Full Length Research Paper

Predominance of N-acetyl transferase 2 slow acetylator alleles in children less than ten years experiencing adverse treatment events following artemisinin-based combination therapy in North and South West Regions of Cameroon

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The human *N*-acetyltransferase II (NAT2) gene may vary between individuals resulting in variability in the incidence of adverse drug reactions. We set out in this *adhoc* analysis to determine the distribution of allele frequencies of *NAT2* gene variants among children less than ten years treated with artemisinin-based combinations in Cameroon. Children with uncomplicated malaria were deparasitized with artemisinin based combination therapy (artesunate-amodiaquine, dihydroartemisinin-piperaquine and artemether-lumefantrine) and followed up for 42 days and adverse events recorded. Blood was spotted on filter paper prior to drug administration for DNA extraction by chelex method. Standard nested PCR followed by restriction enzyme analysis with *KpnI, TaqI*, and *BamHI* for detection of polymorphisms in the *NAT2* was performed. Allelic frequencies and acetylator phenotypes were compared between participants with or without adverse drug events. The prevalence of slow, intermediate and fast acetylators was 54, 34 and 12% respectively. Significant clustering of mutant alleles (*NAT2*5, NAT2*6* and *NAT2*7)* occurred among those who experienced skin rash and tiredness (OR = 5.765, P = 0.046) (OR = 13.280, P = 0.020). No significant difference was observed between fast and slow acetylators with respect to the development of other recorded adverse events. Overall, producers of the slow acetylator alleles were more likely to develop any adverse events (OR = 8.286, P = 0.017) during the study period. Mutant alleles of the wild type NAT2*4 associated with the slow acetylator phenotype were the most predominant NAT2 allelic type and individuals with the phenotype were more likely to develop adverse events to ACTs.

Key words: *N*-Acetyltransferase 2, Artemisinin based combination therapy, adverse events, slow acetylators, allele.

INTRODUCTION

African countries on recommendation by WHO have increasingly adopted artemisinin-based combinations therapies (ACTs) as first line drugs against uncomplicated malaria. In Cameroon, artesunate-

amodiaquine (ASAQ) was adopted as the first line drug against uncomplicated malaria in 2006. Amodiaquine an accompanying drug in these combinations has been associated with adverse drug reactions leading to reduced adherence to this drug (Adjei et al., 2009; AlKadi, 2007). In the light of increase in use of these ACTs, the science and activities relating to the detection, evaluation, understanding and prevention of adverse reactions to medicines or any other medicine-related problems is necessary for evaluating safety risks (WHO, 2002). Pharmacovigilance is important in the resource-constrained settings because patients may present different profiles of adverse events due to genetic, co-morbidity, and other drug interactions (Pirmohammed et al., 2007).

Drug action on the one hand, depends on how drugs are metabolized and how differences in activity of metabolizing enzymes could significantly contribute to the efficacy of these drugs (Ladero, 2008). Mutations in a gene coding for a drug metabolizing enzyme could result in an enzyme with normal, slow, or fast activity (Agundez, 2008). It has been demonstrated that only 30 to 60% of common drug therapy is successful and that adverse drug reactions could cause 7% of all hospital admissions, 4% of withdrawal from new medical entities, and this can cost society an amount equal to the cost of drug treatment itself (Agundez, 2008). The human *N*-acetyltransferase (NAT) is an expression product of a single intronless protein-coding exon which is 870 bp open reading frame located in chromosome 8p22 and encoding 290 amino acids. It is one of the important phase II drug metabolizing enzymes which catalyses the conjugation or *N*-acetylation of arylamine derived therapeutic drugs and carcinogenic compounds (Agundez, 2008). Two functional polymorphic variants of the NAT gene exist; the NAT I and NAT II (Sim et al., 2008). Genetic polymorphisms in the *NAT2* locus influence individual variation in cancer susceptibility, responses to environmental toxins, and the effectiveness of prescribed medications (Ingelman-Sundberg, 2005; Lee et al., 2010). The *NAT2* gene polymorphisms may vary between individuals and ethnicities and results in variability in the incidence of adverse drug reactions (Kang et al., 2009). Several single nucleotide polymorphisms (more than 15 point mutations) exist on the *NAT2* gene that have given rise to at least 50 allelic variants today (Vatsis et al., 1995). A database for nomenclature of the growing number of different alleles or haplotypes of NAT polymorphisms exist (http://nat.mbg.duth.gr/). Polymorphisms in the wild type, (known as NAT2*4) give rise to combinations of 1-4 nucleotide substitutions in characteristic positions in the 870 bp coding sequence of the gene. Thus, wild type

