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**Plasmodium falciparum**: linkage disequilibrium between loci in chromosomes 7 and 5 and chloroquine selective pressure in Northern Nigeria

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**SUMMARY**

In view of the recent discovery (Molecular Cell 6, 861–871) of a (Lys76Thr) codon change in gene pfcr1 on chromosome 7 which determines in vitro chloroquine resistance in *Plasmodium falciparum*, we have re-examined samples taken before treatment in our study in Zaria, Northern Nigeria (Parasitology 119, 343–348). Drug resistance was present in 5/5 cases where the pfcr1 76Thr codon change was seen (100%, positive predictive value). Drug sensitivity was found in 26/28 cases where the change was absent (93%, negative predictive value). Allele pfcr1 76Thr showed strong linkage disequilibrium with pfmdr1 Tyr86 on chromosome 5, more complete than that between pfcr1 and cg2 alleles situated between recombination cross-over points on chromosome 7. Physical linkage of cg2 with pfcr1 may account for linkage disequilibrium between their alleles but in the case of genes pfmdr1 and pfcr1 on different chromosomes, it is likely that this is maintained epistatically through the selective pressure of chloroquine.

Key words: linkage disequilibrium, *Plasmodium falciparum*, chloroquine resistance, malaria, Nigeria.

**INTRODUCTION**

Resistance to chloroquine in *Plasmodium falciparum* developed in South East Asia and South America about 10 years after the introduction of the anti-malarial in the 1950s, and reached Africa by the late 1970s (Peters, 1998). In spite of its reduced efficacy, chloroquine is still the first-line anti-malarial drug in most of Africa for reasons of cost, and also because widespread partial immunity in symptomatic older children and adults enhances the effect of the drug (Sokhna et al. 1997; Djimde et al. 2001).

Nevertheless, resistance is having a major impact. Emergence of chloroquine resistance in Senegal, West Africa, over 12 years was associated with at least a 2-fold higher risk of death from malaria in children under 10 years old (Trape et al. 1998). In East Africa, Kenyan children under 5 admitted to hospital for malaria are reported to have a 33% case fatality rate if given chloroquine treatment in contrast to 11% for sulfadoxine-pyrimethamine, quinine or 5-day cotrimoxazole (Zucker et al. 1996).

In *P. falciparum*, weaker or stronger associations (Foote et al. 1990) are seen between chloroquine resistance and sequence changes in an MDR type protein, Pgh1, localized in the blood-stage parasite’s lysosomal membrane (Cowman et al. 1991), and specified by pfmdr1 on chromosome 5. However, the progeny of a genetic cross showed a link between chloroquine resistance and a locus on chromosome 7 (Wellem et al. 1990). Su et al. (1997) linked resistance to changes in the cg2 gene on this chromosome, which specifies a protein of unknown function (Wellem et al. 1998). Still the correlation with resistance failed to reach 100%, and transfection of mutated cg2 did not transfer it (Fidock et al. 2000a). A lysine to threonine (K to T) change in codon 76 of a new gene, pfcr1 (also on chromosome 7) specifying the lysosomal transmembrane protein PfCRT, gave a complete association with in vitro chloroquine resistance of *P. falciparum* isolates from Africa, South East Asia and South America (Fidock et al. 2000b). Wild-type PfCRT resembles a protein reported to facilitate the transport of organic cations (Zhang et al. 1997) and may normally be involved in the efflux of basic amino acids or short basic peptides (Eggleston, Duffin & Goldberg, 1999) from the lysosome.

Before the discovery of pfcr1, we (Adagu & Warhurst, 1999) examined polymorphisms associated with chloroquine resistance in pfmdr1 and cg2 on chromosomes 5 and 7 in samples taken before chloroquine treatment of a group of children in Zaria, Northern Nigeria. The Asn 86 Tyr codon change in pfmdr1, the Gly 281 Ala mutation and the Dd2-type κ repeat of cg2, were significantly associated, suggesting co-selection by the drug. Polymorphisms examined were highly predictive for drug resistance, but associations were incomplete.

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In order to complete our earlier study, we have re-examined our collection in Zaria for polymorphism at codon 76 of \textit{pfcrt}, determined the value of this polymorphism in prediction of drug resistance in this geographical location, its association with polymorphisms in \textit{cg2} on the same chromosome and with polymorphisms in \textit{pfmdr1} on chromosome 5. We find that \textit{pfcrt} Thr76 on chromosome 7 is highly predictive for chloroquine resistance, and in strong linkage disequilibrium with \textit{pfmdr1} Tyr86 on chromosome 5, more complete than the degree of \textit{pfcrt} 76 linkage with \textit{cg2} alleles situated between recombination cross-over points on the same chromosome. Physical linkage of \textit{cg2} with \textit{pfcrt} may account for linkage disequilibrium between their alleles but in the case of genes \textit{pfmdr1} and \textit{pfcrt}, on different chromosomes, it is likely that, in this geographical location, linkage is maintained epistatically through the selective pressure of chloroquine.

