BRIEF REPORT

High Level of *var2csa* Transcription by *Plasmodium falciparum* Isolated from the Placenta

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***Plasmodium falciparum* parasites that bind to chondroitin sulphate A (CSA) express unique variant surface antigens that are involved in the placental sequestration that precipitates pregnancy-associated malaria (PAM). Two *var* gene subfamilies, *var1csa* and *var2csa,* have been associated with CSA binding. We show here that placental *P. falciparum* isolates highly transcribed *var2csa* but not *var1csa. var2csa* was not transcribed or was only minimally transcribed by parasites isolated from nonpregnant women. Placental parasites that effectively bound to placental chondroitin sulphate proteoglycans transcribed higher levels of *var2csa.* In pregnant women, levels of *var2csa* transcription and plasma anti-VAR2CSA immunoglobulin G were associated. These findings support the idea that VAR2CSA plays a crucial role in PAM and strengthen the rationale for the development of VAR2CSA-based vaccines.**

The expression of variant parasite antigens on the surface of infected red blood cells (iRBCs) in a clonally variable manner is a key factor in the virulence of *Plasmodium falciparum.* These variant surface antigens (VSAs) mediate the sequestration of iRBCs containing mature stages of the parasites in the host microvasculature [1]. Switching of VSA expression is associated with changes in the binding phenotype of the parasites. *P. falciparum* infections are more frequent during pregnancy, with iRBCs accumulating in the placenta. This pregnancy-associated malaria (PAM) causes serious morbidity that affects both the mother and the offspring. Parasites involved in PAM have unique binding phenotypes and predominantly adhere to chondroitin sulfate A (CSA) [2, 3]. The serological phenotype of PAM parasites is also distinct, because, in areas where malaria is endemic, anti-VSA$_{PAM}$ IgG is not present in men and is acquired in a parity-dependant fashion in women [4]. *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) molecules possess multiple adhesive domains that can mediate binding to host cell surface receptors [5]. These proteins are encoded by 50–60 *var* gene copies/haploid parasite genome, and, although several *var* genes are transcribed by ring stages, only 1 gene product is expressed at the iRBC surface [6]. Two subfamilies of conserved *var* genes, *var1csa* and *var2csa,* have been implicated in the pathogenesis of PAM, and PfEMP1 from both are expressed on the surface of CSA-selected parasites [5, 7]. Architecturally, *var2csa* subfamily members consist of 6 Duffy binding–like (DBL) domains, 3 of which do not fit into any of the current domains classification (DBL1-X, DBL2-X, and DBL3-X). Contrary to *var1csa* subfamily members, they lack the DBL-α and the DBL-γ domain, and the latter has been considered to be the main putative vaccine target. Although *var* gene expression has been extensively studied in laboratory parasite lines, the expression profile during PAM is unknown. In the present study, we measured and compared the levels of *var1csa* and *var2csa* transcription by placental parasites and parasites from nonpregnant women.

Patients, materials, and methods. Samples were collected in November 2003 in Guediawaye, a suburb of Dakar, Senegal. The human-experimentation guidelines of both the French and Senegalese Governments were followed. The study was approved by the ethics committee of the Ministry of Health, Senegal. Fifty delivering and 26 nonpregnant women presenting with *P. falciparum* infections were enrolled. *P. falciparum* iRBCs were isolated from placentas by flushing the placenta with 0.1% sodium heparin in PBS [3]. Peripheral ring stages were allowed to mature to trophozoites [3]. Parasites were conserved either in Trizol (Invitrogen) stored at −80°C or as spots on dried Whatmann 3MM filter paper stored at room temperature. Peripheral-blood plasma was stored at −20°C. Genomic DNA was extracted from

Received 28 January 2005; accepted 16 February 2005; electronically published 14 June 2005.

Presented in part: 53rd annual meeting of the American Society of Tropical Medicine and Hygiene, Miami Beach, Florida, 7–11 November 2004 (abstract 971).

Financial support: Commission of the European Union (grants QLK2-CT-2001-01302, PAMVAC, and QLK2-CT-2002-01197, EUROMALVAC2); Danish Medical Research Council (grants 22-02-0220 and 22-03-0333); Institut de Médecine et d'Epidémiologie Africaine. N.G.T.N. is supported by doctoral studentships from the French Ministry of Education and Research, A.S. has been supported by doctoral studentships from the Gates Malaria Partnership, and M.D. is supported by doctoral studentships from the Danish Research Council for Development Research.

