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Amodiaquine Resistance in *Plasmodium falciparum* Malaria in Afghanistan Is Associated with the *pfcrt* SVMNT Allele at Codons 72 to 76▽

Khalid Beshir,[1] Colin J. Sutherland,[1] Ioannis Merinopoulos,[1] Naeem Durrani,[2] Toby Leslie,[1] Mark Rowland,[1] and Rachel L. Hallett[1]*

Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, London, United Kingdom,[1] and Merlin International, House 3, Street 55-A, F6/4 Islamabad, Pakistan[2]

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Mutations in the *Plasmodium falciparum* genes *pfcrt* and *pfmdr1* are selected by amodiaquine treatment in Africa. To examine the importance of these mutations in amodiaquine-treated Asian parasites, we determined pre- and posttreatment genotypes for amodiaquine treatment failures from a clinical trial in Afghanistan. The *pfcrt* codon 72 to 76 haplotype SVMNT was present in all samples tested, both before and after treatment. Amodiaquine did not clearly select for any *pfmdr1* genotype, but a novel mutation, *pfmdr1* N86F, was detected in four samples. We provide *in vivo* data to support the *in vitro* correlation between *pfcrt* SVMNT and increased resistance to the metabolite of amodiaquine.

Amodiaquine (AQ), a 4-aminoquinoline related to chloroquine (CQ), has been used commonly as a monotherapy and now as a partner drug in artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated *Plasmodium falciparum* malaria. In many countries, predominantly in Africa, the *in vivo* efficacy of AQ was found to be good even in the face of increasing CQ resistance (12). However, reports of AQ resistance have come from South America, Asia, and East Africa (7, 8, 10).

Mutations in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) and multidrug resistance gene 1 (*pfmdr1*) have been associated with clinical resistance to both CQ and AQ (13). The presence of the *pfcrt* codon 72 to 76 haplotype SVMNT (Ser-Val-Met-Asn-Thr) correlates with high-level resistance to the AQ metabolite desethylamodiaquine (DEAQ) in *in vitro* tests (14) and has been detected with a high prevalence in parasite populations from Brazil, Papua New Guinea, Laos, Iran, and India (9, 18). Clinical trials in East Africa have also demonstrated high levels of *in vivo* resistance to AQ; in those studies, the parasites carried *pfcrt* codon 72 to 76 haplotype CVIET, and *pfmdr1* polymorphisms 86Y, 184Y, and 1246Y were found to be selected after AQ treatment failure (5, 6, 11).

A clinical trial performed in Nangahar Province, East Afghanistan, in 2002 and 2003 to explore possible replacement treatments for CQ showed very poor efficacies of both CQ and AQ monotherapy (adequate clinical and parasitological responses were seen in 11% and 9% of cases, respectively, by day 42) (4). Our aim in this study was to evaluate pre- and posttreatment samples from patients treated with AQ for *pfcrt* and *pfmdr1* mutations and to determine which, if any, polymorphisms are associated with AQ treatment failure in Afghanistan.

MATERIALS AND METHODS

We analyzed samples collected from AQ-treated participants during a clinical trial of AQ versus CQ versus sulfadoxine-pyrimethamine versus AQ plus artesunate in East Afghanistan between October 2002 and January 2003. The clinical and parasitological results of drug efficacy testing have been reported elsewhere (4). Amodiaquine (Basoquin) was supplied by Parke-Davis. Ethical approval for the *in vivo* study and collection of samples for genotyping was given by the LSHTM Ethics Committee and also locally, by the Ministry of Public Health, Afghanistan. All enrolled participants gave informed consent.

Eighty-three trial participants received AQ monotherapy, 7 were excluded or lost to follow-up, and 69 of the remaining 76 patients (91%) failed treatment, with parasites detected during the 42-day follow-up. We analyzed available blood spots collected on filter paper pretreatment (day 0; $n = 55$) and on the 7th day or later after treatment, when parasites were detected by microscopy (day of failure; $n = 42$). Parasite DNA was extracted from blood spots as previously described (6).

Determination of polymorphisms at *pfcrt* codons 72 to 76 was performed using real-time PCR with double-labeled 26-mer oligonucleotide probes corresponding to the three most common *pfcrt* codon 72 to 76 alleles (16). These are CVMNK (CQ sensitive), CVIET (CQ resistant), and SVMNT1 (CQ resistant [7G8 type]). The assay was run on a Corbett Rotorgene 3000 thermal cycler (Corbett Lifesciences, Qiagen, Germany).

Polymorphisms at codons 86 and 184 of the *pfmdr1* gene were determined by a PCR–sequence-specific oligonucleotide probe assay (6) or by PCR and direct sequencing of amplicons, using BigDye Terminator v3.1 cycle sequencing kits and analysis on an ABI 3730 sequencer (Applied Biosystems). The sequence chromatograms were analyzed using Chromas v2.75.

