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Allele and genotype frequencies of cytochrome P450 2B6 and 2C19 genetic polymorphisms in the Nigerian populations: Possible implication on anti-retroviral and anti-malarial therapy

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The study was carried out to investigate the distribution of polymorphic *CYP2B6* and *CYP2C19* alleles and genotype frequencies in the three major Nigeria ethnic groups in order to evaluate their implications on therapeutic outcome. Three hundred unrelated subjects from the three major Nigerian ethnic groups of Hausa, Ibo and Yoruba who consented to the study were genotyped for *CYP2B6**6, *CYP2C19**2 and *3 alleles using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) and DNA sequencing techniques. The frequencies of the *CYP2B6**6 poor metabolizer (PM) genotype (15631G>T (Q172H) and/or 18053A>G (K262R)) were not significantly different ($p>0.05$) occurring at 21% in Hausa, 17% in Ibo and 17% in Yoruba while the corresponding allele frequencies were 42, 36, and 42%, respectively. The frequencies of the *CYP2C19* PM genotype (*CYP2C19**2/*2) were significantly different ($p>0.05$) and found to be 2.1% in Hausa, 8% in Ibo, and 0% in Yoruba, while for the intermediate metabolizer (IM) genotype (*CYP2C19**1/*2), the frequencies were 20.6, 41.6 and 20.8%, respectively. The defective *CYP2C19**3 allele, prevalent in oriental populations, was not detected in this study. The *CYP2B6**6 allele frequency was being evaluated for the first time in Nigerian populations with no significant variation amongst the three major ethnic groups but occurs at a high frequency. The PM genotype of frequency, *CYP2C19**2/*2 showed significant variations in Nigerian populations, especially the 8% found in the Ibos.

Key words: Genetic polymorphisms, CYP2B6, CYP2C19, genotyping, Nigerian populations.

INTRODUCTION

Genetic variability in drug metabolism is mostly associated with inter-individual and inter-ethnic variation in the pharmacokinetic and pharmacodynamic response to drugs. Polymorphisms in genes encoding particular enzymes may lead to absent or varied enzymatic activity. This variability may translate into the differences in efficacy or toxicity of drug substrates (Grant, 2005).

The cytochrome P450 2B6 (CYP2B6) enzyme, plays an important role in the biotransformation of several therapeutic drugs in humans, including the non-nucleoside reverse transcriptase inhibitor (NNRTI), efavirenz, the cytostatic prodrug, cyclophosphamide; the anti-depressant, bupropion; the narcotics, ketamine and propofol; and the anti-malarial, artemisinin (Ward et al., 2003). The *CYP2B6* gene exhibits extensive polymorphism reflected currently by over 40 allelic variants (Nelson, 2009). Of these, the most common is 15631G>T, (Q172H) and/or 18053A>G (K262R), which

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together define the common *CYP2B6**6 allele. This allele, which is most common in Africans has been associated with higher efavirenz plasma concentrations due to lower clearance rates and may have implications in dosage adjustment for individuals carrying the mutation (Nyakutira et al., 2008). Several amino acid variants were initially found to be associated with decreased expression of CYP2B6 protein in human liver and/or to be deficient in heterologous expression systems (Lang et al., 2001; Lamba et al., 2003).

The clinical relevance of CYP2B6 polymorphisms was sequel to the discovery that the NNRTI, efavirenz, is a specific drug substrate of this enzyme. The oxidative metabolic pathways of efavirenz catalyzed by CYP2B6 results in the production of 8-hydroxy- and 8, 14-dihydroxyefavirenz with CYP3A playing only a minor role (Ward et al., 2003). An extensive inter-individual difference in efavirenz bioavailability resulting in central nervous system toxicity was reported in a population pharmacokinetic study (Csajka et al., 2003).

The CYP2C19 enzyme is an important member of the CYP2C enzyme subfamily which, metabolizes about 20% of important drugs used in clinical practice including, the proton-pump inhibitors (omeprazole, lansoprazole and rabeprazole), sertraline, nelfinavir, fluoxetine diazepam, mephenytoin and proguanil. The polymorphism of CYP2C19 enzyme has been associated with reduced capacity to dispose drugs, the consequences of which may be clinically significant, especially for a prodrug like proguanil that requires bioactivation (Wedlund, 2000).