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The Journal of Infectious Diseases 2005;192:331–5
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0022-1899/2005/19202-0018$15.00

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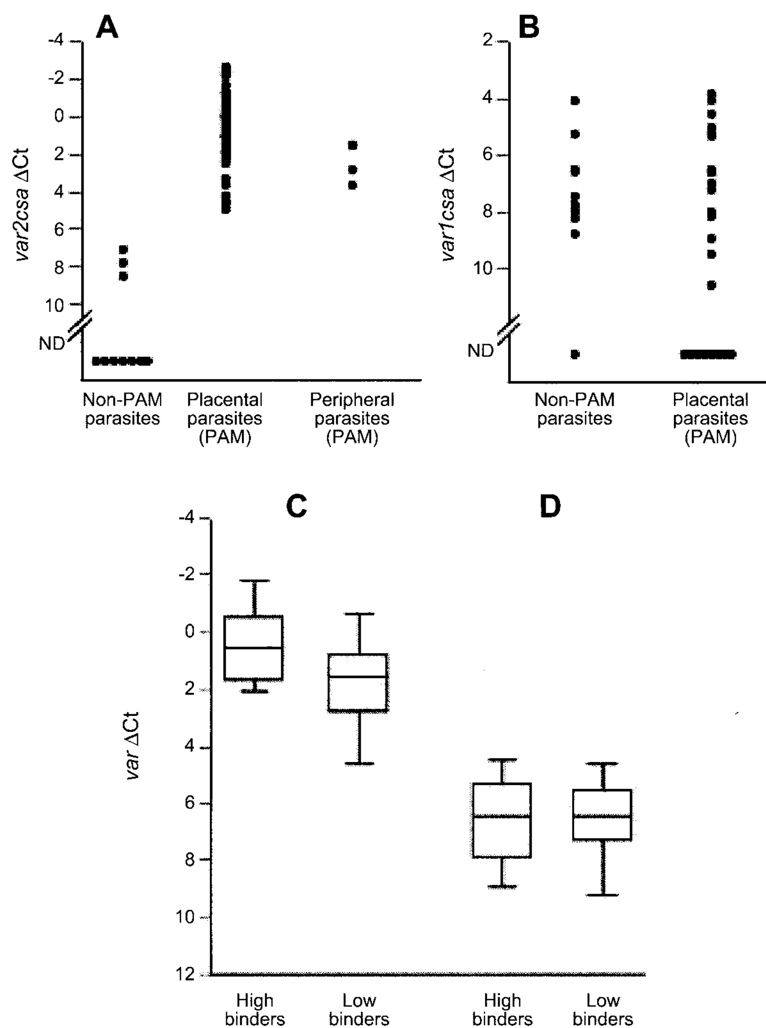

Figure 1. Levels of *var2csa* and *var1csa* transcription by *Plasmodium falciparum* parasites. Shown are levels of *var2csa (A)* and *var1csa (B)* transcription by 42 parasites isolated from the placentas of women with pregnancy-associated malaria (PAM), 4 parasites isolated from the peripheral blood of women with PAM, and 10 parasites isolated from the peripheral blood of nonpregnant women (non-PAM). Transcription levels were estimated as the difference in cycle threshold (Ct) value (ΔCt value) between the *var* target and the fructose-biphosphate aldolase housekeeping gene (control). In panel *B,* only those samples with a Ct value for the housekeeping gene of <25 (24 placental and 10 non-PAM parasites) were included. Also shown are levels of *var2csa (C)* and *var1csa (D)* transcription by 34 placental isolates with high levels (more than the median of 780 parasites/mm^2) and with low levels (less than the median) of binding to purified placental chondroitin sulphate proteoglycans. The difference in *var2csa* transcription was statistically significant ($P < .05$, Mann-Whitney *U* test). ND, not detectable.

the filter spots by use of chelex [8]. Total RNA from placental and matured peripheral iRBCs was prepared with Trizol followed by treatment with DNase 1 (Sigma) for 15 min at 37°C. DNA-free RNA was reverse transcribed with random hexamer primers and Superscript II enzyme (Invitrogen) for 10 min at 25°C, 50 min at 42°C, and 15 min at 70°C.

