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Differential Expression of *var* Gene Groups Is Associated with Morbidity Caused by *Plasmodium falciparum* Infection in Tanzanian Children

Matthias Rottmann,[1]† Thomas Lavstsen,[2]† Joseph Paschal Mugasa,[3] Mirjam Kaestli,[1] Anja T. R. Jensen,[2] Dania Müller,[1] Thor Theander,[2] and Hans-Peter Beck[1]*

Swiss Tropical Institute, Socinstrasse 57, Postfach 4002 Basel, Switzerland[1]; Centre for Medical Parasitology at Institute for Medical Microbiology and Immunology, University of Copenhagen, Panum Institute 24-2, Blegdamsvej 3, 2200 Copenhagen N, Denmark[2]; and Ifakara Health Research and Development Centre, P.O. Box 53, Ifakara, Morogoro, Tanzania[3]

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The *var* gene family of *Plasmodium falciparum* encodes the variant surface antigen *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is considered an important pathogenicity factor in *P. falciparum* infection because it mediates cytoadherence to host cell endothelial receptors. *var* genes can be grouped into three major groups, A, B, and C, and the conserved *var* genes, *var1-4*, according to sequence similarities in coding and noncoding upstream regions. Using real-time quantitative PCR in a study conducted in Tanzania, the *var* transcript abundances of the different *var* gene groups were compared among patients with severe, uncomplicated, and asymptomatic malaria. Transcripts of *var* group A and B genes were more abundant in patients with severe malaria than in patients with uncomplicated malaria. In general, the transcript abundances of *var* group A and B genes were higher for children with clinical malaria than for children with asymptomatic infections. The *var* group C and *var1*-like transcript abundances were similar between the three sample groups. A transcript abundance pattern similar to that for *var* group A was observed for *var2csa* and *var3*-like genes. These results suggest that substantial and systematic differences in *var* gene expression exist between different clinical presentations.

The particular virulence of *Plasmodium falciparum* is linked to the cytoadhesion properties of infected erythrocytes in deep vascular beds leading to multiple complications and symptoms (19). This process of sequestration is thought to be an immune evasion strategy to avoid splenic clearance (2, 8, 14). Infected erythrocytes also form rosettes with uninfected erythrocytes (27) or form larger groups involving platelets, called clumps (25). *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the *var* gene family and expressed on the surfaces of infected erythrocytes, mediates binding to host endothelial receptors and is an important target for protective immunity (1, 7, 9, 13, 31, 34). Each parasite possesses 50 to 60 *var* gene copies, and switching between surface expression of the various *var* gene products results in antigenic variation while maintaining or changing adhesion properties (12, 31). Immunity preventing severe malaria and death develops naturally in exposed populations. In areas of intense transmission, the main burden of malaria morbidity and mortality is among children between 6 months and 5 years of age (32). Adults are often infected asymptomatically, and severe disease is rare.

It has been shown that parasites from patients with severe malaria express a different subset of surface antigens that are more frequently recognized by sera from malaria-exposed individuals, including young children, than parasite antigens from older children with mild malaria (5, 23). It has also been shown that this subset of surface antigens is serologically conserved among different geographical regions (24), and it is therefore crucial to identify the molecular phenotype of such a subset to develop a disease-ameliorating vaccine.

Linking PfEMP1 expression to disease outcome is inherently difficult due to the extensive inter- and intragenomic variation in *var* genes. Previous studies predominantly relied on reverse transcription-PCR using degenerate primers, with subsequent cloning and sequencing (4, 17). While these studies have shown that the transcription of certain DBL1α domains is associated with either severe malaria or rosette formation, they have been unable to identify a clear correlation between *var* gene groups and disease outcomes.

Sequencing of the 3D7 genome revealed that *P. falciparum* parasites contain 50 to 60 *var* genes that can be grouped into three major groups, A, B, and C, and the single-copy intergenomic conserved *var* genes, *var1* and *var2csa*, according to sequence similarities in both noncoding and coding sequences (12, 18, 22, 37). Evidence is emerging for the existence of subgroups of *var* group A, namely, type 3 *var* (18) and type 4 *var* (12, 15) genes, referred to here as *var3* and *var4*, respectively. The functional relevance of this genetic structuring is indicated by the fact that CIDR domains of group B and C PfEMP1 variants bind to CD36, in contrast to CIDR domains of group A variants (26). Parasites selected for chondroitin sulfate A and human bone marrow endothelial cell binding in vitro dominantly express *var2csa* and *var4*, respectively (15, 29), also supporting the notion of functional genetic substructuring.

The genetic organization of *var* genes was exploited to de-

* Corresponding author. Mailing address: Swiss Tropical Institute, Socinstrasse 57, CH 4002 Basel, Switzerland. Phone: 41-61-284 8116. Fax: 41-61-284 8101. E-mail: hans-peter.beck@unibas.ch.

† M.R. and T.L. contributed equally to this work.

