

CONCISE COMMUNICATION

Polymorphisms in the *Plasmodium falciparum* *pfert* and *pfmdr-1* Genes and Clinical Response to Chloroquine in Kampala, Uganda

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The molecular mechanism of chloroquine resistance in *Plasmodium falciparum* remains uncertain. Polymorphisms in the *pfert* and *pfmdr-1* genes have been associated with chloroquine resistance in vitro, although field studies are limited. In evaluations of known polymorphisms in parasites from patients with uncomplicated malaria in Kampala, Uganda, the presence of 8 *pfert* mutations and 2 *pfmdr-1* mutations did not correlate with clinical response to therapy with chloroquine. Most notably, the *pfert* lysine→threonine mutation at position 76, which recently correlated fully with chloroquine resistance in vitro, was present in 100% of 114 isolates, of which about half were from patients who recovered clinically after chloroquine therapy. These results suggest that, although key *pfert* polymorphisms may be necessary for the elaboration of resistance to chloroquine in areas with high levels of chloroquine resistance, other factors, such as host immunity, may contribute to clinical outcomes.

Resistance of the malaria parasite *Plasmodium falciparum* to commonly used antimalarial agents is a large and growing problem. In particular, resistance to chloroquine, which remains the standard therapy for malaria in most of Africa, is an urgent concern [1]. The molecular basis of chloroquine resistance remains uncertain. Chloroquine resistance has been correlated with mutations in a number of *P. falciparum* genes, although some results have been inconsistent [2]. In initial studies of the *P. falciparum* multidrug resistance gene (*pfmdr-1*), an Asn→Tyr mutation at amino acid 86 (N86Y) and other mutations in this gene correlated with chloroquine resistance [3]. However, in several field studies, associations between *pfmdr-1* point mutations and in vivo or in vitro chloroquine resistance were not consistent [2]. More recently, transfection studies showed that the replacement of mutant *pfmdr-1* with the wild-type sequence in resistant parasites decreased chloroquine resistance from high to moderate levels [4]. Thus, it appears that, although mutations

in *pfmdr-1* are not required for chloroquine resistance, polymorphisms may play a role in modulating this phenotype.

In the progeny of a genetic cross between chloroquine-sensitive and chloroquine-resistant strains, the chloroquine-resistance determinant mapped to a 36-kb region of chromosome 7 that did not contain the *pfmdr-1* gene [5, 6]. Analysis of laboratory isolates was complex [5] but eventually demonstrated a clear association between chloroquine resistance and mutations in the newly described *pfert* gene [7]. Chloroquine-resistant isolates uniformly contained the K76T *pfert* mutation and also had additional *pfert* mutations at up to 7 sites. These results suggested that the mutations in *pfert* are the principal determinants of chloroquine resistance.

Although recent studies indicate a primary role for the identified *pfert* mutations in chloroquine resistance as measured in vitro, the role of these mutations in mediating responses after chloroquine therapy remains uncertain. In studies in Mali, in which 86% of patients who presented with malaria responded fully to therapy with chloroquine, the baseline prevalence of the *pfert* K76T mutation was 41% [8]. However, the mutation was present in 100% of infections that occurred within 2 weeks after chloroquine treatment. Thus, many patients infected with parasites containing the *pfert* K76T mutation appeared to respond to therapy with chloroquine, but, when therapy was unsuccessful, chloroquine selected for mutant parasites. It was of interest to evaluate the clinical significance of key mutations in *pfert* and *pfmdr-1* in a region with a higher prevalence of chloroquine-resistant malaria than that found in Mali. Therefore, we evaluated the association between point mutations in the *pfert* and *pfmdr-1* genes and in vivo chloroquine response in Kampala, Uganda.

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Informed consent was obtained from all patients or their adult guardians. The studies were approved by the institutional review boards of Makerere University, Kampala, and the University of California, San Francisco.

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Materials and Methods

Study site. This study used samples from a clinical assessment of chloroquine resistance performed in Kampala, Uganda, from August 1998 through March 1999. Kampala is an urban center where malaria is highly endemic, occurring perennially with peaks during the 2 rainy seasons (Ugandan Ministry of Health, unpublished data). Chloroquine is currently the recommended first-line agent for uncomplicated malaria in Uganda.

Patients. Full details of the clinical study that provided the samples evaluated in this report have been described elsewhere [9]. Consecutive patients ≥ 6 months old with uncomplicated falciparum malaria at a parasite density $\geq 2000/\mu\text{L}$ were enrolled. Patients were treated with standard doses of chloroquine (25 mg/kg base over 3 days) under supervision and were followed up for 14 days. Patient outcomes were classified according to a modified version of the World Health Organization (WHO) 14-day clinical classification system (early treatment failure, late treatment failure, or adequate clinical response) and the WHO parasitologic classification system (sensitive; and RI, RII, and RIII, resistance levels I, II, and III, respectively) [9].