The molecular basis giving rise to the phenotypes for CYP2C19 have been well characterized for orientals and caucasians (Kim et al., 2004) while a number of similar (though limited) genotyping studies have been carried out among African populations (Masimirembwa and Hasler 1997; Dandara et al., 2001), data from the Nigerian population is scanty. The major alleles of *CYP2C19* gene that are responsible for more than 95% of cases of poor metabolism (PM) of the relevant medications are the *CYP2C19**2 (splicing defect) and *CYP2C19**3 (W212X), which occurs due to mutations on the exons 5 and 4 of the *CYP2C19* gene, respectively. However, *CYP2C19**2 allele frequency has been reported in a number of African populations with frequencies of 21.7% in South African Venda, 13% in Zimbabweans (Dandara et al., 2001), 13% in Ethiopians (Herrlin et al., 1998; Aklillu et al., 2002), between 9.7 and 17.9% in Tanzanians (Herrlin et al., 1998; Bathum et al., 1999) and most other populations (Kim et al., 2004). On the other hand the *CYP2C19**3 allele is rare in Black-African populations, with a reported frequency in most cases of 0 to < 1% (Aklillu et al., 2002; Herllin et al., 1998) but it is most common among Asian populations, with frequencies ranging from 4 to 6% (Kimura et al., 1998).

Nigeria is a multiethnic society with the largest population in the African continent and a home land to at

least 250 languages (Robinson, 2004; <http://en.wikipedia.org/wiki/Nigeria>) but it is comprised mainly of three major ethnic groups namely: Hausa, Ibo and Yoruba, which are predominantly found in the Northern, Eastern and Western part of Nigeria, respectively. The three major ethnic groups together constitute over 65% of the Nigerian Population Commission (2006) (<http://www.population.gov.ng>).

This study was intended to provide for the first time the baseline of alleles and genotype frequencies of *CYP2B6**6, *CYP2C19**2 and *3 in the Hausa, Ibo and Yoruba ethnic populations of Nigeria. This was with a view to establish the possible impact of these polymorphisms on the treatment of malaria and HIV/AIDS, which are prevalent diseases in Nigerian populations.

MATERIALS AND METHODS

Subjects

Three hundred healthy, unrelated subjects consisting of 215 males and 85 females, aged 18 to 45 years, who met the study inclusion criteria, were randomly selected, from the three major Nigerian ethnic groups of Hausa, Northern region (N = 98), Ibo, Eastern region (N = 101) and Yoruba, Western region (N = 101) of Nigeria. Details of the study procedures were explained to the potential subjects after which, they were given an opportunity to make an independent decision to participate in the study. Eligible subjects were enrolled after signing the consent form and were classified as belonging to a particular ethnic group based on family history up to two previous generations. The ethics committee of Obafemi Awolowo University Teaching Hospital, Ile-Ife, approved the study.

Sampling and genomic DNA preparation

Qualified personnel withdrew 5 ml of blood sample from each participant using a syringe by veno-puncture into labelled EDTA tubes. After collection, the blood samples were frozen at -20°C until further analysis. The DNA was prepared using the QIAamp DNA Blood Mini kits (Qiagen, Belgium). Each frozen blood sample was thawed at room temperature and transferred to a clean polypropylene tube after pre-purification. Four (4) sets of tubes were labelled for each sample using a convenient labelling system and each set was arranged on a separate tube rack. For the third sets of tube a column was added while for the fourth sets a secure label was made by adding sellotape on label (tubes and columns were provided in the kit). After cell lysis cellular proteins were precipitated by salt precipitation leaving the high molecular weight genomic DNA in solution, which was concentrated and desalted by isopropanol precipitation. The DNA was bound to a column/resin, washed using concentrated ethanol and eluted or dissolved in a buffer solution AE, provided in the isolation kit. After elution stage, the purity of DNA sample was determined by measuring its concentration using a UV spectrophotometer. DNA samples were then stored at 4°C prior to genotyping analysis and aliquot of the samples were stored at -20°C for long-term use.

Genotyping

Genotyping of *CYP2B6**6 and *CYP2C19**2 and *3 were performed using PCR-RFLP techniques in accordance with the method of

Table 1. Genotypes and allele frequencies of *CYP2B6*6* in the Hausa, Ibo and Yoruba of Nigeria.