For the cytoadhesion assays, chondroitin sulphate proteoglycans (CSPGs) were purified from a European woman's placenta by ion-exchange salt gradient chromatography (DEAE-Sephacel), CsBr density gradient ultracentrifugation, and gel filtration chromatography [9]. Bovine CSA (Sigma) and human CSPGs were coated onto Falcon petri dishes [3]. Fresh placental (n = 40) or matured peripheral (n = 10) parasites were used for quantification of adhesion, as described elsewhere [3]. For merozoite surface protein (MSP) 2 genotyping, polymerase chain reaction (PCR) amplification was conducted in an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems) to enumerate and quantify fluorescent fragments, as described elsewhere [10].

Quantitative real-time PCR was performed on cDNA using a Rotorgene thermal cycler system (Corbett Research) [11]. On the basis of an alignment of *var2csa* fragments in a highly conserved part of DBL4-ε, a primer set suitable for real-time PCR was made that targets all *var2csa* genes without bias. The

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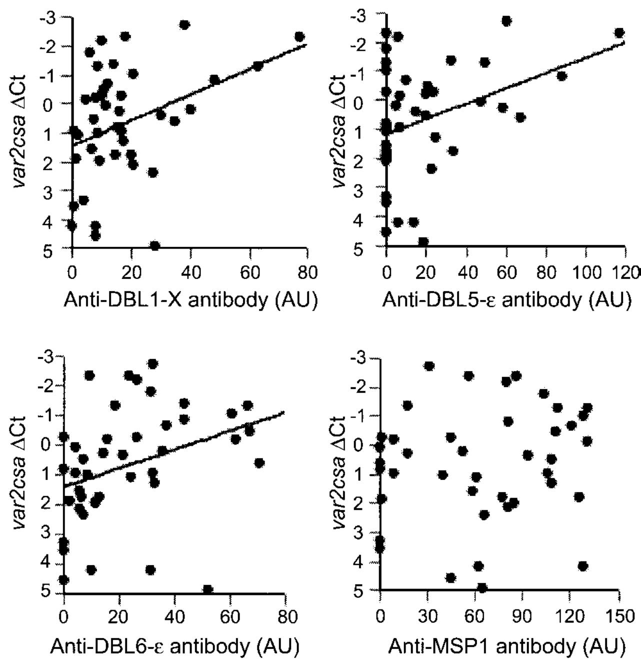

Figure 2. Correlation between *var2csa* transcription (estimated by ΔCt values, as described in figure 1) and plasma levels of IgG (shown in ELISA arbitrary units [AUs]) to 3 different VAR2CSA domains (Duffy binding–like [DBL1]–X, DBL5-ε, and DBL6-ε) but not between *var2csa* transcription and plasma levels of IgG to merozoite surface protein (MSP) 1. For all 3 domains, the levels of anti-VAR2CSA IgG and *var2csa* transcription were correlated (Spearman's ρ, −0.3 to −0.5; $P<.05$); the levels of anti-VAR2CSA IgG between the 3 domains were also strongly correlated (Spearman's ρ, 0.4–0.6; $P<.01$).

var1csa primer set was designed in the conserved upstream region of the *var1csa* gene. Primer bias was tested on serial dilutions of genomic DNA, and specificity was ensured by a melting curve analysis and agarose gel electrophoreses. The *var2csa* primers were 5′-AGCCCAATCGGAAGGTAAGT-3′ (forward) and 5′-TTCATAGCTTCTAGCGCCTT-3′ (reverse); the *var1csa* primers were 5′-TGGCACATCTTTGGTATAAAA-3′ (forward) and 5′-AAACCTTTATATTCCTGTAAAATTCA-3′ (reverse). Reactions were performed in 20-μL volumes with Quantitect SYBR Green PCR Master Mix (Qiagen) and 0.5 mmol/L primers [11]. Quantitative analysis of the *var* gene levels was performed by use of Rotorgene software (version 4.6). The ΔCt (cycle threshold) method was used, in which the Ct value for each specific *var* gene was compared with that for the endogenous fructose-biphosphate aldolase housekeeping gene (control). Samples with Ct values of >30 were not quantified. Because of the relatively low level of expression of *var1csa* in field samples, only those samples with a Ct value for the housekeeping gene of <25 were included, to ensure that enough cDNA was present to measure *var1csa* transcripts within the dynamic range of the PCR.