homozygotes are designated as NAT2*4/NAT2*4 made of two wild type alleles (Vatsis et al., 1995). It has been demonstrated that some single nucleotide polymorphisms (SNPs) in the *NAT2* gene may cause structural protein changes or stability or reduced enzyme quantity (Sim et al., 2008; Walraven et al., 2008). Consequently, segregated gene variants in humans give rise to acetylation phenotypes classified as "rapid" (homozygotes of the NAT2*4 polymorphism), intermediate" (heterozygotes of the NAT2*4 polymorphism) and "slow" acetylation phenotypes (Boukouvala, 2005) (homozygotes of the mutant NAT2*5, NAT2*6, NAT 2*7 alleles) depending on the rate of enzyme activity (Chen et al., 2006; Chen et al., 2007). The effects of genetic polymorphism in the NAT2 gene on N-acetylation activity led to investigations of NAT2 SNPs as genetic markers for therapeutic response with resultant adverse reactions to drugs (Furet et al., 2002). Association studies have linked NAT2 polymorphisms and the acetylation status to individual differences in adverse events (Huang et al., 2002), as well as to development of bladder (Hein, 2002) and colon cancers (Garcia-Closas et al., 2005), rheumatoid arthritis (Kumagai et al., 2004) and systemic lupus erythematosus (Rychlik-Sych et al., 2006). The CYP2A6 and CYP2C8 genes are associated with the metabolism of ACTs. Several authors who analyzed the NAT2 gene also analyzed the CYP2A6 and CYP2C8 genes and have these to be correlated with predominantly slow metabolizers status (Gupta et al., 2013). This evidence indicates that when individuals are slow gene CYP2A6 and CYP2C8 they also present with the same "slow" phenotype for the NAT2 gene and could be at risk of developing adverse events. Achonduh et al. (2013), recently showed that NAT2 slow acetylators alleles clustered with individuals experiencing adverse to ASAQ in a study in the Northwest Cameroon. Therequencies of common NAT2 alleles were investigated to assess the distribution of these alleles among patients responding to ACTs in Cameroon. The aim of this study was thus to determine the genotypic frequencies of common *NAT2* genotypes and investigate their association with adverse events in response to ACT treatment in children less 120 months in Cameroon.

MATERIALS AND METHODS

Study area

The study was conducted at two geographically distinct sites, that fall within two of the three major geo-ecological zones of Cameroon as described by MARA maps (Kleinschmidt et al., 2001) namely; Mutengene is situated at coordinates 04°01'N, 09°11'E. The climate is equatorial with a rainfall of 10,000 mm per annum and a

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temperature average of 25°C. The vegetation is semi-mangrove and tropical wet forest. The study site is limited to the south and south-east by the sea and to the north and north east by mount Cameroon, an active volcano that is 4,100 m above sea level. The population works predominantly on palm and rubber plantations or estates that are owned by the agro-industrial Cameroon Development Corporation (CDC).

Garoua is in the north of Cameroon and lies at the coordinates, 06°24'N, 10°46'E. Garoua serves as a river port in years when the rainfall is abundant. Situated in the river Benue basin, it receives an average annual rainfall of 380 mm. It has about 4 months of rainy season. The mean temperatures for most of the year are about 31°C and the vegetation is guinea-savannah. The population is predominantly of the Fula ethnic group and comprises of cattle grazers. A few have taken to trading in small provision merchandise with neighbouring Nigeria.

