ASSOCIATION OF PLASMODIUM FALCIPARUM CHLOROQUINE RESISTANCE TRANSPORTER VARIANT T76 WITH AGE-RELATED PLASMA CHLOROQUINE LEVELS

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Abstract. The mutation leading to the substitution of a threonine (T76) for a lysine at position 76 of the Plasmodium falciparum chloroquine resistance transporter (PfCRT) was genotyped in 100 Nigerian children with asymptomatic parasitemia. Isolates containing both pfcr1 variants were found to harbor higher numbers of parasite clones (P < 0.002). The prevalence of the pfcr1 T76 variant decreased with age (P < 0.0001) and increased with blood levels of chloroquine (CQ) (P < 0.0001). Whereas the K76 allele was more frequent in individuals without detectable plasma CQ levels (79.7%), only the pfcr1 T76 variant was observed in children with CQ levels > 150 nmol/L. In individuals without detectable plasma CQ, the proportion of those with pfcr1 T76 decreased from 60% in children < 2 years old to 23.5% in children ≥ 6 years old (P < 0.01). The association of actual blood levels of CQ and the occurrence of pfcr1 T76 underlines that the pfcr1 T76 variant is in fact the mediator of CQ resistance.

INTRODUCTION

One of the factors to be considered in the prophylaxis, treatment, and control of Plasmodium falciparum malaria is the resistance of parasite strains that may arise against virtually every drug available. Drug resistance against chloroquine (CQ), the most common drug used for treatment of malaria in many African countries, is of special interest. However, the molecular mechanisms involved in CQ resistance in P. falciparum infections are unclear. In particular, the reasons for the diminished accumulation of CQ in the erythrocytic parasite vacuole of CQ-resistant strains are not completely understood. A possible mechanism could be altered transport of CQ with a reduced influx into or an increased efflux out of the digestive vacuole.1, 2

Recent evidence suggests a crucial role for a point mutation in the P. falciparum chloroquine resistance transporter (pfcr1) gene on chromosome 7 in conferring CQ resistance.3 The mutation results in a substitution of threonine for lysine at position 76 (T76) of the digestive-vacuole transmembrane protein PfCRT. New data on variable haplotypes that all exhibit the pfcr1 T76 resistance genotype suggest that CQ resistance has arisen independently through multiple evolutionary-linked events.4-5 Although gene polymorphisms in the P. falciparum multidrug resistance (pfmdr) gene or other mutations in the pfcr1 gene may also be involved in the development of CQ resistance, pfcr1 T76 is likely the main determinant of CQ resistance.5, 7

Evidence that pfcr1 T76 is a key marker of CQ resistance has been demonstrated in several studies on the selection of this mutation after treatment with CQ8-11 and by varying in vivo and in vitro responses to CQ in different parasite isolates.12-14 Findings on the relationship between blood levels of CQ and the occurrence of pfcr1 76 variants have not been reported.

In the present study, we have analyzed the relationship between the occurrence of the CQ resistance pfcr1 T76 genotype and varying blood levels of CQ in different age groups of clinically healthy children with P. falciparum infections.

MATERIALS AND METHODS

Study group. The study was conducted in Abanla, a village in the rain forest zone of southwestern Nigeria. Malaria is holoendemic in this area.15 Two hundred twenty-eight children without overt signs of malaria were enrolled from a nursery and a primary school during the dry season between December 1996 and May 1997. The study group has been previously described.15 One hundred samples positive for P. falciparum were randomly selected and typed for the two variants at position 76 of the parasite pfcr1 gene. Informed consent was obtained from parents/guardians of children, and the study was reviewed and approved by the Joint Ethical Committee of the University of Ibadan/University College Hospital.

Laboratory examinations. Parasitemia was estimated by standard microscopy (100 oil-immersion fields) and categorized as < 1 parasite/field and ≥ 1 parasite/field. In microscopically negative samples, subpatent parasitemia was detected by a polymerase chain reaction (PCR) assay that differentiated P. falciparum, P. ovale, and P. malariae.16 As an estimate of multilocularity of infection, the minimal number of P. falciparum strains in each individual was determined by assessing the number of alleles of the genes encoding merozoite surface protein 1 (MSP-1) and MSP-2 families. The MSP-1 and MSP-2 alleles were defined by gene family-specific PCR assays and analysis of the resulting length polymorphisms of the PCR products.17 The segment of the pfcr1 gene containing the polymorphism at position 76 was amplified by the PCR. The PCR conditions (primer pfcr1.F: 5'-GACGAGCTTATAGGGAATTA-3' and pfcr1.R: 5'-ATAAAGTTGTAGTTAGGATG-3') were an initial denaturation, followed by 30 cycles at 94°C for 40 seconds, 54°C for 55 seconds, and 72°C for 55 seconds, and a final extension. Enzymatic digestion with Apo I of the resulting 420-base pair (bp) fragment identified parasite strains containing the T76 variant (two fragments: 348 bp and 72 bp) and/or the K76 variant (no fragments).