TABLE 1. SYBR green primers

Primer pair	Sequence (5′–3′)	
	Forward	Reverse
A1	TTGGGRAATBTGTTAGTTAYRGCAA	CTGCAAAACTKCGWGCAAG
A2	AACCCATCTGTRRATGATATACCTATGGA	GTTCCAASGATCCATTRGATGTATTA
A3	AGGTAATGTTTTAGATGATGGTAT	ACCAGAATATACATTATTTGATACATA
B1	CATCCGCCATGCAAGTATAA	CGTGCACGATTTCGATTTTT
B2	ATCAAGGTAATTTCATACATATGTGATA	GTCCGTGCACGATTTCGATTTT
C1	CACATCGATTACATTTTAGCGTTT	TGTGGTAATATCATGTAATGG
C2	GTAGCGACAACCACGRYATCATGG	CATTGTTAACATAGTCTACCATTA
BC1	GACAAAACTTTCACCCAATAGA	AATGATCGGTGTAACCACTATC
BC2	CATCTGTTGCAAATTTATTCCAAATAC	TCAGTAGTATCAGACATAAATGCATA
pvar1utr	TGGCACATCTTTGGTATAAAA	AAACCTTTATATTCCTGTAAAATTCA
pvar2utr	CACGACATTAACAATACATGCAGA	CATTGCATTCACAGACATTGG
pvar3coding	CGTAAAACATGGTGGGATGA	GGCCCATTCAGTTAACCATC

sign primers targeting the conserved regions defining *var1-3* genes and group A, B, and C *var* genes. Using these primers in quantitative reverse transcription-PCR, the transcript abundances of *var* genes were measured in parasites collected from *P. falciparum*-infected Tanzanian children with asymptomatic malaria (AM), uncomplicated malaria (UM), and severe malaria (SM). Our data demonstrate an increase in transcript abundance for group A and B *var* genes in parasites causing severe malaria compared to that in parasites causing uncomplicated malaria.

MATERIALS AND METHODS

Study design and population. The study was conducted in Ifakara, a semirural area in southern Tanzania, from June to September 2003. Ifakara is an area of moderate perennial *P. falciparum* transmission surrounded by areas of more intense transmission.

Of all children seeking medical treatment, 40% were admitted to the hospital due to infection with *P. falciparum*. Malaria is reported to account for a case fatality rate of 2.4% in this hospital (30). Samples were collected from children (aged 4 to 59 months) presenting with malaria at the hospital. Severe malaria cases were defined according to the World Health Organization criteria for severe malaria (38). Uncomplicated malaria was defined as the presence of asexual *P. falciparum*, an axillary temperature of >37.5°C, or symptoms of headache or myalgia but no other signs of severe malaria. Exclusion criteria were confirmed coinfection, malnutrition (mid-upper-arm circumference [MUAC] of <12 cm), or antimalarial treatment during the last 14 days. Asymptomatic patients (presence of *P. falciparum*, axillary temperature of <37.5°C, and no other symptoms) were age-matched as closely as possible to the patients with severe cases by convenience sampling in the same area in January 2005. *P. falciparum* infection was determined by Giemsa-stained blood films, and parasitemia was counted as parasites per 200 white blood cells. Ethical clearance for this study was obtained from the Ifakara Health Research and Development Centre's scientific review board and the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania.

Blood samples. After obtaining written informed consent from parents, 1 to 2 ml of venous blood from children was collected in EDTA tubes. Erythrocytes were separated from serum by centrifugation and washed with 40 ml phosphate-buffered saline, 5 volumes of TRIzol reagent (Invitrogen) was added to the erythrocyte pellet, and the sample was frozen at −70°C. Samples were collected from 52 patients with SM, 56 patients with UM, and 19 AM children. 3D7 parasite lines were generated as described elsewhere (33).

DNA, RNA, and cDNA. Genomic DNAs were isolated from infected red blood cells (100 μl blood was frozen after adding 2 volumes of 6 M guanidine HCl, 50 mM Tris, pH 8.0, 20 mM EDTA) with a QIAamp blood kit (QIAGEN), and total RNAs were extracted by using TRIzol reagent (Invitrogen) twice, as recommended by the manufacturer, and treated with DNase I (Invitrogen) for 30 min at 37°C. The absence of DNA in RNA samples was confirmed by stable base fluorescence after 40 cycles of real-time PCR with seryl-tRNA synthetase primers (29). Reverse transcription was performed using Superscript II (Invitrogen) and random hexamer primers in a total volume of 40 μl according to the manufacturer's recommendations. Hereafter, two different real-time PCR methods were used to quantify *var* transcript abundance, namely, a SYBR green-based assay and a minor groove binder (MGB) probe-based assay.

