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 *pfcrt* 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 *pfcrt* 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 *pfcrt* 76Thr showed strong linkage disequilibrium with *pfmdr1* Tyr86 on chromosome 5, more complete than that between *pfcrt* and *cg2* alleles situated between recombination cross-over points on chromosome 7. Physical linkage of *cg2* with *pfcrt* may account for linkage disequilibrium between their alleles but in the case of genes *pfmdr1* and *pfcrt*, 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 co-trimoxazole (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 (Wellems *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 (Wellems *et al*. 1998). Still the correlation with resistance failed to reach 100%, and transfection of mutated *cg2* did not transfer it (Fidock *et al*. 2000*a*). A lysine to threonine (K to T) change in codon 76 of a new gene, *pfcrt* (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*. 2000*b*). 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 (Eggleson, Duffin & Goldberg, 1999) from the lysosome.

Before the discovery of *pfcrt*, 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 *pfcrt*, determined the value of this polymorphism in prediction of drug resistance in this geographical location, its association with polymorphisms in *cg2* on the same chromosome and with polymorphisms in *pfmdr1* on chromosome 5. We find that *pfcrt* Thr76 on chromosome 7 is highly predictive for chloroquine resistance, and in strong linkage disequilibrium with *pfmdr1* Tyr86 on chromosome 5, more complete than the degree of *pfcrt 76* linkage with *cg2* alleles situated between recombination cross-over points on the same chromosome. Physical linkage of *cg2* with *pfcrt* may account for linkage disequilibrium between their alleles but in the case of genes *pfmdr1* and *pfcrt*, on different chromosomes, it is likely that, in this geographical location, linkage is maintained epistatically through the selective pressure of chloroquine.

MATERIALS AND METHODS

Parasite samples examined in this study were those previously characterized for chloroquine resistance associated sequence variations in *pfmdr1* and *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 $5{\cdot}2 \pm 3$ years). Geometric mean parasitaemia on admission was 20629/mm^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 $76^{AAA \rightarrow ACA}$ ($76^{Lys \rightarrow Thr}$) mutation in *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 *Taq* polymerase (Bioline), dNTPs (200 μM each), primers (1 μM each) and for nest I reaction a sector of glass-fibre membrane DNA source or 1 μl 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 *Apo1* restriction enzyme following the manufacture's protocol and the resulting digest was resolved in a 2% agarose gel.

Analysis of results

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

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

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

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

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

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 χ^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 *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)

MALI	T76 mutant	K76 wild type	Total
Resistant	26·622	2·378	29
	True positives	False negatives	
Sensitive	63·954	107·046	171
	False positives	True negatives	
Total	90·576	109·424	200
% 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 *pfcrt* nor the mutant *pfmdr1*. One sample (F142) was from an infection found resistant *in vitro*. In view of the growing body of evidence unequivocally linking mutant *pfcrt* 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 *pfcrt* 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) recurred 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 reinfections. As argued above, we have also included this result in the calculations. All 28 samples from chloroquine-sensitive infections carried wild-type *pfcrt* codon 76 (76^{AAA}).

Table 3 shows the measures of linkage disequilibrium in paired alleles of *pfcrt, cg2* and *pfmdr1*. Alleles of *pfcrt* and *pfmdr1,* on different chromosomes, show the highest degree of linkage disequilibrium ($D' = 1$ [0·99–1·01], $r^2 = 0{\cdot}81$ [0·68–0·94] $P = 0{\cdot}00002$). This reflects the fact that in all cases where mutant *pfcrt* was seen, there was also mutant *pfmdr1*, although in 1 (chloroquine-sensitive) sample, mutant *pfmdr1* was found without mutant *pfcrt*. Disequilibrium between *cg2* 281 and *pfcrt*76, both on chromosome 7, had a significantly lower value for the more stringent r^2 parameter than was seen between *pfmdr1* 86 and *pfcrt* 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 *pfcrt* 76.

DISCUSSION

The presence of *pfcrt* 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 *pfcrt* 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 *pfcrt* and *pfmdr1*. Sensitivity of the test was 92% and specificity was 63%. In the Mali study, the percentage of mutant *pfcrt* 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 *pfcrt* 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 *pfcrt*, are important for higher levels of chloroquine resistance. Transfection of *pfmdr1* mutants would only enhance

Table 2. Utility of the alleles studied for predicting chloroquine resistance

Resistance and alleles	2×2 Table RES	SENS	*P*	Sensitivity (%) (95% limits)	Specificity (%) (95% limits)	PPV (%) (95% limits)	NPV (%) (95% limits)	PPV + NPV/2
pfcrt 76T	5	0	0·00007	71 (38–105)	100 (99–101)	100 (98–102)	93 (84–103)	96·5 (90–103)
pfcrt 76K	2	28						
Cg2 281A	5	6	0·02	71 (38–105)	79 (63–94)	46 (9–82)	92 (81–102)	69 (47–91)
Cg2 281G	2	22						
Cg2 κDd2	6	7	0·006	86 (60–116)	75 (60–91)	46 (9–83)	96 (77–88)	71 (49–93)
Cg2 κHb3	1	21						
pfmdr1 86Y	5	1	0·0004	71 (38–105)	96 (90–103)	83 (56–111)	93 (84–103)	88 (73–104)
pfmdr1 86N	2	27						

Table 3. Association and linkage disequilibrium between paired alleles of *pfcrt* and *cg2* on chromosome 7, and *pfmdr1* on chromosome 5

			D′ (95% limits)	r^2 (95% limits)	*P*	*n*
Paired alleles	*pfcrt* 76T	*pfcrt* 76K				
pfmdr1 86Y	5	1	1 (0·99–1·01)	0·81 (0·68–0·94)	0·00002	35
pfmdr1 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	*pfmdr1* 86Y	*pfmdr1* 86N				
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				
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				

chloroquine resistance in clones now known to carry mutant *pfcrt* (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 *pfcrt*, both on chromosome 7, and even for *cg2* 281 and the *cg2* κ repeat size polymorphism on the same gene, is less marked in these Northern Nigerian samples than for *pfcrt* with *pfmdr1*, on different chromosomes. Duraisingh *et al*. (2000) reported linkage disequilibrium in Gambian samples between the *cg2* ω repeat size polymorphism and *pfmdr1* 86 ($D' = 0{\cdot}87$: $r^2 = 0{\cdot}27$), 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 *pfcrt*, 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 *pfcrt* with *cg2* and that the stronger linkage we have now shown between *pfcrt* 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 *pfcrt* 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 *pfcrt*) 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 *pfcrt* has been demonstrated in *in vitro* resistant isolates (Fidock *et al*. 2000*b*).

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