Screening and enrollment

Eligible children aged 6 to 120 months with acute uncomplicated falciparum malaria were screened at the outpatient department and informed consent obtained from parents or guardians. Patients meeting the inclusion criteria were randomized to trial allocation and pre-treatment investigations (clinical and laboratory assessments) conducted. Criteria for inclusion were; children 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; ability to ingest tablets orally; willingness and ability to attend the clinic on stipulated follow-up days. 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 696 patients were enrolled for the study and were registered into the three arms of the study at the 2 study sites.

ACT Administration and follow-up

Children were randomized to receive artesunate-amodiaquine, dihydroartemisinin-piperaquine and artemether-lumefantrine in the ratio of 2:2:1 respectively.. The first dose of the antimalarials was administered in the hospital and the rest of the tablets were administered at home, according to the prescription. The medication was administered with water after a meal. Follow up of children was ensured by community health workers (CHW) who visited participants daily in the first three days and reported any complaints from the mother. They also visited these mothers with complaints on other days besides the protocol stipulated days (D7, D14, D28, D35 and D42 after first dose). Adverse events following ingestion of the drugs were recorded on a case report form designed to capture these events. They included but were not limited to any signs and symptoms suggestive of malaria or toxicity, date of onset, duration of symptoms, severity of malaria or other treatment administered and patient outcome. Participants were followed up for 42 days post ACT-administration.

Sample collection and DNA extraction

Finger-prick blood was collected and spotted on filter paper at inclusion and during follow up on days D7, D14, D21, D28, D35& D42 for genomic. Blood spots on the filter paper were excised with a sterile pair of surgical scissors. DNA was extracted from the dried blood spots by bioling in Chelex-100 in buffered Tris-EDTA as previously described (Plowe et al., 1995). The DNA was stored in a Tris-EDTA buffer at -20°C until analysis and allelic discrimination analysis.

Genotyping single nucleotide polymorphisms in NAT2 gene

The most common alleles in Africa population were investigated. They contained following mutations: C481T (*rs1799929*, silent mutation, amino acid change L161L), G590A (*rs1799930*, amino acid change R197Q), A803G (rs1208, amino acid change K268R) and G857A (*rs1799931*, amino acid change G286E). The primers used to amplify the gene were: *NAT2* (+) *5'-GCCTCAGGTGCCTTGCATTT-3'* and *NAT2 (-) 5'-CGTGAGGGTAGAGAGGATAT-3'*. The amplification was carried out using a T3 thermal cycler (Biometra, UK). Each PCR cycle was performed in a total volume of 25 µl containing: nuclease free water, 10Xthermopol buffer, 10 mMdNTPs (200 µM of each deoxyribonucleotide), 20 pmol primer and 5 U/µL Taq polymerase and 3 ng of gDNA. After initial denaturation at 95°C for 5 min, 30 cycles of amplification were carried out with denaturation at 95°C for 50 s, annealing at 55°C for 50 s and extension at 72°C for 50 s, followed by a final extension at 72°C for 5 min. To confirm the presence of *NAT2* alleles, PCR products were electrophoresed on a 2% agarose gel and polymorphisms determined by restriction endonuclease digestion of amplified gene fragments as described (Chen et al., 2007). The amplicons were digested under conditions stipulated for the restriction enzymes *Kpn*I and *BamH*I (New England Biolabs, USA) (37°C for 16 h while *Taq I* digested at 65°C for 16 h). The digestion reaction was followed by inactivation at 80°C for 20 min. Digested and undigested fragments of each sample were electrophoresed on a 2% agarose gel stained with Ethidium Bromide and the pattern of migration analysed by UV trans-illumination. Different alleles and combinations of alleles of the NAT2 gene were determined for each sample according to the migration pattern and information indicated in Table 5.