Blood levels of CQ of study participants were determined in duplicate samples by a modified high-performance liquid chromatography assay as previously described.18 The limit of detection was 17 nmol/L.

Statistical analysis. Statistical analyses were performed using the JMP 4 software (SAS Institute, Inc., Cary, NC). Contingency analyses (chi-square tests) and nonparametric analyses (Kruskal-Wallis tests) were performed. P values < 0.05 were considered statistically significant. Chi-square tests for
trend were used as a more sensitive statistical procedure than standard chi-square tests. This procedure was applied in $2 \times c$ tables when categories (c) of variables had a natural order.$^{19}$

RESULTS

Association of \textit{pfcrt} 76 variants with blood levels of CQ. Of the 100 samples typed for the two \textit{pfcrt} variants, 47% contained exclusively parasites with the wild type variant (K76; putatively CQ sensitive), 39% contained only parasites with the T76 substitution (putatively CQ resistant), and 14% exhibited parasite strains with both the wild type and the mutation. This distribution was clearly related to the blood levels of CQ ($\chi^2 = 34.3$, degrees of freedom [df] = 4, $P < 0.0001$; CQ detected in 31% of the study group) (Figure 1). The proportion of \textit{pfcrt} K76 strains decreased with increasing CQ levels: this variant was predominant in children without detectable blood levels of CQ (79.7%) and never found in children with CQ levels > 150 nmol/L. Mixed isolates (positive for \textit{pfcrt} K76 and T76) were detected in only one child with CQ in plasma (CQ = 26 nmol/L).

Variants of \textit{pfcrt} 76, age, and levels of CQ. Blood levels of CQ were lower in children in higher age groups ($P < 0.0001$, by Kruskal-Wallis test) (Figure 2a). Correspondingly, the proportion of \textit{pfcrt} K76 isolates was positively associated with the age of the individuals ($\chi^2 = 29.7$, df = 6, $P < 0.0001$) (Figure 2b). Isolates carrying both variants were rather observed in older children.

Variants of \textit{pfcrt} 76 and multiclonality of infections. Multiplicity of \textit{P. falciparum} infections was assessed by the number of different bands of the MSP genes in association with the \textit{pfcrt} variants found in the \textit{P. falciparum} isolates. As expected, mixed K76/T76 isolates exhibited higher numbers of alleles of the MSP-1 and MSP-2 families, indicating a higher clonal complexity of infection (median = 5 MSP bands) compared with isolates with either the K76 (median = 4 MSP bands) or the T76 (median = 3 MSP bands) \textit{pfcrt} variant ($P < 0.002$, by Kruskal-Wallis test).

Association of the \textit{pfcrt} variant T76 with age, parasitemia, and multiclonality in children without detectable levels of CQ. To determine whether age and clonality of infections were associated with the occurrence of \textit{pfcrt} T76 independently form actual CQ levels, children without detectable levels of CQ were analyzed ($n = 69$). In CQ-negative individuals, the frequency of \textit{pfcrt} T76 decreased from 60% in children < 2 years old to 23.5% in children ≥ 6 years ($\chi^2 = 7.3$, by chi-square test for trend, $P < 0.01$) (Table 1). The frequency of \textit{pfcrt} T76 was associated with the number of \textit{Plasmodium} spp. ($\chi^2 = 4.1$, by chi-square test for trend, $P < 0.05$) and the degree of parasitemia ($\chi^2 = 5.5$, by chi-square test for trend, $P < 0.02$). Children with subpatent infections carried only the \textit{pfcrt} wild type (Table 1).

