

A MOLECULAR MARKER FOR CHLOROQUINE-RESISTANT FALCIPARUM MALARIA

ABDOULAYE DJIMDÉ, PHARM.D., OGOBARA K. DOUMBO, M.D., PH.D., JOSEPH F. CORTESE, B.S.,
 KASSOUM KAYENTAO, M.D., SAFI DOUMBO, M.D., YACOUBA DIOURTE, PHARM.D.,* ALASSANE DICKO, M.D.,
 XIN-ZHUAN SU, PH.D., TAKASHI NOMURA, M.D., PH.D., DAVID A. FIDOCK, PH.D.,
 THOMAS E. WELLEMS, M.D., PH.D., AND CHRISTOPHER V. PLOWE, M.D., M.P.H.

ABSTRACT

Background Chloroquine-resistant *Plasmodium falciparum* malaria is a major health problem, particularly in sub-Saharan Africa. Chloroquine resistance has been associated in vitro with point mutations in two genes, *pfcr*t and *pfmdr* 1, which encode the *P. falciparum* digestive-vacuole transmembrane proteins PfCRT and Pgh1, respectively.

Methods To assess the value of these mutations as markers for clinical chloroquine resistance, we measured the association between the mutations and the response to chloroquine treatment in patients with uncomplicated falciparum malaria in Mali. The frequencies of the mutations in patients before and after treatment were compared for evidence of selection of resistance factors as a result of exposure to chloroquine.

Results The *pfcr*t mutation resulting in the substitution of threonine (T76) for lysine at position 76 was present in all 60 samples from patients with chloroquine-resistant infections (those that persisted or recurred after treatment), as compared with a base-line prevalence of 41 percent in samples obtained before treatment from 116 randomly selected patients ($P < 0.001$), indicating absolute selection for this mutation. The *pfmdr* 1 mutation resulting in the substitution of tyrosine (Y86) for asparagine at position 86 was also selected for, since it was present in 48 of 56 post-treatment samples from patients with chloroquine-resistant infections (86 percent), as compared with a base-line prevalence of 50 percent in 115 samples obtained before treatment ($P < 0.001$). The presence of *pfcr*t T76 was more strongly associated with the development of chloroquine resistance (odds ratio, 18.8; 95 percent confidence interval, 6.5 to 58.3) than was the presence of *pfmdr* 1 Y86 (odds ratio, 3.2; 95 percent confidence interval, 1.5 to 6.8) or the presence of both mutations (odds ratio, 9.8; 95 percent confidence interval, 4.4 to 22.1).

Conclusions This study shows an association between the *pfcr*t T76 mutation in *P. falciparum* and the development of chloroquine resistance during the treatment of malaria. This mutation can be used as a marker in surveillance for chloroquine-resistant falciparum malaria. (N Engl J Med 2001;344:257-63.)

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FALCIPARUM malaria remains a major cause of disease and death among children and pregnant women in sub-Saharan Africa. During the second half of the 20th century, chloroquine was the antimalarial treatment of choice, because it was safe, inexpensive, and highly effective against susceptible malaria parasites. Chloroquine resistance arose more than 40 years ago in Southeast Asia and South America, and in these regions chloroquine has now been largely abandoned as a treatment for falciparum malaria. Increasing rates of chloroquine resistance contribute to the rising morbidity and mortality from malaria in Africa.^{1,2} Given the lack of affordable alternatives, chloroquine remains the first-line antimalarial agent in most African countries.

Chloroquine acts by interfering with heme metabolism in the digestive vacuole of *Plasmodium falciparum*. In resistant parasites, the accumulation of chloroquine inside the vacuole is diminished.³⁻⁷ Verapamil, which inhibits P-glycoprotein-mediated multidrug resistance (encoded by *mdr*) in mammalian tumor cells, partly reverses chloroquine resistance in malaria parasites grown in vitro.⁸ In *P. falciparum*, *mdr* homologues encoding P-glycoprotein-like molecules have been proposed as determinants of chloroquine resistance, and associations have been reported between chloroquine resistance and amplification or mutation of the *mdr*-like gene *pfmdr* 1, which encodes Pgh1.⁹⁻¹¹ However, the chloroquine-resistance phenotype was dissociated from inheritance of the *pfmdr* 1 gene in genetic studies.¹² Some field studies have found an association between *pfmdr* 1 mutations and chloroquine resistance¹³ and others have not.¹⁴⁻¹⁷ In recent transformation experiments, chloroquine-sensitive *P. falciparum* parasites that acquire *pfmdr* 1 mutations did not become resistant to chloroquine.¹⁸

