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SNPs on ABC Transporters and *in vivo* Malaria Parasite Non Clearance after Chloroquine Treatment in Malian Children

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Authors' contributions

This work was carried out in collaboration between all authors. Author MW wrote the first draft of the manuscript and performed statistical analysis. Authors AHB, MT and AD conducted the fields study and performed the molecular analysis. Author ADjim designed the study and wrote the protocol. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Background*:* *pfcrt K76T* mutation was demonstrated to play a central role in the *P. falciparum* resistance to chloroquine.
Aim: To find any association between mutant alleles of *pfcrt* K76T, *pfmdr1* N86Y, *pfG30* and *pfG47* and the *in vivo* parasite non clearance after chloroquine treatment in Mali.
Methodology: We carried out a chloroquine efficacy study in 196 children suffering from uncomplicated malaria in a rural village of Kollé, Mali, using WHO protocol. Subjects were treated with standard dose of chloroquine and followed for 14 days. Parasite DNA was extracted from finger prick blood blotted onto filter paper and genotypes were analyzed by different PCR methods.
Results: The mutant alleles *pfcrt 76T* and *pfmdr1 86Y* were associated with parasite non clearance with *p=0.00001* and *0.03* respectively.
However, the association of SNPs on *pfG30* and *pfG47* genes with parasite non

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clearance was not statistically significant, $p = 0.43$ and 0.57 respectively. The logistic regression analysis showed that the mutant allele *pfmdr186Y* contributed positively to the *pfcrt 76T* parasites non clearance ($p=0.02$).
Conclusion: These findings have shown that *pfcrt76T* and *pfmdr1 86Y* alleles are associated with the *in vivo* parasite non clearance, but not SNPs on the new putative transporters genes.

Keywords: SNPs; ABC; malaria; drug resistance; chloroquine.

1. INTRODUCTION

Plasmodium falciparum malaria remains one of the major causes of morbidity and mortality in Africa. According to the 2011 WHO report, an estimated 655 000 persons died of malaria in 2010, 86% of the victims were children under 5 years of age, and 91% of malaria deaths occurred in African Region [1]. Chloroquine (CQ) was the best and most heavily used drug in the fight against malaria. However, the effectiveness of CQ has declined with the emergence and spread of CQ-resistant (CQR) *Plasmodium falciparum* parasites [2]. ATP-Binding Cassette (ABC) transporters are efflux pumps frequently associated with multidrug resistance in many biological systems, including malaria. It was shown that several single nucleotide polymorphisms (SNP) in ABC genes modulate *in vivo* and/or *in vitro* drug susceptibility, but the underlying physiological mechanism of the effect of these mutations remains unclear [3]. A mutation on PfCRT, a member of the drug transporter super family, has been demonstrated to be the key factor in the resistance of *P. falciparum* to CQ [4,5]. Other molecules, such as Pgh1, a member of the ABC transporter superfamily encoded by *pfmdr1*, may contribute to CQR [6,7]. However, the implication of *pfmdr1* in CQR remains controversial [8]. Mammalian Pglycoprotein (ABCB1) is, arguably, the best characterized of all ABC transporters and, when overexpressed, confers resistance of cancer cells to a variety of chemotherapeutic drugs [9,10].

A previous study Mu et al. [11] located 49 genes encoding transporters in the *P. falciparum* genome and sequenced these genes in a panel of 97 isolates from worldwide locations. SNPs in 11/49 genes showed significant association with *in vitro* drug resistance to CQ. Apart from *pfcrt* and *pfmdr1*, the remaining nine candidates' genes were new, and among them three genes were associated with the CQ response in African isolates.

In this study, we aimed to find out association of *pfcrt K76T, pfmdr1 N86Y, pfG30* and *pfG47* mutant alleles with the parasite *in vivo* non clearance after CQ treatment in Malian children.

2. MATERIALS AND METHODS

2.1 Study Design

This study was carried out from 2001 to 2002 at the Health Center of a Malian rural village Kollé located at 50 km from Bamako. This is an endemic falciparum malaria area where the preliminary studies have shown the presence of *P. falciparum in vivo* resistance to CQ. The inclusion criteria for this study were those described by the WHO protocol [12]. Briefly, the included subjects have met the following criteria: 6 to 59 months with uncomplicated *falciparum* malaria, infected by 2,000 - 200,000 asexual parasites/μL, and axillary

temperature 37.5ºC - 39.5ºC. Subjects who did not meet these criteria were not included in the study but were treated by adequate drugs.

2.2 Treatment, Assessment of Parasite and Clinical Responses

Standard oral doses of CQ were administered at 25 mg/kg over three days: 10 mg/kg on days 0 and 1, then 5 mg/kg on day 2. To monitor for adverse reactions and to make sure that the medicine was well tolerated; all subjects were observed for at least 60 minutes. Thick and thin blood malaria smears and finger prick blood-onto filter paper samples were collected at the time of enrolment.