DNA extraction. Venous blood was obtained from all patients before treatment and was blotted in $\sim 50\text{-}\mu\text{L}$ aliquots onto filter paper (no. 3 Whatman), which was stored in sealed plastic bags at room temperature. DNA was extracted with Chelex-100 Resin (Bio-Rad Laboratories), as described elsewhere [10]. For control *P. falciparum* DNA, genomic DNA was isolated from schizont-stage parasites (cultured by standard methods) by phenol extraction and isopropanol precipitation [11].

Nested polymerase chain reaction (PCR) for the detection of *pfert* and *pfmdr-1* mutations. Details of PCR reaction conditions are summarized in table 1. Nested PCR protocols were used. The first-round PCR reaction contained $1\ \mu\text{L}$ ($\sim 100\ \text{ng}$) of extracted parasite DNA or $50\ \text{ng}$ of control DNA and $1\ \mu\text{M}$ of each primer in $50\ \mu\text{L}$ of $20\ \text{mM}$ Tris-HCl (pH 8.4), $50\ \text{mM}$ KCl, $2.5\ \text{mM}$ MgCl_2 , and $200\ \mu\text{M}$ dNTP with $2.5\ \text{U}$ Taq polymerase (Life Technologies Gibco BRL). For each series of samples, water was used as a negative control, HB3-strain DNA was used as the wild-type control, and Dd2 DNA was used as the mutant control.

Two nested PCR methods were used for the detection of point mutations: mutation-specific PCR (MS-PCR) and PCR followed by mutation-specific restriction-enzyme digestion (MS-RED). Both techniques were used for the detection of the *pfert* K76T mutation, MS-PCR was used for the detection of the remaining 7 *pfert* mutations, and MS-RED was used for detection of the 2 *pfmdr-1* mutations.

Nested $50\text{-}\mu\text{L}$ MS-PCR reactions contained 1:50–1:50,000 dilutions of the first-round amplicon and $1\ \mu\text{M}$ common and mutant-specific or wild-type-specific primers in $20\ \text{mM}$ Tris-HCl (pH 8.4), $50\ \text{mM}$ KCl, $1.5\ \text{mM}$ MgCl_2 , $200\ \mu\text{M}$ dNTP, and $1.25\text{--}2.5\ \text{U}$ Taq polymerase. The amplified DNA fragments were resolved by electrophoresis in a 1%–2% agarose gel. Wild-type and mutant genotypes were designated on the basis of amplification of products of predicted sizes by the appropriate primers. If bands were present by means of both wild-type and mutant primers, nested PCR reactions using serial 10-fold dilutions of amplicons from the first-round PCR were performed until only 1 band remained. If both

Table 1. Polymerase chain reaction protocols for detection of *pfert* and *pfmdr-1* point mutations.

Primer	Sequence (5'→3')	Size, bp
<i>pfert</i>		
1st Round 74–220		1682
Sense	CCGTTAATAATAAATACAGGCAG	
Antisense	CTTTAAAAATGGAAGGGTGTATAC	
1st Round 271–371		1591
Sense	ACCTGTATACACCCTTCCATTTTT	
Antisense	CCTATAAAGTGTAATGCGATAGC	
74		1182
Common	AACAATTGGAAAAAGGATACC	
Wild	ATTATTAAAGTGATGTGTAATG	
Mutant	ATTATTAAAGTGATGTGTAATT	
75		366
Common	CGAGCGTTATAGAGAATTAG	
Wild	GTTCTTTTAGCAAAAATTGTATT	
Mutant	GTTCTTTTAGCAAAAATTGTTTC	
76		371
Common	CGAGCGTTATAGAGAATTAG	
Wild	TAAAAGTTCCTTTTAGCAAAAATT	
Mutant	TAAAAGTTCCTTTTAGCAAAAATTG	
76 ^a		134
Sense	TGTGCTCATGTGTTTAAACTT	
Antisense	CAAACTATAGTTACCAATTTTG	
220		414
Common	AACAATTGGAAAAAGGATACC	
Wild	TATTAATCTTGCTTAATTAGTG	
Mutant	TATTAATCTTGCTTAATTAGTT	
271		1226
Common	AAATTATAGAACCAAATAGGTAGCC	
Wild	GTATACACCCTTCCATTTTTAAAAC	
Mutant	GTATACACCCTTCCATTTTTAAAAG	
326		694
Common	AAATTATAGAACCAAATAGGTAGCC	
Wild	CTTCGCATTGTTTCTTCTTTAA	
Mutant	CTTCGCATTGTTTCTTCTTTAG	
356		457
Common	AAATTATAGAACCAAATAGGTAGCC	
Wild	TGTATACAAGTCCAGCAAT	
Mutant	TGTATACAAGTCCAGCAAC	
371		310
Common	TCTACCATGACATATACTATTG	
Wild	GAAATCTAATAATCTTGGTCTC	
Mutant	GAAATCTAATAATCTTGGTCTA	
<i>pfmdr-1</i>		
1st Round 86		610
Sense	AGAGAAAAAGATGGTAACCTCAG	
Antisense	ACCACAAACATAAATTAACGG	
Nested 86 ^a		433
Sense	GAGTACCGCTGAATTATTAGA	
Antisense	CAAATTCCTGAACTCACTTG	
1st Round 1246		499
Sense	GTGGAAATCAACTTTTATGA	
Antisense	TTAGGTTCTCTTAATAATGCT	
Nested 1246 ^a		409
Sense	GACTTGAAAAATGATCACATT	
Antisense	GTCCACCTGATAAGCTTTT	