CYB2B6*6 genotypes /allele frequencies	Hausa N/total (%)	Ibo N/total (%)	Yoruba N/total (%)	Mean pooled N/pooled total (%)
EM (G/G)	37/98 (38)	43/101 (43)	34/101 (34)	114/300 (38)
IM (G/T)	39/98 (40)	40/101 (40)	50/101 (50)	129/300 (43)
PM (T/T)	20/98 (20)	18/101 (18)	17/101 (17)	55/300 (18)
Allele (T)	82/196 (42)	72/202 (36)	84/202 (42)	238/600 (40)

Rotger et al. (2005) and de Morais et al. (1994), respectively while *CYP2C19*3* was genotyped by sequencing. The sequences for both forward and reverse primers for *CYP2B6*6* were GGTCTGCCCATCTATAAAC and CTGATTCTTCACATGTCTGCG, respectively, while that of *CYP2C19*2* were AATTACAACCAGAGCTTGGC and TATCACTTTCCATAAAAGCAAG. The *CYP2C19*3* primers sequences were CAGCTAGGCTGTAATTGTTAATTTCG and ACTTCAGGGCTTGGTCAATA. The specificity of the primer sequences for each gene studied was confirmed by a BLAST analysis search and comparison of genomic sequences in the National Center for Biotechnology Information (NCBI) databases (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Identical primers were used for PCR and sequencing techniques.

The PCR reactions were performed in a total of 25 µl mixture containing 5 ng genomic DNA, 200 µM of deoxynucleoside triphosphate mixture (that is, dNTPs: dATP, dCTP, dGTP, dTTP) (Roche Biochemicals, Switzerland), 0.2 µM of each primer, 2.0 mM of MgCl₂, 1xPCR buffer, 1U of Taq polymerase (Roche Biochemicals, Switzerland). All PCR reactions were carried out on MG96G programmable thermal cycler (LongGene Scientific Instruments). The PCR cycles for *CYP2B6*6* consisted of initial denaturation of the DNA by incubating the reaction mixture at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, primer extension at 72°C for 1 min and a final extension at 72°C for 6 min and the product mixture held at 4°C until further use. PCR cycles for *CYP2C19*2* and *3 consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 20 s, primer annealing at 53°C for 30 s, primer extension at 72°C for 30 s and a final extension at 72°C for 6 min. PCR products of *CYP2B6*6* and *CYP2C19*2* upon amplification were digested with *Bsr*NI and *Sma*I restriction enzymes and buffer (New England Biolabs, UK), respectively. Overnight digestion was carried out to ensure complete digestion and accurate interpretation of results.

The digested PCR products were visualized on 2% agarose gel electrophoresis. The agarose gel (2%) was prepared by weighing 6 g of agarose in a conical flask and 200 ml of 1x TAE gel buffer solution was added. The agarose was completely dissolved by heating to boil in the microwave for 1 to 2 min. The gel was allowed to cool (about 55°C) and 30 µl of ethidium bromide was added to the gel and poured onto plate. 5 µl of PCR product was loaded on the gel for analysis of amplified product and 20 µl for digestion product analysis while 10 µl of the diluted molecular weight marker was loaded to the gel. The gel was ran at 100 V, allowing migration of 2.5 to 3 cm. The result of the gel was viewed using a Gel Photo system GFS1000 (Fran Techtum Lab, Sweden).

DNA sequencing

PCR products of 20 DNA samples each from the three ethnic

groups were sequenced for *CYP2C19*3*. Sequencing of *CYP2C19*3* PCR product was performed using an ABI Prism® 3730 DNA analyzer with DNA sequencing analysis software™, version 3.6.1 (Applied Biosystems, Brussels, Belgium). The sequencing reaction mixture consisted of a total volume of 12 µl, which is made up of 5 µl of purified PCR products added to a strip tube containing 2 µl of Big Dye™ terminator version 3.0, 1 µl of 5 x sequencing buffer, 1 µl of sequencing primer (2 µM) and 3 µl of double distilled water. The sequencing cycles consisted of initial denaturation of DNA by incubating the reaction mixture at 96°C for 1 min, followed by 25 cycles of denaturation at 96°C for 10 s, primer annealing at 50°C for 5 s and primer extension at 60°C for 4 min.

