CONCISE COMMUNICATION

Analysis of the Key *pfcrt* Point Mutation and In Vitro and In Vivo Response to Chloroquine in Yaoundé, Cameroon

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The putative key codon (Lys-76 in sensitive parasites and Thr-76 in resistant parasites) of the novel candidate gene for chloroquine resistance, *Plasmodium falciparum* chloroquine resistance transporter *(pfcrt)*, was determined by polymerase chain reaction–restriction fragment length polymorphism from 111 Cameroonian isolates and was compared with in vivo and in vitro responses to chloroquine. The key codon was significantly associated ($P < .001$) with responses in vivo (92% sensitivity and 76% specificity) and in vitro (97% sensitivity and 81% specificity). Some discordant results were due to multiclonal infections. The high, but not perfect, correlation between the *pfcrt* polymorphism and the phenotype implies that a single point mutation in codon 76 of the *pfcrt* gene is the major, but possibly not the sole, determinant for chloroquine resistance.

Despite the spread of drug-resistant *Plasmodium falciparum* in most areas of the world where malaria is endemic, chloroquine is still the first-line drug for the treatment of acute uncomplicated malaria in many African countries. The underlying reasons include its rapid action, high efficacy against the other human *Plasmodium* species, good tolerance, safety in young children and pregnant women, and wide availability at low cost. The mechanism of action of chloroquine has not been fully understood, and various hypotheses have been met with conflicting views. The biochemical basis of chloroquine resistance also is not well understood, but any hypothesis must explain the reduced accumulation of chloroquine in chloroquine-resistant parasites, compared with that in chloroquine-sensitive parasites [1]. Chloroquine resistance is probably associated with the modification of the transport process of chloroquine (enhanced efflux or reduced influx) into the digestive vacuole [2].

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The clinical study was approved by the Cameroonian Ministry of Public Health and the Cameroonian National Ethics Committee. Informed consent was obtained from adult patients or guardians of sick children before enrollment.

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Several genes encoding a candidate protein involved in the transport of chloroquine into or out of the digestive vacuole have been proposed over the past decade: *P. falciparum* multidrug resistance 1 *(pfmdr1)*, *cg2*, and *pfcrt* (or *cg10*). Mutations in *pfmdr1* (localized in chromosome 5), in particular the key amino acid substitution Asn86Tyr, have been suggested as involved in chloroquine resistance [3]. However, subsequent studies failed to confirm this association [4]. Furthermore, genetic cross experiments suggested that the putative chloroquine resistance gene is located in chromosome 7 and not in chromosome 5 [5].

The subsequent discovery of the *cg* gene family in chromosome 7 and analysis of DNA sequences among laboratory-adapted strains of *P. falciparum* suggested a close association between the in vitro chloroquine sensitivity or resistance phenotype and distinct polymorphisms in the *cg2* gene, which is defined by 12 point mutations and 3 repetitive regions exhibiting size polymorphisms [6]. These findings were confirmed in fresh clinical isolates, but the correlation between the polymorphisms and phenotype was not 100% in any of these studies [7–9]. In fact, recent transfection experiments that created allelic exchanges seem to suggest that the role of *cg2* mutations in chloroquine-resistant phenotypes may not be determinant [10]. Moreover, a few laboratory-adapted strains did not display the typical *cg2* pattern associated with chloroquine resistance [6]. These strains and all other chloroquine-resistant reference clones were predicted correctly to be chloroquine resistant by the third candidate for chloroquine resistance gene, *pfcrt*, which was discovered among the genes that belong to the *cg* gene family [11]. There was a perfect correlation between the *pfcrt* mutation and the in vitro chloroquine response of a panel of geographically diverse isolates. Moreover, clinical studies in Mali suggested that the key *pfcrt* amino acid substitution,

Lys76Thr, is selected after chloroquine treatment and is a reliable genetic marker for chloroquine resistance [12].

In the present work, the correlation between the key *pfcrt* mutation at position 76 and in vivo and in vitro response to chloroquine was further evaluated in randomly selected DNA samples from Cameroonian patients who presented with acute uncomplicated malaria. Since 2 other genetic markers, *pfmdr1* and *cg2*, also were characterized in some of these isolates, all available polymorphism and phenotype correlational data from our study site since 1994 were recapitulated, and the concordance among the 3 genetic markers for chloroquine resistance was assessed.

Methods

This study was part of randomized clinical trials conducted in children (≥5 years old) and adults residing in Yaoundé from 1994 through 1998. Parasite DNA was extracted from venous blood samples, as described in our study reported elsewhere [13]. The nested polymerase chain reaction (PCR) protocol used in this study was modified from the techniques provided by Christopher Plowe (University of Maryland) [12]. The amplified fragment of the *pfcrt* gene was incubated with *Apo*I restriction endonuclease (New England Biolabs). The cleavage of the DNA fragment into 2 fragments (34 and 100 bp) indicates the presence of the wild-type codon Lys-76 (nucleotides AAA). If the 134-bp fragment remained undigested, the presence of the mutant codon Thr-76 (nucleotides ACA) was deduced. If the digestion product yielded 2 cleaved fragments (34 and 100 bp) and an uncleaved fragment (134 bp), the presence of mixed alleles was deduced. The wild-type Lys-76 codon has been suggested to be predictive of chloroquine sensitivity, whereas the mutant Thr-76 codon is predictive of chloroquine resistance [11]. In addition to the *pfcrt* gene, 2 polymorphic markers, merozoite surface antigen (MSA)–1 and MSA-2, were amplified to estimate the number of individual parasite populations coinfecting the same patient [13]. A given isolate was considered to be multiclonal if ≥2 distinct bands were observed in ≥1 polymorphic marker. An isolate was considered to be monoclonal if a single band was seen in both markers.

