Mutations in *Plasmodium falciparum* Chloroquine Resistance Transporter and Multidrug Resistance Genes, and Treatment Outcomes in Ghanaian Children with Uncomplicated Malaria

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Summary

The association between the clinical outcome of chloroquine treatment and mutations in the putative *Plasmodium falciparum* chloroquine resistance transporter (*Pf*crt) gene at codon 76 and multidrug resistance gene 1 (*Pf* mdr1) at codon 86 were investigated among 406 children with uncomplicated malaria presenting at five sentinel health centres in Ghana. Presence of mutations in isolates taken at pre-treatment and on day of recurrence of parasites was detected using PCR followed by RFLP techniques. The prevalence of *Pf*crt T76 mutants was 80% at Hohoe, 46% at Navrongo, 98% at Tarkwa, 61% at Sunyani and 46% at Yendi. The prevalence of the mutant *Pf*mdr1 at Hohoe, Navrongo, Tarkwa, Sunyani and Yendi were 78, 58, 95, 53 and 42%, respectively. Significant association between the *Pf*crt mutation and treatment outcome was observed at Hohoe and Sunyani ($p < 0.05$), but not at Navrongo, Tarkwa or Yendi ($p > 0.05$). Similarly, a statistical significant association between *Pf*mdr1 86 and treatment failures was observed at Hohoe and Sunyani ($p < 0.05$) but not at the other three sites. A positive correlation was found between mutant *Pf*crt prevalence only and treatment failures with a Spearman's ρ-value of 0.872 and a p-value = 0.027. All parasite isolates from samples taken at recrudescence from patients with chloroquine treatment failures were found to have both *Pf*crt and *Pf*mdr mutations.

Key words: chloroquine, resistance, genetic markers, *Pf*crt, *Pf*mdr, mutations.

Introduction

The problem of chloroquine resistance in *Plasmodium falciparum* infections has complicated the management of malaria in countries where chloroquine is the first line antimalarial drug. The effect is felt mostly in the malaria endemic countries in sub-Saharan Africa where death due to malaria is reported to be about 3 million annually with the majority being children, aged below 5 years [1].

Chloroquine resistance in Ghana was first reported in 1987 [2]. Since then there have been reports indicating a systematic increase in reduced susceptibility of the parasite to the drug [3, 4]. The problem of antimalarial drug resistance calls for a prompt search for safe, good quality, affordable and acceptable newer antimalarial drugs to replace chloroquine as the drug of choice. It therefore became imperative for the assessment of existing levels of resistance by *P. falciparum* to chloroquine in the country in order to provide field-base evidence to the National

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Malaria Control Program in their efforts to formulate a new malaria treatment policy in Ghana.

Mutations in two genes, the *P. falciparum* chloroquine resistance transporter gene (*Pf*crt) at codon 76 which results in an amino acid change of lysine (K76) to threonine (T76) and at codon 86 of the *P. falciparum* multidrug resistance gene (*Pf*mdr1), that changes asparagine (N86) to tyrosine (Y86) are reported to be associated with chloroquine resistance [5–8]. These mutations have since been used as molecular markers of chloroquine resistance in *P. falciparum* populations [10–13] and found to be an effective epidemiological tool for mapping antimalarial drug resistance [7]. The presence of these mutations can be detected using simple molecular methods namely PCR/RFLP that are highly sensitive and specific [14].

This study investigated the presence of the *Pf*crt 76 and *Pf*mdr1 86 mutations in malaria patients at five sentinel sites namely Hohoe, Navrogo, Tarkwa, Yendi and Sunyani in Ghana using PCR/RFLP. The prevalence of these genetic markers and their association with clinical outcome of chloroquine treatment were established. Findings from this study, among others, became the basis for the replacement of chloroquine with a combination of amodiaquine artesunate as the first line antimalarial drug for the treatment of uncomplicated malaria in Ghana.

