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PHARMACOGENETICS

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CYP2C8 polymorphism frequencies among malaria patients in Zanzibar

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Abstract *Objective*: The determination of the prevalence of the *CYP2C8* main alleles in a typical set of malaria patients in Zanzibar, as these patients represent a typical population exposed to amodiaquine, an antimalarial mainly metabolized by CYP2C8. Also, to determine for the first time the frequencies of *CYP2C8* alleles in native African populations.
Methods: Polymerase chain reaction–restriction fragment polymorphism for the identification of *CYP2C8*1*, *CYP2C8*2, CYP2C8*3* and *CYP2C8*4* on a random population of 165 unrelated malaria patients.
Results: The allele frequencies found were: *CYP2C8*1 (wild type, 83.4%)*, *CYP2C8*2* (13.9%), *CYP2C8*3* (2.1%) and *CYP2C8*4* (0.6%). In terms of genotypes, 70.4% of the patients showed the *CYP2C8*1/CYP2C8*1* genotypes, while heterozygous between the wild type and other minor alleles were seen in 26.0%. Finally, 3.6% of the patients were homozygous for slow metabolizer alleles. The frequencies observed are equivalent to those documented for African-Americans.
Conclusions: *CYP2C8* non-wild type alleles have a significant prevalence in the East African population studied. The consequent frequency of 3.6% of patients homozygous for slow metabolizer alleles represent a significant fraction of the population potentially in higher risk of adverse effects due to a less efficient metabolism of amodiaquine. As approximately 10^6 first-line treatments are currently performed in Zanzibar per year, this represents a non-negligible absolute number of amodiaquine exposures. This information constitutes a background for the pharmacovigilance programs presently being employed in Zanzibar.

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Introduction

Plasmodium falciparum malaria is a major infectious disease, with special relevance in sub-Saharan Africa. Most of the annual 1–2 million lethal cases occur in this region, to a large extent in children under the age of 5 years [1].

Combination therapy using drugs that differ in terms of mode of action has been recently proposed as a global strategy for malaria control [1]. An antimalarial drug that has recently received particular attention as a candidate for use in combination therapy is amodiaquine (AQ). This 4-aminoquinoline is still largely effective against chloroquine and sulfadoxine–pyrimethamine resistance parasites. Its low cost allows for large-scale employ on the African continent.

AQ is rapidly metabolized to a main metabolite, *N*-desethylamodiaquine (DEAQ), among other minor compounds. The conversion of AQ to DEAQ shows significant inter-individual variability in its pharmacokinetic parameters [2, 3]. Recently, it was shown that the reaction in the liver is mainly catalyzed by the polymorphic CYP2C8 [4].

The *CYP2C8* gene locates at chromosome 10q24, in cluster with *CYP2C9*, *CYP2C19* and *CYP2C18* [5]. Among the *CYP2C8* alleles presently known, *CYP2C8*2* (I269F), *CYP2C8*3* (R139K, K399R) and *CYP2C8*4* (I264M) are documented to lead to enzymes with decreased in vitro activity toward the probe drug paclitaxel [6, 7]. This raises the possibility of the CYP2C8 polymorphism being a significant player on the modulation of

AQ metabolism and, thus, on the accumulation of the drug associated with its potential adverse effects. The prevalence of these alleles have been suggested to differ significantly between ethnic groups [6–8].

Starting in 2003, Zanzibar is one of the first areas in Africa employing artemisinine-derivative-based combination therapy on a large scale [9]. The selected combination for the first line treatment is artesunate/amodiaquine.

Amodiaquine is not presently recommended by the World Health Organization as a prophylactic drug, due to a 1 : 2,000 incidence of life-threatening secondary effects, such as agranulocytosis and hepatotoxicity [10]. The drug has, nevertheless, been applied in the treatment of uncomplicated malaria, when the risk posed by the infection outweighs the risk for toxicity. However, the issue of possible side effects from long-term repeated use of this drug remains open, especially in a moment where an increase of the use of AQ in combination therapy is soon expected.

In this work, we have specifically analyzed the heterogeneity of the *CYP2C8* gene in a population of malaria-affected children in Zanzibar, Tanzania, where malaria is the main public health problem, with parasite prevalence rates between 40% and 60% [11]. As children under 5 years represent up to 50% of the deaths, our studied subjects represent a typical population group that will be exposed to AQ in the future, following the new policy of AQ–artesunate as first line treatment.