Changes in genotype prevalence between pre- and posttreatment samples were assessed by two-tailed Fisher's exact test.

RESULTS

The *pfcrt* codon 72 to 76 haplotypes were successfully analyzed in 55 pretreatment samples and 42 posttreatment samples. All samples harbored the SVMNT allele. One day 0 sample carried a mixed infection with parasites carrying the SVMNT allele and the CQ- and AQ-sensitive CVMNK allele.

* Corresponding author. Mailing address: Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom. Phone: 44 (0)20 7927 2338. Fax: 44 (0)20 7636 8739. E-mail: Rachel.Hallett@lshtm.ac.uk.

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TABLE 1. Distribution of *pfmdr1* polymorphisms in day 0 and day-of-failure samples[a]

Sample group	Amino acid at position:		*n*
	86	184	
Day 0 samples	N	Y	29
	N	F	12
	Y	F	7
	Y	Y	1
	F	Y	1
Day-of-failure samples	N	Y	13
	N	F	10
	Y	F	5
	Y	Y	2
	F	Y	3

[a] Genotype changes occurred at codons 86 and 184 only. The 86N allele (wild type) was found in 82.0% of pretreatment samples and 69.7% of posttreatment samples ($P = 0.29$ by Fisher's exact test). The prevalence of 184Y decreased from 62.0% of pretreatment samples to 54.5% of posttreatment samples ($P = 0.65$ by Fisher's exact test). None of the changes showed statistically significant evidence of selection. The sequences at codons 1034 (S), 1042 (N), and 1246 (D) were wild type both before and after treatment with AQ.

The CVMNK allele, however, was not detected after AQ treatment.

We investigated 5 polymorphic codons of *pfmdr1* in 83 samples (50 day 0 and 33 day-of-failure samples). DNAs were of insufficient quality to amplify and sequence all 5 sites from all available blood spots. The prevalences of amino acids at codons 86 and 184 are shown in Table 1. Polymorphisms were seen at codons 86 and 184 only; the sequences at codons 1034, 1042, and 1246 were wild type both before and after treatment with AQ.

Our sequence analysis revealed a novel *pfmdr1* mutation at codon 86. In comparison to the sequence in strain 3D7, a change of 2 nucleotides occurred at this codon, from AAT, which codes for asparagine (N), to TTT, which codes for phenylalanine (F). Therefore, this polymorphism can be annotated N86F. It was observed in 1 day 0 and 3 day-of-failure samples.

When *pfmdr1* codons were analyzed together, 5 distinct haplotypes were found: NYSND, NFSND, YFSND, YYSND, and FYSND. The prevalences of each in day 0 and day-of-failure samples are shown in Fig. 1.

The 3D7 laboratory isolate has *pfmdr1* haplotype NYSND and is defined as the wild type. This haplotype was the most common among the pretreatment and day-of-failure samples in Afghanistan, although the prevalence decreased from 58.0% (29/50 pretreatment samples) to 39.4% (13/33 day-of-failure samples; $P = 0.12$).

DISCUSSION

We have found that the high prevalence of AQ malaria treatment failure *in vivo* in Jalalabad, Afghanistan, is associated with *P. falciparum* parasites carrying the *pfcrt* codon 72 to 76 SVMNT allele. Modest increases in the prevalences of *pfmdr1* 86Y and 184F were observed after AQ treatment failure, but these changes were not statistically significant. There is therefore no evidence of AQ selection on *pfmdr1* in this population of parasites, though almost all AQ-treated individuals had recurrent infections within 42 days of follow-up. Note that the samples tested were collected more than 7 years ago, and it is not known if the *pfcrt* codon 72 to 76 SVMNT allele remains the predominant circulating allele in Afghanistan.

Genetic diversity in this parasite population is low, and the variation within the polymorphic *msp2* gene sequence was insufficient to distinguish between recrudescent and new infections. In 101 randomly selected baseline (day 0) samples, 10 different alleles of *msp2* were detected, although 1 of these was found in almost half of all samples (data not shown). We assume that all recurring parasites were the result of recrudescent infections, as the transmission intensity in the study area during the period of follow-up was low, since samples were collected in the start of the winter season and it was unlikely that trial participants would have received a second infectious bite within 42 days (N. Durrani, unpublished data). However, we recognize that it is a limitation of the study that we could not positively identify recrudescences from new infections by genotyping.

Our findings provide direct *in vivo* evidence that the *pfcrt* codon 72 to 76 allele SVMNT is sufficient to confer AQ resistance, independent of the *pfmdr1* genotype. In contrast, the CQ-resistant CVIET allele is necessary but insufficient to confer AQ resistance *in vivo* (5, 6, 11). *In vitro* studies of natural *P. falciparum* isolates, laboratory reference strains, progeny of genetic crosses, and parasites transfected with allelic replacements support this conclusion (14, 15).