Plasma levels of *P. falciparum*–specific IgG were measured by ELISA with recombinant VAR2CSA or MSP1 (yPfMSP1-19) protein. Three domains (DBL1-X, DBL5-ε, and DBL6-ε) of the synthetic *var2csa* gene that had been previously generated [7] were cloned into the pBAP-TOPO vector (Invitrogen) by PCR. Recombinant proteins from these constructs were produced in baculovirus-infected Sf9 cells and purified [12].

Differences between groups were tested by the appropriate nonparametric test (either the Mann-Whitney *U* test or the Wilcoxon matched pairs signed rank test). Correlations were tested by Spearman's rank sum test. The significance limit was considered to be $P<.05$. Stata software (version 7.0; Stata) was used.

Results. All isolates bound to bovine CSAs and to human CSPGs. The number of iRBCs bound per square millimeter ranged from 46 to 3700 for bovine CSAs and from 37 to 3450 for human CSPGs. Peripheral and placental parasites from 10 women had correlated binding abilities (Spearman's ρ, 0.6–0.7; $P<.05$, for binding to CSAs or to CSPGs).

MOI was assessed on the basis of the number of *msp2* alleles. The MOI was similar in the peripheral (median, 3; range 1–8) and the placental (median, 2; range, 1–6) blood from pregnant women and in the blood from nonpregnant women (median, 3; range, 1–7). In pregnant women, 8 of 50 placental and 10 of 50 peripheral infections were monoclonal, compared with 2 of 26 in nonpregnant women. In polyclonal samples, 1 major allele represented >80% of the overall parasite population in 76% (95% confidence interval [CI], 61%–88%) placental and 58% (95% CI, 41%–73%) peripheral parasites from the pregnant women. In the nonpregnant women, the corresponding value was 42% (95% CI, 41%–73%). In 42 of 50 matched peripheral and placental parasites, the major *msp2* allele was identical.

We first performed PCR on genomic DNA from 40 placental parasites using *var2csa* DBL4-ε–specific primers that were designed on the basis of an alignment of GenBank *var2csa* sequences. *var2csa* was amplified from all 40 of these genomic DNA samples. The PCR fragments were cloned and, on the basis of sequence alignments, a primer set suitable for real-time PCR was identified. In parallel, another primer set based on GenBank sequences that targeted the conserved untranslated promoter sequence (UPS) of *var2csa* was designed. The 2 primer sets amplified genomic DNA from all parasites equally well and provided similar results. For *var1csa* primers, 2 primer sets that targeted the DBL3-γ and UPS regions were designed on the basis of GenBank sequences. The UPS primer set performed best, and PCR products from 38 of 49 parasite samples were identified. Thus, most of the parasites carried *var1csa* in the genome, and *var2csa* was present in all placental parasites. The levels of *var1csa* and *var2csa* transcription were measured by real-time reverse-transcription PCR for all isolates from which DNA-free RNA was successfully extracted. All measurements were related to a housekeeping gene and were quantified

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as the Ct value for the gene-specific reaction minus the Ct value for the fructose-biphosphate aldolase housekeeping gene (ΔCt value). In this system, the transcription level is inversely correlated to the ΔCt value. Data were also normalized against another housekeeping gene (for seryl-tRNA synthetase), with similar results. *var2csa* was transcribed by all placental parasites. *var2csa* transcription in the parasites from the nonpregnant women was either not detectable or very low (figure 1*A*), and there was a marked difference between *var2csa* transcription in the placental parasites and that in the parasites from the nonpregnant women ($P < .001$, Mann-Whitney *U* test). *var1csa* was expressed at comparable levels by the placental parasites and by the parasites from the nonpregnant women (figure 1*B*). In relation to the transcription profiles of *var* genes, the parasites with a high binding ability transcribed higher levels of *var2csa* than did the parasites with a low binding ability ($P < .05$, Mann-Whitney *U* test), whereas the *var1csa* transcription levels did not differ between these 2 groups of parasites (figure 1*C* and 1*D*).

