From *In Vivo* to *In Vitro*: Dynamic Analysis of *Plasmodium falciparum* var Gene Expression Patterns of Patient Isolates during Adaptation to Culture

Qingfeng Zhang¹, Yilong Zhang¹, Yufu Huang², Xiangyang Xue¹, He Yan¹, Xiaodong Sun³, Jian Wang³, Thomas F. McCutchan², Weiqing Pan¹,²*  
¹Institute of Infectious Disease and Vaccine Development, Tongji University School of Medicine, Shanghai, China, ²Department of Pathogen Biology, Second Military Medical University, Shanghai, China, ³Yunnan Institute of Parasitic Diseases, Puer, China

**Abstract**

*Plasmodium falciparum* erythrocyte membrane protein 1 (PFEMP1), encoded by the var gene family, plays a crucial role in disease virulence through its involvement in binding to various host cellular receptors during infection. Growing evidence suggests that differential expression of the various var subgroups may be involved in parasite virulence. To further explore this issue, we have collected isolates from symptomatic patients in south China-Myanmar border, and characterized their sequence diversity and transcription profiles over time of var gene family, and cytoadherence properties from the time of their initial collection and extending through a two month period of adaptation to culture. Initially, we established a highly diverse, DBLα (4 cysteines) subtype-enriched, but unique local repertoire of var-DBL1α sequences by cDNA cloning and sequencing. Next we observed a rapid transcriptional decline of upsA- and upsB-subtype var genes at ring stage through qRT-PCR assays, and a switching event from initial ICAM-1 binding to the CD36-binding activity during the first week of adaptive cultivation *in vitro*. Moreover, predominant transcription of upsA var genes was observed to be correlated with those isolates that showed a higher parasitemia at the time of collection and the ICAM-1-binding phenotype in culture. Taken together, these data indicate that the initial stage of adaptive process *in vitro* significantly influences the transcription of virulence-related var subtypes and expression of PFEMP1 variants. Further, the specific upregulation of the upsA var genes is likely linked to the rapid propagation of the parasite during natural infection due to the A-type PFEMP1 variant-mediated growth advantages.

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* E-mail: wpqan9912@yahoo.com.cn

These authors contributed equally to this work.

**Introduction**

*Plasmodium falciparum* is the most virulent species of malaria that infects humans. Erythrocytes infected with this parasite adhere to different types of endothelial cells in the deep vasculature of the body to avoid being cleared by the spleen [1,2]. The specificity of adherence is mediated by a polymorphic protein, *P. falciparum* erythrocyte membrane protein 1 (PFEMP1), encoded by members of a multi-gene family (the var gene family) [3,4]. It is generally thought that only one member of this family is selectively expressed each generation and migrates to the surface of the infected RBC dictating the cellular receptor to which the erythrocyte will bind [5,6]. There are approximately 60 copies of the PFEMP1 encoded in each genome of 3D7 strain [7,8]. The rate of change in expression may reach ~18% per generation *in vivo* which serves to compromise the immune defense of the host [9,10]. The large, highly variable protein includes several sequence motifs such as the various Duffy-binding-like domains (DBL) and the cysteine rich inter-domain regions (CIDR). All interact with various host cellular receptors such as CD36, ICAM-1, CSA, E-Selectin, VCAM, etc. [1,11,12].

Var gene family can be separated into three major groups based upon conserved sequence features (A, B and C) upstream of the protein coding region [13]. Several studies have investigated the potential association between PFEMP1 expression and presentation of malaria [14,15,16,17,18]. It has been suggested, on the basis of studies conducted in Tanzania and Papua New Guinea, that expression of group A or B/A var genes is associated with severe children malaria, while group C var genes are linked to asymptomatic malaria. In support, large-scale sequence analysis of var-DBL1α domains revealed that the lack of 1 or 2 cysteines in this region, most belonging to group A or B/A var genes, was associated with severe malaria [19,20,21]. Although these investigations indicate that the specific expression of subtype var genes play a crucial role in the pathogenesis of different forms of malaria, little is known of the underlying mechanism.

The individual clonal type of *P. falciparum* contains approximately 60 copies of this gene family, and the collection of clonal types of field isolates that constitute the global population of this parasite seems to be nearly endless. Nevertheless, studies on geographically diverse populations support the existence of
structured var repertoires among various geographic areas, although their sequences are highly diverse within each repertoire [19,22,23,24]. These investigations have established several var repertoires of field isolates in Africa and South America, however, little is known of the parasites in southern China-Myanmar border, a highly epidemic area in Asia [25,26].

In a clonal population the most abundant transcript(s) found in each blood-stage cycle is thought to be expressed and therefore reflective of adherent specificity during in vitro cultivation. For that reason, much of the information we have about var genes comes from transcriptional data. The direct investigation of protein properties would be ideal, but most often such experiments are not done due to the degree of difficulty involved in studying each member and the high number of molecules involved. Further, the study of PEEMP1 in vitro is potentially impacted by artifacts relating both to the effects on the parasite of extended periods in culture and the technical necessity to equate RNA expression with protein presence and hence function. Peters et al have shown such effect on var gene transcription of experimentally infected 3D7 strain during adaptive cultivation [27]. Hence it is important to understand the effects of in vitro adaptation of patient isolates to laboratory populations where the dominant selective forces are different than those affecting the parasite in its native state.