Materials and Methods

Study sites

Five sentinel sites in Ghana; Hohoe, Navrongo, Tarkwa, Sunyani and Yendi were selected for this study. The selection of these sites was based on ecological factors and degree of urbanization. Hohoe (7°9'N, 0°28'E) lies in the middle belt of the country with semi-deciduous forest vegetation. It is an urban community and malaria is hyperendemic. Malaria transmission in this area is perennial with peaks occurring after the major rains during June-October. Navrongo (10°54^{I}N, 1°6^{I}W) is located in the northern part of the Guinea savannah belt. Except for the town centre, which is urban, most parts are rural in character. Malaria is also hyperendemic and transmission is intense, and highly seasonal from June to November. Tarkwa (5°18'N, 1°59'W) is a gold mining town located in the forest zone and is considered an urban setting with easy access to antimalarial drugs. The mining activities have resulted in numerous open trenches containing stagnant water, which serve as breeding grounds for mosquitoes throughout the year. Malaria transmission therefore is perennial with a slight increase during the main rainy seasons in April–November. Sunyani (7°20^{I}N, 2°20^{I}W) lies in the middle belt of the country with forest vegetation. It is an urban community and malaria is hyperendemic. Malaria transmission in this area is perennial with peaks occurring after the major rains in June–October. Yendi (9°26^{I}N, 0°1^{I}W) is located in the northern half of the country in the Guinea savannah belt. It is a rural community and malaria is also hyperendemic. Transmission is intense and highly seasonal occurring mostly between June and November.

Study population and design

Children aged 5 years and below, reporting with uncomplicated malaria to the health centres were recruited to participate in the study. All patients with the symptoms of malaria were screened for inclusion in the study. Informed consent was obtained from parents or guardians of the children both orally (in the local language) or written (in English) where appropriate. Inclusion and exclusion criteria were in line with the WHO (1996) protocol [15]. Briefly, the inclusion criteria are; presence of or history of fever with temperatures of $\geq$37.5°C within the previous 48 h in the presence of parasitaemia of 2000–100 000 μl^{-1} of blood. Blood film from each patient was prepared before treatment (day 0) and then again on days 1, 3, 7 and 14 post-treatment for estimation of parasitaemia. Filter paper blood blots were collected at each time of blood film preparation, dried and stored individually in plastic bags at room temperature for molecular analysis.

Chloroquine was administered at a total of 25 mg kg^{-1} body weight over 3 days (10 mg/10 mg/5 mg). Patients were seen on fixed days up to 14 days post-treatment. Children who had parasitaemia above 25% of pre-treatment level on the third day were considered as having failed treatment and received an alternative treatment of Pyrimethamine-sulphadoxine (Fansidar) at 25 mg kg^{-1} as a single dose.

Parasites were considered to be sensitive to chloroquine if there was initial clearance and no parasitaemia observed on subsequent days up to day 14. Patients were classified as RI if there was initial clearance of parasites but parasitaemia recurred by day 14. Those who had persistent parasitaemia but at <25% of the initial level by day 3 were classified as RII whilst those who had persistent parasitaemia with no reduction in the level of parasitaemia or with a reduction to at least 25% of the initial level by day 3 were classified as RIII type of resistance.

Molecular analysis

DNA extraction. Parasite DNA was extracted from the filter paper blood blots using the methanol fixation method [14]. Briefly 3 mm^2 of the blood blot filter paper was incised into a 0.5 ml microfuge tube, followed by the addition of 50 µl of methanol. The tube was incubated for 15 min at room

temperature, after which the methanol was discarded. Doubled distilled water, 50 µl, was added and the tube heated for 15 min at 95°C with occasional vortexing. The extract after centrifugation, rich in parasite's DNA, was stored at −20°C until used.

*Detection of Pf*crt T76 *and Pf*mdr1 Y86 *alleles*

The detection of *Pf*crt T76 and *Pf*mdr1 Y86 alleles was done using previously described method [14]. This involved nested mutation specific PCR followed by endonuclease restriction. The initial amplification reaction involves the use of the oligonucleotide primer pair, CRTP1 (5′-CCG TTA ATA ATA AAT ACA CGC AG-3′) and CRTP2 (5′-CGG ATG TTA CAA AAC TAT AGT TAC C-3′) to amplify a 537 bp region around the *Pf*crt 76 mutation. A second round of amplification was performed using primers CRTD1 (5′-TGT GCT CAT GTG TTT AAA CTT-3′) and CRTD2 (5′-CAA AAC TAT AGT TAC CAA TTT TG-3′) to obtain a 134 bp fragment for subsequent restriction with *Apo I*. The restriction was carried out using 5 µl of the amplicon and 1U of the enzyme in a final volume of 15 µl, and incubation at 50°C for 6 h.