Classification of acetylator genotype

NAT2 acetylator genotypes were produced according to previously published data (Yokogawa et al., 2001). Homozygotes (*NAT2*4/NAT2*4*) or heterozygotes (*NAT2*4/NAT2*5, NAT2*4/NAT2*6* and *NAT2*4/NAT2*7* combinations) for the dominant *NAT2*4*wildtype allele were classified as fast acetylator genotypes, while homozygotes of the mutant alleles (*NAT2*5, NAT2*6* and *NAT2*7*) were classified as slow acetylator genotypes.

Data analysis

Data was entered on an SPSS version 16.0 (SPSS Inc., USA) statistical software. Frequency of the NAT2 allele and acetylator genotypes in the study population was performed. The association between NAT2 acetylator genotype and adverse events of ACTs in the study population was assessed by binary logistic regression analysis. The odd ratios (ORs) and 95% confidence interval (CIs) were also determined and a *P*-value < 0.05 was considered as statistically significant.

Ethical clearance

Ethical approval was obtained for the study from the National Ethics Committee for Health Research on human subjects in Cameroon and the Ethics Review Committee of the World Health Organisation (WHO-ERC).

RESULTS

Characteristics of study population

Of the 696 participants recruited in the study, the mean

Table 1. Baseline characteristics of the study population.

Variable	Min	Max	Mean	Std. Deviation
Age (months)	6	120	56.41	33.605
Weight (kg)	6.0	43.0	17.185	7.1618
Haemoglobin (g/dl)	5.5	116.0	10.162	4.7411
Parasitaemia	1040	100000	27698.16	29843.326
Temperature (°C)	35.00	40.80	37.97	1.17

Table 2. Adverse events classification, grading and causality for participants on ACT treatment in Garoua and Mutengene.

Adverse event	Percentage of participants showing event	Severity grading (% participants)				Causality with respect to test drug(% participants)				
		Mild	Moderate	Severe	Life-threatening	No related	Unlikely	Possible	Probable	Most probable
Abdominalpain	3.4	2.2	1	0.2	0	0.7	0.2	0.6	0.6	1.3
Anorexia	3	2.3	0.7	0	0	0.2	0	0.4	0.1	1.4
Asthemie	0.4	0.2	0.3	0	0	0	0	0	0.2	0.3
Cough	5.7	3.8	1.4	0.2	0	1.2	0.6	0.57	1.4	0.7
Diarrhea	3	1.9	1	0	0	0.7	0.3	0.4	0.4	0.4
Tiredness	1.9	1.3	0.4	0	0.2	0	0	0.4	0.3	1.2
Fever	1.6	0.4	0.7	0.3	0.2	0.3	0	0.3	0	1
Skin rash	6.3	3	2.7	0.6	0	0.3	0.9	1.2	0.6	2.6
Vomiting	9.5	6.2	2.7	0.3	0.2	0	0.6	4.2	1.2	2.9

age was 56.41 months. Average hemoglobin was 10.162 g/dL and a geometric mean parasitaemia of 27698.16. The mean temperature was 37.97 with an average weight of 17.185 kg (Table 1).

**Adverse reactions following ACTs administration and association with NAT2 alleles**

Adverse events reported during the study were asthenia, diarrhea, skin rash, anorexia, abdominal pain, cough, fever, tiredness and vomiting with severity ranging from mild to life-threatening. The causality of adverse events was ranged from no related to most probable with respect to test drug (Table 2). The frequencies of *NAT2* genotypes and phenotypes in the study population are shown in Figures 1 and 2. Relative to fast acetylators, there was an association between NAT2 slow acetylators and susceptibility to develop skin rash (OR = 5.765, P = 0.046) and tiredness (OR = 13.280, P = 0.020) (Table 3). However, no significant difference was observed between fast and slow acetylators and the development of adverse events. Generally, slow acetylators were more likely (OR = 8.286, P = 0.017) to develop any of the adverse events compared to fast acetylators (Table 4).