Materials and methods

Subjects

The studied population comprised 165 randomly selected children (average age: 2.1 years, SD = 1.1 years, 88 female and 75 male subjects) with uncomplicated malaria from Unguja ($n = 37$) (Uzini Health Care Unit) and Pemba ($n = 128$) (Konde Health Care Unit) between May and December 2001. All patients enrolled in this study were verified for the presence of *P. falciparum* using microscopic analysis and polymerase chain reaction (PCR)-based standard methods (amplification of the *pfdhfr* gene). All the applied protocols were approved by the ethics committee of the Karolinska Institute and the responsible local authorities (Zanzibar National Malaria Program) and followed the recommendations of the Declaration of Helsinki, promulgated in 1964. Blood samples were obtained after written informed consent in the local language (Swahili) from the children's responsible guardians.

Molecular analysis

Peripheral blood samples were obtained by finger pick and preserved in Whatman Chr filter paper. Genomic DNA was extracted as described elsewhere [12].

PCR–restriction fragment length polymorphism (RFLP) methods were applied for the analysis of *CYP2C8* SNPs: CYP2C8 G416A (Arg139Lys) and A1196G (Lys399Arg). Briefly, primers 5′-AGG CAA TTC CCC AAT ATC TC-3′(sense) and 5′-ACTCCT CCA CAA GGC AGT GA-3′(antisense) were used for PCR amplification, followed by restriction with *Bse*RI (New England Biolabs, Beverly, USA) [6] for the analysis of G416A. For the detection of A1196G, a PCR was performed with primers 5′-CTTCCG TGC TAC ATG ATG ACG-3′(sense) and 5′-CTGCTG AGA AAG GCA TGA AG-3′(antisense) followed by restriction with *Bcl*I (New England Biolabs). PCR-RFLP analysis of C792G (Ile264Met) and A805T (Ile269Phe) SNPs was performed with primers 5′-ATGTTG CTC TTA CAC GAA GTT ACA-3′(sense) and 5′-ATCTTA CCT GCT CCA TTT TGA-3′ (antisense) as a modification of the protocol by Bahadur et al. [7]. The resulting amplicon was restricted with *Taq*I (Fermentas, Vilnius, Lithuania) and *Bcl*I (New England Biolabs) for the analysis of C792G and A805T, respectively.

DNA sequencing was applied for confirming the presence of heterozygous patterns, as opposed to incomplete digestion situations. This was performed using dye primer cycle sequencing kit (Applied Biosystems, UK) in an ABI PRISM 377 DNA sequencer.

Statistical analysis

Allelic frequencies and confidence intervals were assessed using the program CIA (Confidence Interval Analysis) [13]. Chi-square (χ^2) testing was performed with Microstat software (Ecosoft Inc, Indianapolis, IN, USA). For the evaluation of Hardy–Weinberg equilibrium on the analyzed SNPs, the GenePop software pack was applied (http://wbiomed.curtin.edu.au/genepop/).

Results

The frequency of alleles that lead to CYP2C8-altered enzymatic activities represent altogether 16.6% of the total set of alleles herein studied (Table 1).

*CYP2C8*2* is the most frequent mutated allele here described (13.9%), while *CYP2C8*3* and *CYP2C8*4* show frequencies of 2.1% and 0.6%, respectively. The two SNPs specific for the definition of the *CYP2C8*3* allele—G416A (R139K) and A1196G (K399R) [6]—were consistently seen in linkage.

In terms of genotypes, 3.6% of individuals were found homozygous for the presence of mutated alleles. When concerning the number of individuals carrying at least one mutated allele, the frequency observed reached 29.6%, more than a quarter of the studied population (Table 1).

The *CYP2C8*2* and *CYP2C8*3* alleles were found to be in Hardy–Weinberg equilibrium ($\chi^2 = 7.8140$, $P = 0.2520$). The low frequency of *CYP2C8*4* did not allow a meaningful test to the equilibrium.

Table 1 Frequencies of alleles observed for *CYP2C8* genotypes in the population of 165 *P. falciparum* malaria patients from the Zanzibar islands

	G416A (R139K)	C792G (I264M)	A805T (I269F)	A1196G (K399R)	*n*	Frequency (95% confidence interval
*1/*1	−/−	−/−	−/−	−/−	116	0.704 (0.633–0.773)
*1/*2	−/−	−/−	+/−	−/−	36	0.218 (0.155–0.281)
*2/*2	−/−	−/−	+/+	−/−	5	0.030 (0.010–0.069)
*1/*3	+/−	−/−	−/−	+/−	5	0.030 (0.010–0.069)
*3/*3	+/+	−/−	−/−	+/+	1	0.006 (0.0002–0.033)
*1/*4	−/−	+/−	−/−	−/−	2	0.012 (0.015–0.043)

Discussion

Scarce information is currently available concerning *CYP2C8* pharmacogenetic variation among different ethnic groups, this being especially true for native African populations. Our work represents the first significant set of available data, in the frame of a rising awareness of the necessity for this type of studies in the developing world [14]. In this study, a non-negligible frequency of 16.6% of variant alleles (**2, *3* and **4*) was found in a population of children representing the typical subjects, which are presently exposed to AQ in Zanzibar. *CYP2C8*2* is the most frequent non-wild type allele in Zanzibar, the *CYP2C8*3* and *CYP2C8*4* being less predominant in this African native population.