From the Malaria Section, Center for Vaccine Development, University of Maryland School of Medicine, Baltimore (A. Djimdé, J.F.C., A. Dicko, C.V.P.); the Malaria Research and Training Center, Faculty of Medicine, Pharmacy and Dentistry, University of Mali, Bamako, Mali (A. Djimdé, O.K.D., K.K., S.D., Y.D., A. Dicko); and the Malaria Genetics Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md. (A. Djimdé, A. Dicko, X.S., T.N., D.A.F., T.E.W.). Address reprint requests to Dr. Plowe at the Malaria Section, Center for Vaccine Development, University of Maryland School of Medicine, 685 W. Baltimore St., HSF 480, Baltimore, MD 21201, or at cplowe@medicine.umaryland.edu.

Drissa Coulibaly, M.D., Malaria Research and Training Center, Faculty of Medicine, Pharmacy and Dentistry, University of Mali, Bamako, Mali, was also an author.

*Deceased.

The locus governing chloroquine resistance in a *P. falciparum* genetic cross has been mapped to a 36-kb segment of chromosome 7. Polymorphisms in one gene, *cg2*, were highly associated with chloroquine resistance,^{19,21} but allelic modification experiments have ruled out a role for this gene in chloroquine resistance.²²

Recently *pfprt*, a gene with 13 exons, was identified near *cg2* on chromosome 7.²³ This gene encodes PfCRT, a transmembrane protein in the digestive vacuoles of malaria parasites. Sets of point mutations in *pfprt* were associated with chloroquine resistance in vitro in laboratory lines of *P. falciparum* from Africa, South America, and Southeast Asia. One mutation, the substitution of threonine (T76) for lysine (K76) at position 76 (K76T), was present in all resistant isolates and absent from all sensitive isolates tested in vitro. Furthermore, genetic-transformation experiments with plasmids expressing mutant forms of *pfprt* conferred chloroquine resistance on three different chloroquine-sensitive clones. These studies point to a key role for the *pfprt* T76 mutation in conferring in vitro chloroquine resistance. The role of these mutations in the failure of chloroquine treatment has not been evaluated in clinical settings.

We conducted haplotype analyses of chloroquine-sensitive and chloroquine-resistant parasites from a drug-efficacy trial in Mali, assessing the relation between chloroquine resistance and mutations in *pfprt* and *pfmdr 1* in parasites from patients with falciparum malaria.

METHODS

Measurement of Chloroquine Efficacy

The study was approved by institutional review boards at the University of Mali, Bamako; the National Institute of Allergy and Infectious Diseases, Bethesda, Maryland; and the University of Maryland, Baltimore. Subjects were enrolled from August through December 1997 in Mopti (population, 60,000) and Bandiagara (population, 12,000) in central Mali, areas where *P. falciparum* is endemic, with intense seasonal peaks.²⁴ Eligible patients were at least two years of age, had a positive blood smear for asexual forms of *P. falciparum*, and were seeking treatment for symptoms consistent with the presence of malaria (fever, chills, headache, and aches). Patients were excluded if they were allergic to chloroquine, had a concurrent nonmalarial febrile illness, had severe malnutrition or another chronic illness, or had severe malaria, defined by the presence of coma, obtundation, seizures, prostration, respiratory distress, shock, protracted vomiting, severe parasitemia (more than 10⁵ parasites per cubic millimeter), a hematocrit of less than 15 percent, or a serum glucose level of less than 40 mg per deciliter (2.2 mmol per liter).