DISCUSSION

Information gathered on the distribution of genetic polymorphism in populations is essential to understand population wide differences in response to treatment and disease risk. The profiles of the NAT2 genotypes/phenotypes in children less than 10 years were determined and
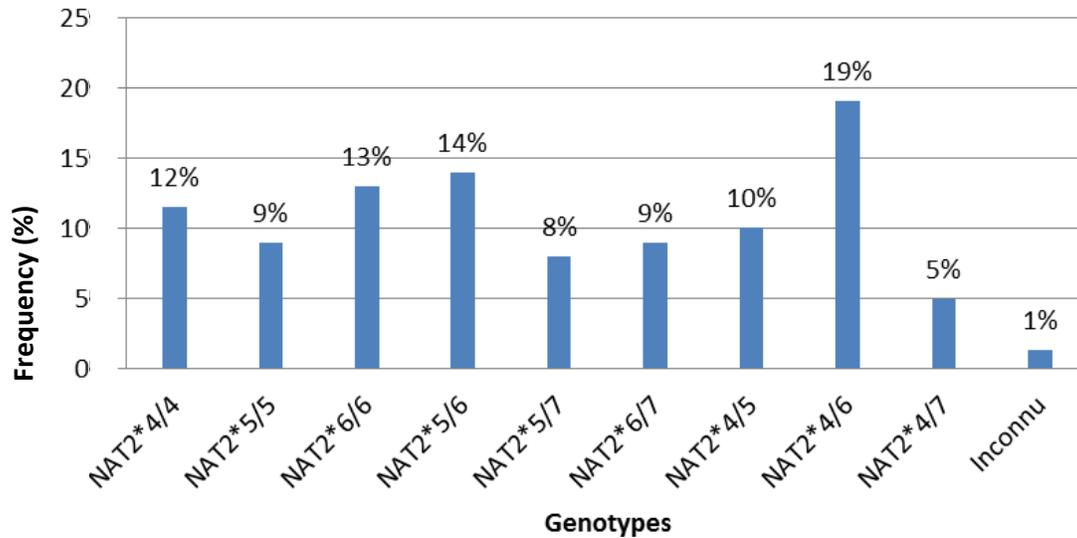
Figure 1. Frequency of NAT2 genotypes in the study population. NAT2*4/4 -fast acetylators; NAT2*5/5, NAT2*6/6, NAT2*5/6, NAT2*5/7, NAT2*6/7- Slow acetylators and NAT2*4/5, NAT2*4/6, NAT2*4/7- Intermediate acetylators.
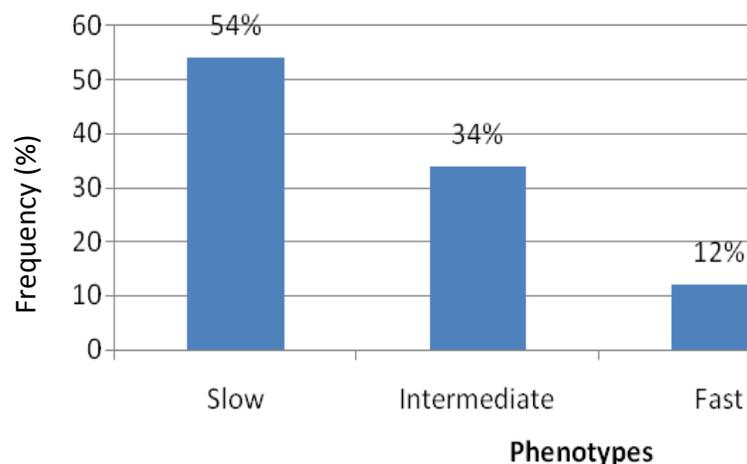
Figure 2. Frequency of NAT2 phenotypes in the study population.

