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# Review: **Genetic diversity of *Plasmodium falciparum*: asexual stages**

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## Introduction

The genetic complexity of *Plasmodium falciparum*, the infectious agent of malignant malaria, and in particular its ability to generate mutant variants, make it a successful pathogen. Genetic variants are involved in pathogenicity and in immune responses and have led to the emergence of resistance against virtually any drug available for causative treatment. Such variants are under strong selective pressure. Analyses of sequence variation in gene segments that are not subjected to selection and of intergenic sequences permit the study of additional mutation patterns, interpopulation diversity of the parasite and its evolutionary origin.

## The genome

The genome of *P. falciparum* consists of 14 linear chromosomes with a total of 25–30 megabases of nuclear DNA with approximately 5000 genes, a mitochondrial fragment of a 6 kb repeat element, and a circular element of 35 kb within the apicoplast. As a consequence of continuous deletions and crossing-over and rearrangement events occurring preferably at their telomeric regions, the chromosomes differ considerably in size (Corcoran *et al.* 1986). The genome is extremely A/T-rich (80%), which has led to difficulties in conventional sequencing strategies because of the instability of genomic fragments in bacterial *Escherichia coli* clones. Meanwhile, several yeast artificial clone (YAC) constructs have been established, allowing for a stable maintenance of *P. falciparum* clone fragments.

Essentially based on YAC contiguous sequences (contigs), the *P. falciparum* genome is now subject of a large DNA-sequencing project, the Malaria Genome Project, which was established in 1996. Contig arrays and restriction maps have been produced for mapping of complete chromosomes (Rubio *et al.* 1995). At present, the majority of the sequences of the 14 chromosomes of the *P. falciparum* clone 3D7 are covered by YAC contigs. Sequencing is performed by a chromosomal shotgun technique with a 10–15× coverage. The project is funded by the Wellcome Trust, the Burroughs Wellcome Fund, the US Department of Defence, and the National Institute of Allergy and Infectious Diseases (US) and performed by Standford University (US), the Sanger Centre (UK), the Institute for Genomic Research (TIGR, US), and contributing groups. Sequencing is close to completion and sequences are published for chromosome 2 and 3 (Gardner *et al.* 1998; Bowman *et al.* 1999; sequence of chromosome 5 not yet published). Gaps still remain to be filled in other chromosomes. The closure of gaps is complicated by the considerable number of A/T-repeats. Sequencing of the cytoplasmatic mitochondrial and apicoplast segments has been completed. A draft sequence of the *P. falciparum* 3D7 genome is expected to be published this year.

Sequence information is being deposited in several clickable databases (Table 1). The information available at present has, by *in silico* analysis of genetic similarity with other organisms, allowed to trace and to assign a function to approximately 40% of the genes. Many of them encode proteins of metabolic pathways. The sequence information finally provided will, after the determination of transcription patterns and the identification of coding sequences, support detailed analyses of relevant proteins, parasite-specific metabolic pathways and, furthermore, support the identification of targets for new drugs and for vaccines. Targets for drugs are now continuously being identified. One example described on the basis of *P. falciparum* DNA sequence information are nuclear genes that are involved in the pathway of lipid synthesis and the subsequent identification of the enoyl acyl-carrier protein (Waller *et al.*

**Table 1** Internet resources providing *Plasmodium falciparum* genome information

| The *Plasmodium falciparum* genome database | http://PlasmoDB.org |
|---|---|
| NCBI malaria genetics & genomics | http://www.ncbi.nlm.nih.gov/Malaria |
| TIGR's the *Plasmodium falciparum* genome database (PFDB) | http://www.tigr.org/tdb/edb2/pfa1/htmls |
| The Sanger Centre's *Plasmodium falciparum* Project | http://www.sanger.ac.uk/Projects/P_falciparum |
| Stanford DNA Sequence and Technology Center's *Plasmodium falciparum* (Pf) Genome Project | http://sequence-www.stanford.edu/group/malaria/index.html |
| EMBL parasite genome databases and genome research | http://www.ebi.ac.uk/parasites/parasite-genome.html |
| Malaria parasite metabolic pathways | http://sites.huji.ac.il/malaria |

1998) which, in the presence of 5-chloro-2-(2,4-dichlorophenoxy)phenol (triclosan), led to *in vitro* inhibition of growth of *P. falciparum* (Surolia & Surolia 2001). Other substances with promising activities against *P. falciparum* are inhibitors of parasitic enzymes involved in heamoglobin metabolism, in particular cysteine proteases (Singh & Rosenthal 2001).

Targets with a potential for vaccines, identified through the support of genome sequence data, are distinct parasite proteins expressed on the surface of red blood cells. Targets include the variable *P. falciparum* erythrocyte membrane proteins (PfEMP), the RIFINs (encoded by repetitive interspersed family (*rif* ) genes), and the products of the subtelomeric variant open reading frame (*stevor*) genes. Other work currently concentrates on sporozoite and hepatocyte surface proteins, on proteins involved in invasion of the parasite into erythrocytes and on proteins that are stage-specific in the life cycle of *P. falciparum*, including the vector stage.

