Published in final edited form as:

Acta Trop. 2011 July ; 119(1): 11–13. doi:10.1016/j.actatropica.2011.01.002.

High Frequency of PfCRT 76T in Two Malian Villages and Its Prevalence in Severe Relative to Non-Severe Malaria

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Abstract

We investigated PfCRT 76T mutation in severe and non-severe malaria in Southern Mali. One hundred and ninety three severe malaria cases were each matched against two non-severe malaria cases. Patients with G6PD deficiency and any known hemoglobin abnormality were excluded. PfCRT 76T was present in 60.8% (n = 386) non-severe malaria cases and in 77.2% (n = 193) severe malaria cases (p < 0.0001). In children 5 years or younger, these proportions were 62.9% (n = 294) vs. 73.5% (n = 147), respectively (p < 0.01). PfCRT 76T was therefore associated with malaria severity in this setting of Mali.

Keywords

severe malaria; non-severe malaria; PfCRT 76T; drug resistance

Introduction

Plasmodium falciparum malaria remains the main cause of child morbidity and mortality in sub-Saharan Africa. The exact mechanisms underlying the pathogenesis of severe and life-threatening malaria are not fully understood. Several factors including age, immunity, hemoglobin types and parasite strain have been reported to be important contributors in these mechanisms [1]. Drug resistance has also been suggested to be a virulence factor for *P.*

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The authors report no conflict of interest.

Presented as a Poster at the Fifth EDCTP conference 12–14 October 2009 in Arusha, Tanzania.

falciparum. A study in Senegal showed that the risk of malaria death among children 0–9 years old increased 5.5 fold with the emergence of chloroquine resistance [2]. High rates of chloroquine resistance have been documented in nearly all sub-Saharan African countries.

Mutations including a K76T substitution in the *P. falciparum* chloroquine resistance transporter (PfCRT) are a key determinant of chloroquine resistance both in laboratory strains and in the field [1,3]. In Mali we and others have detected rates of in vivo chloroquine resistance as high as 80% while prevalence of PfCRT 76T reaches 85% [4]. *In vitro* resistance has been reported to be significantly higher in isolates from patients suffering severe malaria than in isolates from patients suffering non-severe malaria [5]. These resistant parasites may be associated with increased disease severity in non-immune patients [6]. However, Giha and collaborators found in Sudan that the lethal malaria form was rather associated with the wild type PfCRT K76, although the difference in the frequency of the chloroquine resistant parasites in patients suffering from severe malaria and from non-severe malaria was not significant [7].

The few studies that investigated the relationship between PfCRT and malaria severity may have yielded conflicting results because of low sample sizes [8]. Furthermore, proper matching of cases and controls were not performed, nor were confounding factors such as abnormal hemoglobin and G6PD status accounted for. To test whether drug-resistant parasites are associated with increased risk of severe malaria in the field, we conducted a case-control study of the PfCRT 76T marker and disease severity.

Patients and Methods

Study sites

The study was conducted in two rural villages in Southern Mali, Kangaba and Kela with respectively 4,248 and 1,671 inhabitants. Malaria is hyper-endemic in both villages with seasonal peaks of transmission [9].

Study design

This was a case control study in patients aged 6 months or older residing in one of the two villages between June 2001 and December 2003.

Patients in Severe malaria group were included according to the following criteria: asexual parasite density $>500,000/\mu\text{L}$ or parasitaemia of any density plus any one of the following: coma (defined as Blantyre coma score <2), convulsions (witnessed by investigators), severe prostration, severe anemia (hemoglobin $<5\text{ g/dL}$), respiratory distress, hypoglycemia (serum glucose $<40\text{ mg/dL}$), jaundice/icterus, shock (systolic blood pressure $<50\text{ mmHg}$, rapid pulse, cool extremities), cessation of eating and drinking, repetitive vomiting [10].

Non severe malaria cases were recruited based on of following criteria: axillary temperature $>37.5^{\circ}\text{C}$ or history of fever, signs and symptoms of malaria (e.g., headache, body aches, and malaise), asexual parasite density $<500,000$ per microliter of blood, no criteria of severe malaria, and no other etiologies of febrile illness on clinical examination [11].