Chloroquine phosphate was administered orally at a dose of 10 mg per kilogram of body weight per day for two days, followed by a dose of 5 mg per kilogram on day 3. The patients were observed after each dose, and they were given another full dose if they vomited within 30 minutes and a half dose if they vomited within 31 minutes to 1 hour. Clinical follow-up occurred on days 1, 2, 3, 7, and 14 after treatment, with microscopical examination of blood on days 3, 7, and 14 and whenever symptoms were reported or fever (defined as an axillary temperature of at least 37.5°C) was detected.

The outcome of treatment was assessed with the use of classic parasitologic definitions of resistance and sensitivity.²⁵ Class III

resistance was defined as persistent parasitemia with no reduction in the level of parasitemia or with a reduction to 25 percent or more of the initial (pretreatment) level by the third day after treatment. Class II resistance was defined as persistent parasitemia with reduction to less than 25 percent of the initial level by day 3. Class I resistance was defined as the initial clearance of parasites, with recurrence of parasitemia by day 14. An organism was considered to be sensitive to chloroquine if there was clearance of parasites, with no recurrence of parasitemia by day 14.

Molecular Analysis

After DNA had been extracted from dried filter papers that had been soaked in blood obtained from patients before and after treatment, nested mutation-specific polymerase chain reaction (PCR) or nested PCR followed by mutation-specific restriction-endonuclease digestion was used to detect mutations in *pfprt* and *pfmdr 1*. Repeat polymorphisms in the κ region and the Ω region of *cg2* were detected by agarose-gel electrophoresis on the basis of the size of amplified products. The samples were analyzed for the following substitutions and polymorphisms: T76, the substitution of serine (S220) for alanine at position 220 (A220S), the substitution of glutamic acid (E271) for glutamine at position 271 (Q271E), the substitution of serine (S326) for asparagine at position 326 (N326S), the substitution of threonine (T356) for isoleucine at position 356 (I356T), and the substitution of isoleucine (I371) for arginine at position 371 (R371I) in *pfprt*; the substitution of tyrosine (Y86) for asparagine at position 86 (N86Y), the substitution of tyrosine (Y184) for phenylalanine at position 184 (F184Y), the substitution of cysteine (C1034) for serine at position 1034 (S1034C), the substitution of asparagine (N1042) for aspartic acid at position 1042 (D1042N), and the substitution of tyrosine (Y1246) for aspartic acid at position 1246 (D1246Y) in *pfmdr 1*; and size polymorphisms in the *cg2* κ and *cg2* Ω repeats. Direct DNA sequencing was used to detect mutations for which these assays were not available and to confirm results. Microsatellite analysis²⁶⁻²⁸ was performed to determine whether there was genetic similarity among chloroquine-sensitive parasites and among chloroquine-resistant parasites with the use of primers and methods described elsewhere.²⁹ Detailed information on these techniques is available on the Internet at <http://medschool.umaryland.edu/CVD/plowe.html>.

Statistical Analysis

We analyzed samples taken before and after treatment for all patients with chloroquine-resistant infections. Base-line frequencies of mutations were determined from samples taken before treatment that were selected randomly and analyzed without knowledge of the clinical outcome. All samples with class I, II, or III resistance were grouped for analysis. The chi-square test or Fisher's exact test for two-tailed significance ($P=0.05$) was used for univariate comparisons. Multiple logistic-regression analysis was performed with the use of a software program (Stata, College Station, Tex.).

RESULTS

Of the 514 patients who were enrolled, 469 completed follow-up and had an outcome that could be evaluated. The infection was sensitive to chloroquine in 86 percent of the infections, resistant at the class I level in 11 percent, resistant at the class II level in 2 percent, and resistant at the class III level in 1 percent. The median age of the patients was 10 years. The median parasite density was 12,800 per cubic millimeter.

Prevalence of *pfprt* T76 and *pfmdr 1* Mutations

The prevalence of *pfprt* T76 and *pfmdr 1* Y86 was compared in parasites from randomly selected patients before treatment and in parasites from patients

whose infections persisted or recurred 4 to 14 days after treatment. Table 1 shows that these mutations, as well as *cg2* polymorphisms, were more prevalent in samples obtained from patients with a post-treatment infection. Other previously described *pfmdr 1* mutations (F184Y, S1034C, D1042N, and D1246Y) either were not detected or were not more common in samples from patients with a post-treatment infection (data not shown).

