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Relationship Between CYP2C8*2 and *Pfmdr1* N86Y Polymorphisms in Patients with Uncomplicated Malaria in Yaounde, Centre Region of Cameroon

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Abstract: *Background: Plasmodium falciparum* malaria is a major public health problem in Cameroon. It remains endemic and is the leading cause of morbidity and mortality in the most vulnerable groups, including children under five and pregnant women. The fight against plasmodium today faces not only diversity, preventive struggles, but also the spread of resistant parasites to available antimalarials. Several factors, among others, genetic factors, and the immune system predispose patients to develop resistances. The parasite's resistance to antimalarial would continue to be an obstacle to the management of malaria in Cameroon. This study aimed to determine the frequency of the mutation affecting the gene *CYP2C8* (*CYP2C8**2) and its influence on the mutation N86Y of the plasmodial gene *Pfmdr1* in children under 15 years of age suffering from non-complicated malaria in Yaounde. *Methods:* This was a population based, retrospective study in a Cameroonian population. Archived whole blood samples collected from One hundred children infected with *Plasmodium falciparum* malaria were randomly selected. Blood samples spotted on filter papers were used for DNA (plasmodial and human) extraction performed by the chelex-100 method. The *PfmdrI* marker was established by the nested PCR and gene involved in the metabolism of antimalarial by conventional PCR. The RFLP-PCR technique allowed the detection of the polymorphism of these mutations. The restriction enzyme *bclI* was used for the polymorphisms of the cyp2C8 gene and the restriction enzyme *AflIII* for *PfmdrI*. *Results:* the mutant allele CYP2C8*2 had a frequency of 38%. For the *Pfmdr1* gene, 57% of isolates were detected with the mutant 86Y. The application of the Khi2 statistical test showed that patients with the mutant allele CYP2C8*2 were more likely to be infected with the *pfmdrI*-86Y mutant strain (OR: 2,446; P: 0.030). *Conclusion:* This study reported that the mutant allele CYP2C8*2 influences the emergence of *Pfmdr1* 86Y mutants.

Keywords: Resistance, *Pfmdr1*, Plasmodium Falciparum, Antimalarial Metabolism, *CYP2C8*

1. Introduction

The emergence of parasites resistant to antimalarial drugs continues to be a major public health problem in the control of *Plasmodium falciparum* infections. Malaria alone claims at least one life every 30s. The overall burden of malaria can reach 2 million casualties / year, mostly among children aged under 5 years and pregnant women in sub-Saharan Africa [1, 2]. Several molecular markers necessary for the prediction of parasite resistance have a prominent place in the monitoring of antimalarial activity [3]. Thereby, the Pfmdr1 N86Y marker is among the most studied and seems to be involved directly or indirectly in resistance to amodiaquine, chloroquine and quinine and many other antimalarials [4]. Mutations in the *Pfmdr1* gene diminish the ability of the transporter to transport drugs from the cytosol to the digestive vacuole [5]. Almost all drugs developed against *P. falciparum* are metabolized in the human liver through cytochrome P450 enzymes [6]. Genetic polymorphisms of these enzymes determine distinct metabolic phenotypes: slow metabolizer phenotypes accumulate higher plasma concentrations of drugs and show an increased elimination half-life, experiencing adverse side effects; on the other hand, extensive or ultra-rapid metabolizers show lower plasma levels from a standard drug dose [7].

Previous studies have shown that, subfamilies of CYP450 including Cyp 2b, 2c, 2d, 2e, 3a and 4a play a role in the metabolism of ACTs [8, 9]. Human CYP2C8 is involved in the metabolism of important drugs, including amodiaquine (AQ) and chloroquine (CQ) [10]. CYP2C8 is the only enzyme involved in the biotransformation of AQ [10]. CYP2C8 gene is known to be polymorphic with 20 single nucleotide polymorphisms identified thus far. However, only CYP2C8*2, CYP2C8*3, CYP2C8*4 and CYP2C8*5 were reported to be different from CYP2C8*1 in enzyme activity. CYP2C8*1 was the first wild-type CYP2C8 gene sequence reported [11], and the distribution of variant alleles differs among ethnic populations [10]. CYP2C8*2, the variant most common in Africans, is related to a poor metabolizer phenotype (PM) in subjects carrying at least one copy of the defective allele [10, 12]. Subjects who are poor metabolizers experience a longer drug half-life [13] and have increased adverse side effects. In particular, CYP2C8*2 shows six fold lower intrinsic clearance of AQ than wild type [13].

