) available at http://evolution.genetics.washington.edu/phylip .html and by using TreeView (28), available at http://taxonomy.zoology.gla.ac.uk /rod/treeview.html.

Nucleotide sequence accession numbers. The GenBank accession numbers for all of the sequences reported in this study are AY461586 to AY461594, AY464699, AY464700, AY466633 to AY466637, AY466369 to AY466372 AY488868 to AY488880, AY534318, AY534319, DQ286586 to DQ286636, and DQ306266 to DQ306307.

RESULTS

The ability of the degenerate oligonucleotides D3F and D3R1 (Fig. 1a) to amplify both DBLγ and var2csa DBL3x sequences was tested by cloning products amplified from 3D7 genomic DNA. Forty clones were sequenced, yielding 9 of the 14 published 3D7 DBLγ sequences (26) and the var2csa DBL3x sequence. After 40 cycles of amplification, the most frequently amplified sequence (PFD0005w) was detected at 1.5 times the expected frequency using a binomial distribution, as previously described (38) (Fig. 1b). Using the analysis of Taylor et al. (39), these degenerate primers showed a low bias towards amplification of the most frequently detected sequence of <2% (since 1.0240 is >1.5), which is consistent with those of other “universal” DBL domain degenerate primers (8, 24, 38). Nevertheless, the bias may prevent the amplification of some DBLγ sequences. The var2csa DBL3x sequence was amplified at a moderate frequency (Fig. 1b).

The oligonucleotides D3F and D3R1/D3R1.2 were then used to amplify DBLγ and var2csa DBL3x sequences from the cDNAs of 10 placental isolates and 4 isolates from the peripheral blood of children and from the gDNAs of 4 placental
isolates and 1 child's isolate (Table 1, sample group A). No products could be amplified from the cDNAs of a further nine placental and two children's isolates (Table 1, sample group B). The PCR products were cloned and sequenced (Table 1, sample group A). A range of sequences were amplified from the cDNAs of most isolates, but there was an obvious pattern and a detectable first-round product had been amplified using D3R1 (Table 1, sample group A) and on the placental isolates Mplc21 and Mplc22, from which only var2csa sequences had been amplified from the cDNAs of all 10 placental isolates but not from the four children's isolates and nine placental isolates from which no detectable first-round product had been amplified using D3R1.

We wished to extend this analysis beyond previous studies by first determining the endogenously transcribed DBLγ var sequence(s) in each isolate and then quantitating the level of such a sequence in the cDNA of its cognate isolate to compare with the levels of several var sequences that have been implicated in adhesion to CSA in laboratory studies, including var2csa DBL3x. To do this, we needed to clone DBLγ sequences from the isolates. Therefore, we used the degenerate reverse primer D3R2, which could amplify only DBLγ sequences (Fig. 1), in a second round of RT-PCR amplification. The second-round RT-PCR was performed on the two children's isolates and nine placental isolates from which no detectable first-round product had been amplified using D3R1 (Table 1, sample group B) and on the placental isolates Mplc21 and Mplc22, from which only var2csa sequences had been amplified using D3R1.

The second-round primer D3R2 amplified DBLγ sequences from the cDNAs of both children's isolates and six of the nine placental isolates (Table 1, sample group B) but not from the cDNAs of Mplc21 and Mplc22. The second-round primer D3R2 amplified DBLγ sequences from the cDNAs of both children's isolates and six of the nine placental isolates (Table 1, sample group B) but not from the cDNAs of Mplc21 and Mplc22. Combining the results from all degenerate primer PCRs, DBLγ sequences were amplified from the cDNAs of 14 of the 19 placental isolates and all six of the children's isolates (Table 1). There was a wide diversity in the DBLγ sequences amplified from the cDNAs of all samples. Sequences were also amplified by either D3R1 or D3R2 from the gDNAs of a randomly selected group of 10 isolates (Table 1, sample group C).
gDNAs were different from those amplified from the cDNAs of the same isolates, suggesting that the sequences amplified from the cDNAs were detected because of their abundance.

