

Variant surface antigens, virulence genes and the pathogenesis of malaria

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The first Molecular Approaches to Malaria meeting was held 2–5 February 2000 in Lorne, Australia. Following the meeting, Brian Cooke, Mats Wahlgren and Ross Coppel predicted that research into the molecular details of the mechanisms behind sequestration of parasitized erythrocytes would ‘become increasingly more complicated, with further interactions, receptors, ligands and functional domains’. Furthermore, they cautioned that ‘the challenge will be not to lose ourselves in the molecular detail, but remain focused on the role of [the *var* genes and other multigene families] in pathogenesis of malaria’. We contemplate on these statements, following the recent second Molecular Approaches to Malaria meeting, which was held at the same venue on 2–5 February 2004.

Parasites of the genus *Plasmodium* infect a very large variety of animals, including mammals, birds and reptiles. Extensive laboratory research has been conducted on many of these species, including several parasites that infect animal models. Of the species that infect humans, *Plasmodium falciparum* is by far the best studied, both because it is responsible for the large majority of disease episodes and essentially all malaria-related mortality and because it is the only species that can be grown in long-term *in vitro* culture. The virulence of *P. falciparum* has been related to the ability of parasitized erythrocytes to adhere to a range of host receptors in different vascular beds (sequestration), on platelets (clumping or auto-agglutination), on uninfected erythrocytes (rosetting) and on dendritic cells (interference with antigen presentation). These phenomena have all been linked to expression of parasite-encoded, clonally variant antigens on the surface of parasitized erythrocytes [1–3]. Furthermore, variant surface antigens have long been suspected to be important targets of the protective immunity that people living in areas of continuous parasite transmission gradually acquire over years of exposure. This has made these antigens, their relationship both to parasite virulence and the pathogenesis of malaria, and not least the identification of the genes encoding them, major points of interest in the quest to reduce malaria morbidity and mortality.

The genomic organization of virulence genes in *P. falciparum*

Significant advances in understanding the molecular biology of virulence genes in malaria parasites have been achieved since the meeting at Lorne in 2000 [4]. In particular, the publication of the completed and annotated genome sequence of the *P. falciparum* clone 3D7 in 2002 represented a milestone for the entire field of malaria research and has had a significant impact on our view of virulence genes [5]. Three families of variant genes have been characterized in *P. falciparum*: the *var* genes encoding *P. falciparum* erythrocyte membrane protein 1 (PfEMP1); the repetitive interspersed family (*rif*) of genes; and the subtelomeric variant open reading frame (*stevor*) genes. It is now possible to envision the organization of the entire *var*, *rif* and *stevor* gene families, both in terms of the absolute numbers of genes in each family as well as where they are located with respect to each other. Upon viewing the entire genome, the picture that emerges is one that includes 1–3 *var* genes located in the subtelomeric regions of most of the chromosomes, in addition to tandemly arrayed clusters of *var* genes found in the internal portions of the chromosomes (Figure 1). Within the 3D7 genome, there are 59 intact *var* genes, with 149 *rif* and 28 *stevor* genes interspersed near the *var* genes [5].

Whereas *rif* and *stevor* genes appear to be closely related, the *var* genes are unique and are the most extensively studied variant gene family. Close examination and analysis of the *var* gene sequences indicate that most of the family can be divided into three broadly defined classes based on chromosomal location (either subtelomeric or in the central part of the chromosome), direction of transcription (either towards or away from the telomere) and type of upstream flanking region (referred to as UpsA, B or C) (Figure 2) [5–8]. In addition, a pair of ‘strain-transcendent’ *var* genes have been identified that appears to be highly conserved between parasites isolated from different geographical regions [9–12]. One of these genes, referred to as *var2csa*, has generated a great deal of interest because it appears to be crucially involved in the pathogenesis of pregnancy-associated malaria [11]. Sequence analysis has also been used to predict the affinity of the encoded PfEMP1 molecule to bind CD36 [13]. Interestingly, the ability of a particular PfEMP1 molecule to bind CD36 appears to be associated with the location of the encoding *var* gene in the genome [8].