^a Products used for restriction-enzyme digestion.

bands disappeared after a single dilution step, the genotype was designated as mixed.

For MS-RED, $50\text{-}\mu\text{L}$ reactions contained 1:5000 dilutions of the first-round amplicon and $1\ \mu\text{M}$ each primer in $20\ \text{mM}$ Tris-HCl (pH 8.4), $50\ \text{mM}$ KCl, $2.5\ \text{mM}$ MgCl_2 , $200\ \mu\text{M}$ dNTP, and $1.25\ \text{U}$ Taq polymerase. Aliquots of $5\text{--}10\ \mu\text{L}$ of the PCR reaction were incubated with $1\text{--}10\ \text{U}$ of the restriction enzyme in $100\ \text{mM}$ NaCl,

50 mM Tris-HCL, 10 mM MgCl₂, 1 mM dithiothreitol, and 100 μg/μL bovine serum albumin for 6 h at the appropriate temperature. The DNA fragments were resolved by electrophoresis in a 2% agarose gel. For the *pfert* K76T mutation, *ApoI* cut the wild-type but not mutant gene into 34- and 100-bp fragments. For the *pfmdr-1* N86Y mutation, *ApoI* cut the wild-type gene into 245- and 188-bp fragments, and, for the *pfmdr-1* D1246Y mutation, *EcoRV* cut the mutant gene into 210- and 199-bp fragments. For all 3 reactions, mixed genotypes were designated when 3 bands were present after restriction endonuclease digestion.

Statistical analysis. Statistical associations between point mutations and in vivo outcomes were assessed using the Fisher's exact test (2-tailed). *P* < .05 was considered to be significant.

Results

A randomly selected subset of 114 samples from our recent evaluation of chloroquine efficacy in 258 children and adults in Kampala [9] was analyzed for polymorphisms in *pfert* at amino acid 76. These samples were obtained before the initiation of chloroquine therapy and were from patients who subsequently demonstrated a wide range of clinical responses (clinical response was adequate in 56%, treatment failed early in 23%, and treatment failed late in 21%) and parasitologic responses (36% sensitive, 15% RI, 33% RII, and 16% RIII) to the treatment. There was no association (*P* > .2) between selection of samples for molecular analysis and previously identified predictors of chloroquine resistance (age <5 years, initial temperature ≥38°C, or chloroquine use 3–14 days before enrollment). All 114 samples, which were analyzed by using both the MS-PCR and MS-RED methods, contained the *pfert* K76T mutation. In no case was a mixed population of parasites identified. Control HB3-strain parasites demonstrated the wild-type sequence with both the MS-PCR and MS-RED methods. Thus, the wild-type K76 sequence was not found in any parasite DNA from Kampala.

A subset of 30 samples was selected for a case-control study, to assess associations between chloroquine resistance and known mutations in *pfert* and *pfmdr-1*. Case-control samples consisted of 15 patients from each extreme of the parasitologic outcome spectrum (i.e., sensitive and RIII) and included only patients <5 years old, to minimize the effect of acquired immunity. For 6 of the 8 studied *pfert* mutations (M74I, N75E, K76T, A220S, Q271E, and R371I), all of the samples with successful amplification revealed only the presence of the mutant sequence (table 2). At 1 site (amino acid 356), only the wild-type sequence was identified. Polymorphisms were identified at only 1 position (amino acid 326). However, at this site, there was no significant association between the sequence and in vivo outcome. Analysis of control wild-type (HB3) and mutant (Dd2) parasite strains yielded the expected sequences in all cases.