On the basis of transfections and *in vitro* drug tests described by Sidhu et al. (15), Warhurst (17) observed that 50% inhibitory concentrations (IC_{50}s) for CQ in the presence of verapamil showed a correlation with IC_{50} values for DEAQ. Since both CQ-verapamil and DEAQ effects were correlated with the hydrophobicity of *pfcrt* amino acids 72 to 76, he predicted that clinical failure of amodiaquine treatment would be associated with parasites carrying the relatively hydrophilic codon 72 to 76 SVMNT allele.

Recent molecular studies have found that the *pfcrt* SVMNT haplotype predominates in parts of the world historically reporting *in vivo* AQ resistance. Indeed, many of these countries, such as India, Philippines, and Brazil, first introduced AQ as an antimalarial in the late 1940s (14), raising the conjecture that AQ rather than CQ drug pressure selected for the SVMNT haplotype.

In contrast, in many areas where parasites carry the CQ-

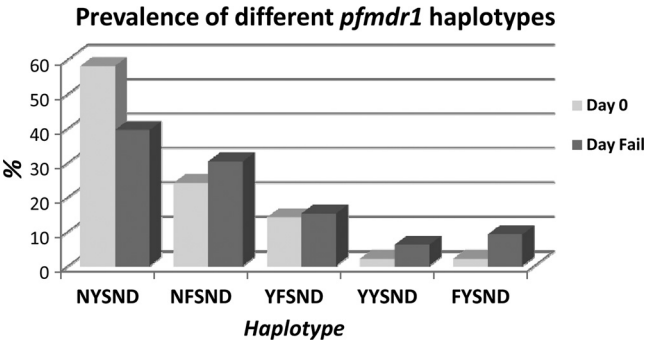

FIG. 1. Prevalences of *pfmdr1* haplotypes at amino acids 86, 184, 1034, 1042, and 1246 before and after treatment with AQ.

selected *pfcrt* CVIET haplotype, such as across sub-Saharan Africa, AQ remains a relatively effective drug, and it is now employed as the partner drug to artesunate as a first-line antimalarial regimen in 19 countries. To our knowledge, *pfcrt* SVMNT allele-carrying malaria parasites have been reported only twice in Africa, in Ghana in 1996–1997 (9) and in Tanzania in 2004 (1). It is clearly of some concern that such parasites would have a survival advantage in areas where AQ is in use.

In parts of east Africa, AQ seems to work less well than in west Africa; clinical trials in Kenya and Tanzania have shown high proportions of *in vivo* AQ treatment failures (5, 10). In the Tanzanian trial, the predominant *pfcrt* codon 72 to 76 haplotype was CVIET (6), and in both trials, significant selection of *pfmdr1* genes encoding 86Y, 184Y, and 1246Y occurred after AQ monotherapy. We postulate that in the presence of high prevalences of *pfcrt* CVIET allele-carrying parasites, AQ can still be effective and that *pfmdr1* mutations are required for the development of clinically significant AQ resistance. It is clear, however, that the correlation between *pfcrt* and *pfmdr1* loci and AQ efficacy is not clear-cut and varies across different geographical settings. Further beneficial modulation of the prevalences of resistant *pfmdr1* haplotypes may occur due to the widespread use of artemether-lumefantrine in sub-Saharan Africa; this drug favors survival of the AQ-sensitive *pfmdr1* 86-184-1246 NFD haplotype (6).

The importance of the *pfmdr1* 86F allele (found in 4 samples) with regard to AQ resistance is not known. The same mutation was recently described for 2 isolates collected in Swaziland in 1999 (3). To our knowledge, this codon sequence has been reported previously only for laboratory isolates under *in vitro* mefloquine selection (2). Similar mutations may have been missed in studies where restriction fragment length polymorphism techniques were used to determine genotypes. The most parsimonious explanation for its appearance would be a single nucleotide change from *pfmdr1* 86Y, rather than 2 nucleotide changes from the wild-type *pfmdr1* 86N allele.

In this study, the predominant *pfmdr1* haplotype before and after AQ treatment failure was the wild-type NYSND haplotype, in contrast to our findings in Tanzania (6). Taken together, the results reported here, the *in vitro* studies highlighted above, and the predictions of Warhurst (17) strongly suggest that carriage of the *pfcrt* SVMNT allele alone is sufficient to predispose a parasite to be clinically highly AQ resistant and lead to the conclusion that ACTs with AQ as the partner drug should not be deployed in parts of the world where parasites with the SVMNT haplotype predominate.

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