An obvious association between placental isolate cDNA and var2csa was revealed by a phylogenetic analysis that included all of the amplified sequences and all of the PfEMP1 domains previously implicated in pregnancy-associated malaria (6, 17, 23, 29, 32, 35) (Fig. 2). There was > 76% identity between 3D7 var2csa and any of the 24 sequences homologous to var2csa DBL3x identified in this study. However, only 36% of all 194 predicted amino acids were identical between all of the 22 full-length var2csa DBL3x sequences (excluding two sequences that had large deletions) (see Fig. S1 in the supplemental material). Four other DBLγ domains associated with malaria in pregnancy formed small, well-supported clusters: FCR3var.CSA DBLγ had at least 70% identity to four sequences from different placental cDNAs; FCR3var.CS4 DBL3γ had > 96% identity with two sequences from different placental isolate cDNAs and one sequence from a child’s isolate cDNA; 3D7chr5 var DBL3γ (32), homologous to 720 (23) and var_common (42), had > 79% identity with three sequences from different placental cDNAs; and var-CS2 had > 97% identity with the only two sequences amplified from a single placental cDNA.

We performed absolute quantitation by Q-RT-PCR to determine quantitative relationships between selected pregnancy-associated var sequences within those isolates for which sufficient cDNA was available (Table 1, sample group D). This group comprised all 6 children’s isolates and 13 of the placental isolates, including all 9 of the placental samples from which no var2csa sequences were amplified with the degenerate primers (Table 1, sample group B). For each cDNA, we quantitated the levels of var2csa DBL3x, var-CS2 DBL2γ, and FCR3var.CS4 DBL3γ. For all six children’s isolates and eight of the placental isolates, we also quantitated the level of an endogenous control DBLγ sequence that had been amplified from the cDNA of the isolate being examined (Table 1 and Fig. 2). This controlled for the possibility that differences between isolates in the levels of transcripts of any of the pregnancy-associated var sequences merely reflected differences between isolates in the levels of general var gene transcription. The selected pregnancy-associated var sequences could not be used to control for this possibility because the presence of these sequences had not actually been established for all isolates. For five placental samples, no endogenous control was used because no sequences could be amplified using the DBLγ degenerate primers. For sample Mplc78, the var-CS2 DBLγ sequence that was cloned from its cDNA was used as the endogenous control.

Q-RT-PCR revealed that var2csa was the most abundant of the transcripts examined for 12 of the 13 placental isolates (sample group D) but that var-CS2 was a more abundant transcript than var2csa in the remaining placental isolate, Mplc78 (Fig. 3). FCR3var.CSA was present at a similar level to that of var2csa in the cDNA from the placental isolate Mplc328. The endogenous control DBLγ sequence was the most abundant of the transcripts tested in four of the six children’s isolate cDNAs, and var2csa was the most abundant of the transcripts tested in the other two children’s isolate cDNAs (Mch1478 and Mch1488) (Fig. 3). To confirm the presence of var2csa sequences in samples analyzed by Q-RT-PCR, we cloned and sequenced var2csa PCR products that were amplified with specific primers from the cDNAs of Mch1478, Mch1488, and eight of the nine placental isolates from which var2csa could not be amplified using the degenerate primers (Table 1, sample group B). All but one of the cloned var2csa sequences were identical to the var2csa sequence of the 3D7 isolate within the region that was used as a template for Q-RT-PCR. The var2csa sequence cloned from Mplc78 had a single mismatch of C versus T at nucleotide 11 of the var2csa DBL3x Q-RT-PCR.
forward primer. Consequently, var2csa cDNA was quantitated in the placental isolate Mplc78 by using both a modified var2csa DBL3x forward primer that had a cytidine residue at nucleotide 11 and a standard curve constructed from the cloned Mplc78 var2csa sequence.