Validation of SYBR green method and quantification of *var* gene transcript abundance. Quantitative real-time PCR using Quantitect SYBR green PCR master mix (QIAGEN) was performed on a Rotorgene thermal cycler system (Corbett Research) as previously described, using the seryl-tRNA synthetase (primer pair p90) and fructose-bisphosphate aldolase (primer pair p61) genes as endogenous controls (23). Primers targeting *var* gene groups A, B, and C were designed based on sequence similarities in the 3D7 *var* repertoire (22). The majority of *var* genes are flanked by conserved upstream region upsA, upsB, or upsC. Primer pairs B1 and B2 target the conserved upstream region of *var* B genes, whereas C1 and C2 target the upstream region of *var* C genes. Attempts to design primer pairs targeting upsA with sufficient amplification in the SYBR green assay were unsuccessful. In strain 3D7, grouping into groups A, B, and C is also maintained in the coding sequences for DBLα and ATS. This was exploited to design primer pairs A1, targeting DBLα of group A *var* genes; A2 and A3, targeting exon 2 of group A *var* genes; and BC1 and BC2, targeting exon 2 of both B and C *var* genes. Additional primers were designed to target conserved regions of *var1* and *var2csa* 5′ untranslated regions (UTRs) and *var3* coding regions (pvar1utr, pvar2utr, and pvar3). Primers designed to target *var4* genes in quantitative reverse transcriptase PCR amplified fragments with the expected melting temperature (T_m) for 3/20 genomic DNAs from field samples only, and this analysis was therefore left out subsequently. Seven annotated genes in 3D7 were predicted not to be targeted by any of these primer pairs. Primers are shown in Table 1, and the 3D7 genes expected to be amplified by the respective primers are listed in Table 2. The primers were validated as follows. Initially, all primers were tested on 10-fold dilutions of 3D7 genomic DNA (gDNA). All primers amplified fragments of the expected size and T_m, and sequencing of one PCR clone from each amplification reaction revealed an expected target sequence. All primers had amplification efficiencies (E) between 1.85 and 2 [$E = 10^{(-1/\text{slope of 10-fold-dilution gDNA standard curve})}$] (data not shown). Cycle threshold (C_T) values for primers targeting multiple genes were compared to those obtained with the control primer p90, targeting a single-copy gene. The observed ΔC_{Ti}($\Delta C_{Ti\text{-obs-3D7}} = C_{Ti} - C_{Tp90}$) was compared to the expected value estimated from the number of predicted genes targeted in 3D7. A ΔC_T value reduction of 1 represents a duplication of targeted gene copies, and the estimated $\Delta C_{Ti\text{-estl}} = -\log(\text{no. of targeted genes})/\log(2)$ (Table 2). Most primer pairs amplified as expected; the primers were then tested on gDNAs from 20 field isolates, and the amplification results were compared to the p90 C_T values ($\Delta C_{Ti\text{-obs-field}} = \mu_{n-20}\Delta C_{Ti} - C_{Tp90}$) (Table 2). All primer pairs yielded fragments with the sizes and T_ms expected from the amplifications of 3D7 gDNA, and all primers amplified with similar efficiencies (95% confidence interval for $\mu_{(n=20)[C_{Ti}-C_{Tp90}]}$, <0.8) from all field isolates, with no significant difference (t test) between DNAs from severe and uncomplicated malaria cases. The amplification efficiency for field isolate gDNA was similar to that for 3D7 gDNA for most primer pairs, except C1, C2, and BC2, indicating that these primers targeted fewer genes in field isolates than in 3D7 and might reflect that a larger variation in *var* group C gene copy numbers exists among parasites from naturally infected individuals. However, none of the primer pairs showed any significant difference in amplification efficiencies between DNAs from severe and uncomplicated malaria cases.

Next, primers targeting *var* groups were tested on cDNAs from isogenic but phenotypically distinct 3D7 parasites with known differential *var* transcript abun-

TABLE 2. Technical characteristics of *var* group primers for SYBR green real-time PCR[a]

Characteristic	Value or description for indicated primer pair				
	A1	A2	A3	B1	B2
Target region	Grp A DBL1α	Group A exon 2	Group A exon 2	upsB	upsB
Targeted genes in 3D7 predicted by alignments	PFD1235w, MAL7P1.1, PF08_0141, PF11_0008, PFD0020c, PF11_0521, PF13_0003, PFA0015c, MAL6P1.314, PFI1820w	PFD1235w, MAL7P1.1, PF08_0141, PF11_0008, PFD0020c, PF11_0521, PF13_0003, PFA0015c, MAL6P1.314, PFI1820w	PF11_0521, PFD1235w, MAL7P1.1, PF13_0003, PFD0020c, PF11_0008	MAL6P1.1, PF07_0139, PF08_0142, PF10_0001, PF10_0406, PF11_0007, PF13_0001, PF13_0364, PFA0005w, PFA0765c, PFB0010w, PFB1055c, PFC0005w, PFC1120c, PFD0005w, PFD1245c, PFE0005w, PFI0005w, PFI1830c, PFL0005w, PFL0935c, PFL2665c	MAL6P1.1, PF07_0139, PF08_0142, PF10_0001, PF10_0406, PF11_0007, PF13_0001, PF13_0364, PFA0005w, PFA0765c, PFB0010w, PFB1055c, PFC0005w, PFC1120c, PFD0005w, PFD1245c, PFE0005w, PFI0005w, PFI1830c, PFL0005w, PFL0935c, PFL2665c
Coverage in 3D7 (group)	All A genes, including *var3* types	All A genes, including *var3* types	All A genes, excluding *var3* types	All B genes	All B genes
Fragment T_m (°C) in 3D7	77.2	73.6	72.1	75.4	75.6
Fragment length (bp)	110–120	100	160	260	190
Fragment T_m (°C)[b] in field isolates (SD) [no. of PCR-negative genomes/total]	77.2 (0.4) [0/20]	73.7 (0.2) [0/20]	72.2 (0.4) [0/20]	75.1 (0.4) [0/20]	75.3 (0.4) [0/20]
$\Delta C_{T_{\text{i-estimated-3D7 gDNA}}}$ [−log(no. of targeted genes)/log(2)]	−3.3	−3.3	−2.6	−4.5	−4.5
$\Delta C_{T_{\text{observed-3D7 gDNA}}}(C_{T_i} - C_{T_{p90}})$	−2.0	−2.5	−1.9	−3.7	−3.9
$\Delta C_{T_{\text{i-mean-observed-field gDNA}}}$ [$\mu(C_{T_i} - C_{T_{p90}})$ (95% CI)][c]	−1.2 (0.4)	−1.9 (0.3)	−1.9 (0.3)	−4 (0.2)	−3.6 (0.2)