These cases and controls were drawn from a larger study which was reported in detail elsewhere [9]. Non-severe malaria was treated with the then first-line drug chloroquine, administered at the doses of 10 mg/kg on day 0 and day 1, and 5 mg/kg on day 2. On post treatment days 1 and 2, symptoms, other medications, temperature, and results of a physical examination were recorded. Finger prick blood was also blotted onto filter paper strips for extraction of DNA and subsequent molecular analyses. Severe malaria cases were treated with intravenous quinine: initially 20mg/Kg in 10% dextrose followed by 10mg/kg every 8 hours until the oral route was tolerated.

Ethical clearance

The protocol was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Pharmacy and Odonto-Stomatology (FMPOS), University of Bamako and the Institutional Review Board of National Institutes of Allergy and Infectious Diseases, National Institutes of Health, USA. Prior to any protocol-specific procedure, written informed consent was obtained from patients or their parents or legal guardian in cases of minor children.

Molecular analysis

P. falciparum DNA was extracted from finger prick blood blotted onto filter paper and *PfCRT K76T* genotypes were analyzed by nested PCR as previously described [12].

Hemoglobin and G6PD deficiencies analysis

Hemoglobin C and S were determined using standard hemoglobin electrophoresis methods as described elsewhere [9]. All samples were analyzed on site in Kela and Kangaba by study investigators. The G6PD (A-) status was determined by nested PCR with two pairs of primers followed by digestion with the enzyme *Hsp* II92 [9]. Patients with G6PD (A-) deficiency and any known hemoglobin abnormality were excluded from both groups for analysis purpose.

Data analysis and statistics

Mixed infections were categorized as mutant *PfCRT 76T*. Data were double-entered, validated using Microsoft Excel® (Microsoft Corporation, Redmond, WA), and analyzed with SPSS version 11.0 (Chicago, IL, USA) or STATA version 8.2 (Stata Corporation, College Station, TX). Significance threshold at $p = 0.05$ was used for significant association between drug resistant allele and malaria severity in matched case-controls.

Results

A total of 579 patients including 193 children with severe malaria and 386 children with non severe malaria, were enrolled in the. The study population included 54.4% ($n = 579$) of females. The population repartition shows that 76.2% ($n=579$) were aged 5 years or younger. (Table 1) Each severe malaria case was matched with two non severe malaria cases by age and sex generating 386 controls.

When all ages were considered the prevalence of *PfCRT 76T* was 77.2% (n = 193) but 60.8% (n = 386) in the severe malaria and non-severe malaria groups, respectively (p = 0.0001). When the analysis was limited to children 5 years old or younger, *PfCRT 76T* was present in 73.5% (n=147) of the severe malaria group but in 62.9% (n=294) of the non-severe malaria group (p = 0.01, Table 2).

When the data was broken down between severe malarial anemia and cerebral malaria, there was no association between the *pfcr*t 76T and these severe malaria sub-phenotypes (p= 0.55 and 0.76, respectively). (Table 3)

We found that fifteen non-severe malaria cases treated with chloroquine developed severe malaria and 14 of them (93.3%) had mutant parasites at inclusion.