In this study, we investigated the composition of var gene transcripts, their transcription patterns during the life cycle of the parasite, and their cytoadherent characteristics from in vivo to in vitro over the course of ~2 months. In so doing we hoped to clarify the influence of the adaptive process on the transcription as well as expression of var genes, and look for potential link between PEEMP1 expression and parasite characteristics starting from each isolate’s collection point, and extending through many generations of in vitro culture. Our data revealed a potential correlation between the predominant transcription of upsA-subtype var genes and the parasitemia of parasites found in the blood when the isolates were collected, which partially reflected the growth rate of parasite in vivo. We therefore suggest that A-type PEEMP1 variants-mediated high parasitemia contributes to the virulence of parasites in natural infection.

Results

Distribution of the conserved cysteine/PolV motifs in DBL1α sequences

Initially, we analyzed the var transcripts from 9 clinical isolates collected from symptomatic malaria adult patients with multi-infection history in the Yunnan province of south China (Table 1 and S1), and compared them with previously reported isolates collected from other regions of the world. It has been shown that all DBL1α sequences can be grouped into 6 subgroups according to the cysteine/PolV structural features within this DBL1α region of transcripts [19,20]. In laboratory 3D7 strain, upsA var transcripts fall into the 1–3 subgroups while most upsB and upsC var genes belong to the 4–6 subgroups. Analysis of our isolates showed that A15, A16 and A19 had a higher proportion of transcripts fitting into the 1–3 subgroups than other samples \((p<0.01)\). In contrast, the overall distribution of these motifs in var transcripts was similar to that of non-severe malaria cases found elsewhere [28], and that in the genome of 3D7 strain (Figure 1A).

According to the number of cysteine residues within the DBL1α domains, the 1–3 subgroups with two cysteines are also referred to as DBL1α subtype, which has been shown to be associated with high virulence of parasites from field isolates, whereas the 4–5 subgroups with four cysteines belong to the DBL1α subtype [28]. Here, we also analyzed the composition of transcript sequences from Yunnan isolates in terms of the summarized classification and compared them to that found in other studies [19,20]. As shown in Figure 1B, Yunnan samples had a similar proportion of DBL1α transcripts to that found in 3D7 genome, but significantly lower than that reported for field isolates from African children as well as the global samples \((p<0.01)\). It is worth noting that the African children analyzed here were generally severe malaria patients including cerebral malaria, whereas the 3D7 clonal type has been adapted for cultivation condition in vitro for numerous generations. This result further demonstrates that the specific transcription of var subgroup with 4 cysteines in their DBL1α domain is associated with non-severe malaria under selection of host immune pressure. Further, a phylogenetic analysis with all of the DBL1α tags from Yunnan isolates revealed a unique cluster corresponding to upsA var genes in 3D7 strain. (Figure 2).

Finally, all DBL1α sequences of the Yunnan isolates were designated in terms of the nomenclature with distinct sequence tags as “PolLV1-PolLV2-PoLV3-(cys)n-PoLV4-length” [28]. In total of 130 var-DBL1α-contigs obtained from the 9 isolates described above, 93 sequences were unique in Yunnan area compared with those of geographic field isolates published elsewhere, suggesting a restricted local repertoire in this area (Table S2) [19,20,22].

Var gene distribution in clinical isolates

To measure the infection complexity of each isolate, we determined the number of genotypes per isolate using a single copy gene, the Merozoite Surface Protein 2 (MSP-2), by PCR-RFLP analysis as described previously [29]. There was a mean of 1.9 infecting clones per isolate indicating a relatively low number of multiple infections of isolates in the area (Table 1). We then analyzed the transcript diversity within each of our isolates. In 9 out of 15 clinical isolates analyzed in this experiment, we obtained more than 50 var-DBL1α cDNA sequence reads per sample by sequencing of clones (Table 1 and S1). Isolates had a mean of 14.4 different DBL1α transcripts per sample of which only 2–3 were representative of abundant transcripts when they had >10 copies (Figure 3). In agreement with similar studies this suggests 1–2 dominant var gene genotypes per population [30] and is consistent with our estimate of genotypes per isolate. The variety of expression profiles raised the possibility that the dosage of var transcription was being regulated at multiple sites during the course of development.

Among these samples, interestingly, three isolates with higher parasitemia, i.e. A15, A16 and A19, exclusively transcribed dominant upsA subgroup var \((p<0.01)\) (Figure 3 and Table 1), while the var-DBL1α sequences cloned from their genomic DNAs did not indicate a higher proportion of upsA var genes (data not shown). Isolates with upsB or upsC dominant var transcripts exhibited much lower parasitemia in peripheral blood.