[a] The following 3D7 *var* genes were not targeted by any primer pair: PFD0635c, PF07_0050, PFL1955w, MAL7P1.55, MAL6P1.4, MAL6P1.316, and PFL0020w (three BA and four BC genes).

[b] Mean *Tm* based on real-time PCR on 20 gDNA and 108 cDNA field isolates.

[c] 95% confidence intervals based on real-time PCR on gDNAs from 20 field isolates. The design and validation of primers and probes for the MGB real-time PCR assay were described by Kaestli et al. (16).

dance patterns. These were nonmanipulated $3D7_{UM}$ and $3D7_{SM}$ selected on hyperimmune serum (15). The transcription measured by group-specific primers was compared to predicted changes calculated from absolute quantifications using gene-specific primers. There was a clear association ($R = 0.904$; $P = 0.0008$ [Pearson correlation]) between results obtained with gene-specific and group-specific primers (Fig. 1).

Assessment of T_ms of fragments generated from cDNAs from collected field samples showed that all primers amplified fragments with the expected T_ms in 90 to 99% of all included samples (not shown), indicating that the primers targeted *var* sequences conserved in the parasite isolates. The C_T values for the two internal control genes showed with cDNAs from field samples that reliable quantification could be performed from the collected samples [$\Delta C_{T_{p90}}(\mu, \sigma) = 21.2$ and 2.6; $\Delta C_{T_{p61}}(\mu, \sigma) = 19.0$ and 2.5]. As expected, there was a negative association between parasitemia and control gene p90 C_T values (representing the overall amount of cDNA) (regression coefficient$_{[\log2(\text{parasitemia/blood sample volume}): C_{T_{p90}}]}$, −0.58; $P = 0.005$).

Validation of MGB method and quantification of *var* transcript abundance. Quantitative real-time PCR using MGB probes was performed using an ABI PRISM 7200 sequence detection system (Applied Biosystems) as described by Kaestli et al. (16), with few modifications. Briefly, cDNAs were synthesized from total RNA, and a primary PCR with 16 cycles over the *var* 5′ UTR-DBL1α target sequence was performed prior to real-time PCR for *var* groups A, B, and C. Primers and probes targeting upsA, -B, and -C for the MGB probe assay were described by Kaestli et al. (16) (upsA-probe, upsB-probe, and upsC-probe). The seryl-tRNA synthetase internal control gene was used for relative quantification without prior amplification. The primers and probe were designed using Primer Express software 2.0 (Applied Biosystems) and had the following sequences: primer p90Probe_for, 5′-ACCTCAGAACAACCATTATGTGCTT-3′; primer p90Probe_rev, 5′-TGTGCCCCTGCTTCTTTTCTAA-3′; and p90Probe, 5′-6-carboxyfluorescein-AGGTTACCACTCAAATACGCTGGATTCTCATCTTG-6-carboxytetramethylrhodamine–6-carboxyfluorescein-3′.

Data analysis. After all samples had been subjected to real-time PCR, the data set was cleaned for subsequent statistical analysis. Data points were not considered if the T_m diverged more than 1°C from the expected value or if the C_T value was above 30. Transcript abundances were compared between clinical groups after normalization to internal controls (yielding ΔC_T values) (Fig. 2). Based on these, *x*-fold changes were calculated by the $\Delta\Delta C_T$ method (see Table 4). Comparisons between groups were made with one-way analysis of variance and Intercooled Stata 8.0 analysis software.

RESULTS

Sample collection and clinical data. Samples were collected from 52 children admitted to hospital with SM and 56 children with UM. *var* gene transcription analysis was performed on cDNAs from 42 SM and 52 UM cases. Twelve samples from 19 children with asymptomatic *P. falciparum* infections collected during a village survey could be analyzed for *var* transcript abundance. Clinical characteristics of all children from whom cDNAs were available are presented in Table 3.

Comparison of *var* transcript abundance profiles. The *var* group A transcript abundance was lowest in AM cases, higher in children with UM, and highest in children suffering from SM (Fig. 2; Table 4). Similar findings were obtained with primers targeting the upsB upstream region, whereas data obtained with primers targeting group C *var* genes indicated that group C *var* genes were transcribed at the same level in the three groups of children.

The BC1 primer pair was predicted to predominantly amplify fragments of B *var* genes, whereas BC2 primers were expected to amplify a smaller subset of genes consisting of group B, BC, and C *var* genes (Table 2). The BC1 primer results showed that the targeted genes were transcribed at higher levels by SM parasites than by UM or AM parasites. No significant changes were observed in transcription of genes targeted by the BC2 primer pair.