Recombinant proteins from 3 VAR2CSA domains (DBL1-X, DBL5-ε, and DBL6-ε) were used to assess the plasma levels of anti-VAR2CSA IgG. For all 3 domains, the levels of anti-VAR2CSA IgG and parasite *var2csa* transcription were correlated (Spearman's ρ, -0.3 to -0.5; $P < .05$) (figure 2). The levels of anti-VAR2CSA IgG between the 3 domains were also strongly correlated (Spearman's ρ, 0.4–0.6; $P < .01$, for all correlations), confirming that the recombinant VAR2CSA proteins are capable of being recognized by naturally acquired antibodies. There was no correlation between *var2csa* transcription and plasma levels of anti-MSP1 IgG. Thus, parasites that transcribe *var2csa* induce a specific immune response in infected pregnant women.

Discussion. Placental parasites and CSA-selected lines express unique surface antigens [4]. Although the *var1csa* and *var2csa* gene subfamilies are expressed by CSA-selected parasite lines [5, 11], their expression has never been quantified in a large set of parasites isolated from the placenta and the peripheral blood. *var2csa* was transcribed by all placental parasites, and the mean ΔCt value for 42 placental parasites was 0.8, which was almost similar to that for the housekeeping gene. Interestingly, parasites from the peripheral blood of pregnant women also transcribed high levels of *var2csa* (mean $\pm$ SD ΔCt, 2.3 $\pm$ 1.1). These are high levels of transcription, given that the ΔCt value for CSA-selected parasite lines in which VAR2CSA is the dominantly expressed PfEMP1 on the surface of infected erythrocytes is ~5 [11].

var1csa was expressed at comparable levels by placental parasites and parasites from nonpregnant women. This finding agrees with those from a previous study that detected *var1csa* mRNA transcription in a high proportion of parasites from nonpregnant women [13]. Compared with those of other *var* genes, the transcription profile of *var1csa* is unusual [14], and it is doubtful that its transcription always results in protein production. Indeed, in the present study, *var1csa* transcripts were often undetected in the placental parasites, suggesting that the protein is not required for placental sequestration. Direct comparisons of ΔCt values obtained with different primer sets are confounded by primer effectiveness and primer bias. However, it is noteworthy that *var1csa* transcription seemed to be higher than *var2csa* transcription in the parasites from nonpregnant women, whereas *var2csa* transcription exceeded *var1csa* transcription in the placental parasites.

Placental infections are often polyclonal, but a dominant genotype is more often present among placental parasites than among parasites from nonpregnant women. This suggests that placental infections are under some clonal restriction, which is in line with the idea that binding is mediated by an antigen that shows allelic polymorphisms, as both VAR1CSA and VAR2CSA do. Although both VAR1CSA [5] and VAR2CSA [15] domains can bind to CSA in vitro, the association between *var2csa* transcription level and CSPG binding ability argues for the direct involvement of VAR2CSA in placental sequestration. Parasites that transcribe *var2csa* are capable of inducing a specific immune response in pregnant women, and the strong correlation between *var2csa* transcription and plasma levels of IgG to the 3 VAR2CSA domains confirms that VAR2CSA is architecturally conserved.

In conclusion, the present study has shown that *var2csa* is highly and specifically transcribed by malarial parasites infecting the placentas of pregnant women. Although other molecules might also be involved in placental binding, the relationship between *var2csa* transcription and CSPG binding of placental parasites suggests that VAR2CSA mediates binding to CSPGs in the placenta. Because the ability of parasites to bind to CSPGs plays an important role in the pathogenesis of PAM and the disease's clinical consequences, such as low birth weight [3], the present results strengthen the rationale for the development of VAR2CSA-based vaccines for the prevention of PAM.

Acknowledgments

We acknowledge the women who participated in this study, the maternity staff in Guediawaye, and the staff of the Institut de Recherche pour le Développement research unit in Senegal, especially Souleymane Doukoure and Mamadou Ngom. We are grateful to Valerie Briand and Gilles Cottrel, for advice on statistical analysis.

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