Identification of SNPs was carried out using the novoSNP v2.1.9 software package (Weckx et al., 2005). Reference sequence was NC_000010.9 for *CYP2C19*. The identified SNPs were compared with the NCBI Single Nucleotide Polymorphism database (dbSNP) (<http://www.ncbi.nlm.nih.gov/SNP>). As SNPs can cause the introduction of pre-microRNA (miRNA) sites, this was included as part of the annotation in the novoSNP analysis procedure. Frequencies of SNPs were calculated using Genepop (Raymond and Rousset, 1995).

Statistical analyses

Allele and genotype frequencies were obtained by direct counting and were tested for Hardy-Weinberg equilibrium based on the chi-square (χ^2) test of observed versus predicted using the Stata intercooled statistical software version 9.0, rejecting the null hypothesis if $p < 0.05$.

RESULTS

The genotype and allele frequencies of *CYP2B6*6* are shown in Table 1. The poor metaboliser (PM) genotype frequency (T/T) was not significantly different ($p > 0.05$) in the Hausa, Ibo and Yoruba populations. The genotype and allele frequencies distribution in these populations showed no deviation from the Hardy-Weinberg expectations.

The detection of *CYP2C19*2* allele following analysis on 2% agarose gel electrophoresis is shown in Figure 1. The gel picture shows fragmentation patterns of the PCR products with restriction enzyme *Sma*I, which indicates that the PCR products from subjects carrying exon 5 mutation were not fragmented, hence a 169 bp was obtained (NGH19). The PCR products lacking the exon 5 mutation were digested by *Sma*I into two fragments of 49

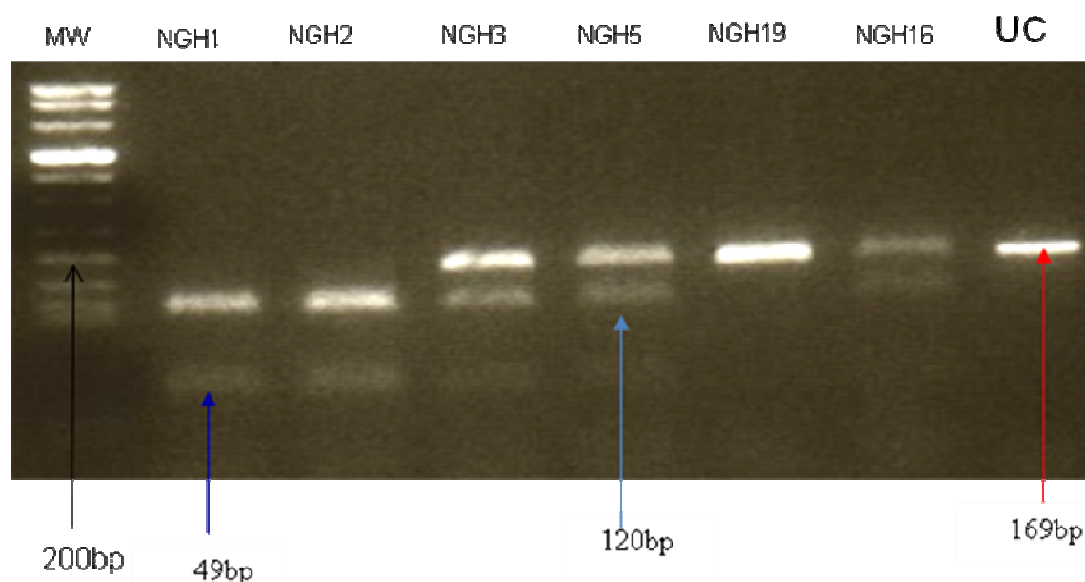


Figure 1. PCR-RFLP analysis of the *CYP2C19*2* allele (exon 5 mutation), MW: molecular weight marker, UC: undigested positive control.

Table 2. *CYP2C19* genotype and allele frequencies in the Hausa, Ibo and Yoruba ethnic groups of Nigeria.