TABLE 2—*Continued*

Value or description for indicated primer pair						
C1	C2	BC1	BC2	pvar1utr	pvar2utr	pvar3
upsC subtype PFD0625c, PFL1960w, PF07_0048, PFD0630c, PF07_0049, PF07_0051, PFD0615c, PFD1015c	upsC subtype PFD0615c, PF07_0051, PFD0995c, PFD1000c, PF07_0049, PFD1015c, MAL6P1.252	Group B and C exon 2 PF08_0142, MAL6P1.1, PFA0765c, PFC1120c, PFC0005w, PFE0005w, PF10_0406, PFB1055c, MAL7P1.56, PF08_0140, PF13_0364, PF13_0001, PFL2665c, PFI0005w, PFB0010w, PF11_0007, PF07_0139, PFD1005c, PFD1000c, PF08_0107, PFD0005w, PFL0005w, PFD1015c, PFI1830c, PF07_0050, PF10_0001	Group B and C exon 2 MAL7P1.50, PF07_0048, PF08_0103, PF08_0106, PFA0005w, PFD1245c, PFL0935c, PFL1950w	*var1* 5′ UTR PFE1640w	*var2* 5′ UTR PFL0030c	*var3* coding PFA0015c, MAL6P1.314, PFI1820w
8/13 C	7/13 C	17/22 B, 4/13 C, 1/4 BA, 2/9 BC	4/9 BC, 3/22 B, 1/13 C	*var1*	*var2*	*var3*
74.1	73.5	75.9	75.5	69.6	74	75.5
106	120	110	170	87	184	155
74.2 (0.4) [0/20]	73.5 (0.3) [0/20]	75.9 (0.3) [0/20]	75.6 (0.3) [0/20]	69.6 (0.2) [0/20]	74.3 (0.4) [0/20]	75.6 (0.2) [0/20]
−3	−2.8	−4.7	−3	0	0	−1.6
−2.2 0.3 (0.8)	−0.7 0.9 (0.5)	−4.5 −4.55 (0.2)	−1.67 0.7 (0.3)	2.8 2.6 (0.2)	−0.8 0.3 (0.3)	−1.8 ND

The primer pairs targeting the *var1* and *var2csa* (Table 1) gene family showed no significant difference in transcript abundance between the cohorts, although a trend of increased *var2csa* transcript abundance with increased severity of disease was observed. In contrast, primers targeting the *var3* family showed significantly higher transcript abundance in SM than in AM samples and a trend of higher transcript abundance with increased severity of disease (Fig. 2). *var3* belongs to the group A *var* genes, and a correlation between the transcript abundances of *var3* and group A *var* genes would be expected. The strongest correlation of *var3* transcript abundance was found with transcripts measured by A2 ($R_{\text{pvar3:A2}} = 0.482$; $P = 0.0012$ [Spearman rank]).

High transcript abundances of group A and B *var* genes are associated with severe disease. The association between transcript abundance and clinical presentation of malaria was tested in logistic regression models in which the dependent variable was the clinical presentation (SM or UM) and the independent variables were age, MUAC, parasitemia, sex, and transcript abundance measured by the respective primer pair. The logistic regression models were built for primer sets exhibiting statistically significant differences in transcript abundance (Table 4) and showed that young age and increased MUAC significantly increased the risk of severe disease. No significant association was found for parasitemia and sex (data not shown). According to the model, the risk of severe malaria is increased 20 to 61% with a twofold increased *var* group A or *var* group B transcript abundance (Table 5).

Seven severe cases were classified as cerebral malaria due to Blantyre scores of ≤3 (data not shown). Parasites from these children showed a trend towards a larger abundance of *var* group A transcripts than those for all other clinical cases (for upsA-probe, $P = 0.1660$; for A2 primer, $P = 0.0077$). Primer pairs A1 and A3 showed no difference in transcript abundance between the groups (data not shown).

Associations of *var* transcript abundance with other clinical features. Linear regression models showed that increased *var* group B transcript abundances measured by both primer pairs B1 and B2 were positively associated with parasitemia. This was also the case when corrected for age [$R_{(\text{parasites/200 leukocytes}):\Delta C_T(\text{B1/B2})} = -226/-223$; $P = 0.045/0.019$; an increase of parasitemia by 226 parasites/200 leukocytes resulted in a twofold increase in *var* group B transcript abundance (1 C_T value decrease)]. In contrast, there was a nonsignificant trend for *var* group C transcript abundance, as measured by C1 or C2 primers, to be negatively correlated with parasitemia [$R_{(\text{parasites/200 leukocytes}):\Delta C_T(\text{C1/C2})} = 224/207$; $P = 0.091/0.074$). There was also no association between *var* group A transcript abundance and parasitemia, but *var* group A transcript abundance tended to decrease with age among the UM cases ($R_{\text{age (months)}:\Delta C_T(\text{A1/A2/A3})} = 1.58/1.67/1.72$; $P = 0.050/0.058/0.081$). No such trends were found with *var* group B or C.