Smears were Giemsa-stained and parasitemia was measured by counting asexual parasites relative to 300 white blood cells. The blood Filter papers were air-dried and stored at room temperature for molecular analysis. After enrolment on day 0, subjects were followed actively on days 1, 2, 3, 7 and 14 [12] (WHO, 2000) and smears as well as filter paper strips were obtained at these days. The identified *in vivo* resistance cases and severe malaria cases were fully evaluated and treated with appropriate medical care including parenteral quinine.

2.3 Molecular Analysis and Statistics Analysis

P. falciparum DNA was extracted from finger prick blood blotted onto filter paper at days 0 and 14 as described by Plow and collaborators Plowe et al. [13]. The *pfcrt K76T* and *pfmdr1 N86Y* genotypes were analyzed by nested PCR using the primers as described elsewhere [14,15]. For SNPs on *pfG30* and *pfG47*, mutation specific PCR was used with primers described by Mu et al. [11].

3. RESULTS AND DISCUSSION

Clinical results and a part of molecular analysis of this study have been published by Tekete et al. [16]. In this study, we considered 196 children aged 6–59 months, suffering from uncomplicated malaria, treated and followed up for 14 days. Among them 54 subjects (27.5%) did not clear their parasites at D14. The mutant alleles *pfcrt 76T* and *pfmdr1 86Y* were associated with parasite non clearance with statistically significance $P<.0001$ and $P=.03$ respectively (Table 1).

However, the association of the mutant alleles of *pfG30* and *pfG47* with parasite non clearance was not significant with $P=.43$ and $P =.57$ respectively (Table 1).

These results are consistent with those found by Cojean et al. [17] who found an association of *in vitro* CQR with SNPs on genes *pfcrt* and *pfmdr1* but not on putative transporters genes (*pfG7*, *pfG25*, *pfG30*, *pfG49*, *pfG54* and *pfG70*).

The study of Anderson found no association between eight putative transporter genes and the response to eight different antimalarials including CQ [18].

Table 1. Association of mutant alleles with parasite non clearance after CQ treatment

Mutant alleles	D0 Analyzed filter papers	% of positive cases	D14 Analyzed filter papers	% of positive cases	*P*
pfcrt 76 T	158	72.78	54	100	.00001
pfmdr1 86Y	143	56.64	50	74	.03
pfG30 mutant	141	44	43	37.2	.43
pfG47 mutant	142	59.2	39	64.1	.57

The logistic regression analysis was performed on parasites caring *pfcrt 76T* allele, among them some carried also different mutant allele such as: *pfmdr1*, *pfG30* and *pfG47*. It showed that the mutant allele *pfmdr1 86Y* contributed positively to the *pfcrt 76T* parasites non clearance (*P=.02*). However, mutant alleles of *pfG30* and *pfG47* did not contribute to the *pfcrt 76T* parasites non clearance, *P= .22* and *P =.13* respectively (Table 2). In different *in vitro* and *in vivo* studies *Pfmdr1* gene has been described as a modulator of CQR [19,20,21].

Mu et collaborators did not observed the impact of ABC SNPs in *in vitro* CQR (IC_{50}> 100 nM) parasites (n=6) carrying *pfcrt 76T*. They have located the SNP on pfG30 on intronic part of the gene and found that the association was not statistically significant with SNP on *pfG47* (p = .4) (n=15). However, they have reported an association between SNP on *pfG7* and *in vitro* response to artesunate [11].

Table 2. Association of SNPs with *pfcrt 76T* parasite non clearance after CQ treatment

Mutant alleles with *pfcrt 76T*	Odds ratio	*P*	Confidence intervalle
pfmdr1 D0	2.48	.02	1.15 - 5.34
pfG47 D0	1.58	.22	0.75 - 3.33
pfG30 DO	0.53	.13	0.24 - 1.20

These previous results were in favour of the hypothesis that CQR could result from a multi-gene process, *pfcrt* mutations being necessary but not sufficient to acquire CQR. This hypothesis is supported by strong linkage disequilibrium between variants in *pfcrt* and *pfmdr1* [11,22] perhaps involving a functional interplay between these proteins.

4. CONCLUSION

In conclusion, our data showed that the parasite non clearance in children after chloroquine treatment is strongly associated with *pfrct 76T* and *pfmdr1 86Y* but not with SNPs on the new putative transporters genes.

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ETHICAL APPROVAL

The protocol of study and informed consent document were reviewed and approved by the ethical committee of the Faculty of Medicine and Dentistry of University of Bamako, Mali. Parental or guardian written informed consent was obtained before performing any protocol specific procedure. A child was withdrawn from the study if she/he developed severe malaria, or if his/her parent or guardian requested it.

All authors declare that 'written informed consent was obtained from the parents or guardians for publication of this case report..

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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