\textbf{Materials and Methods}

Parasite samples examined in this study were those previously characterized for chloroquine resistance associated sequence variations in \textit{pfmdr1} and \textit{cg2} genes (Adagu & Warhurst, 1999). The samples were from Zaria, an area of North Central Nigeria located in the Guinea Savannah belt. Malaria in this area is holoendemic and in 1993 when the samples were collected, 20\% of infections showed resistance to chloroquine, mainly at the RI level (Adagu et al. 1995). Symptomatic children were aged from 7 months to 11 years (mean 5.2 ± 3 years). Geometric mean parasitaemia on admission was 20629/\textmu m$^3$, ranging from 1000 to 55353. Samples F91, F130, F170 and F183 (3 sensitive and 1 resistant) examined in our previous study (Adagu & Warhurst, 1999) were no longer available. A total of 35 samples remained.

Professor C. Plowe kindly gave us the protocol for a nested PCR/RFLP (Djimde et al. 2001: GenBank accession number AF233068) for detection of the 76$^{\text{AAA}}$→$^{\text{ACA}}$ (76$Lys$→$Thi$) mutation in \textit{pfcrt}. Nest I primers, 5\'-CCG TTA ATA ATA AAT ACA CGC AG 3\' (forward) and 5\'-CGG ATG TTA CAA AAC TAT AGT TAC C 3\' (reverse) and the nest II primers, 5\'-TGT GCT CAT GTG TTT AAA CTT 3\' (forward) and 5\'-CAA AAC TAT AGT TAC CAA TTT TG 3\' (reverse) were used as described in the protocol. The PCR mix contained standard KCl buffer, 1.25 U \textit{Taq} polymerase (Bioline), dNTPs (200 \muM each), primers (1 \muM each) and for nest I reaction a sector of glass-fibre membrane DNA source or 1 \muL of nest I product for nest II reaction. Nest I reaction (94\°C, 30 s; 56\°C, 30 s and 60\°C, 1 min) was cycled 45 times with initial denaturation and final extension steps of 94\°C, 3 min and 60\°C, 3 min respectively. Nest II reaction (94\°C, 30 s; 48\°C, 30 s and 65\°C, 3 min) was cycled 30 times with an initial denaturation and final extension steps of 94\°C, 5 min and 65\°C, 3 min respectively. Nest II product was restricted at 50\°C with 0.5 U of \textit{Apol} restriction enzyme following the manufacturer’s protocol and the resulting digest was resolved in a 2\% agarose gel.

\textbf{Analysis of results}

The diagnostic parameters of sensitivity, specificity, and positive and negative predictive value were calculated as follows.

\textbf{Sensitivity}. Percentage of resistant outcomes correctly predicted by the positive test result. \((\text{TP}/\text{TP} + \text{FN}) \times 100\), (where \text{TP} = True Positives; \text{FN} = False Negatives; \text{TN} = True Negatives; \text{FP} = False positives).

\textbf{Specificity}. Percentage of sensitive outcomes correctly predicted by the negative test result \((\text{TN}/\text{TN} + \text{FP}) \times 100\).

\textbf{Positive predictive value}. Percentage of positive tests correctly predicting a resistant outcome. \((\text{TP}/\text{TP} + \text{FP}) \times 100\).

\textbf{Negative predictive value}. Percentage of negative tests correctly predicting a sensitive outcome. \((\text{TN}/\text{TN} + \text{FN}) \times 100\).

Linkage disequilibrium values were calculated by a pairwise analysis of the loci studied using the method described by Maynard Smith (1989) for \(D'\), and that of Hill & Robertson (1968) for \(r^2\). Both \(D'\) and \(r^2\) have values of −1 to +1, and the closer they approach to −1 or 1, the greater the linkage disequilibrium between loci. Prevalences were used to estimate frequencies by assuming that the presence of a single allele in a sample is indicative of infection with a single clone. Thus mixed alleles were excluded from the analysis. Significance of associations was estimated using 2 by 2 tables for \(\chi^2\) from the EpiInfo 6 StatCalc Program.