The clone 3D7, originally derived from the isolate NF54 in Holland in 1979, has since undergone continuous culture and nothing is known about genome alterations such as chromosomal deletions or others that occurred during the period of culture. Additional sequencing of other clones than the 3D7 clone will be required for a more detailed analysis of *P. falciparum* polymorphisms and, thus, lead to a more refined characterization of potential drug and vaccine targets. Furthermore, the availability of a large panel of genetic markers will essentially contribute to the understanding of parasite–host interactions and to the fine resolution in association studies.

## Polymorphism

The inherent variability of *P. falciparum* provides multiple effective immune evasion and drug resistance mechanisms for the parasite. Many of the studies on the parasite's polymorphism have focused on variants exhibiting mutations that lead to amino acid substitutions (non-synonymous mutations) that are likely subjected to selection, such as immunogenic proteins and resistance phenotypes. Other studies have examined sequences which are rather unlikely to be subjected to adaptive pressure and therefore allow considerations on the phylogeny and the age of the parasite.

Polymorphism of the *P. falciparum* genome has mainly evolved through DNA rearrangements such as gene duplication events, gene conversions, translocations, deletions and insertions (Wellems *et al.* 1990; Kemp 1992; Deitsch *et al.* 1997). Single nucleotide polymorphisms (SNPs) contribute largely to the variability. Alignment of sequences based on information obtained from multiple geographical locations have shown that synonymous mutations are scarce in most of the parasite's genes. Genes encoding proteins that are expressed on the surface of sporozoites or merozoites are, in general, more variable than housekeeping genes or genes expressed during the sexual stages and, thus, constitute a major obstacle in targeting them in the design of vaccines. Comparison of the rather selectively neutral synonymous and the more frequent non-synonymous substitutions indicates that natural selection may account for most polymorphisms observed at functional gene loci (Escalante *et al.* 1998). The diversifying mechanisms of single non-synonymous mutations (SNP) resulting in the substitution of amino acids and, as a second process, rapid intragenic recombination observed in gene segments with repeat motifs have been proposed to underlie the polymorphism in those genes that are subject of adaptive selection (Rich & Ayala 2000).

Non-synonymous SNPs are found at high frequencies in genes that are under strong selective pressure, in particular those genes encoding proteins linked to immune evasion or drug resistance. Point mutations affecting, for example, drug resistance have been found in the dihydrofolate reductase-thimidylate synthase (DHFR-TS) and the dihydropteroate synthetase (DHPS). These genes are associated with resistance to pyrimethamine and sulfadoxine, respectively (Cowman *et al.* 1988; Wang *et al.* 1997). Other SNPs have been claimed of being responsible for chloroquine (CQ) resistance (Fidock *et al.* 2000).

Genes where polymorphism has arisen through intragenic recombination in repetitive segments are characterized by repeat motifs with length variability differing between strains. Among these genes are those encoding the *P. falciparum* circumsporozoite protein (Arnot *et al.* 1993), a glutamate-rich protein (GLURP) (Borre *et al.* 1991), two merozoite surface proteins (MSP-1 and MSP-2) (Kimura *et al.* 1990; Fenton *et al.* 1991) and the apical membrane antigen AMA-1 (Marshall *et al.* 1996).

Some polymorphic genes encode proteins that are specialized in generating extensive antigenetic diversity such as members of the *var* gene family, encoding *P. falciparum* erythrocyte membrane proteins (Pf EMP1) (Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995).

Polymorphism of the parasite genome is not restricted to expressed genes. Intergenic and intronic DNA regions contain simple sequence repeats of intraindividually variable length (microsatellites). Typical repetitive sequences are $(TA)_n$, $(CA)_n$, or $(A \text{ or } T)_n$ motifs, most of them leading to intraspecies length polymorphism of genes (Su & Wellems 1996). These DNA segments are particularly useful in the molecular characterization of the parasite population structure and parasite epidemiology, because microsatellites provide a panel of selectively neutral markers.

A systematic approach to the analysis of genetically determined factors is provided through segregation analyses of experimentally derived parasite pedigrees. These experiments are based on cross-fertilization of gametozytes of different parasite strains within mosquitoes and subsequent transmission to chimpanzees. The erythrocyte-stage parasites from the chimpanzees are cloned *in vitro* and progeny clones are used for subsequent analyses. So far, crosses between the HB3 and 3D7 clones (Walliker *et al.* 1987) and between the HB3 and Dd2 clones (Wellems *et al.* 1990) have been produced. Large-scale detection of progeny clones by microsatellite markers can be achieved by methods such as the lactate dehydrogenase (LDH) technique (Goodyer & Taraschi 1997) or the colour detection technique (Kirkman *et al.* 1996).

The recognition of parasite polymorphisms and their association with distinct properties has made considerable progress in the last years. This is largely because of the advents provided by polymerase chain reaction (PCR)-based assays. Dependent on the type of a mutation, SNPs are assessed by either specific digestion assays with restriction enzymes, by DNA hybridization techniques using specific oligonucleotides, by allele specific amplification, or by sequencing of PCR products. Mass-spectrometry assays will certainly, in the near future, essentially enhance the resolution of polymorphism. Variant internal repeat motifs and microsatellites are determined by multiplex PCR amplification using a set of primers flanking the length-variant sequences and subsequent electrophoresis.