Monitoring the var gene family of isolates over the course of cultivation in vitro

In the work described above, we observed a potential link between the dominant upsA var transcripts and higher density of ring-stage parasites in peripheral blood. This raises the question of whether the transcription status of upsA and other var genes are constant during adaptation to culture. Previously it has been reported that the overall abundance of upsA, -D and -E transcripts of ex vivo samples declined dramatically after a short period (the first ~10 days) of in vitro cultivation [27]. This indicated that a selective process specifically affects those subgroups during parasite cultivation. To clarify these issues, we monitored the transcription patterns of var gene families at both ring and trophozoite stages of individual isolates over a two month period of in vitro cultivation.
transcription profile was a reduction in and 5). It was notable that the only dramatic change in the sub-clonal proportion of each isolate due to their different growth (Figure 4B). To avoid the probable bias coming from the change of transcripts at the initial stage, but they returned by the fourth cycle upsBC1 produced the most abundant transcripts at ring stage, while parasites during the period of adaptive cultivation in the figures.

In most isolates, three subtype var genes, upsA, upsB and upsBC1, produced the most abundant transcripts at ring stage, while upsBC1 subtype transcription alone dominated in trophozoite-stage parasites during the period of adaptive cultivation in vitro (Figure 4 and 5). It was notable that the only dramatic change in the transcription profile was a reduction in upsA- and upsB-subtype var transcripts at the initial stage, but they returned by the fourth cycle (Figure 4B). To avoid the probable bias coming from the change of sub- clonal proportion of each isolate due to their different growth advantages during the adaptive process, we measured the composition of sub-clones for the 6 isolates at various time points in vitro in comparison with that of the collection time by genotyping with microsatellite markers [31,32]. Result showed that three isolates (YN3, YN8, YN29) with significant transcriptional decline of upsA- and upsB- var had only a single clone and maintained the same one from in vivo to in vitro in two weeks. While a selection effect was observed in another 2 isolates (YN11 and YN27), in which one sub-clone had taken over the culture in vivo, no significant decrease of the two above-mentioned subtypes had occurred in these samples (Figure 4B and Table S3). Therefore, it implies that the first several developmental cycles are crucial to the adaptive process of parasites from field isolates, and that the transcriptional fluctuations of the two var-subtypes are reflective of this process.

In ring-stage parasites, two different transcription patterns of var gene family were observed. 12 out of 14 field isolates exhibited dominant transcripts of upsBC1 subtype, whereas two isolate, i.e. YN3 and F08B-33B, showed that the upsA subgroup was dominantly transcribed in rings (Figure 4B and 5A). All var genes were transcribed at much lower levels in trophozoites than in ring stage parasites except the upsBC1 subgroup, which was exclusively transcribed in most cultures with a similar level to that in rings. In

| Isolates | Age(ys)/ gender | Strain (stage) | Multi- infection | Parasitemia (/μl blood) | Symptom | Dominant var transcripts (ups) |
|----------|-----------------|----------------|-----------------|------------------------|---------|-----------------------------|
| A2*      | 50/M            | Pf (R)         | 1               | 1620                   | fever, headache | B, C ¹                   |
| A3*      | 24/M            | Pf (R)         | 2               | 9120                   | fever, headache | B, C ¹                   |
| A7*      | 34/M            | Pf (R)         | 1               | 36300                  | fever, systemic ache | B, C ¹               |
| A15*     | 37/M            | Pf (R)         | 3               | 209400                 | fever         | A, B ¹                   |
| A16*     | 38/M            | Pf (R)         | 2               | 194400                 | fever, headache | A, C ¹                   |
| A19*     | 36/M            | Pf (R)         | 2               | 122100                 | fever         | A, B, C ¹                |
| A20*     | 35/M            | Pf (R)         | 2               | 7500                   | fever, headache | B, C ¹                   |
| A21*     | 34/M            | Pf (R)         | 2               | 11280                  | fever, systemic ache | B, C ¹               |
| A22*     | 42/M            | Pf (R)         | 2               | 8580                   | fever, systemic ache | C ²                  |
| F07-4²   | 14/M            | Pf (R)         | 1               | 38157                  | fever         | ND                        |
| F08B-5²  | 18/F            | Pf (R)         | 1               | 186667                 | fever, headache | ND                        |
| F08B-9²  | 25/F            | Pf (R)         | 2               | 10952                  | fever         | ND                        |
| F08B-33² | 16/M            | Pf (R)         | 1               | 10099                  | fever, headache | ND                        |
| F08B-34² | 24/F            | Pf (R)         | 2               | 975                    | fever         | ND                        |
| LZF22²   | 26/F            | Pf (R)         | 2               | 2068                   | fever         | ND                        |
| LZF25²   | 29/M            | Pf (R)         | 1               | 17950                  | fever, headache | ND                        |
| LZF26²   | 19/F            | Pf (R)         | 1               | 3980                   | fever         | ND                        |
| YN3³     | 32/M            | Pf (R)         | 1               | 1733360                | ague, coma    | A, B, BC ³               |
| YN8³     | 47/M            | Pf (R)         | 1               | 10320                  | fever, ague   | B, BC ³                 |
| YN11³    | 17/M            | Pf (R)         | 2               | 22160                  | fever, ague   | BC ³                     |
| YN27³    | 6/M             | Pf (R)         | 2               | 131200                 | fever, ague   | BC ³                     |
| YN29³    | 16/M            | Pf (R)         | 1               | 273760                 | fever, ague, coma | BC ³               |
| YN53³    | 18/M            | Pf (R)         | 1               | 620000                 | fever, ague   | BC ³                     |