\begin{figure}
\centering
\includegraphics{figure1}
\caption{Proportion of children with parasite strains exhibiting only the \textit{Plasmodium falciparum} chloroquine resistance transporter (\textit{pfcrt}) K76 variant (solid bars), only the \textit{pfcrt} T76 variant (open bars), and each of the variants (dotted bars) in relation to chloroquine levels ($P < 0.0001$); chloroquine level 0 nmol/l, $n = 69$; 1–150 nmol/l, $n = 24$; > 150 nmol/l, $n = 7$.}
\end{figure}

\begin{figure}
\centering
\includegraphics{figure2}
\caption{a, plasma chloroquine levels as a function of age ($P < 0.0001$). Individual values are shown as dots. b, Proportion of children with parasite strains exhibiting only the \textit{Plasmodium falciparum} chloroquine resistance transporter (\textit{pfcrt}) K76 variant (solid bars), only the \textit{pfcrt} T76 variant (open bars), and each of the variants (dotted bars) as a function of age ($P < 0.0001$).}
\end{figure}
TABLE 1
Distribution of the Plasmodium falciparum chloroquine resistance transporter 76 variants and influence of various factors in children without plasma chloroquine

<table>
<thead>
<tr>
<th>Number of children (n = 69)</th>
<th>T76 mutation (%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>5</td>
<td>60.0</td>
</tr>
<tr>
<td>2–3</td>
<td>26</td>
<td>57.7</td>
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<td>4–5</td>
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<td>23.8</td>
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<tr>
<td>≥6</td>
<td>17</td>
<td>23.5</td>
</tr>
<tr>
<td>Parasitemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subpatent</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>&lt;1 parasite/field</td>
<td>28</td>
<td>32.1</td>
</tr>
<tr>
<td>≥1 parasite/field</td>
<td>34</td>
<td>50.0</td>
</tr>
<tr>
<td>Number of Plasmodium spp.†</td>
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<td></td>
</tr>
<tr>
<td>Single infection</td>
<td>38</td>
<td>47.4</td>
</tr>
<tr>
<td>Double infection</td>
<td>19</td>
<td>36.8</td>
</tr>
<tr>
<td>Triple infection</td>
<td>12</td>
<td>16.7</td>
</tr>
</tbody>
</table>

* By chi-square test for trend
† P. falciparum, P. malariae, and P. ovale.

DISCUSSION

Our results show that the prevalence of the pfcrt T76 variant increases substantially with the CQ blood concentrations measured in isolates from asymptomatic P. falciparum-infected children. Correspondingly, the proportion of children harboring at least one clone with pfcrt K76 was highest in children without detectable levels of CQ. This observation supports the view that the pfcrt polymorphism at position 76 is in fact a significant factor of CQ resistance, as shown in previous studies from Cameroon, Mali, Mozambique, Nigeria, Sudan, Uganda, Madagascar, Laos, Papua New Guinea, and Thailand.

The proportion of children infected with parasite clones carrying the pfcrt T76 variant decreased significantly with age and correlated with the age-associated decrease in CQ levels. The higher levels of CQ in younger children might result from the fact that these children are more frequently given CQ when they are febrile during an assumed malaria attack. An age-dependent mode of CQ metabolism appears rather unlikely. Determinants of the occurrence of both pfcrt 76 variants were the blood concentration of CQ (negatively correlated), age (positively correlated), and the multiclonality of P. falciparum infection (positively correlated). All determinants were dependent on each other.

Other gene variants putatively mediating CQ resistance, especially pfmdr and cg2, have been extensively investigated. It has been found that the pfcrt T76 and the pfmdr1 Y86 alleles are closely associated in CQ-resistant strains. However, all studies comparing the associations of the pfmdr1 Y86 variant and the pfcrt T76 variant have shown that the impact of the pfcrt gene was stronger than that of the pfmdr1 gene. Although an association of a cg2 gene polymorphism with CQ resistance has been described, allelic modification experiments have excluded a significant role of this gene in CQ resistance. It has been suggested that the degree of CQ resistance is further modulated by factors linked to genes other than pfcrt or pfmdr, but the nature of such factors is still unclear.

Consistent with other studies, one can assume that the prevalence of pfcrt T76 is a function of the actual CQ level and, thus, of age, and possibly influenced by acquired immunity and natural resistance factors of the host. Furthermore, one can assume that CQ intake contributes essentially to the selection of the pfcrt T76 allele. Based on our observations, CQ appears to have an extended influence on the distribution of the pfcrt polymorphism in an isolate. Thus, subtherapeutic blood levels of CQ not only promote the emergence of drug resistance by direct selection, but also appear to influence the age-dependent clonal composition of a particular isolate for a longer period of time, even when blood levels of CQ are no longer detectable.

REFERENCES