When comparing the observed allele frequencies with other studies available in the literature, significant differences were found with Caucasian populations (Table 2). These include the observed for *CYP2C8*2* ($\chi^2 = 26.515$, $P < 0.001$), **3* ($\chi^2 = 25.497$, $P < 0.001$) and **4* ($\chi^2 = 15.704$, $P < 0.001$) in the North East England population studied by Bahadur et al. [7] as well as for the three alleles in a study in Germany [15]—*CYP2C8*2* ($\chi^2 = 21.453$, $P < 0.001$), **3* ($\chi^2 = 23.632$, $P < 0.001$) and **4* ($\chi^2 = 15.807$, $P < 0.001$) and in a recent study performed in Central Spain limited to the *CYP2C8*3* allele ($\chi^2 = 26.786$, $P < 0.001$) [16] (Table 2). These differences are due to the fact that *CYP2C8*2* allele is rare among these populations, with the **3* and **4* being more prevalent, the inverse of that observed in Zanzibar. No significant differences were found when comparing our results with a study performed in African-Americans [6], a similar intra-ethnic trend as observed among Caucasian populations, suggesting a limited diversity among populations belonging to the same broad ethnic group. This is in contrast to what has been observed with other genes, such as *CYP2D6* [17].

In addition, our results reinforce the probable singularity of the Japanese population previously shown to be almost devoid of *CYP2C8* polymorphism [8].

Recently, the *CYP2C8*3* allele has been shown to be significantly linked to *CYP2C9*2* in a large study [18]. Extrapolating these findings to our data, it is expected for *CYP2C9*2* to be present in a frequency of 2–3%, a value in line with previously documented prevalence of this allele in East African regions (0–4%) [19, 20].

AQ toxicity has been associated with the formation of a quinoneimine reactive moiety able to irreversibly bind to cellular proteins, as well as to induce an immune response [21, 22]. DEAQ shows significantly reduced capacity to generate this quinoneimine, when compared with AQ [23], suggesting the formation of DEAQ to be a pathway associated with a decreased risk of the advent of quinoneimine-related cytotoxic effects. It is, therefore, expected that polymorphisms in the *CYP2C8* gene might modulate the risk of AQ adverse effects.

Table 2 Comparison of *CYP2C8* SNP frequencies in different populations

Allele	Population origin	Number of subjects	Allele frequency (95% confidence interval)	Reference
2C8*2	Zanzibar	165	0.139 (0.102–0.177)	–
	US African-Americans	82	0.183 (0.124–0.242)	[7]
	North East England	116	0.004 (0.0001–0.024)	[8]
	West Germany	244	0.016 (0.005–0.041)	[13]
	Japan	360	0.000	[15]
2C8*3	Zanzibar	165	0.021 (0.009–0.043)	–
	US African-Americans	82	0.018 (0.004–0.053)	[7]
	North East England	107	0.150 (0.102–0.197)	[8]
	West Germany	244	0.140 (0.096–0.183)	[13]
	Sweden	1468	0.095 (0.009–0.106)	[16]
	Central Spain	130	0.150 (0.107–0.193)	[14]
	Japan	360	0.000	[15]
2C8*4	Zanzibar	165	0.006 (0.001–0.022)	–
	North East England	107	0.075 (0.043–0.119)	[8]
	West Germany	244	0.074 (0.044–0.114)	[13]
	Japan	360	0.000	[15]

In Zanzibar, approximately one million doses of AQ–artesunate are employed per year as first-line treatment for uncomplicated malaria (A. Bhattarai, Karolinska Institute, personal communication). As such, and in accordance with our data, 30,000–40,000 events of a CYP2C8 slow metabolizer being exposed to AQ are expected to also occur per year.

In conclusion, we observed that the main population exposed to AQ in Zanzibar shows a non-negligible incidence of *CYP2C8* poor metabolizer alleles, in particular, if taking into account the very high prevalence of malaria in these islands; further studies are warranted to clarify the relationship between side effects of AQ and *CYP2C8* genetic polymorphism. These observations should represent a background in frame of the pharmacovigilance program ongoing in the Zanzibar islands, following the launch of AQ–artesunate as the first-line combination therapy.

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