The *pfprt* T76 mutation was present in all 60 samples from patients with a post-treatment infection that were analyzed for this mutation (Fig. 1). In contrast, 14 percent of the 56 samples analyzed from patients with post-treatment infections carried only the wild-type allele at position 86 of *pfmdr 1* (N86) and 16 percent carried a mixture of the wild-type and the mutant allele.

We performed microsatellite analysis in 8 samples from patients with chloroquine-sensitive infections in which the parasites had the K76 *pfprt* allele and in 22 samples from patients with chloroquine-resistant infections in which the parasites had the T76 mutant allele. This analysis (data not shown) confirmed the diversity of the genetic backgrounds of *P. falciparum* parasites in all infections, and there was no evidence that a clone or strain of parasite was responsible for either sensitive or resistant infections.

Prevalence of Other *pfprt* Mutations in Association with T76

Seven other *pfprt* mutations have been identified in association with T76 in parasites from Africa and

Asia: I74, E75, S220, E271, S236, T356, and I371.²³ In randomly selected samples obtained from patients before treatment and patients with post-treatment infections, all but the T356 mutation were significantly selected for in vivo by chloroquine treatment (Table 1). This result is consistent with the lack of association of T356 with in vitro chloroquine resistance.²³

In most pretreatment infections with chloroquine-sensitive parasites that had the K76 *pfprt* allele, which is associated with sensitivity to chloroquine, the parasite also carried wild-type *pfprt* alleles at other positions that are associated with sensitivity type at A220 (30 of 31 samples), Q271 (35 of 35), N326 (29 of 34), I356 (35 of 36), and R371 (27 of 29).²³ Among parasites with the T76 mutation, there was no significant difference in the prevalence of *pfprt* I74, E75, S220, E271, S326, T356, or I371 mutations in the infections that cleared after chloroquine treatment and those that did not clear after chloroquine treatment. In all parasites tested for their presence, the *pfprt* mutations I74, E75, S220, and I371 accompanied T76.

Association between *pfprt* and *pfmdr 1* Mutations and Treatment Outcome

To determine whether the presence of *pfprt* and *pfmdr 1* mutations at the time of treatment was associated with subsequent treatment failure, we compared the prevalence of these mutations in infections that failed to clear and in infections that cleared with chloroquine treatment. The mutations *pfprt* T76 and *pfmdr 1* Y86, as well as the polymorphisms in *cg2* κ and *cg2* Ω repeats that are associated with resistance,

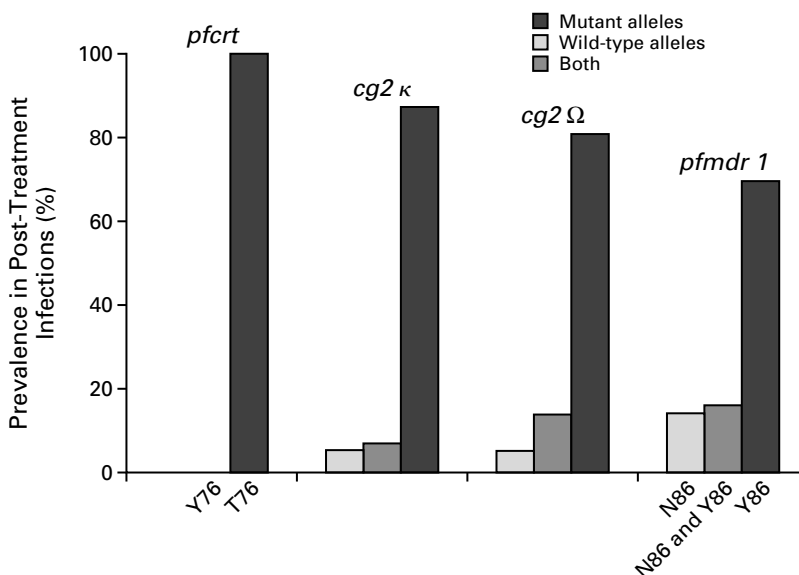


Figure 1. Prevalence of Alleles with the *pfprt* T76, *cg2* κ , *cg2* Ω , or *pfmdr 1* Mutations Associated with Resistance to Chloroquine, Wild-Type Alleles Associated with Sensitivity to Chloroquine, or Both in Samples from Patients with a Persistent or Recurrent Infection after Chloroquine Treatment.