Polymorphic CYP2C8 is the metabolizer of Amodiaquine to Desethylamodiaquine which is the active metabolite. Variations in the functions of drug metabolizing enzymes can affect the treatment outcome of the patient due to alterations of its bioavailability [14, 15]. This gene variant may therefore have important implications in malaria treatment and possibly contribute to the selection of drug resistant parasite. We evaluated, the following 2 parameters: (1) prevalence of *Pfmdr1* N86Y polymorphisms and (2) frequency of the human cytochrome P450 CYP2C8*2 allele. Moreover, to evaluate the possible relationship between cytochrome P450 CYP2C8*2 and *Pfmdr1* N86Y polymorphisms in patients with uncomplicated malaria in Yaounde, Centre Region of Cameroon.

2. Materials and Methods

2.1. Study Area

This study was conducted in Yaounde. Yaounde is the capital of the Centre Region of Cameroon with an estimated population of 2.5 million people. Malaria transmission in Yaounde is perennial with two main seasons: the long wet season from February to November (with more intense rains between September and November) and a short dry season from June to July and December to January [16].

2.2. Screening and Enrolment

Eligible patients aged 6 months to 15 years with acute uncomplicated falciparum malaria were screened at the outpatient department and informed consent obtained. Criteria for inclusion were; patients of either gender, suffering from acute uncomplicated *P. falciparum* malaria confirmed by microscopy, or presenting with fever (axillary temperature ≥ 37.5°C) or having a history of fever in the preceding 24 h. A presentation was made of the anticipated risks and benefits, the discomfort to which the subjects were exposed, as well as the right to interrupt the participation at any time on their own free-will. A total of 100 patients were enrolled for the study.

2.3. Sample Collection and DNA Extraction

Finger-prick blood was collected and spotted on filter paper at inclusion for genomic (or molecular analyses). Blood spots on the filter paper were excised with a sterile pair of surgical scissors. DNA was extracted from dried blood spots heating at 100°C in Chelex-100 in buffered Tris-EDTA as previously described [17]. The DNA was stored in a Tris-EDTA buffer at -20°C until allelic discrimination analysis was done by PCR-RFLP.

2.4. Genotyping Single Nucleotide Polymorphisms in CYP2C8 and Pfmdr-1 Genes

Amplification of the *CYP2C8* gene was done according to the adapted approach of Dai *et al.,* [18] using primers (*cyp2C8*F: 5'-AAGATACATATATCTTATGACATG-3 *&* *cyp2C8*R: 5'- ATCCTTAGTAAATTACAGAA GG-3'). For a total of 25µl, the reaction mixture (NEBiolabs) was composed of PCR water, buffer (10X thermopol buffer), 10mM dNTPs (200µM of each deoxyribonucleotide), 0.8µM of each primer, 1.25U/µl of Taq polymerase DNA and 5µl of DNA extract. The T3 thermal cycler (Biometra, UK) was used for the PCR amplification of target genes. Cycling conditions were as follows: 94°C for 5 minutes (pre-denaturation), 45 cycles of 94°C for 20 secs (denaturation), 55°C for 20 secs (annealing), and 72°C for 20 secs (extension). Final extension of amplicons was carried out at 72°C, after which PCR products were stored at 4°C for immediate use or -20/80°C for long-term use. The restriction enzyme Bcl I was used in

digesting the *CYP2C8* gene according to a previously described protocol [18].

