Clinical Probe of Cyp2C8*2 Mutants in a Malaria Hyperendemic Zone: Evidence from North-Central, Nigeria

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ABSTRACT

Background: A tremendous level of success has been achieved since the introduction of chloroquine and the combination of amodiaquine and artemisinin for the treatment of both complicated and uncomplicated malaria infections in sub-Saharan Africa. However, the recent discovery of drug resistant strains of *Plasmodium falciparum (P. f.)* and the ability of the parasite to ingest CYP2C8 into its digestive vacuole is of great public health concern. This study probes the occurrence of CYP2C8*2 allelic mutant amongst malaria patients in North-Central Nigeria.

Methods: Three hundred and eighty five (385) unrelated study participants were screened for current malaria episodes using routine microscopy and/or rapid diagnostic test strips (RDTs). Chelex extraction method was used for single nucleotide polymorphisms (SNPs) and identification of CYP2C8*2 (805A > T) variant respectively. Wild-type (A) and the defective allele (T) were differentiated with the use of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The results obtained were further validated with Sanger sequencing of a few samples and thereafter, the genotype data were statistically processed. All alleles obtained were in Hardy Weinberg equilibrium.

Results: Out of the 385 participants (45.5% Male and 54.5% Female) genotyped for SNPs, 75 (19.5%) had the autosomal recessive mutant trait. Occurrence of mutant traits was gender and ethnic independent ($p > 0.05$). Yoruba ethnic group recorded a reduction in proportion of genotypic defective CYP2C8*2 allele (T) (1 in every 8 persons) with a carrier percentage of 13.3% compared with Hausa (26.62%); Igbo (25.37%) and other minority ethnic groups (17.6%).

Conclusions: A remarkable inter-ethnic differences in autosomal recessive CYP2C8*2 allele was observed. By implication, there is a gradual incursion of genetic drift for poor CQ and AQ-Artemisinin metabolizers among the inhabitants.

KEYWORDS

Plasmodium falciparum; Chloroquine; Amodiaquine-Artemisinin combination therapy; CYP2C8*2; Hausa, Igbo, Yoruba, Nigeria

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INTRODUCTION

Infections arising as a result of *Plasmodium falciparum* is the major cause of malaria-related deaths and it has been reported to be the most common of the four human malaria parasites across sub-Saharan Africa (1). Despite wide documented chloroquine (CQ) and Amodiaquine (AQ) resistance; majority of the populace still rely on therapeutic CQ and AQ medications. The increasing failure of these drugs against falciparum malaria constitutes a notable setback in the eradication efforts of malaria in many African countries (2–4). Previous studies have established that host genetic variations with respect to cytochrome P450 (CYP) 2C8 (CYP2C8) as drug metabolizers is responsible for the metabolism of about 20–50% clinical drugs and endogenous substances. The emerging mutations with respect to these metabolites is one of the main risk factors for the drug resistant strains of *P. falciparum* in Africa (5). Genotype-inferred low metabolizers were reported in 1–4% of African populations corresponding to millions of expected exposures to AQ (6). Recent studies further revealed that resistant strain are often characterized with the genetic defective variant (CYP2C8*2) identified as being responsible for the hepatic metabolism of CQ and AQ, consequently altering chloroquine flux or reduced drug binding to hematin inside the parasite digestive vacuole. This mechanism culminates into *Plasmodium falciparum* chloroquine resistance transporter (PfCRT) point mutations (5, 7). Similarly, quinoline ring in 4Qs is resistant to degradation by cytochrome P450 enzymes (CYP) CYP2C8 and CYP3A4, with potentials to mediate 80% of the total metabolism of 4AQs (8). However, the common occurrence of genetic variant CYP2C8*2 in malaria infected host has been linked to the presence of drug-resistant parasites in the infected host (*pfcrt-76Y* and *pfmdr1-86Y P. falciparum* alleles). This anomaly is documented as a strong factor that is chronically hindering the efficacy of CQ and AQ in Africa. A recent longitudinal study in Africa reported that the prevalence of the defective allele "CYP2C8*2" is statistically insignificant among ethnic groups in Nigeria, although comparable with what was obtained in Senegal and Madagascar (9). However, it is pertinent to further explicate the presence of this allele in other African descents, because of the crucial role it plays in the epidemiology of falciparum infections. This study will investigate the occurrence and determine the allele frequencies of CYP2C8*2 amongst residents in a malaria high transmission zone of North-Central Nigeria. This is to guide intervention for better understanding of the metabolic mechanism of CQ and AQ artemisinin-based combination therapy (ACT) in the study area.