Currently, genetic variants of *P. falciparum* are being typed in many studies, aiming at describing parasite population dynamics and at the identification of vaccine targets. This involves the determination of allele prevalence of variable *Plasmodium* genes in different human populations exposed to malaria, including community based comparisons, but also the determination of clonal multiplicity in individuals. It has to be considered in such studies that parasite variability within communities, but also within individuals, is subject to extensive fluctuation, depending on the seasonal and epidemiological situation and on variable exposure patterns, properties of the mosquito vector, and even the time of day of sample collection.

Genotyping of polymorphisms is not only a tool for the description of a distinct clonal parasite strain, but also for defining multiplicity of infections with clonally variable *P. falciparum* strains. Genotyping in field studies mostly allows only an approximation to the estimation of different strains in individual infections. The precise estimation of the number of parasite clones is complicated by the high proportion of low-parasitemic *P. falciparum* infections (e.g. Roper *et al.* 1996; May *et al.* 2000).

PCR-based techniques have shown high prevalence of mixed infections (Brown *et al.* 1992; Snounou *et al.* 1993; Zhou *et al.* 1998). Several experimental studies in humans (reviewed by Richie 1988) and rodents (Snounou *et al.* 1992; Dennison & Hommel 1993) reported interactions between different parasite species infecting the same vertebrate host, affecting factors such as mortality, pathology and infection dynamics. Attenuation of *P. falciparum* disease severity had been reported in such cases (Black *et al.* 1994; Maitland *et al.* 1996; Luxemburger *et al.* 1997; Price *et al.* 1997). Species might have evolved to take advantage of a situation of mixed infections through enhanced transmission by suppression of one species by the other (Butcher 1998). In fact, some studies have reported alterations in gametocyte production in mixed infection (Graves *et al.* 1988; Price *et al.* 1999).

## Variability of non-coding sequences

Intergenic microsatellites are scattered throughout the *P. falciparum* genome. They differ in the length of the repeated motif, the interindividually variable number of repeat motifs and, thus, in the length of the respective allele. The length of a distinct allele can be determined by PCR amplification and subsequent gel electrophoresis. The recent development of a high-resolution *P. falciparum*

**Table 2** Selected studies using microsatellite markers (ms) for *Plasmodium falciparum* typing

| Repeat | No. of ms | Populations | No. of samples | References |
|---|---|---|---|---|
| Tri-nucleotides | 114 | Laboratory lines | 12 | Anderson *et al.* (2000b) |
| Various | 10 | *Anopheles gambiae* | 2 | Anthony *et al.* (2000) |
| Repeats of three bases and more | 12 | Nine ethnic groups | 465 | Anderson *et al.* (2000a) |
| cg2 omega | 1 | South Africa | 47 | McCutcheon *et al.* (2000) |
| NOS2 pentanucleotide | 1 | Five ethnic groups | 1393 | Xu *et al.* (2000) |
| NOS2 pentanucleotide | 1 | Tanzania | 185 | Levesque *et al.* (1999) |
| Various | 2 | India | 15 | Okoyeh *et al.* (1999) |
| Repeats of three bases and more | 12 | Papua New Guinea | 57 | Anderson *et al.* (1999) |
| PfRRM | 1 | Laboratory lines | 16 | Su *et al.* (1998) |
| var1 (PJ/3) | 1 | Laboratory lines | 12 | Su & Wellems (1997) |
| Various | 901* | Laboratory lines | | Su *et al.* (1999) |
| Various | 25 | Six ethnic groups | 8 | Volkman *et al.* (2001) |
| Various | 224 | Laboratory lines | 12 | Su & Wellems (1996) |

* Including RFLP markers.

linkage map consisting of hundreds of selectively neutral genetic microsatellite markers allows an appropriate genomic approach in understanding the relationship between genetic variations and biological phenotypes (Su *et al.* 1999; Ferdig & Su 2000).

Restriction fragment length polymorphisms (RFLPs) and microsatellite polymorphisms have been used for the determination of intergenic diversity in plasmodia (Su & Wellems 1996; Walker-Jonah *et al.* 1992; Ferdig & Su 2000). Microsatellites evolved through sequential mutational processes. Sequence repeats of microsatellites are found frequently in the *P. falciparum* genome and occur every 2–3 kb in coding and non-coding regions (Su & Wellems 1996). Advantages of microsatellite markers over RFLP determination are rapidity and automatization of analysis, but also smaller amounts of DNA required for typing. Typing of microsatellite markers allow to screen for loci which are under selective pressure and to characterize population structures and divergent evolution of the parasites.

Microsatellites have been, for example, applied in describing interpopulation diversity in allele and haplotype frequencies of the *Pfs48/45* gene on chromosome 13 (encoding a candidate for a transmission blocking vaccine) (Conway *et al.* 2001), in the determination of the recent origin of *P. falciparum* from a single ancestor (Volkman *et al.* 2001), in the analysis of mutation patterns of the *P. falciparum* genome (Anderson *et al.* 2000a), and in the detection of drug-resistance promoting loci. Some relevant microsatellite analyses are listed in Table 2.

### Variability of coding sequences

After the observation that the clinical manifestation of iatrogenically induced malaria, employed in the treatment of *Treponema pallidum* infections, varied depending on the isolates obtained from different continents and resulted in species-specific and isolate-specific immunity, the concept of plasmodial diversity arose for the first time. Allozymes and antigenic proteins, in particular variants of the parasite's glucose phosphate isomerase (GPI) and LDH (Carter & McGregor 1973) were then the first plasmodial variants to be recognized. Variants of these enzymes were detected in individual malaria patients, proving the existence of mixed infections with differing *P. falciparum* clones in single individuals. Allelic variation and geographical differences of several genes were then demonstrated in a study on *P. falciparum* isolates from Asia, Africa and South America (Creasey *et al.* 1990), and variant proteins could be distinguished with monoclonal antibodies.