For de *Pfmdr-1* gene, DNA samples were amplified by nested polymerase chain reaction–restricted fragment length polymorphism (PCR-RFLP) technique to identify N86Y point mutation in *Pfmdr*1. For outer, the primers used to amplify were: *MDR1F* 5'-GCG CGC GTT GAA CAA AAA GAG TAC CGC TG-3'and *MDR2R* 5'-GGG CCC TCG TAC CAA TTC CTG AAC TCAC-3'. A total volume of 25 µl and the reaction medium was composed of PCR water, buffer (10X thermopol buffer), 10mM dNTPs (200 µM of each deoxyribonucleotide), 0.8 µM of each primer, 1.25 U/µl of Taq DNA polymerase and 3ng of DNA extract for each amplification. The amplification program previously recorded in the thermocycler (T3 thermal cycler, Biometra, UK) proceeded as follows: the pre-denaturation at 95°C for 5 minutes followed by 45 amplification cycles of which each cycle is a succession of denaturation at 92°C for 30 seconds, annealing at 48°C for 30s, elongation at 65°C for 1 minute. The final extension which comes at the end of these cycles was carried out at 65°C for 5 minutes. For the inner amplification we used the following primers to amplify the *Pfmdr-1* gene: *MDR3F*: 5'-TTT ACC GTT TAA ATG TTT ACC TGC-3 and *MDR4R*: 5'-CCA TCT TGA TAA AAA ACA CTT CT-3'. A total volume of 25 µl and the reaction medium was composed of PCR water, buffer (10X thermopol buffer), 10mM dNTPs (200 µM of each deoxyribonucleotide), 0.8 µM of each primer, 1.25 U/µl of Taq DNA polymerase and 1µl of amplicons from the outer amplification was used. The amplification program previously recorded in the thermocycler (T3 thermal cycler, Biometra, UK) proceeded as follows: the pre-denaturation at 95°C for 5 minutes followed by 20 amplification cycles of which each cycle is a succession of denaturation at 92°C for 30 seconds, annealing at 48°C for 30s, elongation at 65°C for 1 minute. The final extension which comes at the end of these cycles was carried out at 65°C for 5 minutes. The expected band was 291bp, according to the protocol already available. The PCR product was then incubated with *AFlIII* enzyme. Digested and undigested fragments of each sample were separated on a 2% agarose gel stained with ethidium bromide.

2.5. Statistical Analysis

Data were analyzed using the statistical programs SPSS for Windows version 10.01 (SPSS, Chicago, IL). P values of the association of *CYP2C8*2* and *Pfmdr-1.* were obtained by Chi square test. Unadjusted odds ratios (ORs) were calculated with 95% confidence intervals (CIs). All tests of significance were two-tailed. P values < 0.05 indicated statistical significance.

3. Results

3.1. Characteristics of Study Population

Of the 100 participants recruited in the study, the mean age was 6,13 years. The mean temperature was 37.807°C with an average weight of 22.19 kg (Table 1).

3.2. Allelic and Phenotype Frequencies of the CYP2C8 and Pfmdr1 Genes

For CYP2C8, we determined that there were 2 alleles in our study population with the predominance of the wild-type CYP*2C8*1* allele, at a frequency of 62%. And for *Pfmdr1*gene the most predominant allele was 86Y (57%) (Table 2).

3.3. CYP2C8 and Pfmdr-1 Genes

To investigate whether the *CYP2C8*2* variant affects the risk of being infected by *P. falciparum* resistant strains, we estimated the prevalence of drug resistance marks in infections according to the *CYP2C8* alleles. As shown in Table 3, trends for a higher risk of carrying *pfmdr1-86Y* resistant *P. falciparum*, in subjects carrying the *CYP2C8*2* allele. Considering the whole sample, the presence of the *CYP2C8*2* allele was associated with a higher risk of harboring a Multi-drug resistant *P. falciparum* infection (OR, 2.446; 95% CI, 1.080 - 5.536; Pv.03).