MATERIALS AND METHODS

STUDY AREA, DESIGN AND PROTOCOLS

The study was conducted within Ilorin metropolis, an urban area, in the North-Central zone and the capital of Kwara State, Nigeria. It is located on longitude 4°35′E and latitude 8°35′N. It covers an area of about 38 square miles, with an estimated population of 1.4 million people. The area is associated with intense rainfalls from April to October and daily temperature of between 23 °C and 37 °C. Inhabitants are mostly farmers, civil servants, traders and students. Out-patients from four randomly selected hospitals (Civil Service Hospital, Temitope Hospital, Children Specialist Hospital and University of Ilorin Health Centre) in Ilorin were used for the study. A simple structured questionnaire was administered to volunteers after written informed consent was sought and approved to obtain some basic information on ethnicity and Knowledge about usage of antimalarials like CQ, AQ and ACTs (viz; Artemether-lumefantrine, Artesunate-mefloquine and Dihydroartemisinin-piperaquine). Only volunteers with a record of past CQ and AQ medications were considered for the present study. Intravenous blood samples of subjects were spotted on Whatman number 3 filter papers, air dried and separately stored in sealed plastic containers. Routine malaria diagnosis was initially performed by microscopic examination of Giemsa-stained thick blood smears and/or a rapid diagnostic test (Malaria Antigen *P. f.*, Standard diagnostics, INC. Ingbert, Germany). Single nucleotide polymorphisms in CYP2C8*2 was screened for according to Marwa et al. (10). DNA extraction and subsequent identification of CYP2C8*2 (805A > T) variant was carried out using Chelex extraction method and Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) respectively as described by Paganotti et al. (11). Briefly, 2µl of DNA template was amplified by PCR, at 107bp fragment for the CYP2C8 gene forward primer (i.e. at 5′-GAACACCAAGCATCACTGGA-3′) and reverse primer (i.e. at 5′-GAAATCAAAATACTGSTCTGTTGC-3′). The products from PCR analysis was incubated with Bcl 1 enzyme that cuts the wild type allele only (A); undigested products then represent the variant allele (T). In order to detect the size polymorphisms, both types were allowed to run on a metaphor 3% gel. Controls for human genotyping were then utilized after sequencing of the PCR product obtained from each different genotype. Genotyping errors were avoided by double checking for the heterozygous samples.

ETHICAL APPROVAL

This study was performed according to the Declaration of Helsinki and the procedure followed was part of a study approved by the University of Ilorin Ethical Consideration with protocol approval number: UERC/ASN/2012/221. Consent form was administered and collected from the volunteers before the commencement for the study.

STATISTICAL ANALYSIS

Data obtained were analyzed with SigmaPlot for Windows version 12.0 (Systat. Software, Inc.). The prevalence of recorded alleles (i.e. wild and mutant alleles) were subjected to Chi-square ($\chi 2$) analysis and statistical significance was set at $p < 0.05$. The results obtained were further validated with Sanger sequencing of a few samples and thereafter, the genotype data were statistically processed. Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$) was used to estimate the frequency of the carrier state (2pq) for autosomal recessive trait among the study population (12).

Tab. 1 Characteristics of samples taken for CYP2C8*2 analysis.