The complete absence of parasites with the wild-type Y76 allele for *pfprt* at position 76 after chloroquine treatment indicates selection for the T76 mutation in vivo.

TABLE 1. PREVALENCE OF MUTATIONS IN SAMPLES OBTAINED FROM PATIENTS BEFORE CHLOROQUINE TREATMENT AND FROM PATIENTS WITH PERSISTENT OR RECURRENT INFECTION AFTER TREATMENT.

MUTATION*	BEFORE TREATMENT	AFTER TREATMENT	P VALUE
	no. of mutations/ total no. of samples (%)		
<i>pfprt</i> I74	2/8 (25)	6/6 (100)	0.01
<i>pfprt</i> E75	2/8 (25)	6/6 (100)	0.01
<i>pfprt</i> T76	47/116 (41)	60/60 (100)	<0.001
<i>pfprt</i> S220	16/43 (37)	23/23 (100)	<0.001
<i>pfprt</i> E271	14/44 (32)	22/23 (96)	<0.001
<i>pfprt</i> S326	13/46 (28)	13/22 (59)	0.03
<i>pfprt</i> T356	1/44 (2)	2/24 (8)	0.59
<i>pfprt</i> I371	16/44 (36)	23/23 (100)	<0.001
<i>pfmdr 1</i> Y86	58/115 (50)	48/56 (86)	<0.001
<i>cg2</i> κ	47/117 (40)	52/55 (95)	<0.001
<i>cg2</i> Ω	43/108 (40)	55/58 (95)	<0.001

*The *pfprt* I74 and E75 mutations were determined by DNA sequencing of parasites from a limited subgroup of infections. Mutations at all other positions were determined by allele-specific PCR, restriction-endonuclease digestion, or both of parasites from randomly selected patients before treatment and patients with persistent or recurrent infection after treatment.

were all associated with in vivo chloroquine resistance in univariate analyses. Overall, parasites carrying *pfprt* T76 were the most likely to be resistant to chloroquine treatment (odds ratio, 18.8; 95 percent confidence interval, 6.5 to 58.3). The additional presence of *pfmdr 1* Y86 and of polymorphisms in *cg2* that are associated with resistance did not strengthen the association between *pfprt* T76 and in vivo resistance.

Adjustment for age showed in almost all cases that the strongest associations between genotypes and resistant infections were found in children younger than 10 years of age, which was the median age of the patients (Table 2).

Multiple logistic-regression analysis confirmed that *pfprt* T76 (odds ratio for resistance, 16.1; 95 percent confidence interval, 5.7 to 45.7) and *pfmdr 1* Y86 (odds ratio, 2.5; 95 percent confidence interval, 1.1 to 5.8) were independently associated with an increased likelihood of resistance, although there was no interaction between these two mutations. The *cg2* polymorphisms were not associated with outcome independently of *pfprt* T76. Increasing age was confirmed to be protective against resistance, whereas the presence or absence of fever and the level of parasitemia were not significantly associated with outcome.

Effect of Age on the Association between Mutations and Outcome

The base-line prevalence of *pfprt* T76 was higher than that of clinical chloroquine resistance: 41 percent of the parasites obtained from 116 randomly selected patients before treatment had T76, whereas the parasites from only 14 percent of 469 patients exhibited in vivo resistance. Partial immunity develops with prolonged exposure to malaria, and older persons in endemic areas have protection against the disease.³⁰ To determine whether immunity contributed to the ability to clear infections by parasites carrying *pfprt* T76, we compared the proportion of infections by parasites carrying T76 that cleared in children younger than 10 years with the proportion of infections by parasites carrying T76 that cleared in older children and adults. In the younger group, 68 percent of 73 pretreatment infections by parasites