### LSA-1

LSA-1, a prime candidate for a subunit vaccine, is a 200-kDa protein expressed during the liver stage of *P. falciparum* and accumulated in the parasitophorous vacuole. Two epitopes of LSA-1 have shown to induce cytotoxic T-cell responses in carriers of particular HLA-B variants. The gene coding for LSA-1 has a large central repeat region and non-repetitive sequences encoding the N and C terminal ends of the protein. Sequence data of the coding gene are limited; it is known, however, that non-synonymous mutations occur at the N-terminal and C-terminal ends.

### EBA-175

The *P. falciparum* EBA-175 (175 kDa erythrocyte-binding protein), a member of the *ebl* (erythrocyte binding-like) gene family of ligands involved in recognition of host cell

receptors, consists of several domains (regions I-VII) and binds with its region II to sialic acid and glycophorin A of human erythrocytes. The outer domain of EBA-175 is, with 20 amino acid differences among several strains sequenced, highly conserved. Allelic families are also observed among other regions of EBA-175 (Ozwara *et al.* 2001).

## MSP

The merozoite surface proteins MSP-1 (Kimura *et al.* 1990) and MSP-2 (Fenton *et al.* 1991) with numerous alleles and differing in the length of the genes have been extensively studied and their genetic polymorphisms were used to describe clonality of infections in a large number of studies. Length variability mainly results from repeat sequences. The alleles of MSP-1 belong to the allelic groups K1, MAD20 and RO33 with high variability when comparing the groups, but less variability within them. Dependent on the degree of amino acid substitution (highly variable, semiconserved, conserved), MSP-1 has been categorized into 17 blocks (Tanabe *et al.* 1987). Block 2 is of particular interest, as it exhibits repetitive trinucleotides and appears to be subjected to rapid intragenic recombination processes, comparable to those of the *csp* gene (see below). It has been shown that IgG antibodies against the most frequent subtypes of block 2 of MSP-1 are important in acquired antimalaria immunity (Conway *et al.* 2000). A further study, where distinct MSP alleles were associated with distinct HLA class II alleles, points also to an immune selection of MSP variants by the human host (May *et al.* 1999).

MSP-2 with the allele groups FC27 and 3D7 has been considered the most informative marker of clonality and has been genotyped in many studies to assess the degree of multiple infections. FC27 consists of a 96 nucleotide sequence (possibly several copies) and a variable number of 36 nucleotide repeats. Allele 3D7 is characterized by tandem repeats of 12–30 nucleotides (Felger *et al.* 1994). In a recently published comparative multicentric study of laboratory-cloned lines the MSPs proved to be only partly useful as genetic markers of clonality (Färnert *et al.* 2001). A high specificity for the number of alleles, but varying sensitivity, was achieved. In particular, the detection of low-density clones in mixed infections was hampered. The number of alleles identified per isolate varied considerably between laboratories, mainly as a result of differing typing procedures.

MSP-3 is polymorphic as well; the protein is, most likely, not a surface protein but secreted in the plasma of the erythrocyte or into the parasitophorous vacuole. Variability of MSP-3 appears to be limited (Huber *et al.* 1997).

## GLURP

Variability of the glutamine-rich protein (GLURP; Borre *et al.* 1991), exhibited by length polymorphism, has also been described and determined in studies on multiclonal *P. falciparum* infections. However, GLURP variability plays a rather minor role in the resolution of polyclonal infections, compared with MSP-typing.

## CSP

The circumsporozoite protein (CSP) of *P. falciparum* is highly immunogenic and it is considered one of the prime vaccine candidates (Nardin *et al.* 1999). The gene encoding CSP (*csp*) has two non-repetitive 5′- and 3′-ends and a central region. In the non-repetitive 5′- and 3′-regions non-synonymous nucleotide substitutions can be identified in segments coding for B- and T-cell epitopes; synonymous mutations are completely absent (Rich *et al.* 1997). The central region is characterized by a variable number of 40–50 tandem repeats consisting of 12 nucleotides. The repeats code for the amino acid sequences 'NANP' and 'NVDP' only. Allelic variability of the central region is attributable to deletions and duplications of repeat sequences, but also to synonymous mutations. Analysis of the *csp* gene, but also of other genes, has greatly contributed to estimations on the recent origin and the phylogeny of *P. falciparum* populations (Rich *et al.* 1997; Rich & Ayala 2000).

## AMA-1

The apical membrane antigen-1 (AMA-1) has also been regarded as a component of a subunit vaccine (Hodder *et al.* 2001). The 82 kDa protein is located in apical organelles of *P. falciparum* and, after cleavage, expressed on the surface of released merozoites. Parts of the AMA-1 DNA sequence are conserved among parasite strains, while other sequence elements exhibit point mutations at 68 positions (Marshall *et al.* 1996). As a marker in genotyping of parasite strains in mixed infections, AMA-1 typing has not been applied widely and appears to be of limited value.