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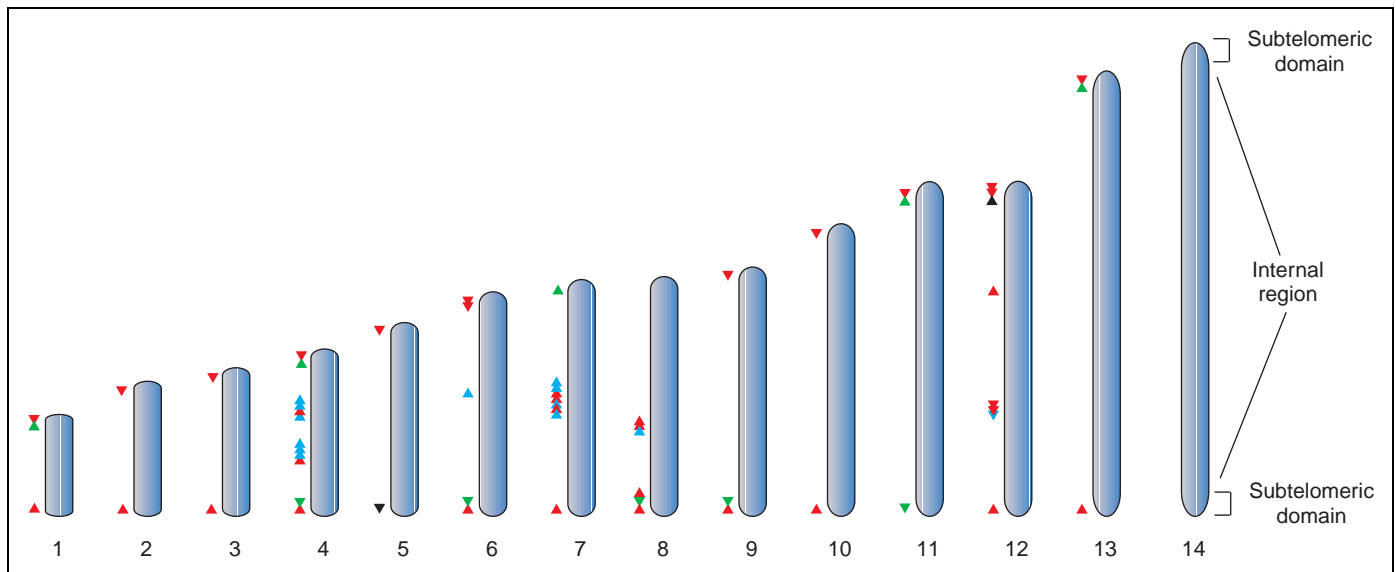


Figure 1. Organization of the *var* gene family in the *Plasmodium falciparum* 3D7 genome. Each chromosome is numbered, and each arrowhead represents a single *var* gene (not drawn to scale) with the direction of the arrowhead indicating the orientation of the gene. Note the clusters of tandemly arranged *var* genes in the internal regions of the chromosomes, as well as one to three copies in the subtelomeric regions, transcribed in either direction. Genes flanked by UpsA (green) are found near the telomeres, those flanked by UpsC (blue) are located in the internal portions of the chromosomes, and those flanked by UpsB (red) are found in both regions. Two strain-transcendent *var* genes that possess unique flanking regions are indicated by black arrowheads.

Specifically, all subtelomeric *var* genes that are transcribed towards the telomere are predicted not to bind CD36. These findings have led to the hypothesis that *var* genes are organized into separate evolving groups based on gene location and orientation that limit recombination possibilities [7,8]. Several groups are now extending this work to determine whether *var* gene organization has implications for patterns of expression over the course of an infection and for predicting the severity of disease.