Table 1. *Study population demographics.*

Variables	Min	Max	Mean ± standard deviation
Age (years)	1	15	6,13 ± 3,60
weight (kg)	7	49	22,19 ± 10,95
Temperature (°C)	36	40,5	37,807 ± 1,18

Table 2. *Frequency of the CYP2C8 and Pfmdr-1 gene.*

***CYP2C8* ALLELE**	**FREQUENCY**	***Pfmdr-1* ALLELE**	**FREQUENCY**
*CYP2C8*1 (Fast)*	62%	*N86 (Wild-type)*	43%
*CYP2C8*2 (Slow)*	38%	*86Y (Mutant)*	57%

Table 3. *Association between CYP2C8*2 allele and 86Y mutation of Pfmdr-1.*

		Genotype *Pfmdr*-1		**Odds Ratio**	**95% CI**	***P value***
		N86 (*Wild-type*)	**86Y (*Mutant*)**			
Genotype *CYP2C8*	CYP2C8*1 *(Fast)*	25	23	1.693	1.693- 1.037	.050
	CYP2C8*2 *(Slow)*	16	36	2.446	1.080 - 5.536	.030*
Total		41	59			

OR, Odd ratio; CI, confidence interval; *, significant correlation.

4. Discussion

The discovery of functional variability in genes encoding drug metabolizing enzymes has contributed significantly to the understanding of the inter-individual variability in dose-concentration relationships and drug response. Knowledge of polymorphisms in genes encoding enzymes that metabolize anti-malarial drugs, as well as their associations with N86Y *Pfmdr*1 resistance marker, can be useful in designing dosage regimens and modulating drug therapy that is safe, effective and therefore less likely to select for pathogen's drug resistance.

In this work the mutant allele CYP2C8*2 had a frequency of 38% which is similar to what was observed in the study in Tanzania [19], and Ghana [20]. High prevalence of mutation 86Y (57%) *Pfmdr1* obtained in our study population corroborates the results of work carried out in Equatorial Guinea [21]. But it is even higher compared to studies conducted in Bangui in the Central African Republic [22] and south-eastern India [23]. The increase in the frequency of the 86Y mutation of *Pfmdr1* could have been due to the adoption of ACT as the first line of treatment for *P. falciparum* malaria in malaria-endemic countries, which would probably have contributed to the increase in drug pressure thus promoting the selection of AQ resistant strains. This frequency of 86Y SNP is to be taken seriously because this mutation is associated to resistance of chloroquine, quinine and amodiaquine which are antimalarial used in the artemisinin based combination therapies [24]. The high prevalence of this mutation in our study may affect the effectiveness of ACTs.

This study has shown that there is an association between mutant allele *CYP2C8*2* and 86Y mutant allele of *Pfmdr-1* gene. *CYP2C8* is the enzyme that metabolizes antimalarial drugs in phase 1, so a mutation of the latter will make the duration of the half-life of the drug longer. *Pfmdr1* gene is involved in transporting the active ingredient into the vacuole of the parasite to destroy it. The *CYP2C8*2* mutation will cause a drug pressure and the concentration of the active ingredient of the drug carried by the *Pfmdr1* necessary to kill the parasite will not be available, the parasite will find a way to survive by simply creating a mutation on its *Pfmdr1* [13]. The presence of these mutant *CYP2C8*2* alleles in our study population could thus be responsible for the other observed adverse effects associated with the administration of AQ.

5. Conclusion

At the end of this study we show that *P. falciparum* resistant *Pfmdr1* 86Y allele is associated with CYP2C8*2 mutation. The apparition of the latter may have thus been influenced by the presence of the former leading to the selection of AQ-resistant strains present in the study population. This study thus reiterates the potential role played by CYP2C8*2 mutations in AQ resistance and warrants the continuous monitoring of these mutations as well as resistance-associated genotypes of *Pfmdr1* in malaria endemic areas where AQ is used as partner drug in ACT regimens.

Authors' Contributions

WFM, JPKC, PMN contributed to the design of the study. JPKC, PMN, coordinated the study. CMM, CTF, NLN supervised the sample collection. JPKC, AHME, CTF performed the molecular analysis. PMN, AHME, CMM performed data analysis. JPKC, PMN, NLN drafted the manuscript. All authors contributed in the revision of the manuscript and approved the final version of the manuscript prior to submission.

Conflict of Interest

All the authors do not have any possible conflicts of interest.

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