a. Total RNA extracted from Yunnan field isolates were used in diversity and distribution analysis of var-DBLs sequence at the time point of collection ("0" hr).
b. Field isolates from Yunnan and Myanmar used in adaptive cultivation in vitro for ~2 month, and measured of transcription profile and cytoadherent features during the course.
c. Myanmar field isolates used in adaptive cultivation in vitro for ~16 days, and measured of transcription profile in rings and trophozoites.
d. The dominant var transcript at collection time.

1. The dominant var transcripts were identified by cDNA cloning-sequencing strategy.
2. Not done.
3. The dominant var transcripts were identified by qRT-PCR with var subtype-specific primers.

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Table 1. Clinical assessment of symptomatic malaria patients recruited in this study.
contrast to that in ring-stage parasites, the upsBC1-subtype transcripts in trophozoite stage remained the dominant and constant over the course of in vitro culture (Figure 5B). Due to the exon 2-specific amplification by the degenerated upsBC1 primers, the dominant transcripts of late-stage parasites are expected to be derived from the intron promoter of var genes [33].

In addition, the transcription profiling of the var gene family in the first cycle in vitro revealed that only isolate YN3 had a higher transcriptional abundance of the upsA-subtype var compared with other members during the ring stage (Figure 4C). It is notable because this isolate exhibited a relatively higher parasitemia at the collection time (Table 1). This phenomenon further supports the

**Figure 1. Classification of var-DBL1α transcript sequences of Yunnan isolates in comparison with other field isolates and 3D7 strain.**

(A) DBL1α amino acid sequences derived from cDNA were divided into six sequence groups in terms of the conserved “cysteine/PoLV” sequence tag within the DBL1α region. The distribution of different subsets of individual isolate and overall cDNA sequences (Aall) of the 9 Yunnan isolates were shown in each bar with 3D7 strain (genomic sequence) as reference. The 95% confidence values (p) for the χ² test between DBL1α (subgroup 1~3) and others was: p<0.01*. (B) The distribution of each subgroups of DBL1α sequence (cDNA) based on the number of cysteines in Yunnan symptomatic malaria isolates were analyzed with 3D7 (gDNA), Africa (cDNA) and global sequences (cDNA) as references. The subgroup var genes were labeled as follows: red, cys2 (DBLα1); green, cys4 (DBLα2); white, cysX (else).

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correlation between the specific upA subtype var transcription with the increased parasitemia in vivo.

**Cytoadhesion feature of isolates during cultivation in vitro**

Finally, we tried to investigate the cytoadherence properties of each isolate for interaction with host cellular receptors such as CD36, ICAM-1, CSA and HA for a period from the point of collection and extending to the first two months of adaptation. Initially, we attempted to establish the association between transcription of specific var subtypes and cytoadhesion phenotypes in the laboratory FCC1/HN strain [34]. In this unselected culture, the link between upBC and CD36 or Var2CSA and CSA phenotype were observed (Figure S1). The first has been described by Kraemer and Robinson [35,36], and the second association was well studied with parasites involved in pregnancy-associated malaria (PAM) [37,38]. In this study, CD36 was the main cytoadhesion feature in all cultivated isolates except the initial stage of F08B-33, and the CD36-binding property was retained over the two months in vitro (Figure 6).

The cytoadhesive patterns of various receptors used here were relatively constant during the cultivation in vitro, except a few time points. ICAM-1 binding was detectible at a few time points for isolates F08B-9 (30 days), F08B-34 (30 days) and LZF22 (45 days).
Interestingly, isolate F08B-33 bound to ICAM-1 at the start of the study and then switched to CD36 over the first week in culture. Combined with the transcription analysis of these isolates corresponding to these timepoints, we observed a potential link between the ICAM-1 binding phenotype and dominant transcription of upsA-subtype var genes (Figure 5A and S2).

