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Limited artemisinin resistance-associated polymorphisms in *Plasmodium falciparum K13-propeller* and *PfATPase6* gene isolated from Bioko Island, Equatorial Guinea

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ABSTRACT

Objective: With emergence and geographically expanding of antimalarial resistance worldwide, molecular markers are essential tool for surveillance of resistant *Plasmodium* parasites. Recently, single-nucleotide polymorphisms (SNPs) in the PF3D7_1343700 kelch propeller (*K13-propeller*) domain are shown to be associated with artemisinin (ART) resistance *in vivo* and *in vitro*. This study aims to investigate the ART resistance-associated polymorphisms of *K13-propeller* and *PfATPase6* genes in *Plasmodium falciparum* isolates from Bioko Island, Equatorial Guinea (EG).

Methods: A total of 172 samples were collected from *falciparum* malaria patients on Bioko Island between 2013 and 2014. The polymorphisms of *K13*-propeller and *PfATPase6* genes were analyzed by Nest-PCR and sequencing.

Results: Sequences of *K13-propeller* and *PfATPase6* were obtained from 90.74% (98/108) and 91.45% (139/152) samples, respectively. The 2.04% (2/98) cases had non-synonymous *K13-propeller* A578S mutation but no found the mutations associated with ART resistance in Southeast Asia. For *PfATPase6*, the mutations were found at positions N569K and A630S with the mutation prevalence of 7.91% (11/139) and 1.44% (2/139), respectively. In addition, a sample with the mixed type at position I723V was discovered (0.72%, 1/139).

Conclusions: This study initially offers an insight of *K13*-propeller and *PfATPase6* polymorphisms on Bioko Island, EG. It suggests no widespread ART resistance or tolerance in the region, and might be helpful for developing and updating guidance for the use of ART-based combination therapies (ACTs).

1. Introduction

The emergence of *Plasmodium falciparum* resistance to antimalarial drugs has been threatening the world's malaria control and elimination efforts (Young et al., 1963). Currently, *P. falciparum* have developed resistance/tolerance to antimalarial drugs chloroquine (CQ) and sulfadoxine-pyrimethamine (SP). Although World Health Organization (WHO) has recommended artemisinin (ART)-based combination therapies (ACTs) as the first-line treatment for uncomplicated *P. falciparum* malaria, *P. falciparum* are becoming insensitive to ART and its derivatives (Harinasuta et al., 1965; Wongsrichanalai et al., 2002; Amaratunga et al., 2014). At present, virtually all malaria endemic countries in sub-Saharan Africa are adopting either Artemether-Lumefantrine (AL) or Artesunate-Amodiaquine (AS-AQ) as the front-line ACTs. AS-AQ and the ART derivative dihydroartemisinin-piperaquine (DP) are used in

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Equatorial Guinea (EG) (Barrette and Ringwald, 2010).

Emerging evidence indicate that *P. falciparum* has been developing the resistance to ART and its derivatives. In Pailin of western Cambodia and other Southeast (SE) Asia area, parasite clearance was delayed following the treatment with ART monotherapy or ACTs (Noedl et al., 2008; Dondorp et al., 2009; Amaratunga et al., 2012; Miotto et al., 2013; Ashley et al., 2014). Our recent study showed the presence of high prevalent mutations in *Pfmdr1* (91.39%) and *Pfcrt* (98.67%) which markers for antimalarial drug resistence in *P. falciparum* clinical isolates on Bioko Island, EG (Li et al., 2015). It is globally threatening for malaria prevention and treatment (Wootton et al., 2002; Roper et al., 2004). It is imperative to conduct surveillances to identify areas that are potentially developing drug resistance.

Several molecular markers for antimalarial drug resistance has been identified (Wongsrichanalai et al., 2002; Barrette and Ringwald, 2010). Polymorphisms of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase ortholog in *P. falciparum* (*PfSERCA* or *PfATPase6*) has been associated with ART resistance, although the association of SNPs in *PfATPase6* with resistance to ART and the underlying mechanism remains to be confirmed (Jambou et al., 2005; Afonso et al., 2006; Mugittu et al., 2006; Cui et al., 2012).