Associations between gene polymorphisms and chloroquine resistance were also determined by analysis of 2 × 2 tables using StatCalc. It was not possible to calculate all diagnostic parameters for pre-treatment data from the paper of Djimde \textit{et al.} (2001) on the basis of information supplied for the whole population. However, data in Table 2 and in other parts of the text in their publication were usable. Assuming a group of 200 patients, it can be deduced that treatment would fail in 29 (14.5\%) and succeed in 171 (85.5\%). A diagnostic comparison table can then be drawn as follows.

The calculated sensitivity of 91.8\% is reported as 92\% in their table, validating the calculation.
Table 1. Diagnostic table calculated from Djimde et al. (2001)

<table>
<thead>
<tr>
<th></th>
<th>T76 mutant</th>
<th>K76 wild type</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistant</td>
<td>26726</td>
<td>2378</td>
<td>29</td>
</tr>
<tr>
<td>Sensitive</td>
<td>63954</td>
<td>107046</td>
<td>171</td>
</tr>
<tr>
<td>Total</td>
<td>90576</td>
<td>109424</td>
<td>200</td>
</tr>
</tbody>
</table>

% Sensitivity 91.8
% Specificity 62.6
% PPV 29.4
% NPV 97.8

RESULTS

The chloroquine susceptibility and the pfmdr1/cg2 profiles of the parasites have been reported elsewhere (Adagu et al. 1995; Adagu & Warhurst, 1999). Table 2 shows the 2×2 tables and analyses of the association between resistance and polymorphisms. Two samples from patients with ‘resistant’ infections are of particular concern, because they showed neither the mutant pfcr nor the mutant pfmdr1. One sample (F142) was from an infection found resistant in vitro. In the view of the growing body of evidence unequivocally linking mutant pfcr with in vitro chloroquine resistance, it is unlikely that this is a correct assignment. However, Djimde et al. (2001) have reported that parasites from 8% of failed treatments in their study in Mali did not reveal the pfcr mutation in the pre-treatment sample, although it appeared in all the recurring infections examined. We have no valid reason to exclude this infection from our calculations. The other (F211) recurrent on day 28 and could have been a re-infection. As reported by Adagu & Warhurst (1999), lack of post-treatment samples in our study did not permit PCR analysis which could have indicated cases where recurrences were re-infections. As argued above, we have also included this result in the calculations. All 28 samples from chloroquine-sensitive infections carried wild-type pfcr codon 76 (76AA).

Table 3 shows the measures of linkage disequilibrium in paired alleles of pfcr, cg2 and pfmdr1. Alleles of pfcr and pfmdr1, on different chromosomes, show the highest degree of linkage disequilibrium (D' = 1 [0.99–1.01], r² = 0.81 [0.68–0.94] P = 0.00002). This reflects the fact that in all cases where mutant pfcr was seen, there was also mutant pfmdr1, although in 1 (chloroquine-sensitive) sample, mutant pfmdr1 was found without mutant pfcr. Disequilibrium between cg2 281 and pfcr76, both on chromosome 7, had a significantly lower value for the more stringent r² parameter than was seen between pfmdr1 86 and pfcr 76 on different chromosomes. Surprisingly, both linkage measures between cg2 281 and cg2 κ, on the same gene, were significantly lower than those between pfmdr1 86 and pfcr 76.

DISCUSSION

The presence of pfcr 76Thr predicted chloroquine resistance in our study (100% positive predictive value). Specificity and positive predictive value using either of 2 loci on cg2 were significantly inferior to those obtained using pfcr 76 or pfmdr1 86. In their treatment trial in endemic malaria in Mali, Djimde et al. (2001) examined the association between chloroquine resistance and mutations in pfcr and pfmdr1. Sensitivity of the test was 92% and specificity was 63%. In the Mali study, the percentage of mutant pfcr test results predicting treatment failures (positive predictive value) is estimated by us as 29.4%, whilst 98% of non-mutant test results predicted successful treatments (negative predictive value is 98%). When pfcr 76Thr and pfmdr1 86Tyr were considered together, specificity increased to 78%, but sensitivity fell from 92% to 73%. Positive predictive value improved appreciably when patients under 10 years of age were considered separately.