The absolute quantitation data were normalized to compare the cDNA levels of specific var sequences between isolates and to indicate the abundances of specific var sequences within an isolate. The quantitative data should ideally be normalized to the total level of var mRNA to control for fluctuations between isolates in the levels of var gene transcripts caused by either differences in the temporal stages of the parasites sampled or

FIG. 2. Phylogenetic tree of sequences amplified from cDNAs and gDNAs of parasites isolated from placentas and from peripheral blood of children. The candidate CSA-binding PfEMP1s encoded by var2csa (35), FCR3.varCSA (6), var-CS2 (29), MAL6P14 (17), 3D7chr5var (32), 482, 498, 485, 720, and 732 (7, 23) are included, and those shown in bold are in the clusters described in the text. Bootstrap values of >50 are indicated at tree branches leading to clusters of sequences. The sequences used as endogenous DBLγ controls for Q-RT-PCR are indicated with stars. The sequences were named for the samples from which they were amplified, followed by a space and then a different number for each different sequence from a single Mple or Mch sample or a different letter for each different sequence from an Rple sample. The suffix "-g" following an Mple or Mch sequence indicates that it was amplified from gDNA.
differences in the peak levels of var mRNA transcribed by allogeneic isolates. This was an issue in the current study because the peripheral blood samples from children contained predominantly ring-stage parasites at the peak of var transcription while the placental isolates were composed mainly of mature trophozoites, in which var transcription is reduced. However, the concentration of total, functional var cDNA cannot be determined by Q-RT-PCR because the only sequences that are sufficiently conserved between all var genes to be used for this purpose are the second-exon sequences, which are also present at high levels in mature parasites as sterile, truncated transcripts. Consequently, to normalize the var Q-RT-PCR data, we used two genes, namely, the skeleton binding protein 1 gene (SBP) (PFE_0065w) and a hypothetical protein gene (PF11_0505), because their temporal transcriptional profile closely approximated that of the var multigene family in allogeneic parasite strains 3D7, HB3, and Dd2 (4, 27). Therefore, the normalized data presented in Fig. 4b represent the levels of cDNAs for specific var genes in equivalent amounts of total var cDNA from each isolate, as approximated by two control genes.

We used a time course of CS2 parasites, in which var2csa is the dominant var transcript (9), to validate our choice of genes for normalization (Fig. 4a). The levels of the normalizing genes were compared to the levels of var2csa in the 8-h postinvasion ring-stage CS2 parasites and the 36-h postinvasion mature trophozoite-stage CS2 parasites, using the following ratio: ring-stage var2csa cDNA/ring-stage control gene cDNA:mature trophozoite-stage var2csa cDNA/mature trophozoite-stage control gene cDNA. This gave ratios of 2.5:1 for SBP and 1.3:1 for PF11_0505. Thus, we assumed that the mature trophozoites in the placental isolates would contain 2.5-fold more var transcripts than were calculated by using SBP for normalization and 1.3-fold more var transcripts than were calculated by using PF11_0505 for normalization, and we adjusted the normalized data accordingly. The levels of 18S rRNA were also quantitated in the CS2 parasites to show that the similar transcriptional profiles of var2csa, PF11_0505, and SBP did not merely reflect the use of decreased parasite cDNA in the mature trophozoite Q-RT-PCR (Fig. 4a). The levels of SBP in the 12 placental and 6 children’s isolates analyzed (Table 1, sample group D) correlated with the levels of PF11_0505 ($R^2 = 0.8812$), corroborating the evidence of their similar temporal transcriptional profiles in 3D7, HB3, Dd2, and CS2 parasites. The placental sample Mplc345 was omitted from the normalized data analysis because it contained insufficient levels of cDNA from either normalizing gene.

