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var gene transcription dynamics in *Plasmodium falciparum* patient isolates☆

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ABSTRACT

A major feature of *Plasmodium falciparum* parasitized red blood cells (pRBC) is their capacity to sequester in the microcirculation. The binding is mediated by PfEMP1 (*P. falciparum* erythrocyte membrane protein 1), a variable protein encoded by the *var* gene family. *P. falciparum* avoids the host antibody response generated against previously used variants by switching the expression of PfEMP1, which may affect the disease outcome. We have here studied *var* gene transcription over time within the life cycle of the parasite by semi-quantitative PCR and sequencing by employing three sets of degenerate primers to the 5-prime end of the *var* genes (corresponding to the DBL1α-domain). To accurately determine transcript levels, subsequent in-depth analysis was made by amplifying the 10 most frequently expressed *var* sequences identified in each developmental stage by quantitative PCR (Q-PCR). The maximum peak in *var* gene transcription seems to vary in time among parasites. In five out of seven parasites, *var* gene transcription was found to be higher or equal at 22–26 h post-invasion compared to 4–10 h post-invasion. Our data indicate that the intra-isolate *var* gene transcription dominance order may change between different developmental stages. The transcription of *var* genes in field isolates is more complex than in laboratory strains and often changes after *in vitro* adaption of the parasite. By using semi-quantitative PCR employing degenerate primers combined with quantitative-PCR using specific primers it is possible to monitor *var* gene transcription in detail during the life cycle of the parasite. The work presented here suggests that trophozoite pRBC is likely to be the optimal source of RNA for predicting the translated *var* gene species.

1. Introduction

More than one million children succumb every year to *Plasmodium falciparum* malaria infections. A central *P. falciparum* virulence factor is the rosetting and cytoadherence of parasitized red cells (pRBC) with uninfected RBCs, other pRBCs and endothelial cells in the microvasculature. The sequestration process occurs in the context of cytokine release and may cause obstruction of the blood flow and induction of further inflammatory cascades in the neighboring tissues, resulting in severe disease if binding is pronounced [1–6]. The adhesion is mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a multi-domain protein of 200–250 kDa expressed at the surface of the pRBC [7–9]. PfEMP1 is encoded by the *var* gene family, which comprises approximately 60 copies per haploid genome [9,10]. The mechanism of *var* gene regulation is not clearly understood and there is conflicting evidence regarding the number of short- and full-length *var* genes that are transcribed within the course of the erythrocytic life cycle. It has been claimed that a cloned, single *P. falciparum* parasite simultaneously transcribes short- and full-length *var* genes in early ring stages but

Abbreviations: pRBC, parasitized red blood cell; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; DBL, duffy binding-like domain; CSA, chondroitin sulfate A; RT-PCR, reverse transcriptase PCR; Q-PCR, quantitative PCR.

☆ *Note:* Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers GE639566–GE647878.

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that transcripts of one *var* gene dominates in the later trophozoite stages [11–14]. Others argue that a single full-length *var* gene is transcribed both in ring and trophozoite stages in clonal parasites [15–18]. Still, overall it is agreed that one *var* gene transcript is dominantly translated into a single species of PfEMP1 in late ring stage parasites and mature trophozoite stages.

Over the course of an infection, *P. falciparum* switches expression of PfEMP1 to avoid the host antibody response generated against previously expressed variants [19,20]. Re-arrangements and recombinations [21,22] generate a vast pool of antigenically diverse genes, and numerous studies have confirmed that the global *var* gene repertoire is wide and that there is little genomic overlap of *var* genes between different *P. falciparum* isolates [23–30]. Still, the *var* genes can be subdivided into five distinct classes (A–E) and two intermediate groups (B/A and B/C) depending on their 5′ upstream sequences and chromosomal position [31–33]. Recombination is therefore likely more prone to occur within rather than between groups, thereby globally maintaining the nature of the distinct *var* gene groups [34,35].

Different adhesive properties of PfEMP1 have been described in parasites cultured *in vitro*. Indeed, the different groups display different binding properties, where the PfEMP1s of group A *var* genes do not bind to CD36, while the other groups do [36]. The grouping might have clinical relevance, since associations have been made between group A and B *var* gene transcription and severe malaria in children [37–40]. Still, the majority of *var* genes identified have been placed within groups A or B, making it necessary to further explore the role of each group of genes in the pathogenesis of the disease.

The parasites also carry and transcribe sterile *var* transcripts in mid to late stage development. These group D *var* genes, named var_{COMMON}/$var1_{CSA}$, are globally conserved and appear in a limited number of sequence variants [41,42]. The function of this transcript remains unknown. The second exception to the high *var* gene sequence variability is the group E *var* gene, *var2CSA*. The PfEMP1 encoded by *var2CSA* has been implicated in pregnancy associated malaria. In addition, long-term propagated adhesive clones of 3D7 have been shown to successively and spontaneously switch to *var2CSA* transcription [43]. This was matched by the loss of PfEMP1 surface expression, suggesting that the presence of *var2CSA* transcripts is a default variant in these particular parasites.