The role of telomeres

Extension of the genome sequence information into the regions of the chromosomes that surround *var* genes has been particularly interesting with regard to the subtelomeric *var* genes. The location of *var*, *rif* and *stevor* genes in close proximity to telomeres has led some to speculate that the condensed, heterochromatic structure of the telomeres could play a role in controlling (specifically in silencing) the expression of these genes [14]. In addition, the telomeres have been shown to cluster together in the nuclear

periphery [15]. Such clustering should bring the subtelomeric *var* genes into alignment, thus facilitating recombination events between genes that are positioned similarly along the chromosome [15,16]. This model is supported by the sequence analysis mentioned above that provides evidence for *var* genes being divided into separately evolving groups based on gene location and orientation [7,8]. The clustering of the telomeres appears to be mediated by the sequence repeats found there. This was demonstrated by showing that transfected episomes that include the *rep20* repeat co-localize with the telomeric clusters [17]. These experiments highlight the fact that the nucleus has a highly organized structure, and that this structure might play an important role in regulating such nuclear functions as transcription and chromatin assembly. One proposal is that the nuclear periphery might represent a place of high concentration of proteins involved in chromatin condensation and gene silencing (i.e. the SIR family of proteins). Thus, the localization of a large number of *var*, *rif* and *stevor* genes in the nuclear periphery as a

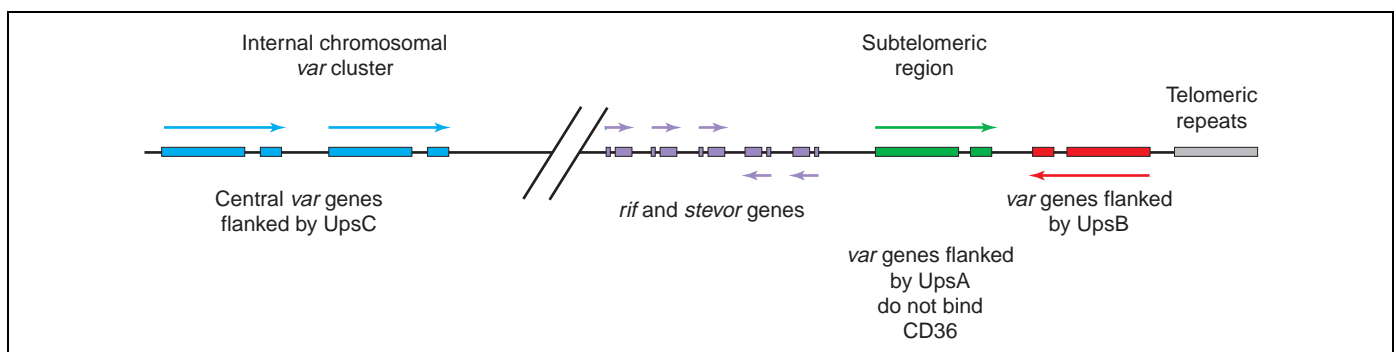


Figure 2. Typical arrangement of *Plasmodium falciparum* *var*, *rifin* and *stevor* genes. Within the sequenced 3D7 genome, internal chromosomal clusters of *var* genes vary between one and seven copies found on five different chromosomes. Subtelomeric regions contain zero to three *var* genes, found in either orientation, whereas the *rif* and *stevor* genes can be in either orientation in close proximity to *var* genes. Three types of flanking sequences, referred to as UpsA, UpsB or UpsC, are found upstream of *var* genes depending on their location and orientation. The subclass of *P. falciparum* erythrocyte membrane protein 1 (PfEMP-1) molecules that are encoded by subtelomeric *var* genes transcribed towards the telomere and flanked by UpsA are predicted not to bind CD36.

result of their close proximity to the telomeres might indeed play a role in the transcriptional silencing of these genes. The condensed chromatin structure that forms at telomeres could potentially spread into the portion of the chromosome where the subtelomeric *var* genes are located, and consequently influence their expression. The extent to which the spreading of telomeric chromatin structure affects *var*, *rif* and *stevor* expression awaits further work.