**Discussion**

The worldwide high diversity of var primary sequences through frequent recombination among field strains, and the switching expression of var genes in different generations contribute to the complexity of the malaria pathogen [19,22,24,39,40,41]. To explore the sequence polymorphism and its association with different malaria symptoms, the var gene sequences have been extensively investigated in terms of conserved features of functional regions as DBL1α domain in highly epidemic area in Africa or South America [19,20,28]. However, little is known of the filed isolates in southern China-Myanmar border, the representative malaria epidemic area in Asia [25,28]. In this study, 93 out of 130 different DBL1α transcripts identified in 9 field isolates of clinical adult patients (most were of mild symptoms) from Yunnan-Myanmar border were novel compared with those of other areas, suggesting a restricted local var gene repertoire in this area with limited overlap to other populations. This observation points to the existence of geographic population structures in various areas as previously observed by analysis of microsatellite or single nucleotide polymorphism [22,42]. Structure feature analysis of the DBL1α sequences in terms of the conserved cysteine motif revealed a major distribution of four-cysteines subtypes (~75%) reflective of a relatively lower virulence in these clinical isolates. Recent studies reported that the expression of different var gene types was under selection of host immune pressure [21,43,44,45]. Unlike the non-immune volunteers or severe malaria in children investigated in other studies, the symptomatic malaria adult patients from Yunnan area had undergone multiple infections in past years suggesting that previous exposure afforded a measure of natural immunity.
Figure 4. Transcription profile of subtype var genes in six isolates during the initial cultivation. (A) Two assays were designed according to the requirement of the material amount needed for different analysis. R, ring; T, trophozoite. (B) Transcriptional level of three major subtypes of var genes respectively in six field isolates cultivated in vitro for 17 days measured by qRT-PCR. The time points were shown as days post thawing, and...
Although there are some inconsistency among those studies on the association between var gene expression and severity of malaria disease investigated in several geographical isolates from Africa, it is recognized that the expression of upsA- and partial upsB (B/A)-subtype var genes correlates to the severe malaria disease [15,16,18,46,47]. Nevertheless, the underlying mechanism is far from being understood. In the present study, through sequence similarity analysis of DBL1a domain, upsB and upsC var were appeared to be overlapping, however, upsA var could be distinguished from other subtypes. It was therefore feasible to investigate the possible link between upsA var and parasite virulence. Interestingly, we found that the upsA var transcription had a potential link to the higher parasitemia of isolates in peripheral blood by different strategies (cDNA cloning/sequencing and qRT-PCR). Since these samples are collected from those patients under the same criteria including the symptom, age, sampling location and time point after the occurrence of disease, infection history, etc., it is likely that the expression of group A PfEMP1 confers the more effective growth rate on the parasites, probably due to better sequestration to avoid the clearance by the host immune system. This point is supported by another independent study with ex vivo samples which showed that NF54 parasites expressing group A and B var had expanded more effectively in vivo compared to those expressing other var genes in experimentally infected humans [48]. Therefore, this finding suggests A-type PfEMP1 variant-mediated high parasitemia may contribute to the virulence of parasites in patients. However, since the difference of parasitemia in patients might be due to some other factors (patient genetic background, infection history, and parasite factors), the dominant expression of upsA var would not be the only determinant to control the growth rate of parasites in natural infection. As we observed, the isolate F08B-33 exhibited a dominant transcription of upsA var as well as ICAM-1-binding activities, however, it did not show a significantly higher parasitemia compared with other isolates. Hence, further experiments using more malaria isolates with various severities are needed to confirm the correlation between in vivo growth rate and var gene expression pattern (transcription and protein level) and the underlying mechanism. In addition, dynamic transcription analysis by qRT-PCR at different developmental stages in individual cycles revealed a link between the ICAM-1 binding phenotype and transcription of upsA var, which supports the previously observed association between upsA-subtype var and severe malaria since ICAM-1-mediated cytoadhesion has been implicated in pathogenesis of severe malaria [49,50,51].

**Figure 5. Transcription profile of subtype var genes at different stages in individual cycles.** The ring-stage (A) and trophozoite-stage (B) transcription patterns of the var gene family of two representative isolates (F03B-9 and F03B-33) in culture for ~2 months. The Seryl-tRNA synthetase gene was used as the endogenous control.

![Figure 5](https://www.plosone.org/doi/10.1371/journal.pone.0020591.g005)
In a homogeneous laboratory *P. falciparum* clone selected by a monoclonal antibody against a specific PIEMP1 or a unique receptor as CSA, it has been generally accepted that only one *var* gene is dominantly transcribed throughout the blood-stage cycle, and translated into the specific PIEMP1 protein which is exported onto the surface of the iRBC and mediates the cytoadhesion phenotype of the culture [30,52,53]. In other words, the dominantly transcribed *var* gene in a single cycle is expected to be translated into the PIEMP1 mediating a specific phenotype. However, due to the qRT-PCR technique with subtype-specific *var* primers for *var* transcription profiling had been validated only in rings, the transcriptional dominance order among various *var* in trophozoites might not reflect the actual status. It has also been demonstrated that *var* transcriptional levels in trophozoites are 10–100 times lower than that in rings [53], resulting in very dubious quantification of transcript levels in the trophozoite states. This is contrary to our transcription data where the *upsBC1* level was almost similar between rings and trophozoites. Considering that the *upsBC1* and *BC2* primer pairs are targeting the exon 2 region of *var* genes [15], it is likely that these high transcriptional signals are from sterile *var* transcripts due to the intron promoter activity as reported previously [33,54]. While the transcription level of the active genes in trophozoites are much lower compared with rings, the quantification of *var* gene transcription profile in this stage might be biased by the sterile transcripts. Therefore, since the PIEMP1 product appeared on the iRBC surface at 16 hr post invasion [52], it is rational to speculate the dominant *var* transcripts in rings, including the *uspA var* s, would be responsible for the cytoadhesion phenotypes in the late stage, which is consistent with our observation that the higher transcription level of *uspA var* s in rings is linked to the higher parasitemia in vivo and ICAM-1-binding in vitro.