TABLE 2. UNIVARIATE ANALYSIS OF THE ASSOCIATION BETWEEN THE PRESENCE OF VARIOUS MUTATIONS BEFORE TREATMENT AND THE LIKELIHOOD OF CLINICAL CHLOROQUINE RESISTANCE, ACCORDING TO AGE.*

MUTATION	PREVALENCE IN CHLOROQUINE-SENSITIVE INFECTIONS	PREVALENCE IN CHLOROQUINE-RESISTANT INFECTIONS	ALL AGES		AGE <10 YR		AGE ≥10 YR	
			ODDS RATIO (95% CI)	P VALUE	ODDS RATIO (95% CI)	P VALUE	ODDS RATIO (95% CI)	P VALUE
			% (no./total no.)					
<i>pfprt</i> T76	37 (40/107)	92 (56/61)	18.8 (6.5–58.3)	<0.001	20.7 (5.9–80.3)	<0.001	16.8 (2.0–373.3)	0.001
<i>pfmdr 1</i> Y86	49 (51/104)	75 (46/61)	3.2 (1.5–6.8)	<0.001	3.5 (1.4–8.7)	0.002	4.5 (0.8–32.9)	0.05
<i>cg2</i> κ	37 (39/105)	71 (40/56)	4.2 (2.0–9.1)	<0.001	4.8 (1.8–12.8)	<0.001	2.5 (0.6–10.8)	0.13
<i>cg2</i> Ω	39 (39/100)	77 (47/61)	5.3 (2.4–11.5)	<0.001	6.9 (2.6–19.0)	<0.001	4.6 (1.0–24.8)	0.02
<i>pfprt</i> T76 and <i>pfmdr 1</i> Y86	22 (22/102)	73 (43/59)	9.8 (4.4–22.1)	<0.001	13.3 (4.5–40.2)	<0.001	12.5 (2.1–95.5)	<0.001
<i>pfprt</i> T76, <i>pfmdr 1</i> Y86, <i>cg2</i> κ, and <i>cg2</i> Ω	16 (14/90)	55 (28/51)	6.6 (2.8–15.9)	<0.001	10.5 (3.1–37.7)	<0.001	3.9 (0.8–20.8)	0.06

*CI denotes confidence interval.

with the T76 mutation failed to clear, whereas in older patients, only 34 percent of 35 pretreatment infections with the T76 mutation failed to clear ($P < 0.001$).

DISCUSSION

The T76 mutation in *pfert*, which encodes a transporter protein of the *P. falciparum* digestive vacuole, was found in 60 samples from patients with falciparum malaria infections that recurred or persisted after treatment with oral chloroquine, indicating the absolute selection for this mutation in parasites capable of surviving in the presence of chloroquine. Parasites harboring *pfert* K76, which is associated with chloroquine sensitivity in vitro,²³ were not detected in any of these post-treatment infections. In contrast to the total absence of *pfert* K76, *pfmdr 1* N86, the form associated with sensitivity, was detected in parasites from 30 percent of patients whose infections persisted or recurred after chloroquine treatment. The presence of *pfert* T76 at the time of treatment was also strongly associated with subsequent resistance to chloroquine in vivo. These data, combined with the genetic evidence of Fidock et al.,^{22,23} support the idea that *pfert* is an essential determinant of chloroquine resistance in clinical falciparum malaria.

Chloroquine therapy cleared some infections by parasites carrying *pfert* T76. This result is consistent with those of previous field studies in which in vitro drug resistance was more common than in vivo resistance.³¹⁻³³ The association we observed between age and successful treatment reflects the gradual acquisition of partial immunity in this highly endemic area, and this immunity helps in the clearance of resistant parasites.

In some infections that were resistant to chloroquine treatment, *pfert* T76 was not detected at the time of treatment but was detected in the parasites that survived treatment. Although this result could have been due to reinfection or to the failure to achieve adequate chloroquine levels in blood, reinfection should be rare during a 14-day follow-up period, and subtherapeutic chloroquine levels owing to poor compliance are unlikely with directly observed therapy. A more likely explanation is that these were mixed infections consisting predominantly of sensitive parasites along with minute populations of resistant parasites whose levels were below the threshold of detection by PCR or restriction-endonuclease methods. During exposure to chloroquine, sensitive parasites would have been cleared as the resistant parasite population expanded, resulting in treatment failure.