Controlling expression

One of the most interesting and unanswered questions regarding the *var*, *rif* and *stevor* gene families is how expression is limited to one or a very few genes while the remaining copies are somehow silenced. Most work has focused on the *var* gene family, looking at the level of individual genes and the elements that control their expression. Early work indicated that expression was controlled at the level of transcription: neither the chromosomal position nor the DNA sequence change when comparing an individual *var* gene in its active and silent state [18,19]. This implies that the gene regulation is epigenetic in nature, probably controlled at the level of chromatin structure. Transfection of cultured parasites using recombinant plasmids containing reporter genes has allowed promoter elements to be mapped in the upstream regions of *var* genes [6,20,21], and accompanying gel shift assays using nuclear extracts have identified elements that are bound by protein complexes [21]. This approach has also been used to identify a silencing element that exists within the introns of *var* genes [22,23]. This silencer was found to silence an associated *var* promoter in an S-phase-dependent manner, a characteristic typical of silencing that is mediated through chromatin assembly. Interestingly, the promoter elements identified by gel shift assays were bound by protein complexes extracted from nuclei during S-phase, again emphasizing the importance of this portion of the cell cycle for controlling *var* gene expression. Consistent with these findings, a 'strain-transcendent' *var* gene has been identified that is missing a substantial portion of the intron [9,24]. Northern blot analysis demonstrated that this *var* gene is transcribed constitutively in many laboratory parasite lines [25], and RT-PCR studies found the gene to be actively transcribed in ~60% of field isolates [12], indicating that silencing of this gene might be impaired.

Virulence genes in other species of *Plasmodium*

Although the most extensive work on virulence genes has been carried out on the multicopy gene families in *P. falciparum*, variant gene families have also been identified in all other malaria parasite species investigated. Whereas some of these genes have been shown to be located in subtelomeric regions of the chromosomes, many are distinctly different in structure and sequence from those identified in *P. falciparum*, indicating that they might have evolved independently and have different functions. The *vir* gene family was identified in *Plasmodium vivax*, and homologs have been found in the rodent parasites *Plasmodium yoelii* (*yir*), *Plasmodium chabaudi*

(*cir*) and *Plasmodium berghei* (*bir*) [26–30]. Although the exact function of the proteins encoded by these gene families remains uncertain, it has been proposed that they are involved in antigenic variation or some other aspect of the host–parasite relationship. A gene family called *Py235* has also been characterized in *P. yoelii* and encodes proteins that are located in the merozoite apical complex [31,32]. In the monkey parasite *P. knowlesi*, the schizont-infected cell agglutination variant antigen (*SICAvar*) family has been identified, and a switch in expression was extensively characterized [33]. This switch was shown to be associated with a recombination event that resulted in an altered 3' end of the gene and 3' untranslated region (UTR) sequence. 3' UTR sequences have been shown to be involved in translational regulation, thus raising the possibility that *SICAvar* expression could be controlled at the level of translation rather than transcription [34]. Such a finding, if confirmed, would have significant implications for current models being proposed for controlling *var* gene expression and antigenic variation in *P. falciparum*, although the extensive differences between the gene families in the different species raises the possibility that mechanisms for controlling regulation might not be entirely similar.

Variant surface antigens and the pathogenesis of malaria

The four years since the first Lorne meeting has also witnessed several studies using non-genomic approaches to characterizing the parasite-encoded variant molecules on the *P. falciparum*-infected erythrocyte surface. These studies have served to strengthen the link between these antigens, the pathogenesis of *P. falciparum* malaria and the acquired protective immunity to the disease. Particularly rewarding in this respect have been studies of the relationship between variant surface antigen expression on the one hand and severe malaria in children and pregnancy-associated malaria on the other.

Switching between different *P. falciparum* variant surface antigens occurs both *in vitro* and *in vivo* – in some cases at remarkably high rates [35,36]. This would seem to lead to rapid exhaustion of the repertoire compatible with parasite survival in the face of acquired immunity, and the fact that this does not occur has been the topic of much speculation. It has recently been shown in cultured parasites that 'on' and 'off' switching rates for different *var* genes can vary drastically, and that these switch rates seem to be an intrinsic property of individual genes [37]. This implies that the probability of activation or silencing of a particular gene is determined by the surrounding DNA. Evidence from infected individuals suggests that most parasites in a given malaria patient at a given time express variant surface antigens that are similar to each other in terms of antibody recognition, but that expression changes with time at the population level from one predominant type to another predominant type and so on [36]. Furthermore, it is clear that variant surface antigen expression is related to disease severity, and that it depends to a large degree on the level of immunity in the patient [38–41]. Interestingly, the variant