	CYP2C19 genotype <i>CYP2C19*<i>X</i></i> (<i>X</i> = allele no.)	Hausa** N/total (%)	Ibo N/total (%)	Yoruba N/total (%)	Mean pooled N/pooled total (%)
EM	*1/*1	75/97 (75.8)	51/101 (50.5)	80/101 (80)	206/299 (68.9)
IM	*1/*2	20/97 (20.6)	42/101 (41.6)	21/101 (21)	83/299 (27.8)
PM	*2/*2	2/97 (2.1)	8/101 (8)	0/101 (0)	10/299 (3.3)
	*3/*3	0/20 (0)	0/20 (0)	0/20 (0)	0/60 (0)
Alleles	<i>CYP2C19*1</i>	170/194(88)	144/202(71)	181/202(90)	495/598 (82.8)
	<i>CYP2C19*2</i>	24/194 (12)	58/202 (29)	21/202 (10)	103/598 (17.2)
	<i>CYP2C19*3</i>	0/40 (0)	0/40 (0)	0/40 (0)	0/120 (0)

Note: **data was available only for 97 subjects.

bp and 120 bp (NGH1 and NGH2) while the PCR products carrying heterozygous mutation at exon 5 gave a cleavage pattern of 120 bp and 169 bp (NGH3 and NGH5). Table 2 shows the *CYP2C19* genotype and allele frequencies in the Hausa, Ibo and Yoruba populations. In this study the *CYP2C19*1* allele was used as the wild type, which predicts extensive metabolism (EM) in homozygous state while *CYP2C19*2* as one which predicts poor metabolism (PM) in homozygous individuals. The sequencing result showed that *CYP2C19* (636 G>A), the major single nucleotide transition that defines the defective *CYP2C19*3* allele was found to be absent in this study. Therefore, the genotypic PMs in the Nigerian populations were mainly due to the defective *CYP2C19*2*. The genotype and allele frequencies of

*CYP2C19*2* were significantly ($p<0.05$) different in the Nigerian populations, especially, the 8% *CYP2C19* PM genotype found in the Ibo, although there was no deviation from the Hardy–Weinberg expectations.

DISCUSSION

Genetic variability in drug metabolizing enzymes has been assessed in different ethnic groups and a number of functional variants discovered, among these, the 15631G>T, (Q172H) and/or 18053A>G (K262R) SNPs, defines the *CYP2B6*6*, a null allele most prevalent in African populations (Klein et al., 2005). *CYP2B6* polymorphisms affect the pharmacokinetics of the NNRTI,