## PfEMP1

Evidence for plasmodial molecules expressed on the surface of infected erythrocytes came from early studies using the schizont infected cell agglutination test where serum from infected monkeys led to the agglutination of parasitized, but not of uninfected erythrocytes. Later, it was shown that antiparasite immunity was dependent on the presence of strain-specific antibodies (Bull *et al.* 1998). Meanwhile, it is established that the immunogenic

PfEMP1 are expressed in a mutually exclusive manner at the surface of infected erythrocytes. These proteins are subjected to continuous clonal antigenic variation with a switching rate of 2% per generation (Roberts *et al.* 1992). This group of variable proteins is crucially involved in the rapid and continuous generation of diversity.

The expression of PfEMP1 on the erythrocyte membranes does, at a first view, not appear to be advantageous for the parasite, because the parasite displays immunogenic peptides outside of its protected environment. However, these surface molecules are capable of preventing elimination of infected erythrocytes in the spleen. They mediate, in conjunction with the oligomorphic (Kant & Sharma 1996; Hirawake *et al.* 1997) knob-associated histidine rich protein (KAHRP), the adhesion of erythrocytes to the endothelium cells in different organs, preferably under the conditions of blood flow. Many ligands of endothelial cells, among them the intracellular adhesion molecule ICAM-1, chondroitin sulphate A, CD31, CD36, thrombospondin, selectins and others bind to PfEMP1 with different PfEMP1 domains binding to different endothelium ligands. Genetic variability of some of these ligands appears to play an important role in the pathogenesis of malaria. While adherence to thrombospondin and CD36 is observed for all *P. falciparum* strains, binding to other receptors depends on particular properties and does not happen with all strains. A clinically dramatic and frequently lethal (up to 30%, even of appropriately treated cases) situation arises when parasitized cells adhere in the vascular bed of the brain, leading to the manifestation of cerebral malaria.

PfEMP1 proteins are encoded by members of the *var* gene family, a group of 40–50 variable genes per haploid genome of the parasite and extensively differing among strains. The size of the genes is between 6 and 15 kb and they are mostly, but not exclusively, found in telomeric localizations of all chromosomes. The telomeric position of the genes appears to favour their variability. The *var* genes have two exons, whereby the 5′-exon encodes the polymorphic extracellular part and the 3′-exon encodes the more conserved transmembrane and the intracellular domains of the proteins. The mature trophozoite expresses, in a manner of mutual allelic exclusion, after activation of a single *var* locus only a single *var* gene that determines the particular adhesion phenotype, although in earlier stages abortive transcripts of different *var* genes can be detected. The function of these early transcripts remains to be established. The *var* genes recombine at frequencies much higher than those expected from homologous crossover events alone (Freitas-Junior *et al.* 2000; Taylor *et al.* 2000), also as a consequence of their mostly telomeric position. As a consequence of their extensive variability, *var* products are key molecules in immune evasion (Bull *et al.* 1998).

It is, for several reasons, difficult to assess to what extent distinct *var* gene variants could be responsible for particular manifestations of clinical malaria. Firstly, the multigenic occurrence of *var* genes does not allow for an isolated PCR-amplification of a single *var* gene from genomic DNA. Secondly, the repertoire of *var* genes in a given isolate does not allow to predict the actual variant expressed at a given time of the infection. Finally, multiple infections do not allow to assign *var* genes to one of the infecting parasite strains. For these reasons, current experimental designs aim at cloning panels of *var* genes and expressing their products (Oguariri *et al.* 2001). Monoclonal antibodies raised against the products will then allow to determine the variant protein in individual infections.

A substantial review on the structure and the evolution of *var* genes and their products has recently been published by Kyes *et al.* (2001).

## RIFINs

Erythrocytes infected with particular parasite strains may be adhesive to uninfected erythrocytes and mediate rosette formation, possibly facilitating invasion of parasites into yet uninfected cells and/or protecting infected cells from immune attacks. In addition to PfEMP1 proteins, rossetins are involved in erythrocyte rosetting, some of them being encoded by repetitive interspersed family (*rif*) genes coding for RIFINs. The function of RIFINs that are not involved in rosetting is unclear so far. RIFINs are expressed on the surface of erythrocytes and approximately 200 variant members of the *rif* gene family have been recognized to date (Fernandez *et al.* 1999). Parasite populations expressing a single *var* gene can express multiple RIFINs, most likely even in an individual erythrocyte (Kyes *et al.* 1999).

## Stevor/Pf60

Although variable as well, the subtelomeric variant open reading frame (*stevor*) genes, members of the *rif* gene superfamily, are more conserved. Their role is not entirely understood. This applies also to the *Pf60* multigene family, which is highly similar to the second exon of *var* genes. *Pf60* genes are characterized by stop codons and frameshifts, a fact that has meanwhile led to the assumption that they might be pseudogenes.

## *Clag*

The family of *P. falciparum* cytoadherence-linked asexual genes (*clag*) has only recently been recognized. *Clag* genes identified on chromosomes 2 and 3 (*clag* 2, 3.1, 3.2) are

divergent. So far, no information is available with regard to polymorphisms of *clag* 2, 3.1, 3.2. *Clag 9* on chromosome 9 has a high degree of similarity among different isolates with only a limited number of synonymous and non-synonymous substitutions (Manski-Nankervis *et al.* 2000).