The potential turn over of *var* expression pattern in culture *in vitro* was considered as an obstacle in evaluating their functions. A recent study with "*ex vivo*" 3D7 samples had shown a rapid down-regulation of overall transcription level of *var* genes at ring stage,
especially the upsA, -D and -E had a significantly faster rate of transcriptional reduction in the first ~10 days of culture [27]. It is worthy of note that the dominant transcripts throughout the cultivation duration in their samples were only upsB var s. In addition, the transcription profiling in trophozoites were not performed. In the present study, transcription measurement of var genes at both ring and trophozoite stages over the course of ~2 month post-thaw did undergo a rapid turn over event for upsA subset as well as upsB/A var s in the second cycle, indicating the virulence-related var subtype are more sensitive to the absence of immune pressure or other signals in host during the first cycle in vitro. Surprisingly, the declined var s were up-regulated again immediately after the fourth cycle in culture, which was not observed in the previous “ex vivo” 3D7 samples. It seems that the naturally infected isolates are able to adapt to the in vitro culture condition more rapidly than the parasites of passive infection.

It is well known that the CD36-binding phenotype was the most common in cultivated field isolates from patients with various symptoms, and group B and C var genes encode CD36-binding PfEMP1 variants in 3D7 strain [36,55]. Similarly, the cytoadherent phenotypes of the cultivated field isolates from patients with symptomatic malaria in the Yunnan area in our study maintained the CD36 receptor-binding activity as the major phenotype during the ~2 months of culture. However, it is worthy of note that the initial phenotype at collection time point might be different, e.g. the apparent ICAM-1 binding of F08B33 isolate during the first week in culture (Figure 6). This suggests an expression switching event among PfEMP1 variants occurred rapidly during the initial adaptive cultivation in vitro even though no apparent difference of var transcription in trophozoites was observed, which may be also linked to the role of upsA var transcripts in rings.

In summary, we conducted a systematic analysis of sequence diversity of the var repertoire, a dynamic transcription profile of the different subsets of var genes, and a cytoadherence analysis of parasites from malaria patients during a adaptive cultivation in vitro. Our results showed a restricted var-DBL1α repertoire in this area, and a greater proportion of var genes containing a Cys4 DBL1α domain than seen in similar studies in Africa. This work extends the global database of var gene repertoire in various geographic isolates. Further, our data raised a new role of the ring-stage transcribed subset of var genes (upsA subtype) in development of malaria parasite in patients. Here we suggested the A-subtype PfEMP1 variants might be linked to the rapid growth rate of isolates in vitro. Finally, it should be cautious in interpreting the experimental data and understand the nature of var gene family from the in vitro cultivated parasites due to the observation of a rapid switching of var transcription and cytoadherent phenotype of field isolates during the initial cultivation process.

Materials and Methods

Ethics Statement

In this study, patients who sought medical care at the local Center for Disease Control and Prevention (CDC) in Yunnan from 2006 to 2008 were recruited with written informed consent, and the clinical protocol was approved by the Internal Review Board of Second Military Medical University, China.

Culture of Plasmodium falciparum laboratory line FCC1/HN

Plasmodium falciparum FCC1/HN line was isolated from Hainan Island, China, established as a laboratory culture in 1979 and subsequently adapted to cultivation in RPMI 1640 medium (Invitrogen) containing 25 mM Heps, 2 mM L-glutamine, and supplemented with 0.1 mM hypoxanthine (Sigma), 20 μg/ml gentamicin (Sigma), 15% rabbit serum and 2-4% (v/v) type O® erythrocytes under the condition of 5% CO₂ at 37°C [56,57].