All parasites with the *pfert* T76 mutation also had the *pfert* I74, E75, S220, and I371 mutations. The presence of several of these mutations may be required to maintain native PfCRT function and at the same time confer chloroquine resistance. Simultaneous acquisition of several mutations by a single PfCRT

molecule would be an extremely rare event. This may explain the slow rate of emergence and contiguous pattern of the geographic spread of chloroquine resistance in South America and Southeast Asia³⁴ and the different sets of *pfert* mutations found in chloroquine-resistant isolates from these regions.²³ Among chloroquine-sensitive parasites without the T76 mutation, some had the S220, S236, T356, or I371 mutation or more than one of these mutations. This result is consistent with the finding of Fidock et al. that some chloroquine-sensitive clones do not have the T76 mutation but do have other *pfert* mutations,²³ and it further supports the idea that T76 has an essential role in chloroquine resistance.

Our finding of a significant association between *cg2* polymorphisms and chloroquine resistance is consistent with the findings of others,^{20,21} but in the light of recent genetic-transformation studies,²² this result is almost certainly due to the proximity of *cg2* and *pfert* on chromosome 7 and not to any causal role of *cg2* in chloroquine resistance.

We found that the *pfmdr 1* mutation Y86 was significantly selected for by chloroquine treatment, as previously reported.¹³ Because *pfmdr 1* and *pfert* are on different chromosomes, their coselection cannot be attributed to physical linkage. Rather, *pfmdr 1* Y86 may confer some advantage to the parasite in the presence of chloroquine, either by compensating for fitness lost because of *pfert* mutations or by augmenting the level of resistance.

Our study does not support the idea that *pfmdr 1* has a primary role in conferring chloroquine resistance in *P. falciparum*. This result is consistent with those of previous studies, which reported the absence of an association between the presence of other *pfmdr 1* mutations and chloroquine resistance in vivo¹⁴⁻¹⁷ and showed that resistant infections in vivo can be due to parasites with no *pfmdr 1* mutations at position 86.^{14,19,35} Although there is some evidence that *pfmdr 1* may modulate the level of in vitro resistance,¹⁸ the presence of *pfmdr 1* Y86 in parasites obtained from patients before treatment did not strengthen the association between *pfert* T76 and treatment failure. Since these two mutations could affect each other only if they occurred in the same parasite, these data do not rule out the possibility that *pfmdr 1* modulates chloroquine resistance in areas where polyclonal infections are common. However, because most parasites in our study had only mutant forms of both *pfert* and *pfmdr 1*, any strong interaction should have been detected. It is possible that any additive or epistatic effects of *pfmdr 1* Y86 or other genetic factors on chloroquine resistance would be more apparent in areas where the level of immunity, the prevalence of chloroquine resistance, or the genetic complexity of infections is different.

Molecular assays for detecting *pfert* mutations are potentially important tools for identifying chloro-

quine-resistant *P. falciparum* malaria. Our results suggest that *pfcr* T76 will be most predictive of clinical chloroquine resistance in nonimmune populations, such as travelers or residents of areas with low or unstable rates of malaria transmission. In areas such as our study site, where the prevalence of *pfcr* T76 exceeds that of clinical resistance, indicating low specificity of *pfcr* T76 as a clinical test, determining the ratio of the prevalence of T76 mutations to the prevalence of chloroquine resistance may permit the prediction of clinical resistance rates. Surveys to determine the prevalence of *pfcr* T76 will be useful not only in areas that still rely on chloroquine, but also in regions where the failure rates of drugs that replaced chloroquine are now increasing. The finding of a decreasing prevalence of *pfcr* mutations in these areas would provide a rationale for considering the reintroduction of chloroquine, ideally in combination with other antimalarial drugs, so as to prevent the reemergence of resistance.³⁶ A better understanding of the specific host factors that contribute to the clearance of parasites with resistance-conferring *pfcr* mutations will be needed in order to improve the ability of molecular markers to predict in vivo resistance in semi-immune populations.

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