DISCUSSION

Studies on the development of natural acquired immunity have suggested a genetic structuring of the PfEMP1 protein family leading to niche characteristics with regards to host receptor binding (6, 7, 9, 13). This is supported by *var* expression analyses of in vitro manipulated parasites (15, 31). The present study aimed to analyze the expression of *var* genes in naturally infected individuals presenting with different forms of malaria. Based on the sequence of the *P. falciparum* clone 3D7, it appears that most *var* genes fall into one of three main groups, A, B, and C, according to both coding and noncoding regions. Four interclonally conserved *var* genes (*var1-4*) have also been identified. This genetic structuring might reflect

functional differences of the encoded PfEMP1 proteins, a notion that is supported by observations with regards to CD36 binding properties of CIDR domains (26), differences in survival rates in vivo (21), and variant surface antigen serotypes of genotypically identical parasites with diverse PfEMP1 expression profiles (15, 29). However, only a few studies have aimed to directly correlate *var*/PfEMP1 expression in naturally infected individuals with different presentations of malaria. One study showed that parasites from Chinese children suffering from cerebral malaria expressed larger PfEMP1s than did parasites from other malaria patients (3). This may indicate an involvement of group A genes in severe malaria, as large size is characteristic of but not unique to group A PfEMP1s (22).

PfEMP1 variants of group A were also associated with severe malaria in a study with Brazilian children. The dominant DBL1α transcripts were determined by their amplification frequencies upon reverse transcription-PCR using degenerate primers (17). However, it has been shown to be difficult to reliably reproduce results using this approach (10). Bull et al. (4) used a similar approach to study *var* gene expression in 12 clinical isolates from Tanzania. Although they were able to identify unique short DBLα sequence markers for *var* group A that correlated with the formation of rosettes, no association between *var* group expression and disease outcome was found. Recently, Kaestli et al. (16) analyzed parasites from Papua New Guinean children with asymptomatic, uncomplicated, or severe malaria infections by using quantitative real-time PCR to investigate changes in the proportion of *var* A, B, or C transcripts, using primers targeting the corresponding upstream regions. This study showed a significant increase in proportions of *var* group B transcripts in clinical cases, whereas *var* group C transcript levels were increased in asymptomatic cases. No particular involvement of *var* group A was reported.

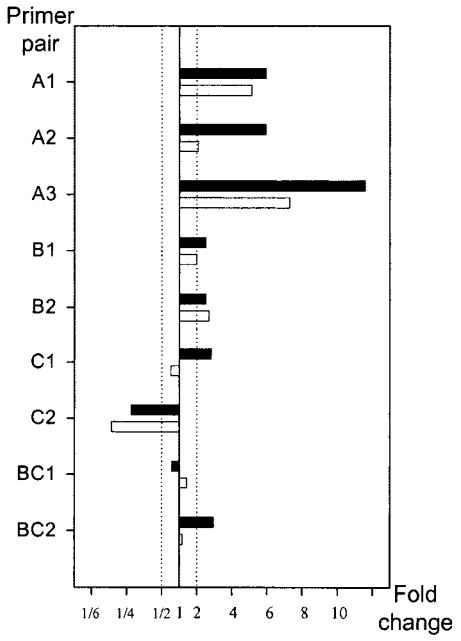

FIG. 1. Differences in *var* transcript abundance (*x*-fold changes) between $3D7_{UM}$ and antibody-selected $3D7_{SM}$ parasites (15). Transcript abundance was measured by using primers targeting *var* gene groups (white) or primers targeting single *var* genes (black) and are summarized corresponding to *var* gene groups. A twofold change in *var* transcript abundance (dashed lines) was arbitrarily defined as the cutoff for biologically significant changes in *var* transcript abundance.

The above-mentioned primers and probes were also applied in the present study, but to allow for relative comparisons to internal control genes, a new set of primers with specificity for the three main groups (A, B, and C) and *var1-3* was designed. By using these primers, *var* transcript levels were measured in cDNAs from 106 Tanzanian children with asymptomatic, uncomplicated, or severe *P. falciparum* infections.

Similar to the results of the study by Kaestli et al. (16), a larger transcript abundance of *var* group B genes was observed with an increasing degree of disease severity. Importantly, a similar pattern of transcript abundance was found for the *var* genes of group A (including *var3*). Conversely, *var* genes of group C were found to be transcribed at the same level in all sample groups. The differential transcript abundance patterns determined with the BC1 and BC2 primers corresponded to the transcript abundance patterns measured by B and C group-specific primers, respectively. These conclusions were supported by data generated by two quantitative PCR methods with primers targeting both the 5′ and 3′ ends of the genes. Although we cannot exclude the possibility that the difference in PfEMP1 expression patterns in asymptomatic children and younger symptomatic children is due to the marked age difference or the severity of infection, the data suggest that *var* group C genes are not involved in severe childhood malaria. The fact that no *var* gene group was detected at higher transcription levels in AM and UM than in SM samples is puzzling. This could be explained in several ways. Firstly, the categorization of patients into AM, UM, or SM is operational, and other host and parasite factors, including other variant surface antigens, might play equally important roles in disease outcome. Secondly, since the current knowledge of the global *var* sequence repertoire is limited, unknown transcripts not targeted by our primers may be excluded from the analysis. Finally, there is a possibility that the present primers result in biased amplification of a subset of predicted target genes. In particular, the last case might be true for the *var* group C primer pairs C2 and BC2, as indicated by the relatively large differences between estimated and observed C_T values for the 3D7 genomic DNA amplifications. Alternatively, group A or B *var* genes might be expressed in relatively larger abundance than *var* group C genes. This explanation would assume that not only the type of adhesion ligand, but also the amount of ligand, determines the adhesion phenotype. Future studies will test this hypothesis.