Investigation of 2 known mutations in the *pfmdr-1* gene revealed polymorphisms and a number of mixed genotypes (table

Table 2. Correlation of chloroquine resistance outcomes with *Plasmodium falciparum* genotypes.

Mutation, ^a outcome	No. of genotypes				<i>P</i> ^b
	Mutant	Wild type	Mixed	No result	
<i>pfert</i>					
M74I					
Sensitive	11	0	0	4	—
RIII	14	0	0	1	
N75E					
Sensitive	15	0	0	0	—
RIII	15	0	0	0	
K76T					
Sensitive	15	0	0	0	—
RIII	15	0	0	0	
A220S					
Sensitive	15	0	0	0	—
RIII	15	0	0	0	
Q271E					
Sensitive	13	0	0	2	—
RIII	15	0	0	0	
N326S					
Sensitive	3	9	2	1	.21
RIII	2	13	0	0	
I356T					
Sensitive	0	15	0	0	—
RIII	0	15	0	0	
R371I					
Sensitive	15	0	0	0	—
RIII	15	0	0	0	
<i>pfmdr-1</i>					
N86Y					
Sensitive	13	1	1	0	1.0
RIII	10	0	5	0	
D1246Y					
Sensitive	11	3	1	0	1.0
RIII	8	2	5	0	

NOTE. Case-control samples consisted of 15 patients from each extreme of the parasitologic outcome spectrum (i.e., sensitive to chloroquine and resistance level III [RIII]; see [9]).

^a Each mutation is labeled (in the following order) with the wild-type amino acid, amino acid number, and mutant amino acid, using the single-letter code.

^b Fisher's exact test (2-tailed).

2). However, no sequence at either position was associated significantly with either a sensitive or a resistant clinical outcome.

Discussion

In this study, we report on the correlation between polymorphisms in the *pfert* and *pfmdr-1* genes of *P. falciparum* field isolates and in vivo chloroquine response. Our samples were obtained from an area of intense transmission, where chloroquine resistance rates are high [9, 12]. In 114 samples from patients with a wide range of clinical and parasitologic outcomes after therapy with chloroquine, the *pfert* K76T mutation was ubiquitous. All parasite isolates, which were obtained before chloroquine therapy, contained the K76T mutation. A case-control analysis that evaluated 8 known *pfert* mutations and 2 key *pfmdr-1* mutations failed to identify any sequence that predicted a sensitive or resistant response to chloroquine therapy in Kampala.

Our results suggest that, although the presence of the *pfert* K76T mutation (and perhaps others) may be necessary for the expression of chloroquine resistance, other factors, including host immunity, may play a significant role in determining clinical outcomes after administration of chloroquine. Other recent studies also have shown that many patients with apparently sensitive responses to chloroquine therapy were infected with mutant parasites. At a number of sites in Mali, the prevalence of the K76T mutation was consistently 2–3 times higher than the prevalence of clinical chloroquine resistance (A. Djimde and C. V. Plowe, University of Maryland School of Medicine, Baltimore, personal communication). In Kampala, where the prevalence of clinical chloroquine resistance is higher than in Mali, a similar ratio of genotypic resistance to clinical resistance would predict the presence of the K76T mutation in all parasites, as was seen in our study. Our findings are probably explained by the influence of host immunity on clinical outcomes. In areas of intense transmission, where immunity is high, some patients appear to be able to clear their parasitemia even in the presence of the *pfert* K76T mutation. It has been argued that the emergence of chloroquine resistance only after many years of widespread chloroquine use suggests that multiple mutations are required to produce the chloroquine resistance phenotype [13]. Such mutations might be the multiple identified *pfert* mutations, which may improve the fitness and stability of parasites containing the K76T mutation. Alternatively, our findings are consistent with the possibility that mutations in plasmodial genes other than *pfert* and *pfmdr-1* may be important in the development of the chloroquine-resistant phenotype.

In summary, our results confirm that PCR-based techniques provide a simple, rapid method of detecting polymorphisms in genes that may affect resistance to chloroquine. However, the predictive value of these tests in areas where levels of clinical chloroquine resistance and transmission are high remains unclear. The development of simple molecular tests to help analyze antimalarial drug resistance in field settings remains an important goal. Therefore, further studies are needed to assess the role of new molecular techniques in the surveillance of antimalarial drug resistance in various epidemiologic settings.

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