## Drug resistance

Parasite antimalarial multidrug resistance is a main reason for treatment failure and recrudescence. Resistance to CQ, mefloquine, halofantrine, quinine, sulpha drugs (e.g. sulfadoxine), folate antagonists (e.g. pyrimethamine, pro-/cycloguanil), as well as to the recently launched atovaquone has been reported. Although recurrent parasitemias are being observed after the application of artemisinine derivatives, resistance has not been described so far. Some recent reports of artemether therapeutic failure have, however, raised that issue (Gogtay *et al.* 2000).

Research on drug resistance, supported by observations of the growth of *P. falciparum in vitro*, have allowed the

**Table 3** Markers of antimalarial drug resistance

| Gene | Resistance | Mutations proposed to be associated to drug resistance | Selected references and comments |
|---|---|---|---|
| *pfmdr1* | Chloroquine<br>Mefloquine | Asn86Tyr (**A**AT → **T**AT)[1–7]<br>Tyr184Phe (**TA**T → **TT**T) [6]<br>Ser1034Cys (**A**GT → **T**GT)[6]<br>Asn1042Asp (**AA**T → **GA**T)[1,5–7]<br>Asp1246Tyr (**GA**T → **TA**T)[1,2,5–7] | [1]Cox-Singh *et al.* (1995); [2]Frean *et al.* (1992); [3]Basco and Ringwald (1998); [4]Grobusch *et al.* (1998); [5]Adagu and Warhurst (1999); [6]Duraisingh *et al.* (2000b); [7]Flueck *et al.* (2000) |
| *pfcrt* | Chloroquine | Lys76Thr (**AA**A → **AC**A)[1] | [1]Djimde *et al.* (1999) |
| *pfdhfr* | Pyrimethamine<br>Pro/cycloguanil | Ser108Asn[a](**AGC** → **AAC**)[2,3,4,9]<br>(**AGC** → **AAT**)[1],<br>Ser108Thr(**AGC** → **ACC**)[2,9]<br>Ala16Val[b](**GCA** → **GTA**)[2,4,9]<br>Ala16Ser[3] (**GCA** → **TCA**)<br>Leu46Ser [c,11]<br>Cys50Arg (**TGT** → **CGT**)[3,5,8]<br>Asn51Ile[b,d](**AAT** → **ATT**)[2,3,9,10],<br>Asn51His (**AAT** → **CAT**)[1]<br>Cys59Arg[d](**TGT** → **CGT**)[2,3,9,10]<br>Val140Leu (**GTT** → **CTT**)[3,7]<br>Ile164Leu[d](**ATA** → **TTA**)[2,9]<br>Gly-Lys-Lys-Asn-Glu (GGG AAA AAA AAT GAG)[c,5] | [1]Kaneko (1999); [2]Parzy et al. (1997); [3]Wang *et al.* (1997); [4]Eldin de Pecoulas (1995); [5]Plowe *et al.* (1997); [7]Zindrou *et al.* (1996); [8]Urdaneta *et al.* (1999); [9]Jelinek *et al.* (1999); [10]Khan *et al.* (1997)<br>[a]Considered essential for pyrimethamine resistance<br>[b]Linked to Ser108Asn, these SNPs are associated with enhanced resistance to pyrimethamine<br>[c]Nucleotide sequence not yet available<br>[d]With Ser108Asn, these substitutions contribute to cross-resistance of pyrimethamine/cycloguanil<br>[e]'Bolivian insertion' (Plowe *et al.* 1997) |
| *pfdhps* | Sulfadoxine<br>Dapsone | Ser436Phe[f](**TCT** → **TTT**)[1,4],<br>Ser436Ala[e](**TCT** → **GCT**)[2,3]<br>Ala437Gly[f](**GCT** → **GGT**)[1,3,4]<br>Ala581Gly[g](**GCG** → **GGG**)[1,3,4]<br>Lys540Glu[f](**AAA** → **GAA**)[3]<br>Ala613Ser[f](**GCC** → **TCC**)[1,4]<br>or Ala613Thr[f](**GCC** → **ACC**)[1] | [1]Jelinek *et al.* (1999); [2] Brooks et al. (1994); [3]Wang *et al.* (1997); [4]Wang *et al.* (1995)<br>[f]The combination of these SNPs also causes SDX resistance<br>[g]A single mutation causes SDX resistance |
| *pfGR* | Quinoline drugs? | Lys281Asn (AA**G** → AA**T**)[1]<br>Glu283Gly (G**AA** → G**GA**)<br>Arg335Thr (A**GA** → A**CA**)[1] | [1]PCR-RFLP method (JP Gil, unpublished) |
| *Cytb1* | Atovaquone | Met133Ile (AT**G** → AT**A**)[1]<br>Tyr268Ser (**TA**T → **TC**T)<br>Lys272Arg (**AA**A → **AG**A)<br>Pro275Thr (**CC**A → **AC**A)<br>Gly280Asp (G**GT** → G**AT**)<br>Val284Lys (**GT**A → **AA**A) | [11]PCR-RFLP method (JP Gil, unpublished) |

Nucleotides shown in bold indicate mutations.

isolation of several polymorphic genes coding for products putatively mediating resistance. This has been evaluated and confirmed in epidemiological studies and, more recently, by gene transfection techniques (Wu *et al.* 1996; Fidock *et al.* 2000; Reed *et al.* 2000).