In logistic regression models, there was a statistically significant association between the risk of developing severe malaria and the transcript abundance of group A or B *var* genes. Thus, a twofold increase in *var* group A or B transcript abundance was associated with an increase of 20 to 61% in the risk of developing severe malaria. This observation does not indicate any difference between *var* group A and B transcript abundances in relation to disease severity. However, some indications of functional differences in group A and B *var* genes may be found in the linear regression models of *var* transcript abundance and clinical features. The association of increased parasitemia with *var* group B transcript abundance suggests that the parasites expressing these genes caused the severe

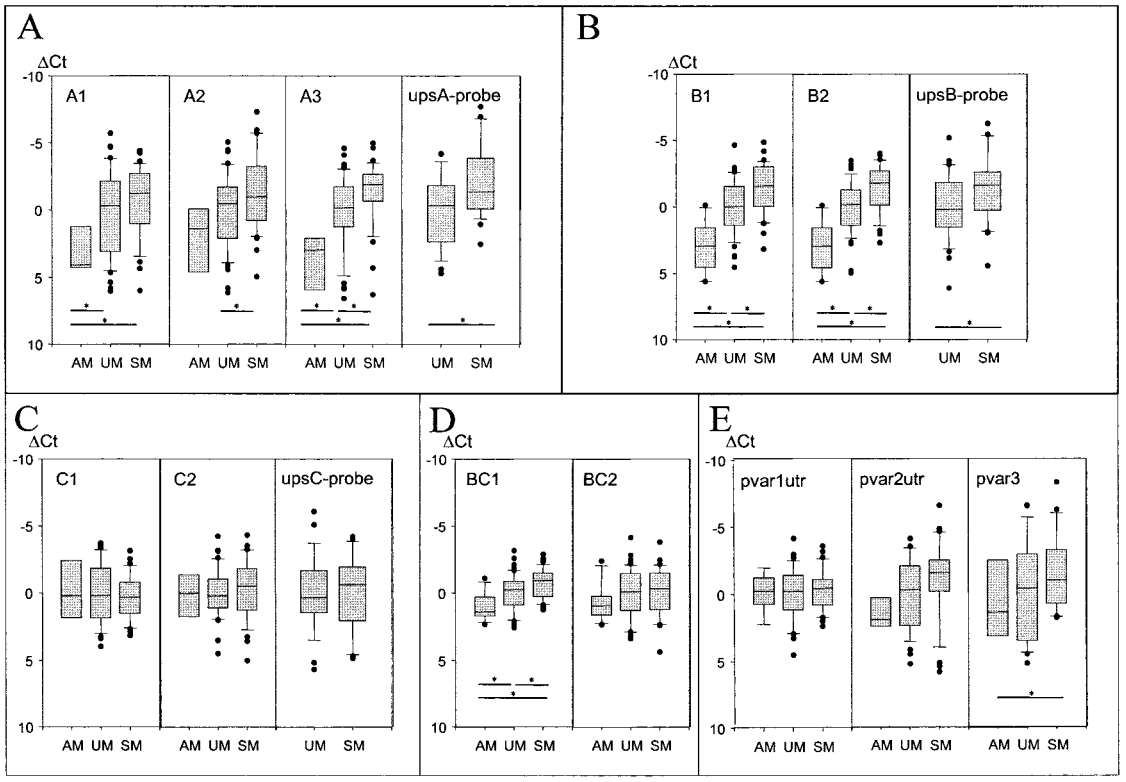

FIG. 2. Transcript abundances of *var* gene groups in parasites from children with AM and from children suffering from UM or SM. Transcript abundances are shown relative to the average abundances in uncomplicated cases (ΔC_T values). Panel A shows *var* group A transcript abundances measured with primers A1-3 in quantitative PCR and with upsA-probe in the MGB assay. Similarly, panels B and C show the transcript abundances of group B and C *var* genes, respectively. Panel D shows the transcript abundances measured with primers BC1 and BC2, targeting group B and C genes. Panel E shows transcript abundances measured with primers targeting the conserved *var* genes *var1-3*. Boxes outline 25th to 75th percentiles, with medians indicated as a line inside each box and whiskers illustrating the 5th and 95th percentiles. Horizontal lines with asterisks below the plots indicate statistically significant differences in transcript abundance between groups (one-way analysis of variance; $P < 0.05$ after Bonferroni correction).

infections in these children. In contrast, the lack of association between *var* group C transcription and parasitemia or clinical presentation supports a previous finding which suggested that group C *var* genes may be involved in establishing chronic infections (16). Similar to the case for *var* group B transcripts, a positive correlation between *var* group A transcript abundance and parasitemia would be expected in these data and in previous findings indicating the involvement of group A *var*