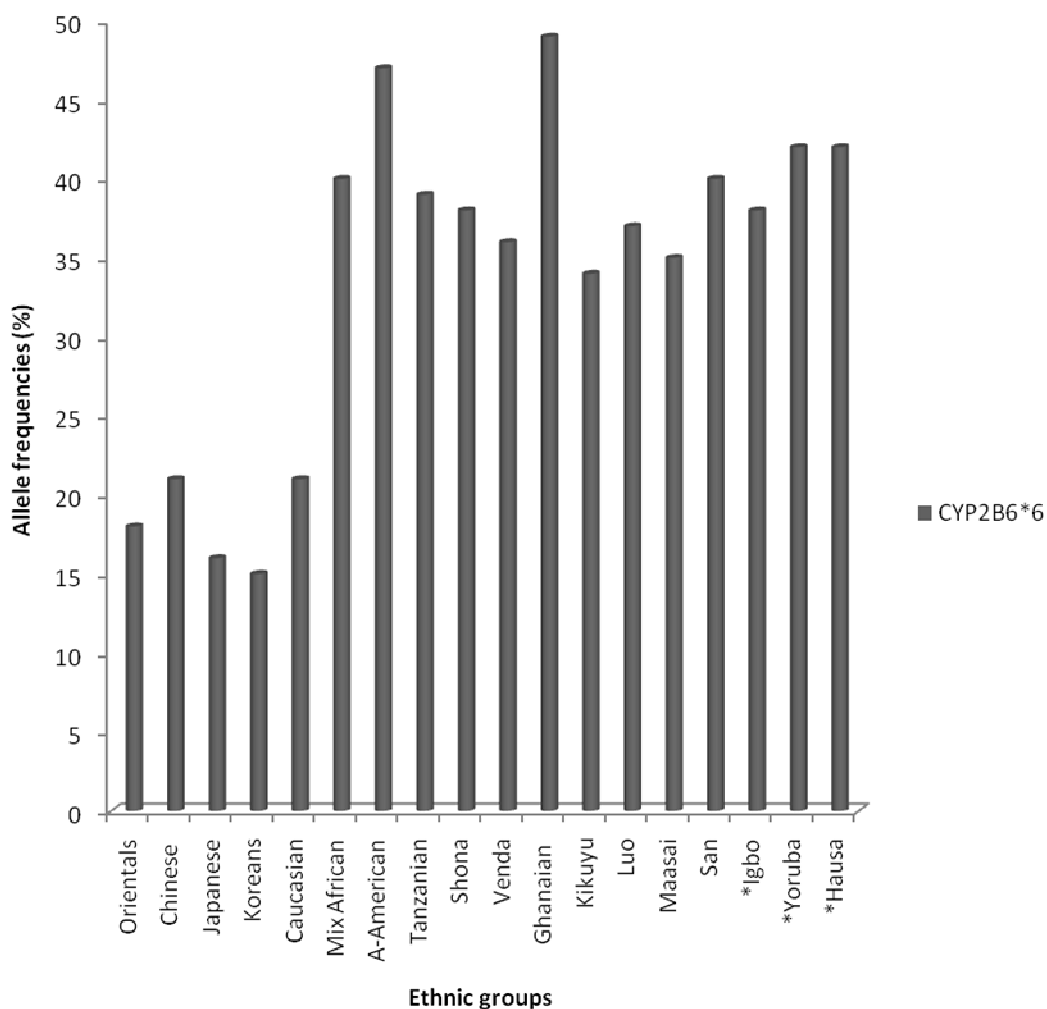


Figure 2. Allele frequencies of CYP2B6*6 in the Hausa, Ibo and Yoruba populations of Nigeria in this study and other African populations, as well as other ethnic/race populations in literature (* ethnic groups in this study).

efavirenz with possible implications of drug toxicity due to high plasma levels and drug resistance resulting from drug levels below minimum effective concentration in individuals undergoing HIV/AIDS treatment when given a normal dose (Rodriguez-Novoa et al., 2006).

The *CYP2B6**6 genotypes and allele frequencies showed no significant deviations from Hardy-Weinberg equilibrium ($P > 0.05$) in the Nigerian populations. Moreover, the mean *CYP2B6**6 allele frequency of 40% obtained in this study was consistent with those found in other African populations with frequencies up to 49 and 47% in Ghanaians and African Americans, respectively, but higher when compared to frequencies of 25 and 18% in Caucasians and Orientals (Klein et al., 2005; Rodriguez-Novoa et al., 2006) as shown in Figure 2. This could partly explain the clinical observations that many

patients of African origin experience more and severe side effects to efavirenz compared to Caucasians (Nyakutira et al., 2008). With the increasing use of NNRTIs in African countries and Nigeria in particular due to high prevalence of HIV/AIDS, it has become imperative to provide for the first time the genotype and allele frequencies of *CYP2B6**6 in Nigerian populations as a baseline study to enable further evaluation of the functional significance of this allele on NNRTI therapy in different sub-populations. There are currently many studies being conducted in Africa to evaluate the clinical implications of the *CYP2B6* genotypes in the use of efavirenz and these data may be useful in extrapolating the findings to other African populations.

The genotyping study of *CYP2C19* alleles in the Hausa, Ibo and Yoruba ethnic groups were predictive of