Box 1. Linking virulence gene expression patterns to pathogenesis

As the molecular understanding of virulence genes and their expression patterns improves, the prospect of linking the expression of specific genes to distinct pathogenic phenotypes becomes a possibility. Recent advances in the understanding of pregnancy-related malaria provide perhaps the most tangible example. Parasites that accumulate in the placentas of pregnant women display a distinct binding affinity to proteoglycans such as chondroitin sulfate A (CSA) [45,46]. Placental and many CSA-adhering parasites also express variant surface antigens that are serologically distinct from those expressed by other parasites [47–49]. Genomic analysis of parasites with both these properties indicates that they predominantly express a single, strain-transcendent *var* gene called *var2csa* [11]. Development of pan-specific *var*-based vaccines might represent a daunting exercise based on the size and variability of the entire gene family, but a vaccine based on a single, strain-transcendent *var* gene (such as *var2csa*) that specifically targets pregnancy-associated malaria might represent a more approachable possibility. Malaria scientists are currently busy searching for putative additional conserved subfamilies of *var* genes that are specific for certain pathogenic phenotypes (i.e. cerebral malaria) and offer the possibility of other syndrome-specific vaccines. The observation that the subclass of *var* genes flanked by UpsA does not bind CD36 [8] indicates that parasites expressing such genes have similar cytoadhesion and pathogenic phenotypes, and recent data do indeed suggest a relationship between *var* gene structure [7], variant surface antigen phenotype [53] and the pathogenesis of severe malaria in children [52]. If this trend holds true for several subclasses of *var* genes, the strategy of vaccines based on individual or small numbers of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) molecules might indeed prove fruitful.

surface antigens expressed by parasites causing severe disease and by parasites infecting young children with little immunity are more often recognized by plasma IgG from malaria-exposed individuals than parasites from non-severe cases and asymptomatic infections in older, more immune individuals, and also appear to be more antigenically conserved between isolates [42]. Some variant surface antigens are indeed remarkably conserved between isolates, and the whole issue of interclonal diversity (which has been assumed to be vast but remains somewhat understudied) clearly needs to be studied in more detail [11,42,43]. The apparent inverse relationship between disease severity and interclonal diversity of variant surface antigens [42] is in agreement with earlier observations that protection from severe disease is acquired more rapidly than resistance to infection as such [44], and raises the hope that syndrome-specific vaccines based on variant surface antigens might be feasible (Box 1). The first of these could well be against pregnancy-associated malaria, where progress has been particularly rapid. The syndrome is caused by massive placental accumulation of parasites that can adhere to proteoglycans such as chondroitin sulfate A [45,46]. These parasites have distinct adhesive and antigenic characteristics related to the variant surface antigens they express [47–49], and IgG to these antigens, which appear to show limited interclonal diversity [47], are strongly related to protection from the adverse consequences of placental infection [50,51].

Linking virulence genes, surface antigens and pathogenesis

As outlined above, knowledge regarding virulence genes has expanded at a tremendous rate since the first MAM meeting in 2000. At the same time, investigations of the variant surface antigens expressed by field and laboratory-adapted parasites, and the immune responses against them, have also yielded substantial insights. However, there has been a shortage of studies attempting to link molecular data regarding virulence genes to variant antigens on the infected erythrocyte surface and not least to pathogenesis and protection. An important lesson that has been learned in the past few years, including from studies of pregnancy-associated malaria, is that such links can be instrumental. Recent detailed molecular analyses of the relationship between *var* gene expression, *var* gene organization and variant surface antigen phenotype [8,11,13,52], made possible by the mapping of the entire 3D7 genome [5] and the ability to manipulate variant surface expression of this parasite *in vitro* [53], are examples of this approach.

Where we are and what to do

Today, the challenge, as it was expressed by Cooke, Wahlgren and Coppel after the first MAM meeting [4], remains largely unchanged, although it can now be phrased more specifically: we must exploit our molecular insights to establish causal links between events at the genome level and pathogenesis/clinical outcome. To meet this challenge, we must keep our minds open and not shy away from re-interpreting existing evidence when it is indicated by new insights.

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