The in vitro drug sensitivity assay was performed to determine in vitro resistance to chloroquine ($IC_{50} \geq 100$ n*M*) [14]. We used the 14-day test of therapeutic efficacy, developed by the World Health Organization in 1996, to assess the in vivo response to chloroquine [15]. The in vivo response was classified as early treatment failure, late treatment failure, and adequate clinical response. The current definition of an "adequate clinical response" includes patients with no clinical symptoms related to malaria, notably fever, but with a positive blood smear on day 14. In the present study, those patients with an initial clearance of parasitemia but with the reappearance of asexual parasites on day 14 were considered to have resistant cases.

Parasite DNA samples were selected randomly from the list of patients who were treated with chloroquine or other antimalarial drugs. The results obtained from molecular techniques were expressed qualitatively as chloroquine sensitive or chloroquine resistant and were presented on a 2 × 2–contingency table. The *pfcrt* allele that predicts chloroquine sensitivity (Lys-76) or chloroquine resistance (Thr-76) was gauged against both in vitro and in vivo tests of chloroquine resistance to calculate the odds ratios. Since the in vitro and in vivo prevalence of chloroquine resistance from a representative sample of residents in Yaoundé is known and is relatively high (~50%), the sensitivity, specificity, and positive predictive value of the molecular markers were calculated if the *P* value of the odds ratio was <.05. Some samples were analyzed for polymorphisms in the *pfmdr1*, *cg2*, and/or *pfcrt* genes. We previously described the results on the characterization of *pfmdr1* and *cg2* polymorphisms of the Cameroonian isolates [7–9].

Results

In total, 111 DNA samples were selected randomly from the list of patients who completed the 14-day follow-up and were analyzed, to determine the key codon of the *pfcrt* gene. Of these samples, the in vitro chloroquine sensitivity or resistance level was characterized in 93 isolates, and the in vivo response to chloroquine was available from 67 patients. The other 44 patients were assigned to amodiaquine, pyronaridine, or sulfadoxine-pyrimethamine treatment. Of the patients treated with chloroquine, 42 (63%) had an adequate clinical response (6 with an asymptomatic parasitemia on day 14); 18 and 7 responded with late and early treatment failure, respectively. Overall, the parasitologic and clinical failure rates were 46% (31 of 67) and 37% (25 of 67), respectively. The in vitro assay showed that 40 (43%) of 93 isolates were chloroquine resistant ($IC_{50} \geq 100$ n*M*); 53 (57%) of 93 were chloroquine sensitive ($IC_{50} < 100$ n*M*).

The *pfcrt* mutation was significantly associated ($P < .001$) with both in vivo and in vitro responses to chloroquine (table 1). When gauged against the in vivo response, the sensitivity, specificity, and positive predictive value of the *pfcrt* mutation to predict chloroquine sensitivity and resistance were 92%, 76%, and 75%, respectively. The sensitivity (97%), specificity (81%),

Table 1. Analysis of association between putative resistance marker and in vivo and in vitro response to chloroquine.

	In vivo response			In vitro response		
Marker[a]	*n*	OR (95% CI)	*P*	*n*	OR (95% CI)	*P*
pfcrf	59	37.5 (6.3–294)	<.001	84	152 (17.7–3396)	<.001
pfmdr1	36	0.6 (0.02–9.91)	NS	96	2.96 (0.6–15.6)	NS
cg2κ	42	3.0 (0.7–13.5)	NS	44	17.0 (3.0–110.9)	<.001
cg2ω	46	12.8 (2.4–76.2)	<.001	75	12.0 (3.6–42.4)	<.001
cg2 mutations	33	1.2 (0.2–6.0)	NS	37	18.0 (2.7–146)	<.001

NOTE. CI, confidence interval; OR, odds ratio.