Factor	No examined (%)	Allele frequency (%)		
		Mutants	Wild Type	p-value
Total no examined	385	75 (19.5)	310 (80.5)	
Gender				0.814
Male	175 (45.5)	35 (46.7)	140 (45.2)	
Female	210 (54.5)	40 (53.3)	170 (54.8)	
Age group				0.003
0–5	100 (26.0)	20 (26.7)	80 (25.8)	
6–15	90 (23.4)	10 (13.3)	80 (25.8)	
16–25	50 (13.0)	20 (26.7)	30 (9.7)	
26–35	35 (9.1)	5 (6.7)	30 (9.7)	
36–45	60 (15.6)	10 (13.3)	50 (16.1)	
> 45	50 (13.0)	10 (13.3)	40 (12.9)	
Ethnic group				0.102
Yoruba	195 (50.6)	45 (60.0)	150 (48.4)	
Hausa	40 (10.4)	10 (13.3)	30 (9.7%)	
Igbo	45 (11.7)	5 (6.7)	40 (12.9)	
Others*	105 (29.9)	15 (20.0)	90 (29.0)	

* Idoma (30, 7.8%), Fulani (10, 2.6%), Nupe (40, 10.4%), Igala (10, 2.6%) and Benue/Igede (15, 3.9%).

RESULTS

Three hundred and eighty five (385) individuals consisting of 175 (45.5%) male and 210 (54.5%) female with a past record of CQ and AQ use voluntarily participated in this study. Seventy five (75 (19.5%)) was analysed to have recessive mutant traits of CYP2C8*2 allele. Mutant population with respect to gender and ethnic group were not statistically significant ($p > 0.05$). However, the defective CYP2C8*2 allele in comparison with the wild dominant allele was significant with respect to distribution among the respective age groups sampled ($p = 0.003$) (Table 1).

The genotype and allele frequencies in the Nigerian major and minor ethnic groups domicilied in the study area was assessed with Hardy-Weinberg equilibrum calculator (12). The genotype frequencies obtained obeyed the assumptions layed down for the principle. For instance, the allele frequency for this generation was done by pooling together the alleles from each genotype of the same generation according to the expected contribution from the homozygote and heterozygote genotypes. Yoruba ethnic group recorded a reduction in proportion of CYP2C8*2 allele (T) frequency (1 in every 8 persons) with a carrier percentage of 13.3% despite the large sample size screened (N = 195) compared with others (viz; Hausa: 26.62%; Igbo: 25.37%; Others: 17.6%) (Table 2).

DISCUSSION

The recent discovery of molecular markers for drug resistance in genomic studies is gradually eliciting various dimension (13). Investigation on defective CYP2C8*2 is essential to evaluate emergence of antimalarial drug resistance markers (*P. falciparum*) population among the three major Nigerian ethnic groups. In this study, a non-negligible frequency (19.5%) of autosomal recessive CYP2C8*2 mutants was obtained among malaria patients. This outcome is similar to the report of Adehin et al. (14) in South-west Nigeria. However, this study reported a lower

Tab. 2 Genotypes for CYP2C8*2 and T allele frequency among the studied Ethnic groups.

Ethnic groups	CYP2C8*2 (rs11572103, A > T)					
	Genotype frequencies				Allele frequency	
	N	AA	AT	TT	T	Carrier (%)
Yoruba	195	0.862	0.133	0.005	1 in 8	26 (13.3)
Hausa	40	0.709	0.266	0.025	1 in 4	11 (26.62)
Igbo	45	0.724	0.254	0.022	1 in 4	11 (25.37)
Others*	105	0.814	0.176	0.010	1 in 6	18 (17.61)

* Idoma (30, 7.8%), Fulani (10, 2.6%), Nupe (40, 10.4%), Igala (10, 2.6%) and Benue/Igede (15, 3.9%). AA – homozygous wild-type; AT – heterozygous carrier; TT – homozygous mutant; T – Phenotype.