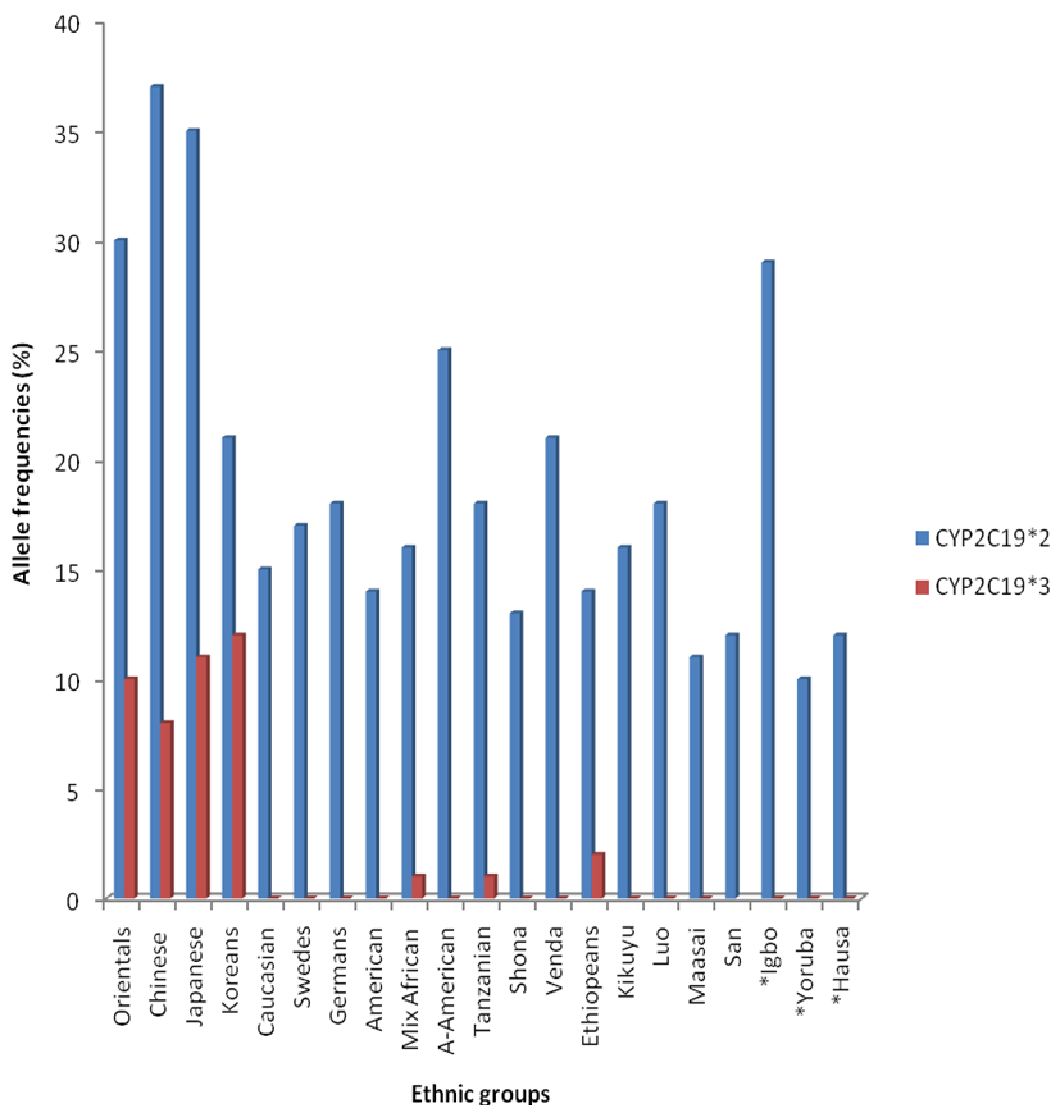


Figure 3. Allele frequencies for CYP2C19*2 and *3 in Hausa, Ibo and Yoruba populations of Nigeria in this study and other African populations, as well as other ethnic/race populations in literature (* ethnic populations in this study).

low poor metabolizer (PM) genotype frequency. The results indicate that the PM status was due to the defective *CYP2C19**2 allele. Among the Hausas, Ibos and Yorubas, the frequencies of *CYP2C19* genotypic PMs were 2, 8, and 0%, respectively. These frequencies are comparable to the 3 to 5% *CYP2C19* genotypic PMs reported for other African populations such as: South Africa Vendas, Tanzanians, Zimbabweans and Beninese (Dandara et al., 2001; Allabi et al., 2003). These frequencies are also similar to the 3 to 7% PMs reported among the Caucasians as shown in Figure 3 but much lower than the 14 to 25% PMs reported among the Orientals and the over 70% reported among the Vanuatu