The detection of *Pf*mdr1 86 alleles also followed the method previously described [14]. Primary amplification of a 500 bp fragment around the mutation at codon 86 was performed using the oligonucleotide primer pair, MDR1 (5′-GCG CGC GTT GAA CAA AAA GAG TAC CGC TG-3′) and MDR2 (5′-GGG CCC TCG TAC CAA TTC CTG AAC TCA C-3′). Nested amplification reaction used primer pair MDR3 (5′-TTT ACC GTT TAA ATG TTT ACC TGC-3′) and MDR4 (5′-CCA TCT TGA TAA AAA ACA CTT CTT-3′), which amplified a 300 bp fragment for further restriction with *Afl III*. The restriction analysis was carried out using 5 µl of the amplicon and 1U of the enzyme in a final volume of 15 µl and incubated at 37°C for 6 h. The sizes of PCR products were determined by comparison with a 100 bp molecular weight marker (Roche, UK) upon 2% agarose gel electrophoresis.

Recrudescent malaria was distinguished from re-infection using previously described methods [16].

Ethical clearance for this study was obtained from the Ethical Committee of the Noguchi Memorial Institute for Medical Research (NMIMR).

Data analysis

The demographic data and the linear regression analyses were carried out using SPSS version 11 (SPSS Inc., USA). The score (i.e. either mutant or wild-type) of each sample was used to generate the prevalence of alleles for each codon. The 2×2 chi-square test was used to determine the association between the prevalence of genetic mutations and treatment failure and the Spearman's rho used to test the correlation between the two parameters.

Results

Overall 406 patients were recruited to participate in the study; 130 from Hohoe, 105 from Navrongo, 42 from Tarkwa, 62 from Sunyani and 67 from Yendi. The mean ages (in months) of the patients were 27.5 (±14.8), 28.7 (±15.0), 30.3 (±14.9), 24.7 (±13.2) and 28.7 (±15.9), whilst the geometric mean initial parasitaemia were 15 348, 22 089, 19 337, 24 596 and 34 957 for Hohoe, Navrongo, Tarkwa, Sunyani and Yendi, respectively. Cases of treatment failures were 81/130, 38/105, 23/42, 25/62 and 26/67 for Hohoe, Navrongo, Tarkwa, Sunyani and Yendi, respectively. Out of these treatment failures RI, RII and RIII types of resistance were 52, 31 and 17% at Hohoe, 45, 32 and 23% at Navrongo, 52, 22 and 26% at Tarkwa, 52, 24 and 24% at Sunyani and 35, 19 and 46% at Yendi.

The baseline prevalence of the *Pf*crt T76 from parasites was 80% (104/130) at Hohoe, 46% (48/105) at Navrongo, 98% (41/42) at Tarkwa, 61% (38/62) at Sunyani and 46% (31/67) at Yendi. The distribution of *Pf*crt T76 alleles from the five sites are shown in Table 1. Parasites from post-treatment samples all revealed the presence of the *Pf*crt T76 mutants at all the sites. *Pf*crt T76 was also associated with chloroquine treatment failure at Hohoe

TABLE 1
Distributions of Pfcrt and Pfmdr1 alleles (in percentages) at the five sentinel sites

Alleles	Study sites				
	Hohoe ($n=130$)	Navrongo ($n=105$)	Tarkwa ($n=42$)	Sunyani ($n=62$)	Yendi ($n=67$)
K76	20	54	3	38	54
T76	45	40	83	60	45
K76T	35	6	14	2	1
N86	22	42	5	47	58
Y86	60	53	62	39	30
N86Y	18	5	14	14	12
T76 + Y86	68	35	95	48	28

(OR = 2.69, $p = 0.02$) and Sunyani (OR = 3.00, $p = 0.05$) contrasting with lack of association at Navrongo (OR = 1.11, $p = 0.79$), Tarkwa (OR = 1.28, $p = 0.86$) and Yendi (OR = 1.65, $p = 0.32$).

The prevalence of *Pf*mdr1 Y86 mutants was 78% at Hohoe, 58% at Navrongo, 95% at Tarkwa, 53% at Sunyani and 42% at Yendi. Significant association between the *Pf*mdr1 Y86 prevalence and levels of treatment failures was observed at Hohoe (OR = 2.92, $p = 0.01$) and Sunyani (OR = 2.79, $p = 0.05$), but not at Navrongo, Tarkwa and Yendi ($p > 0.05$ in all cases).

The prevalence of the double mutations among parasite populations was 68, 35, 95, 48 and 28% at Hohoe, Navrongo, Tarkwa, Sunyani and Yendi, respectively. There was, however, a lack of association between treatment outcome and the presence of both mutations at Navrongo, Tarkwa and at Yendi ($p > 0.05$ in all cases) whilst it was strong at Hohoe (OR = 2.73, $p = 0.008$) and Sunyani (OR = 2.92, $p = 0.04$).

