CONCISE COMMUNICATION

Analysis of the Key pfcr 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].

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 (pfdnr1), eg2, and pfcr (or cg10). Mutations in pfdnr1 (located 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 determinantal [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, pfcr, 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 pfcr7 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, pfmdrl 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 pfcr7 gene was incubated with ApoI 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 pfcr7 gene, 2 polymorphic markers, merozoite surface antigen (MSA)-1 and MSA-2, were amplified to estimate the number of individual parasite populations coexisting the same patient [13]. A given isolate was considered to be monoclonal 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 (IC50 > 100 nM) [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 pfcr7 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 pfmdrl and cg2, and/or pfcr7 genes. We previously described the results on the characterization of pfmdrl 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 pfcr7 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 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 (IC50 > 100 nM); 53 (57%) of 93 were chloroquine sensitive (IC50 < 100 nM).

The pfcr7 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 pfcr7 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.

<table>
<thead>
<tr>
<th>Marker</th>
<th>n</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>n</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>pfc7f</td>
<td>59</td>
<td>37.5 (6.3-294)</td>
<td>&lt;.001</td>
<td>84</td>
<td>152 (17.7-336)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>pfc7f3</td>
<td>36</td>
<td>0.6 (0.02-9.91)</td>
<td>NS</td>
<td>96</td>
<td>2.96 (0.6-15.6)</td>
<td>NS</td>
</tr>
<tr>
<td>gc27</td>
<td>42</td>
<td>3.0 (0.7-13.3)</td>
<td>NS</td>
<td>44</td>
<td>17.0 (5.0-110.9)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>cg2e</td>
<td>46</td>
<td>12.8 (2.4-76.2)</td>
<td>&lt;.001</td>
<td>75</td>
<td>12.0 (3.6-42.4)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>cg2Z</td>
<td>33</td>
<td>1.2 (0.2-6.0)</td>
<td>NS</td>
<td>37</td>
<td>18.0 (2.7-146)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

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

* Results on pfmdrl and cg2 polymorphisms have been described elsewhere [7–9]. Genetic characterization was based on codon 76 of the pfcr7 gene, codon 86 (chloroquine sensitive, Asn-86; chloroquine resistant, Tyr-86) of the pfmdrl gene [5], and codon 275 (chloroquine sensitive, 11–13 repeat units; chloroquine resistant, 14 repeat units) and w (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, Gin- or His-275A/As- or Gin-281A/– or Cin-299, except for combination Gin-275A/As-281A/Gin-299, which represents the chloroquine-resistant allele) [8]. Sensitivity, specificity, and positive predictive value of the cg2e 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 pfcr7 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 IC₅₀ 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 IC₅₀ values (48–88 nM) for chloroquine. Two mutant isolates had relatively low IC₅₀ values (25 and 36 nM). Five of 9 mutant isolates were multiclonal. One monoclonal isolate with a wild-type pfcrt was characterized by a near borderline IC₅₀ (125 nM), but the patient had an adequate clinical response.

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 (IC₅₀ = 100 nM) 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 vitro 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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