Plasmodium falciparum isolate samples

The malaria patients participating in this study were residents in the border area of south China (Yunnan province) and Myanmar. Malaria in this area is subject to seasonal variation with an increased incidence level occurring from July to September. Adult male farmers are at higher risk for P. falciparum infection than the rest of the population because their occupation leads to exposure to Anopheles minimus, a potent malaria vector. Blood samples came from patients who sought medical care at the local Center for Disease Control and Prevention (CDC) in Yunnan from 2006 to 2008. Malaria diagnosis was done by microscopic examination of blood smears. Parasitemia was measured relative to leukocyte numbers per μl of blood, with an average of 5000 leukocytes/μl of blood. In this study, we selected symptomatic malaria patients who were >5 years of age (most were adult). In general, they had P. falciparum in peripheral blood with parasitemia >1000 infected red blood cells (IRBC) per μl of blood, an axillary temperature <10°C, and a history of fever. Basically, patients with severe malaria, including cerebral and placental malaria, were treated immediately and hence excluded from this study except two samples with symptom between mild and severe malaria (YN3 and YN29 in Table 1). The sample size and the way of sample collection from patients were in accord with different analysis in this study:

Analysis 1: samples in this assay were used to analyze the sequence diversity of var-DBL1α domain by cDNA clone and sequencing. Hence, the fresh blood samples from patients were mixed with anticoagulant, EDTA and RNAlater solution, a RNA protection reagent (Ambion). Then the samples were frozen at ~80°C until total RNA extraction. Totally 22 samples were collected for this aim, and 15 isolates were randomly selected for total RNA extraction. However, only 9 samples were analyzed for var gene diversity since others did not produced sufficient var-DBL1α sequences by cDNA cloning with total RNAs of poor quality (see Figure 3 and Table S1).

Analysis 2: isolates used in this assay were aimed to measure the dynamic var gene transcription patterns both at ring- and trophozoite-stage, and cytoadhesion assay over time during the adaptive cultivation of ~2 months in vitro (shown in Figure 4A). Hence, fresh blood samples were cryopreserved in glycerol (Baxter), and frozen in liquid nitrogen. Totally 33 isolates were collected for this assay, and the actual number of samples used in this assay was described in the result section. We attempted to cultivate 10 field isolates for assay 1 whereas 13 isolates for assay 2 (Figure 4A), which were randomly selected to be thawed. Among these samples, 6 isolates (assay 1) and 8 isolates (assay 2) reached ~1% parasitemia within the first 5–10 days and subsequently adapted the growth condition in vitro (see Table 1 and S1).

Cultivation of patient isolates in vitro

Frozen isolates from malaria patients were thawed for analysis as described previously [44], and cultured in the RPMI 1640 medium (Invitrogen) containing 25 mM Heps, 2 mM L-glutamine, and supplemented with 0.1 mM hypoxanthine (Sigma), 20 μg/ml gentamicin (Sigma), 0.5% Albumax II, 2% human serum (type AB±). Cultures were grown in media with type O® erythrocytes at a 3% hematocrit under 2% O₂, 5.5% CO₂, 92.5% N₂ at 37°C [57]. The ring-stage parasites were synchronized with 5% sorbitol or Percoll alternatively.
Total RNA extraction and complementary DNA (cDNA) synthesis for cloning

Total RNA was isolated both directly from frozen patient blood samples and from in vitro cultured samples. For the cultivated parasites, the rings of 10–20 hr or trophozoites of 25–35 hr post invasion were harvested, respectively. RNA was extracted with Trizol (Invitrogen) according to the manufacturer’s instructions with modification as described elsewhere [58]. Subsequently, all of the RNA extracts were treated with Dnase I (Ambion) to remove the potential contamination of genomic DNA. RT-PCR amplification, cloning and sequencing of var-DBL1α domain DBL1α sequences were amplified using the following primers: DBL2aAF, 5′-GCACG(A/C)AGTTT(C/T)GC3′, and DBL2aBR, 5′-GCCC-ATTG(G/C)/TCGAACCA3′ [19]. Negative control reactions were performed in the same conditions without reverse transcriptase. DBL1α sequences from each sample were isolated from a 50 μl PCR reaction containing 2 μl of cDNA template (32 cycles: 30 s at 94°C, 30 s at 42°C and 30 s at 65°C followed 5 min at 6 5°C). The amplified products of DBL1α amplification reactions were separated by electrophoresis in 1.5% agarose and the pattern after electrophoretic separation of the reaction mix. The amplified products were cloned into a TA vector as described by the manufacturer (Promega). For each original isolate, we sequenced 50–100 cDNA clones, and only those isolates with >50 sequence reads were used in our analysis.

Sequence analysis

Sequences edited with DNAsstar software (version 6) aligned in batches using ClustalW analysis (http://www.ebi.ac.uk/clustalw/) using the default settings (Gonnet230 matrix, gap opening penalty = 10.0, gap extension penalty = 0.2, gap closing penalty = 1, gap separation penalty = 4). Unrooted phylogenetic trees (based on amino acid sequences) were constructed by p-distance/neighbor-joining (NJ) method with 1000 bootstrap replicates using MEGA version 3 [59]. Observed clusters from each tree were confirmed visually on alignments.