Recent evidence suggests that changes in pfmdr1 taking place on a background of another determinant, presumably a change in pfcr, are important for higher levels of chloroquine resistance. Transfection of pfmdr1 mutants would only enhance
chloroquine resistance in clones now known to carry mutant pfcr (Reed et al. 2000). Unfortunately, this possibility has not been tested directly for codon 86 allelic forms of pfmdr1. We have shown here that linkage disequilibrium between cg2 alleles and pfcr, both on chromosome 7, and even for cg2 281A and the cg2 κ repeat size polymorphism on the same gene, is less marked in these Northern Nigerian samples than for pfcr with pfmdr1, on different chromosomes. Duraisingh et al. (2000) reported linkage disequilibrium in Gambian samples between the cg2 κ repeat size polymorphism and pfmdr1 86 (D’ = 0.87: r2 = 0.27), strikingly this was higher than the linkage between pfmdr1 codons 86 and 184, separated by only 296 base pairs. Duraisingh et al. (2000) concluded that the linkage disequilibrium between the alleles of genes pfmdr1 and cg2 indicated that these or closely related loci are important determinants of chloroquine resistance, their linkage being maintained epistatically through selection by chloroquine. Adagu & Warhurst (1999) had also reported linkage between pfmdr1 86 and cg2 281 and κ polymorphisms, with the same conclusion. However, our additional linkage analysis includes both cg2 and pfcr, which are located approximately 10K base pairs apart (0·6 centimorgans: Su et al. 1999) on chromosome 7, in a 36 K base-pair sequence between recombination cross-over points mapped in the Hb3×Dd2 genetic cross (Su et al. 1997). This location suggests that any linkage disequilibrium between their alleles is related to physical linkage and not to epistatic factors. It is highly likely that the linkage disequilibrium we demonstrated earlier between pfmdr1 and cg2 depends on the physical linkage of pfcr with cg2 and that the stronger linkage we have now shown between pfcr and pfmdr1 on chromosome 5 is maintained epistatically by chloroquine. Selection for pfmdr1 86Tyr by chloroquine and amodiaquine was shown in a treatment trial (Duraisingh et al. 1997). The recent study by Djimde et al. (2001) confirmed selection of both pfmdr1 86Tyr and pfcr 76Thr by chloroquine treatment.

It must be emphasized that an association between pfmdr1 and chloroquine resistance has not been confirmed in all studies especially those from other geographical areas (for example Sudan–Awad El Kariem, Miles & Warhurst, 1992: Thailand–Wilson et al. 1993). Currently it appears that West and Central Africa (Adagu et al. 1996; Adagu & Warhurst, 1999; Basco et al. 1995; Djimde et al. 2001; Duraisingh et al. 1997, 2000; Grobusch et al. 1998) are the main areas where an association (not absolute and presumably depending on the presence of mutated pfcr) between pfmdr1 codon changes and chloroquine resistance can reliably be demonstrated. In Brazil too, where chloroquine resistance has been present about 20 years longer than in Africa, pfmdr1 with altered codons is predominant (Povoa et al. 1999) and mutated pfcr has been demonstrated in in vitro resistant isolates (Fidock et al. 2000b).

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| Table 3. Association and linkage disequilibrium between paired alleles of pfcr and cg2 on chromosome 7, and pfmdr1 on chromosome 5 |
|---|---|---|---|---|
| Paired alleles | pfcr 76T | pfcr 76K | D’ (95 % limits) | r2 (95 % limits) | P | n |
| pfdm1 86Y | 5 | 1 | 1 (0·99–1·01) | 0·81 (0·68–0·94) | 0·00002 | 35 |
| pfdm1 86N | 0 | 29 | | | | |
| cg2 281A | 5 | 6 | 1 (0·99–1·01) | 0·36 (0·20–0·52) | 0·001 | 35 |
| cg2 281G | 0 | 24 | | | | |
| cg2 κ Dd2 | 5 | 3 | 1 (0·99–1·01) | 0·55 (0·37–0·73) | 0·0004 | 30 |
| cg2 κ Hb3 | 0 | 22 | | | | |
| Paired alleles | pfcr 86Y | pfcr 86N | D’ (95 % limits) | r2 (95 % limits) | P | n |
| cg2 281A | 6 | 5 | 1 (0·99–1·01) | 0·45 (0·29–0·62) | 0·0003 | 35 |
| cg2 281G | 0 | 24 | | | | |
| cg2 κ Dd2 | 5 | 3 | 0·77 (0·62–0·92) | 0·41 (0·23–0·59) | 0·002 | 30 |
| cg2 κ Hb3 | 1 | 21 | | | | |
| Paired alleles | cg2 281A | cg2 281G | D’ (95 % limits) | r2 (95 % limits) | P | n |
| cg2 κ Dd2 | 7 | 1 | 0·50 (0·32–0·68) | 0·41 (0·23–0·59) | 0·001 | 30 |
| cg2 κ Hb3 | 4 | 18 | | | | |

REFERENCES