prevalence of CYP2C8*2 status when compared with several early studies reports in African populations (10, 11, 15, 16). In a similar vein, CYP2C8*2 allele was successfully genotyped in 75% (213/285) of children in Congo Brazzaville. The CYP2C8*2A allele had a frequency of 63%, whereas the CYP2C8*2T allele had a frequency of 37%. Genotypes CYP2C8*2AA (rapid metabolizer), CYP2C8*2AT (intermediate metabolizer), and CYP2C8*2TT (poor metabolizer) were reported in 44%, 38%, and 18% of the investigated participants, respectively (17). This suggests that mutations in specific *P. f.* genes may confer resistance to antimalarial drugs, climaxing into sustained drug pressure (18). Also, this finding may serve as an important tool at predicting the level of resistance to CQ and AQ + Artemisinin combinations drugs. It is suffice to mention that in sub-Saharan Africa, people carrying CYP2C8*2 C.805A > T (CYP2C8*2; rs11572103) allele suffer impaired amodiaquine metabolism, increased risk of amodiaquine-related adverse events, and may promote the selection of drug-resistant parasite strains (17). CYP2C8 accounts for the metabolism of > 20% of drugs used in the treatment of varying ailments with over 60 clinically important therapeutic agents of which malaria is one (19). However, CYP2C8*2T allele occurs mostly in people with a sub-Saharan Africa ancestry (19%) and it is less frequent (≤ 1%) in individuals of European, Asian, or American origin (20). The 4-Qs become resident in the acidic digestive vacuole, where they are believed to bind b-hematin and interfere with heme detoxification (21). In human, AQ is mainly metabolized in the liver, and CYP2C8 is the main hepatic isoform that catalyzes the formation of N-desethylaminodiaquine (DEAQ) (22). From the aforementioned, it is obvious that the defective allele give rise to a number of different point mutations affecting the heme and or substrate binding ability of CYP2C8 as it is the most abundant form expressed in the liver and other extrahepatic tissues (23). In our study, genotypic data obtained showed that the Yoruba ethnic group' chances of outcome with the defective allele was one in eight which appears to be the least because the carrier frequency was 13.3%, amongst the studied population. The observed variance may be due to the differences in population sampled or it may be ascribed to activity impacting nature of the SNPs playing less relevance in some descents (10, 14). CYP2C8 also metabolizes arachidonic acid and the anticancer drug paclitaxel, and CYP2C8 variants have been shown to be defective in the metabolism of both substrates (22). The emergence and spread of drug resistance depends, in part, on the number of mutations required to encode resistance and their effects on parasite fitness (24). Specific multiple point mutations is however very important in a gene make up of a resistant marker for an antimalarial drug (20, 25). Many adverse reactions are attributable to reduced CYP2C8 expression, but yet unreported during clinical trials. The expression often leads to any one or all of the following, viz; poor metabolizer phenotypes, hepatoxicity and a severe reduction in white blood cell count (22). The aforementioned may result in the risk of both mild and severe adverse clinical outcomes associated with AQ treatment (16). Furthermore, the presence of this defective allele in our population is suggestive of possible inter-population differences in clinical outcomes associated with ACT drugs.

CONCLUSION

Currently, the increasing knowledge in genomic revolution is significantly improving our understanding of reasons why individuals and populations differ in their susceptibility to multiple diseases. The occurrence of inter-ethnic differences in the frequencies of clinically relevant CYP450 variants is the real reason populations don't maintain the stable allele frequencies predicted by the Hardy-Weinberg equilibrium. A remarkable inter-ethnic differences in autosomal recessive CYP2C8*2 allele was observed. By implication, there appears to be a gradual incursion of genetic drift for poor CQ and AQ-Artemisinin metabolizers among the inhabitants. Further studies are required to evaluate the toxicological significance of this poor metabolizer in our settings and give insights to the adverse effect on the drug pharmacokinetics of CQ and AQ-ACT drugs.

AUTHORS CONTRIBUTION

OSH, OAB, OIA and MKS designed, did statistical analysis and contributed to the manuscript write-up, OAO supervised the collection of the samples, OBO and OF provided patients in their respective hospitals, OMA and MIF collected dried blood spot samples, AE, OAI and AO carried out the PCR and RFLP analysis. OIA edited the final manuscript.

COMPETING INTEREST

The authors of this study declare no competing interest. All authors partook in the design, implementation and the write-up.

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