Drug resistance can be either caused by mutations of genes of the drug targets (*pfdhps*, *P. falciparum* dihydropteroate synthase; *pfdhfr*, *P. falciparum* dihydrofolate reductase; *pfcytb*, *P. falciparum* cytochrome b), or by parasite strategies of drug metabolism, transport, or the modification of intracellular conditions (*pfmdr1*, *P. falciparum* multidrug resistance 1; *pfcrt*, *P. falciparum* chloroquine resistance transporter; *pfgr*, *P. falciparum* gluthathione reductase).

*pfdhps*

Sulpha drugs (e.g. sulfadoxine) bind to the dihydropteroate synthase (pfDHPS). This enzyme catalyses one of the steps of the folic acid pathway that is essential for DNA synthesis. Several SNPs have been described in the *pfdhps* gene, leading to the expression of proteins with significantly diminished drug binding capacities, while retaining their enzymatic activity. Resistance has been associated to a single mutation (Ala581Gly) or to combinations of other SNPs. The main mutations in the *pfdhps* gene are given in Table 3. A further mechanism of resistance is the tandem amplification of the *pfdhfr* gene (Inselburg *et al.* 1987; Thaithong *et al.* 2001).

Recently, alternative pathways for evading sulpha drug effects through the active influx of exogenous folates from the host have been suggested (Wang *et al.* 1999). This action has been preliminary associated to a new marker at a 50 kb region in chromosome 4 of *P. falciparum* (J. Hyde in Macreadie *et al.* 2000).

*pfdhfr*

pfDHFR (*P. falciparum* dihydrofolate reductase) is also an enzyme of the folic acid pathway, located downstream of *pfdhps*, and target for several folate antagonists such as pyrimethamine and cycloguanil. The molecular basis of resistance is similar to that in *pfdhps*: a series of point mutations modifies the protein structure and leads to weaker drug binding. The main mechanism of resistance has been proposed to be a Ser108Thr substitution in the protein (Peterson *et al.* 1990). Resistance is significantly increased by additional sequence changes at positions 51 and 59. Ile164Leu has been related to pyrimethamine-pro/cycloguanil cross-resistance (Peterson *et al.* 1991; Plowe *et al.* 1995). Besides SNPs, an intraopen reading frame 15 bp repeat at codon 30 ('Bolivian insertion', Table 3) has been shown to cause pyrimethamine resistance as well (Plowe *et al.* 1997).

*cytb1*

Atovaquone, an hydroxynaphtoquinone, was introduced in the late 1990s as a fixed combination with proguanil hydrochloride, representing 'the first new antimalarial therapy in more then 40 years' (Looareesuwan *et al.* 1999). The substance exhibits a novel mode of action, by binding to the cytochrome *bc1* complex and inhibiting the mitochondrial electron transport system (Fry & Pudney 1992). Development of parasite resistance to atovaquone has been predicted (Looareesuwan *et al.* 1999) and confirmed *in vitro* as well as in the field (Korsinczky *et al.* 2000). Analysis of the *cytb1* gene from an atovaquone resistance isolate has revealed the substitution Tyr268Ser. *In vitro* drug exposure of the sensitive clone *P. falciparum* K1-1D4 selected for a number of atovaquone resistant parasite populations, and the identification of a series of mutations giving rise to haplotypes associated to different levels of resistance (Korsinczky *et al.* 2000).

*pfmdr1*

The *pfmdr1* gene codes for a parasite homologue Pgh (P-glycoprotein homologue) of the human Pgp (P-glycoprotein), an antigen binding cassette transporter (Foote *et al.* 1989; Wilson *et al.* 1989). The protein is mainly localized in the membrane of the digestive vacuole, its function being not yet fully determined. The gene is polymorphic with SNPs at positions 754, 1049, 3598, 3622 and 4234 (Foote *et al.* 1990). Two alleles have been shown to be associated with quinoline resistance, and in particular to chloroquine in field isolates (Foote *et al.* 1990; Reed *et al.* 2000): (i) the K1 type, coding for Asn86Tyr, and (ii) the 7G8 type characterized by the Ser1034Cys/Asn1042Asp/Asp1246Tyr haplotype. K1 and 7G8 are predominant types in isolates from South America and Southeast Asia, respectively. This distribution is in accordance with previous suggestions of a 'two foci origin' of chloroquine resistance (Payne 1987). Molecular epidemiology studies of this gene yielded contradictory results, with some demonstrating a positive correlation between the presence of certain alleles and the *in vivo* or *in vitro* resistance phenotype, while others did not find any difference in the *pfmdr1* alleles with regard to the drug susceptibility phenotype. These data probably reflect a multigenic basis of resistance to quinolines.

Current models, supported by results of parasite transfection studies, suggest that *pfmdr1* might be a secondary factor in chloroquine resistance (Fidock *et al.* 2000; Reed *et al.* 2000), but possibly a more important one in quinine, mefloquine and halofantrine sensitivity (Reed *et al.* 2000). Reports suggesting a role of this gene in the modulation of a potential artemisinine derivative resistance are of interest (Duraisingh *et al.* 2000a, b; Reed *et al.* 2000). In addition

to SNPs, other types of polymorphisms have been identified in this locus, in particular a repetitive motif at the 3′ untranslated region of the gene (Foote *et al.* 1990) and a full tandem amplification of the gene (Foote *et al.* 1989). The tandem sequence appears to correlate with quinine, mefloquine and halofantrine resistance (Barnes *et al.* 1992; Cowman *et al.* 1994), leading to the view that this might be a contributing, but not exclusive event for the development of these phenotypes (Lim *et al.* 1996).