In data normalized with either control gene (Fig. 4b), there was significantly more var2csa cDNA in the placental isolates than in the children’s isolates (Table 2) (Mann-Whitney test; $P = 0.0043$ [SBP] or 0.017 [PF11_0505]) but no significant difference in the levels of var2csa associated with gravidity. There was significantly more var2csa cDNA than endogenous control DBLγ cDNA in the placental isolates (Table 2) (Mann-Whitney test; $P = 0.0009$ [SBP] or 0.0023 [PF11_0505]) but not in the children’s isolates (Table 2) (Mann-Whitney test). The median levels of endogenous control DBLγ sequences were higher for cDNAs from the children’s isolates than for those from the placental isolates, but the difference was not significant (Table 2) (Mann-Whitney test). The most abundant var transcript in CS2 parasites is var2csa (9), so the level of var2csa in CS2 cDNA indicates the abundance of specific var sequences in the cDNAs of the other samples (Fig. 4b). The abundance of var2csa cDNA in the child’s isolate Mch1478 was apparent in the normalized data, but only a low level of var2csa was present in cDNA from the other child’s isolate (Mch1488) in which var2csa was the most abundant of the analyzed var transcripts (Fig. 4b).

The abundance of var-CS2 in the placental isolate Mplc78 was apparent in the normalized data (Fig. 4b). The placental
isolate Mplc328 contained similar but low normalized levels of var2csa DBL3x and FCR3var.CSA DBL3γ cDNAs (Fig. 4b). The median values for FCR3var.CSA DBL3γ sequences were higher for placental cDNAs than for children's cDNAs, but the difference was not significant (Table 2) (Mann-Whitney test).

**DISCUSSION**

Our degenerate primer RT-PCR analysis revealed a similar diversity of transcribed DBLγ domains in placental isolates to that reported in previous studies (15, 22, 23, 32) but also revealed that var2csa was transcribed in all 10 placental isolates from which D3F and D3R1/D3R1.2 could amplify a product (Table 1, sample group A). This apparent dominance of var2csa DBL3x domains over DBLγ domains in the mRNAs of placental isolates was corroborated by the evidence of a low bias of primers D3F and D3R1 towards amplification of var2csa from 3D7 gDNA and by their ability to amplify var2csa from the cDNAs but not the gDNAs of the four placental isolates tested (Table 1, sample group A).
Further quantitative analysis revealed that var2csa was the most abundant of the analyzed var transcripts in 12 of the 13 placental isolates examined (Table 1, sample group D), supporting the association between var2csa transcripts and parasites sequestered in the placenta (35, 40). The significantly higher levels of var2csa than of endogenous DBLγ sequences in the placental cDNAs suggested that the high levels of var2csa cDNA in these placental isolates did not simply reflect high levels of total var gene cDNA. This conclusion was supported by the lower levels of endogenous DBLγ sequences in the placental than in the children’s isolate cDNAs. Although this difference was not significant, it suggested that, if anything, the placental than in the children’s isolate cDNA (15, 32). However, this domain is part of an unusually conserved subset of var genes (34) that are frequently transcribed by parasites infecting nonpregnant individuals (42), and unlike all other domains implicated in pregnancy-associated malaria, recombinant proteins derived from FCR3var.CSA DBLγ do not bind CSA (6, 18, 19). These sequences were amplified from the Rplc placental cDNAs that had also previously been used to survey DBLα domain transcription (32). Unfortunately, there was insufficient material available from the Rplc samples to quantitate these sequences by Q-RT-PCR.

We concluded that var2csa was probably the principal var gene transcribed by parasites sequestered in the placentas of most of the participants in this study. However, the abundance of var-CS2 in one placental isolate suggests that parasites transcribing var genes other than var2csa can be sequestered in the placenta. Larger studies must determine whether var2csa is the dominant var transcript in placental isolates from around the globe if var2csa is to be explored as a possible candidate for vaccination against malaria during pregnancy. Also, note the diversity within the small sample of var2csa DBL3x sequences isolated from one locale and examined in this study. This indicates that it may be difficult to develop a vaccine from a single var2csa DBL3x sequence, and thus it is essential to identify the most conserved regions of var2csa that elicit functional immunity.

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REFERENCES


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