Discussion

During the malaria transmission seasons from 2001 to 2003, in a matched case-control study conducted in Kangaba and Kela, we included 193 cases of severe malaria and 386 non-severe malaria cases. We observed a statistically significant association between the presence of the mutant allele *PfCRT 76T* and malaria severity. This association was less significant in the age group 0 – 5 years than in the general population. The association of *PfCRT* with disease severity was no longer seen when the phenotype was broken down into severe malarial anemia and cerebral malaria although this could be due to the limited number of cases with each of these sub-phenotypes of severe malaria. Sufficient power for the detection of such associations would have required larger numbers than were available in our study. Our results confirm observations made in India and The Gambia. In India, Ranjit and collaborators found that the *PfCRT76T* genotype was significantly higher (p = 0.01) in severe malaria cases compared to non-severe malaria [8]. Similarly, Meerman and collaborators used a case-control study in 2002 in Gambian children and found significant association between carriage of *PfCRT 76T* and *Pfmdr1 86Y* and severe malaria (p = 0.01 for each marker) [3]. However, Giha and collaborators found in Sudan that the lethal malaria form was rather associated with the wild type *PfCRT K76* although the difference in the frequency of the chloroquine resistant parasites in patients suffering from severe malaria and from non-severe malaria was not significant [7]. Similarly, in a more recent study in Congo, Mayengue and collaborators did not find any relation between the severity of the disease and the presence of the mutant allele *PfCRT 76T* [13]. We note that in the Sudanese study, the sample size (n = 47) was rather small and not sufficient to support the hypothesis of increased virulence of chloroquine sensitive strain. The *PfCRT 76T* allele was almost 100% in the Congolese study area, so their finding would be expected. These conflicting results may also be due to conflicting influences of *pfcr*t 76T on disease severity. On one hand, chloroquine resistance mutations are likely to decrease the fitness and/or virulence of pathogens as shown by reports showing the decrease of *pfcr*t76T only a few years after CQ was withdrawn from Malawi [17]. On the other hand, resistance in the setting of continued use of chloroquine would be expected to allow progression to severe malaria. The interplay of these two factors might explain somewhat conflicting results between different studies.

The reason for this observed association in our study is not clear. Chloroquine was widely available in the study area and was the first treatment given to any patient with any fever in these villages. Yet, the prevalence of PfCRT76T was as high as 80%. The administration of chloroquine to children with drug-resistant infections could have delayed their arrival to the clinic and therefore acted as a confounding factor for severe malaria. This effect in just a fraction of the children could account for the slightly higher rates of PfCRT 76T found in the children with severe vs. non-severe malaria. The fact that 14/15 (93.3%) of those who progressed from non-severe to severe malaria carried PfCRT 76T at baseline supports this idea.

The biological role of the PfCRT protein is still not known [14]. There are no data in the present literature implicating PfCRT in the virulence of *P. falciparum* or the pathogenesis of *falciparum* malaria. However, the *pfert* gene resides near a cluster of *var* genes on chromosome 7 and this *pfert*-*var* linkage disequilibrium has been reported from some island regions [15]. Disease-associated *var* genes may also occur [16]. It is unclear whether recombinationally-active villages like Kangaba and Kela might allow linkage disequilibrium for any significant period and therefore could support a meaningful association of PfCRT 76T with malaria severity. Testing of this idea might be possible in the context of datasets of larger studies.

Acknowledgments

We thank the local guides of Kangaba and Kela, the Village councils, the entire population of Kangaba and Kela for their help and support during the study. We thank Drs Madani Ly, Abdoul Madjid Traore, Awa Traore, Mamadou Mounkoro for technical support.

Financially supported by grants from International Atomic Energy Agency (IAEA) RAF/6025; MIM-UNICEF-UNDP-World Bank- WHO Special Programme for Research and TDR Grant # A20238, NIAID/NIH Supplement Award 5 RO1 AI44824- 03, European and Developing Countries Clinical Trial Partnership Senior Fellowship (Grant # 2004.2.C.f1), Howard Hughes Medical Institution International Scholarship (Grant # 55005502), Training grant from University of Bamako, Mali and PER-AUF Agence Universitaire de la Francophonie, and the Intramural Research Program of the NIH, NIAID, USA.

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Table 1

Distribution of clinical malaria cases by age categories

	[0 – 5] years	6 years	Total
Non-severe malaria	294	92	386
Severe malaria	147(76.2%)	46(23.8%)	193

Table 2
Distribution of PfCRT alleles among non-severe and severe malaria cases of malaria

	Non-severe malaria		Severe malaria		p-value
	N	PfCRT 76T (%)	N	PfCRT 76T (%)	
All ages	386	60.8	193	77.2	0.0001
0 – 5 years	294	62.92	147	73.46	0.01

Table 3

Distribution of PfCRT alleles among severe malaria sub-phenotypes

Severe malaria sub-phenotypes	Uncomplicated malaria		Severe malaria		P-value
	Wild allele	Mutant allele	Wild allele	Mutant allele	
Severe Anemia	5	12	7	11	0.6
Cerebral Malaria	13	27	19	45	0.8