*pfcrt*

After cloning of the *pfmdr1* gene, several studies of the *pfmdr1* K1 and 7G8 alleles showed an incomplete, and sometimes not significant correlation with chloroquine resistance. Moreover, in a cross between the CQ resistance clone Dd2 and the CQ sensitive clone HB3, the verapamil-reversible CQ resistance phenotype (Martin *et al.* 1987) was shown to be linked to a locus at chromosome 7, and not at chromosome 5, the chomosome where *pfmdr* had been localized (Wellems *et al.* 1990). Further RFLP-based linkage mapping localized the CQ resistance associated locus to a segment of 36 kb in chromosome 7 (Wellems *et al.* 1991). A subsequent open reading frame search on this region allowed the isolation of a new gene, named *pfcrt* (Su *et al.* 1997; Fidock *et al.* 2000).

*pfcrt* codes for a 424 amino acid, 48.6 kDa transmembrane protein, localized in the digestive vacuole membrane. With its 13 exons the gene is unusually complex for *P. falciparum*. Twelve SNPs in the coding region have been recognized (Fidock *et al.* 2000; Cooper *et al.* 2000), leading to amino acid substitutions mainly localized in, or nearby, transmembrane domains of the protein. One of these mutations, Lys76Thr, has been linked to chloroquine resistance with a 100% concordance in *P. falciparum* lines from diverse geographical locations (Fidock *et al.* 2000). That *pfcrt* Lys76Thr is in fact a key marker of CQ resistance has been demonstrated by several studies on selection of this mutation after CQ treatment (Djimde *et al.* 2001; Dorsey *et al.* 2001; Mayor *et al.* 2001; Pillai *et al.* 2001) and by varying *in vivo* and *in vitro* responses to CQ in different parasite isolates (Babiker *et al.* 2001; Basco & Ringwald 2001; Chen *et al.* 2001). An extensive study of children affected by uncomplicated malaria performed in Mali confirmed this trend (Djimde *et al.* 1999). These epidemiological results were further supported by *P. falciparum* transfection studies (Fidock *et al.* 2000), leading to the view that *pfcrt* Lys76Thr is in fact the main determinant of CQ resistance (Carlton *et al.* 2001).

Some new data re-emphasize, however, the possible multigenic nature of CQ resistance. Recent re-analysis of the Dd2 × 3D7 cross progeny indicated unequivocal linkage with a locus located at chromosome 5, again suggesting the involvement of *pfmdr1*, and of another gene at chromosome 13 (J. Wootton, in Macreadie *et al.* 2000). In addition, several studies revealed the presence of *pfcrt* Lys76Thr carriers among CQ resistant isolates and reported *in vivo* therapeutic failure of CQ (Dorsey *et al.* 2000).

The molecular mechanisms of CQ resistance are still unclear. In particular, the reasons for the diminished accumulation of CQ in the erythrocytic parasite vacuole of CQ-resistant strains are not well understood. A possible mechanism could be a modification of CQ transport with a decreased influx into or an increased efflux out of the digestive parasitophorous vacuole (Krogstad *et al.* 1992). The *pfcrt* SNP associated with CQ resistance is given in Table 3.

*pfgr*

The tripeptide glutathione (L-γ-glutamyl-L-cysteinyl-glycine, GSH), in its reduced state, is important in redox and xenobiotic stress responses. This low mass thiol has been proposed to be of importance in the detoxification of toxic free haeme by participating in the reduction of $H_2O_2$ produced by iron released from heme (Atamna & Ginsburg 1995; Ginsburg *et al.* 1998). Cloroquine and amodiaquine were shown to inhibit GSH-mediated heme degradation, leading to the accumulation of heme in the parasite membrane and disturbing ion homeostasis. This suggests a role of the GSH metabolism in drug response, and consequently, as in other biological systems, in resistance.

This has been recently demonstrated by the involvement of GSH-associated enzymes, such as the glutathione S-transferase (GST), in *P. falciparum* drug resistance (Srivastava *et al.* 1999). Adequate levels of parasite intracellular GSH are needed during drug exposure, and these levels are maintained by glutathione reductase (pfGR) because of its capacity of reducing oxidized glutathione back to GSH. The gene encoding this enzyme has been cloned (Farber *et al.* 1996) and proved to be polymorphic, giving rise to at least two enzymatic variants, GR2 (derived from Pf K1) and GR3 (derived from Pf3D7). The variants have significantly different kinetic characteristics. GR2 has a fivefold higher catalytic activity than GR2 (Gilberger *et al.* 2000). The variation results from three SNPs (Table 3) that are possibly further genetic markers of drug resistance.

The expected completion of the Malaria Genome Project, supplemented by sequence information of additional clones and isolates, and the subsequent establishment of applicable molecular typing systems, including microarrays, proteomics, and SNP and linkage analyses will provide the informational base for further approaches to address the pressing biological and medical problems of human malaria.

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