Validation of var subtype-specific qRT-PCR primers in Yunnan isolates

Primers specific for subtype or individual var genes were described previously [15,60]. These var subtype-specific primers had been validated in 3D7 and southern Tanzania isolates. To validate these primers in the samples from Yunnan area, all primers were tested on genomic DNA from our isolates. The genomic DNAs were extracted with the GenElute™ Mammalian Genomic DNA Miniprep Kit (sigma). All primers had amplification efficiencies (E) between 1.80 and 2. The relative copy numbers of amplified subtype var genes by these primers were compared to the internal single-copy control gene, Seryl-tRNA synthetase. The amplification efficiency for field isolate gDNA was similar to that for 3D7 gDNA for upcA1, A2, A3, B2, BC1, C2, Var1 (upsD) and Var2 (upsE). Because there are overlapping members between BC1 and BC2, C1 and C2, respectively, we used the primers of upcA2, B2, BC1, BC2, C1, C2, D and E subgroup in this study.

qRT-PCR

Parasite cultures were tightly synchronized by sorbitol lysis for total RNA extraction. Total RNA of synchronous parasite culture was extracted using Trizol reagent (Invitrogen) according to the protocol as described previously [58]. The potential genomic DNA contamination were removed by Dnase I treatment with DNA-free kit (Ambion), and the resulting RNA samples were tested by PCR of 35 cycles with primers of the housekeeping gene seryl-tRNA synthetase (PF07_0073) to confirm the lack of genomic DNA contamination. 500 ng total RNA was subjected to reverse transcription reaction with a mixture of oligo dT and random hexamer primers as reverse primers in a 20 μl reaction according to the manufacturer’s recommendations (Invitrogen). Negative control reactions were performed in the same conditions without reverse transcriptase. All runs were performed using an Applied Biosystem 7300 detection system in a 20 μl reaction contained 0.5 μl cDNA, 1×SYBR Green 1 Mastermix, 0.2 μM specific primer pair for individual gene or var gene subtypes tested, and each reaction was run in duplicate. Seryl-tRNA synthetase was used as endogenous control gene. The qRT-PCR reaction conditions were as follows: 95°C for 10 s, followed by 40 cycles of 94°C for 20 s, 50°C for 20 s and 62°C for 20 s.

Transcripts were quantified as follows: the threshold cycle (Ct) is defined as the cycle number at which the quantity of fluorescence product passes a pre-determined threshold. The relative amounts were calculated using the equation: \( \frac{2^{-ACt}}{2^{-ACt_{\text{ref}}}} = \frac{2^{ΔCt}}{2^{ΔCt_{\text{ref}}}} \). Dissociation curves were generated and the homogeneity of the products were verified by observing banding pattern after electrophoretic separation of the reaction mix.

Cytoadherence assays on immobilized receptors

Cytoadherence assays were carried out as described [61]. Briefly, host receptors including CD36, ICAM-1, CSA and Hyaluronan (HA) were used for panning of parasites. Plastic Petri dishes were coated overnight at 4°C with 1xPBS containing either 1 mg/ml CSA sodium salt from bovine trachea (Sigma), 100 μg/ml HA sodium salt from bovine vitreous humor (Sigma), 10 μg/ml recombinant human CD36 (R&D Systems), 10 μg/ml recombinant human ICAM-1 (R&D Systems) or 1% BSA. These receptors were spotted onto a 150 mm petri dish (BD Falcon) in duplicates and incubated in a humid box overnight at 4°C. Trophozoite-stage parasites (24–30 hr post-invasion) infected erythrocytes were enriched by Percoll and resuspended in adhesion medium containing RPMI 1640 and 25 mM Hepes, pH6.8. Binding assays were incubated at 37°C for 1 hr without agitation. Dishes were gently washed 3 times with adhesion medium and once with 1xPBS. The bound cells were fixed with 2% glutaraldehyde (Amresco) in PBS and stained with Giemsa solution (Gibco). The average numbers of parasites adherent to each receptor in duplicate spots (cells per mm²) were counted in 25 continuous fields by light microscopy.

Statistic analysis

SPSS16.0 software was used to perform Mann-Whitney U test to evaluate the difference of parasitemia between upcD and other group. Difference was considered significant if P value was <0.05 (2-tailed). The distribution analysis of var-DBL1α sequences were were compared using \( \chi^2 \) test. *p* <0.01 between tested group and control.

Supporting Information

Figure S1 Transcriptional pattern and cytoadherent features of unselected P. falciparum FCC1/HN line. (A) Synchronized trophozoite-stage parasites were used to measure the transcriptional level of various subtype var genes. (B) Cytoadherence assay of FCC1/HN line with CD36, ICAM-1, CSA, and HA purified receptors with BSA as negative control. (TIF)

Figure S2 The ring-stage transcription profile of var gene family in LZF22 isolate. The analysis was started after two weeks post-thaw based on the stuff amount. The Seryl-tRNA
synthesize gene was used as the endogenous control. The time point when the "871c" was significantly associated with the outcome of interest was determined using the same approach.

Table S1 Clinical assessment of symptomatic malaria patients recruited in this study (supplementary to Table 1).

**Table S2** Plasmodium falciparum DBL1α contigs of var transcripts in field isolates from symptomatic patients in Yunnan-Myanmar area.

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