population of Melanesia (Herrlin et al., 1998; Kaneko et al., 1999). However, in comparison with other African population, relatively high PM frequency was found in the Nigerian Ibo. In contrast, the data present no homozygous *CYP2C19* mutations in the Yoruba population of Nigeria suggesting that the prevalence of *CYP2C19* PM genotype in Yoruba is lower than that of the Ibo and Hausa. The finding of non-homozygous *CYP2C19* genotypic PMs in the Yoruba is in agreement with a previous report in the Beninese, a population with high proportion of Yoruba (Allabi et al., 2003). The PM status is often mainly due to the defective *CYP2C19**2 allele since the *CYP2C19**3 was found to be absent in

this study. This was in agreement with previous findings that the *CYP2C19*3* have been found to be virtually absent in most African populations (Dandara et al., 2001). This means that the PM of *CYP2C19* would largely be due to *CYP2C19*2* or other rare SNPs, which remain to be ascertained.

The most common defective *CYP2C19* allelic variant among the three major Nigerian ethnic groups was *CYP2C19*2* with frequencies ranging from 10 to 29% which is comparable to values of 13 to 22% obtained in other African populations and 12 to 15% in Caucasians but lower than the 19 to 40% in Asians (Kim et al., 2004). The absence of *CYP2C19*3* allelic variants in Nigerian population is similar to the findings in other African population with frequencies less than 1% but much lower than those reported in Asians with frequency ranging from 11 to 29% (Aklilu et al., 2002). Therefore, *CYP2C19*3* could be said to be of Asian origin. However, *CYP2C19*2* has been reported in almost all the ethnic groups so far studied pointing to its possible existence before the divergence of human populations. *CYP2C19*3* on the other hand may have evolved in Asians in response to environmental pressures (Kim et al., 2004). The mean frequency of approximately 3% *CYP2C19* genotypic PMs in the three major Nigerian ethnic groups in this study is in agreement with the 4% phenotypic PMs reported in a previous phenotyping study in Nigerians (Bolaji et al., 2002). Similarity in mean frequencies of the common *CYP2C19* allelic variants amongst the Nigerian ethnic groups and other African populations may further confirm the genetic relatedness of African populations. The genotype and allele frequencies of *CYP2C19* were in concordance with the Hardy-Weinberg equilibrium.

The therapeutic implication of the defective *CYP2C19* alleles is exemplified in the administration of proguanil, a pro-drug substrate of *CYP2C19*, which requires bio-activation to its active metabolite, cycloguanil. It is widely used for chemoprophylaxis in malaria and it is the drug of choice for malaria suppression in sickle cell patients. Nigeria accounts for one of the highest incidence of sickle cell anaemia cases in the world with currently over 6 million people suffering from the disease (Aliyu et al., 2008). A high frequency of *CYP2C19* PM genotype in its population may be detrimental to these high risk individuals with sickle cell anaemia on proguanil prophylaxis. Besides, resistance to first-line anti-malarial drugs (for example, chloroquine) has necessitated the use of anti-malarial drug combinations in the treatment of *Plasmodium falciparum* malaria, with proguanil being an essential component of some of the combinations for example, proguanil with dapson (Lapdap®) and proguanil with atovaquone (Malarone®). It is also noteworthy that artemisinin-based combination anti-malarial have now become the main stay in malaria treatment with *CYP2B6* playing a major role in the metabolism of artemisinin.

This study was essentially undertaken to provide baseline allele and genotype frequencies of *CYP2B6* and *CYP2C19* in the Nigerian populations. There is an ongoing investigation on the clinical and functional significance of *CYP2B6* polymorphism on the efavirenz pharmacokinetics in HIV/AIDS patients. Also, it may be necessary to assess the *CYP2C19* genotype in sickle cell anaemia patients on chemoprophylaxis with proguanil, especially in patients with frequent bouts of sickle cell crises.

Conclusion

The allelic distribution of *CYP2B6*6* appears comparable in the Nigerian populations and other African populations but significantly higher than the Caucasian and Asian populations indicating that Nigerian populations may be at risk of adverse reactions, if given similar dose of *CYP2B6* substrate (for example, efavirenz), as in Caucasians and Asians. Also, the genotype status of *CYP2C19* showed variations within the Nigerian populations studied most especially the 8% PM frequency obtained for the Ibo with possible implication on sickle cell anaemia chemoprophylaxis with proguanil.

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