TABLE 3. Clinical and parasitological details of subjects

Parameter	Value for indicated group[a]			Significant relationship(s) between groups (*P*)[b]
	AM (n = 12)	UM (n = 52)	SM (n = 42)	
Mean age (mo)	46 (38, 54)	30 (26, 34)	28 (24, 32)	AM > UM/SM (<0.001)*
Sex (no. of males/no. of females)	6/6	22/30	18/24	
Mean parasitemia (parasites/200 leukocytes)	336 (144, 527)	867 (1,067, 2,206)	2,105 (1,883, 3,230)	AM < UM < SM (<0.001)*
Mean PCV (%)	29.28 (25.60, 32.95)	27.98 (26.32, 29.64)	25.37 (23.34, 27.4)	AM > UM/SM (<0.001)*
Mean lactate (mmol/liter)	3.82 (2.79, 4.84)	2.67 (2.4, 2.9)	2.9 (2.6, 2.3)	AM > UM (0.02)*
Mean glucose level (mmol/liter) (95% CI)	4.97 (4.22, 5.71)	5.1 (4.62, 5.58)	5.24 (4.68, 5.81)	0.491*
No. of days of illness	Not applicable	2.6 (2.3, 2.9)	2.8 (2.5, 3.1)	0.526**
Mean MUAC (cm)	Not determined	15.96 (15.02, 15.71)	15.36 (15.56, 16.36)	0.01**
No. of patients with prostration/ total no. of patients	0/12		40/42	
No. of patients with impaired consciousness, coma, or neurological alterations (Blantyre score of ≤3)/total no. of patients	0/12	0/52	7/42	

[a] Values in parentheses are 95% confidence intervals.
[b] *, analysis of variance/Kruskal-Wallis test; **, *t* test.

TABLE 4. Comparison of *var* group transcript abundances in parasites from patients with SM or UM

Primer pair	Fold change[a] (by $\Delta\Delta C_T$ method)	Confidence interval	*P* value (*t* test)
A1	1.6	0.6, 3.9	0.3067
A2	2.6	1.2, 5.6	0.0175
A3	2.6	1.2, 5.6	0.0148
UpsA-probe	4.3	1.6, 11.5	0.0051
B1	2.7	1.5, 4.9	0.0014
B2	2.5	1.4, 4.5	0.0020
UpsB-probe	2.8	1.2, 6.5	0.0208
C1	0.8	0.4, 1.5	0.4779
C2	1.1	0.6, 2.1	0.6836
UpsC-probe	1.1	0.4, 3.1	0.8494
BC1	1.7	1.2, 2.4	0.0050
BC2	1.1	0.6, 1.8	0.7928
Pvar1utr	1.1	0.5, 2.7	0.4375
Pvar2utr	2.0	0.8, 4.8	0.1383
Pvar3coding	2.6	0.7, 10.2	0.1611

[a] Transcript abundance in SM parasites/transcript abundance in UM parasites.

TABLE 5. Logistic regression model showing the risk of severe malaria for a twofold increase in transcript abundance of specific *var* gene groups after correcting for the effects of age, MUAC, parasitemia, and sex

Primer pair	Odds ratio	95% Confidence interval	*P*
A2	1.28	1.06, 1.56	0.012
A3	1.37	1.08, 1.75	0.010
upsA	1.35	1.02, 1.79	0.037
B1	1.47	1.08, 2.00	0.014
B2	1.52	1.14, 2.04	0.006
BC1	1.61	1.06, 2.44	0.023
upsB	1.20	0.93, 1.56	0.170

genes in severe malaria (3, 15, 17). The lack of such an association might be explained if group A PfEMP1 variants confer the strongest cytoadhesion in naïve individuals only and if group A variants only dominate in first malaria infections. Since the average age of the children enrolled in this study was 29 months, most children would have undergone several, sometimes severe, infections and would have developed some immunity against PfEMP1 variants of group A. This is supported by a trend towards a lower *var* group A transcript abundance with increasing age in UM cases.

The observed trend of more abundant *var* group A transcripts in cerebral malaria cases than in all other cases might indicate that these *var* genes play a specific role in cerebral malaria. *var2csa* has been identified as the main chondroitin sulfate A binding ligand in pregnancy-associated malaria (28). The difference seen in *var2csa* transcript abundance between the disease groups was therefore unexpected. However, for all samples, the *var2csa* transcript abundance was >100-fold lower (data not shown) than that reported for placental parasites (35) or parasites selected in vitro on CSA (29). In addition, while *var2csa* transcription appears to be controlled by similar mechanisms to those controlling group A *var* genes (11), the translation of *var2csa* transcripts, unlike that of other *var* transcripts, seems to be controlled by translation of an upstream open reading frame (22; K. W. Deitsch, personal communication). Thus, *var2csa* is most likely not responsible for the disease outcomes of these children.

var1-like genes are unique among *var* genes because they are highly conserved between parasite genomes and appear to be controlled by a unique 5′ region (36), which might indicate a specialized function for *var1* products similar to the *var2csa* gene in pregnancy-associated malaria. However, the similar abundances of *var1* transcripts in all three groups gave no indications of the function of *var1* products. This, together with the observed constitutive *var1* transcription throughout the intraerythrocytic stages in isogenic but phenotypically different parasite lines (20, 21, 29), leaves the function of *var1* molecules enigmatized.

In conclusion, the data presented here show an association between disease outcomes and the transcription of *var* subtypes in African areas where malaria is endemic. Of specific importance, the association between severe malaria in young children and *var* group A and B transcription is demonstrated and supports the notion that a vaccine based on selected PfEMP1 molecules might be feasible.

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