Much scientific effort has been concentrated on the Duffy binding-like domain-α (DBL1α) located in the N-terminal head structure of PfEMP1 since it is relatively conserved and mediates the virulence associated interactions. It brings about rosetting and endothelial adhesion of pRBC through binding to different receptors, including heparan sulfate, complement receptor 1 and the blood group A antigen [10,44,45]. Further, certain degenerate amino acid motifs in DBL1α have been found overrepresented in parasites causing severe disease or rosetting and there is evidence of antigenic cross-reactivity in between structural elements in distinct DBL1α domains [40]. The cysteine content of the DBL1α domain covaries with the upstream grouping of the *var* genes. Group A has one or two cysteines, while non-group A has three to five cysteine residues within a defined area on the DBL1α [46]. A low cysteine content in transcribed DBL1α domains has been shown to correlate with rosetting [25,40] and also to the occurrence of severe malaria [27,40]. The binding characteristics of the PfEMP1 variants that are dominantly expressed in an infecting parasite population are therefore likely to determine the disease outcome.

The diversity of PfEMP1 has been closely studied through sequencing of the encoding *var* genes, but the mechanism of *var* gene transcription is still not clearly understood. We have here used a novel approach to study *var* gene transcription over time within the life cycle of the parasite to elucidate *var* transcription in clinical isolates both before and after cryopreservation. We present a method to monitor relative *var* gene transcription in clinical isolates using semi-quantitative PCR and sequencing followed by an in-depth analysis with quantitative PCR (Q-PCR). Our data suggest that intra-isolate *var* gene transcription dominance order may vary between developmental stages and that *var* gene switches can be detected as early as after six generations post-thawing in *in vitro* propagated cultures. Accurate transcription mapping through Q-PCR is made possible through the use of the combined sequence information gained by three semi-quantitative PCRs.

2. Materials and methods

2.1. Parasites and cultivation

In all 21 *P. falciparum* isolates and strains were used in this study. Of these, 14 were clinical isolates collected in Uganda in 2002 and 2003 which have been described elsewhere [40]. In brief, venous blood was drawn from children under the age of five with different states of active *P. falciparum* infection. Patients were recruited in two locations in Uganda: at the district hospital in Apac, which is situated in a malaria holoendemic area [47] 250 km north of Kampala, and at the Mulago hospital, located in the capital. Informed consent was obtained from the parents of the patients. Patient data is summarized in the supplemental information (SI) Table 1. Ethical permissions for the study have been obtained both in Sweden (permission 03/095) and in Uganda (permission MV717). All isolates were cultivated around 20 h (zero generations) for the study of transcripts in trophozoite pRBC. Additionally, nine of the isolates were also sampled directly at ring stage (4–10 h). Five isolates were thawed, *in vitro* adapted and studied as ring- (4–10 h) and mid-stage trophozoites (22–26 h) after 6–11 generations *in vitro*. A time course of *var* transcription of five time points over the life cycle was established for one of the isolates (UAS31). In addition seven laboratory strains were used in the study including TM284, FCR3, FCR3S1.6, 3D7AH1, 3D7AH1.S2, 7G8 and DD2. For details of the included isolates and strains, see SI Table 1.

All 21 isolates and strains were cultivated using standard methods [48] with the modifications that all *in vitro* adapted Ugandan isolates were cultivated in AB+ non immune serum and that gassing with a mixture of 90% NO_2, 5% O_2 and 5% CO_2 and shaking incubation replaced the static candle jar technique. Parasites were kept tightly synchronous using 5% sorbitol (v/w). Time post-invasion was estimated by evaluating the parasite morphology including size within the pRBC with acridine orange [49]. Moreover, for the laboratory strains and *in vitro* adapted isolates timing of parasite invasion was also done in order to correctly estimate time post-invasion. The isolates were frozen using the Stockholm sorbitol method and thawed in a sodium chloride gradient as described [50].

2.2. RNA extraction, reverse transcription, PCR and sequencing of *var* sequences

RNA was extracted from both ring (4–10 h) and trophozoite (22–26 h) stage parasites using the Qiagen RNeasy mini kit according to the manufacturer's instructions (Qiagen, Düsseldorf, Germany) with minor modifications. To remove any contaminating gDNA the extracted RNA was treated with TURBO DNAse (Ambion, Austin, TX, USA) for 30 min at 37 °C. Total RNA was reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA, USA) with random hexamers and oligo(dT)12–18 (300 ng/ml and 25 ng/ml, respectively) at 25 °C for 10 min and 50 °C for 120 min followed by 70 °C for 15 min. For each cDNA synthesis reaction, a control reaction without reverse transcriptase (RT-) was performed with identical amounts of template. The DBL1α domain of the *var*