[a] Results on *pfmdr1* and *cg2* polymorphisms have been described elsewhere [7–9]. Genetic characterization was based on codon 76 of the *pfcrt* gene, codon 86 (chloroquine sensitive, Asn-86; chloroquine resistant, Tyr-86) of the *pfmdr1* gene [3], κ (chloroquine sensitive, 11–13 repeat units; chloroquine resistant, 14 repeat units) and ω (chloroquine sensitive, 10–32, but not 16, repeat units; chloroquine resistant, 16 repeat units) repetitive domains of the *cg2* gene, and a combination of 3 putative key codons, 275, 281, and 299, of the *cg2* gene (chloroquine sensitive, Gln- or His-275/Ala- or Gly-281/Gln- or His-299, except for combination Gln-275/Ala-281/Gln-299, which represents the chloroquine-resistant allele) [6]. Sensitivity, specificity, and positive predictive value of the *cg2ω* repeat units were 86%, 68%, and 69% to predict in vivo response to chloroquine and 81%, 74%, and 75% for in vitro response to chloroquine, respectively. See text for measures of validity for the *pfcrt* gene.

and positive predictive value (80%) to predict chloroquine resistance were even higher when gauged against the in vitro response to chloroquine. The distribution of chloroquine IC_{50} values in relation to the *pfcrt* mutation is illustrated in figure 1. Of 10 isolates with discordant results between the key *pfcrt* codon and phenotype, 9 had the mutant Thr-76 allele but were chloroquine sensitive in vitro. Most ($n = 7$) of these isolates displayed near borderline IC_{50} values (48–88 n*M*) for chloroquine. Two mutant isolates had relatively low IC_{50} values (25 and 36 n*M*). Five of 9 mutant isolates were multiclonal. One monoclonal isolate with a wild-type *pfcrt* was characterized by a near borderline IC_{50} (125 n*M*), but the patient had an adequate clinical response.

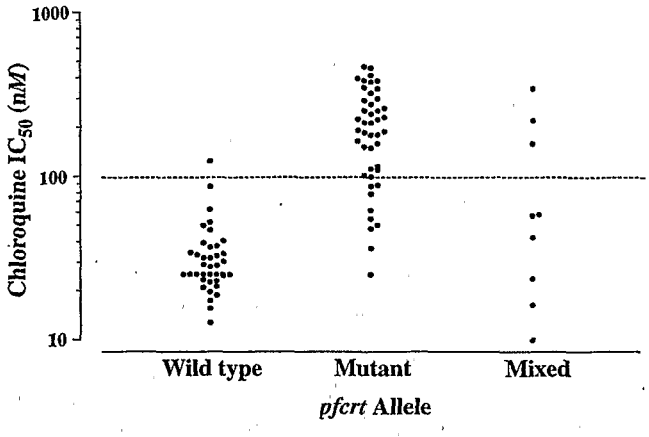

Figure 1. Distribution of IC_{50} values of chloroquine and *pfcrt* mutation at codon 76 in 93 Cameroonian clinical isolates. Lys-76 is associated with chloroquine sensitivity and Thr-76 with chloroquine resistance [11]. In vitro resistance threshold level was estimated to be an IC_{50} of 100 n*M* *(dashed line)*.

Discussion

These results lend further support to the hypothesis that the *pfcrt* gene may be the key parasite gene that determines the chloroquine sensitivity and resistance phenotype. This association was more evident with the in vitro than with the in vivo response to chloroquine. However, the correlation between the *pfcrt* and the in vitro chloroquine resistance was not perfect.

The first possible explanation involves the in vitro drug sensitivity assay. The inaccuracy of the cutoff value ($IC_{50} = 100$ n*M*) for in vitro chloroquine resistance was not validated by in vivo/in vitro correlational studies in nonimmune patients and may lead to an underestimation of the proportion of chloroquine-resistant isolates. There may be other technical problems in performing the in vitro assay, including poor adaptation of a given isolate to in vitro growth conditions and the presence of trace amounts of antimalarial drugs that may influence parasite growth.

The second factor that seems to play an obvious role in an erroneous prediction of the in vitro response to chloroquine, based on the *pfcrt* mutation, is multiclonality with mixed chloroquine-sensitive and chloroquine-resistant parasite populations. In Yaoundé, 57% of symptomatic children and adults are infected with ≥2 distinct parasite populations (as shown by the presence of multiple MSA-1 and MSA-2 alleles) [13]. Moreover, age (between 5 and 60 years old), initial parasitemia, and multiclonality were not correlated with response to drug treatment. An isolate characterized to be sensitive in vitro to chloroquine may be composed of a minority of parasite populations that are chloroquine resistant, or vice versa, and amplification of mixed alleles by the PCR may or may not yield sufficient amplification product and digested fragments that are visible on an agarose gel, depending on the proportion of mixed parasite populations.

A third explanation for discordance is the possible influence of other *pfcrt* mutations on the chloroquine-resistant phenotype. Eleven other codons may undergo mutation in a chloroquine-resistant parasite [11]. The sequences of these codons were not determined in our study. Last, it cannot be ruled out at present that another gene (e.g., *cg2*) or genes may be involved in the change of phenotype from chloroquine sensitivity to chloroquine resistance.

The definition of the role of chloroquine at primary and secondary care centers requires a regular update on its therapeutic efficacy in different regions of Africa. The use of in vitro drug sensitivity assays and molecular markers as additional tools to map areas of chloroquine resistance seems to be warranted, even if the correlation between the in vivo response to chloroquine and the in vitro assay or molecular markers was not perfect in our study. Further studies are needed, especially in young children (≤5 years old), to assess the association between molecular markers and in vivo response. Nevertheless, the determination of the key codon of the *pfcrt* gene, with or without *cg2* alleles, may have a practical use for the description of the epidemiology of chloroquine-resistant *P. falciparum*.

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