their association with adverse reactions to ACTs as fixed dose combination following deparasitization analysed. Functional genetic polymorphisms of xenobiotic metabolizing enzymes have been associated with therapeutic response differences (Wilkinson, 2005) and disease risk susceptibility (Wilkinson, 2005; Nebert, 1997). In this study, it was observed that the slow acetylator phenotype was an independent risk factor for susceptibility (OR = 6.974, P = 0.017) to any adverse drug event, that is, asthenia, diarrhea, skin rash, anorexia, abdominal pain, cough, fever, tiredness and vomiting compared to those with fast acetylator phenotypes. This could be due to slow elimination of drugs from the system resulting in prolonged exposure

and subsequent adverse events. Relative to other adverse events, tiredness and skin rash were observed to be significantly associated with slow acetylator phenotype. The question has been raised why African would preferentially maintain a mutation that places them at a disadvantage of adverse events during therapy. It is probable that over many centuries the pathophysiology allowed for minimal elimination of herbal drugs that accumulated in the body at sub-optimal concentration so that infections could be cleared by the presence of these active ingredients. Selectively the African population with slow metabolism is not exposed to drugs administered at high enough concentrations to usher in adverse events by preferential drug retention.

Table 3. Association between NAT2 phenotype and all adverse events.

Adverse event	Phenotype	Yes	No	OR	95% IC	P-value
Skin rash	Fast	6	14	1.902	0.690 - 5.247	0.166
	Slow	118	105	5.765	1.487 - 8.739	0.046*
Abdominal pain	Fast	2	18	1.069	0.232 - 4.928	0.587
	Slow	21	202	0.728	0.306 - 1.731	0.309
Anorexia	Fast	1	19	0.534	0.068 - 4.203	0.465
	Slow	20	203	0.711	0.288 - 1.754	0.303
Cough	Fast	5	15	1.790	0.611 - 5.244	0.216
	Slow	35	187	0.764	0.386 - 1.509	0.273
Diarrhea	Fast	1	19	0.534	0.068 - 4.203	0.465
	Slow	20	203	0.573	0.228 - 1.436	0.165
Tiredness	Fast	0	20	0.942	0.911 - 0.973	0.318
	Slow	78	145	13.289	4.686 - 15.646	0.020*
Fever	Fast	1	19	1.121	0.136 - 9.233	0.619
	Slow	10	213	2.713	0.702 - 10.482	0.116
Vomiting	Fast	4	16	0.649	0.209 - 2.018	0.323
	Slow	62	160	1.469	0.830 - 2.599	0.119

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

Table 4. Association between NAT2 phenotype of participants with or without adverse event.

Phenotype	Adverse event				
	Yes	No	OR	95% IC	P-value
Fast	30	63	0,555	0.327 - 0.943	0.466
Slow	309	390	8,286	5.713 - 11.331	0.017

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

Table 5. Restriction sites and lengths of the nucleotide fragments produced by the digestion of the 535 base pair (bp) amplification products with *Kpn I*, *Bam HI* and *Taq I* enzymes.

NAT2 alleles	Position (mutation)	Restriction enzymes	Recognition motif	Fragmentpattern (bp); alleles (wt)	Fragment pattern (bp); point mutation
NAT2*5	481 (C→T)	*Kpn I*	G-GTAC'C	483;52	535
NAT2*6	590 (G→A)	*Taq I*	T'CG-A	205;170;160	330;205
NAT2*7	857 (G→A)	*Bam HI*	G'GATC-C	428;107	535

A, Adenine; C, Cytosine; G, Guanine; NAT2, N-acetyltransferase 2; T Thymine; wt, Wild type

Results presented herein demonstrate a higher prevalence of *NAT2* slow acetylators in this population with the like likelihood to develop adverse events. These findings indicate that *NAT2* polymorphism may be considered a good genetic marker for safety assessment of inter-population differences when populations are exposed to drug such as when assessing the safety profile of newly developed drugs in phase 3 and 4 clinical

trials in Africa.

Conflict of Interests

The authors have not declared any conflict of interests.

Abbreviations

NAT2, *N*-Acetyltransferase II; **PCR,** polymerase chain reaction; **ACTs,** artemisinin-based combinations therapies; **ASAQ,** artesunate-amodiaquine; **WHO,** world health organisation; **CYP,** cytochrome P450 enzymes; **EDTA,** ethylene diamine tetraacetic acid; **DNA,** deoxyribonucleic acid.

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