There was a statistically significant positive correlation between the prevalence the *Pf*crt mutation and percent treatment failures (Spearman's $\rho = 0.872$, $p = 0.027$) (Fig. 1) but not for the *Pf*mdr1 mutation (Spearman's $\rho = 0.6$, $p = 0.142$).

Discussion

This study found high frequencies of the *Pf*crt mutant gene at Hohoe, Sunyani and Tarkwa, which reflected, the high rates of *in vivo* chloroquine treatment failures. The likely reason for this observation is that easier access to the drug in these urban areas with the attendant higher drug pressure had led to the selection of resistant parasites strains. Moreover, significant association was found between treatment outcome and the prevalence of *Pf*crt T76 at Hohoe and Sunyani. The observed associations at these sites were similar to those of other studies carried out in Mali, Mauritania, Sudan and Cameroon, where the high prevalence of *Pf*crt mutants was associated with treatment failures [7, 10–12].

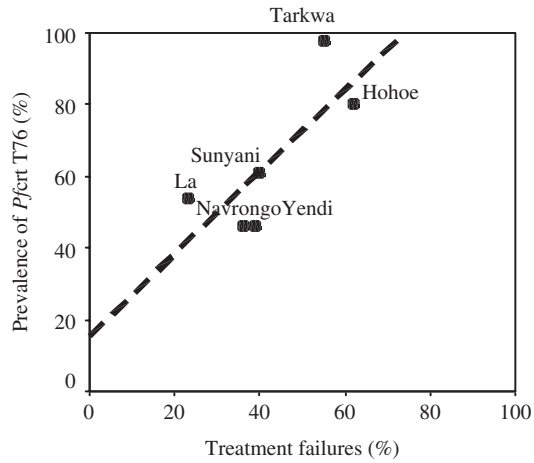

FIG. 1. Relationship between *Pf*crt T76 mutant prevalence and treatment failure levels at the five study sites. The Spearman's rho correlation coefficient was 0.872 ($p < 0.05$).

At Navrongo and Yendi, which are more rural, a different scenario of no association between the mutation and clinical outcome was observed. However, parasite mutant alleles were present in all patients with treatment failures. Also at Tarkwa, where very high prevalence of *Pf*crt T76 (98%) was recorded no association was found between the prevalence of mutant alleles and chloroquine treatment failure. Similarly, as found in these studies, all the parasites isolated from patients at post-treatment had the *Pf*crt T76, which lends support to the fact that this mutation is essential in the evolution of chloroquine resistance. It also suggests that it is likely to be the main mechanism in chloroquine resistance in Ghana. However, 79/406 patients who were carrying mixed population responded satisfactorily to chloroquine. The probable reason for this is that the hosts' immune status as well as the additive effect of chloroquine treatment could have accounted for the clearance of the mutant parasites.

Another observation made in this study was that in few cases the *Pf*crt T76 mutant was not detected at pre-treatment but was found in their post-treatment samples. This could be due to resistant parasites existing in very low numbers below detectable limit by PCR during pre-treatment but then dominated after the susceptible strains had been cleared. The positive association between the *Pf*mdr1 Y86 and chloroquine treatment outcome in Hohoe and Sunyani supports other reported findings [10] whilst the contra-observation in Navrongo, Yendi and Tarkwa has also been reported in Mali and Cameroon [7, 13]. It is therefore not surprising that no association exist between the presence of both mutations, *Pf*crt T76 and *Pf*mdr1 Y86, and treatment outcome in Navrongo, Yendi and Tarkwa.

The presence of both mutations of the *Pf*crt 76 and *Pf*mdr1 86 were found to be strongly associated with treatment failure in Hohoe and Sunyani. Since *Pf*mdr1 and *Pf*crt are on different chromosomes, their selection could not be attributed to physical linkage. Rather, it could be that *Pf*mdr1 confers some advantage to the parasite in the presence of chloroquine by augmenting the level of resistance due to *Pf*crt mutation (additive effect). The present study has also shown that the *Pf*crt prevalence could be useful in predicting the level of chloroquine resistance in Ghana, evidenced by the observed statistically significant positive correlation.

Moreover, the observation that, all the treatment failures harboured *Pf*crt T76 parasites seems to implicate this mutation as the major mechanism involve in chloroquine resistance in the country. It must, however, be pointed out that new approaches for understanding the relationship between mutations and antimalarial drug resistance have been suggested [17].

Data presented here form the baseline for molecular markers profile for Ghana and was partly used to support the decision by the Ghana Malarial Control Program to replace chloroquine with a combination of amodiaquine and artesunate as the first-line drug. It also opens the possibility of continuous monitoring for changes in drug susceptibility at the molecular level in Ghana.

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