Single-nucleotide polymorphisms (SNPs) in the PF3D7_1343700 kelch propeller (*K13-propeller*) have been identified to be a key causal determinant of ART resistance in SE Asia (Mok et al., 2015). From 2009 to 2015, a series of studies associated with *K13*-propeller has been published particular in Asia (Talundzic et al., 2015; Tun et al., 2015; Wang et al., 2015) and WHO Africa region (Cooper et al., 2015; Ouattara et al., 2015). In Africa, limited mutations of *K13*-propeller were found in Dakar (Torrentino-Madamet et al., 2014), Uganda (Cooper et al., 2015), Mali (Ouattara et al., 2015), and even 12 countries from sub-Saharan Africa (Kamau et al., 2015). These studies showed the mutational loci in African countries were different from those of SE Asia (Kamau et al., 2015).

Malaria is a serious health problem in EG, especially on Bioko Island. However, whether the ART resistance has been developed on the Bioko Island remains unclear. In this study, we surveyed polymorphisms in *K13-propeller* and *PfATPase6* genes in clinical isolates collected from Bioko Island, EG. Our findings may provide a clue to prevent and treat malaria using ART on Bioko Island, EG.

2. Materials and methods

2.1. Study area

Bioko Island belongs to EG and is located in the Gulf of Guinea, about 100 km off the coast of southern Nigeria and 160 km northwest of continental EG (Fig. 1). The island has a population of 266 000 inhabitants (2001 census) and a humid tropical environment. The launch of the Bioko Island Malaria Control Project (BIMCP) have had a marked impact on malaria transmission, malaria due to *P. falciparum* is still the major public health problem on the island. The entomological inoculation rates (EIRs) in Bioko Island ranged from 163 to 840, with the outdoor EIRs reaching more than 900 infective mosquito bites yearly and a malaria prevalence of 52% under the age of five years (Overgaard et al., 2012; Rehman et al., 2013).

2.2. Samples collection

Blood samples (3 ml) were collected from the confirmed malaria cases between September 2013 and March 2014. Approximately 300 μl of blood was aliquoted on 3 MM Whatman® filter paper (Whatman International Ltd., Maidstone, England), and air dried. These filters were then stored individually in Ziplock bags containing silica desiccant beads and kept at −20 °C. These samples were examined using the ICT malaria *P.f.* Cassette Test (ICT Diagnostics, South Africa) and Giemsa-stained thick and thin peripheral blood smear examination with microscope. For quality control, archived malaria positive slides were re-examined and parasitaemia was recorded. The *Plasmodium* spp. was confirmed by *Plasmodium* malaria real time PCR diagnostic kit (Shanghai Liferiver Bio-Tech Corp, China). This study was approved by the ethics committees of Malabo Regional Hospital. The informed consent was obtained from all participated subjects.

2.3. DNA extraction from blood samples

Genomic DNA (gDNA) was extracted from dried filter blood spots (DBS) by following Chelex-100 extraction procedure described in our previous report (Li et al., 2014). An 18S-rRNA-based RT-PCR was used to evaluate the quality of *P. falciparum* gDNA.

2.4. Genotyping

Nucleotide and amino-acid sequence of *K13-propeller* and *PfATPase6* used in current study has been reported in PlasmoDB (http://plasmodb.org) under Gene ID: PF3D7_1343700 and PF3D7_0106300. In order to illustrate the mutations of *K13*-propeller and *PfATPase6*, one segment of *K13*-propeller gene and one fragment from *PfATPase6* gene were amplified by a nested PCR (Zhang et al., 2008; Li et al., 2014), respectively.

The *K13-propeller* and *PfATPase6* genes were amplified by nested PCR using the primers in Table 1. For first round PCR, 0.5 μl of DNA was amplified with 10 μl 2 × NovoStar Green PCR Mix (1.25 U/μl NovoStar Taq DNA Polymerase, 0.4 mM dNTP Mixture, 2 × PCR Buffer, and 4 mM Mg^{2+}), 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), and sterile ultrapure water to a final volume of 20 μl. For the second round PCR, 0.5 μl primary PCR products were amplified with 40 μl reaction system, including 20 μl 2 × NovoStar Green PCR Mix, 1.0 μl forward primer (10 μM), 1.0 μl reverse primer (10 μM), and H_2O (up to 40 μl).

PCR reaction conditions were listed in Table 1. All PCR products were analyzed using 1.0% agar gel electrophoresis and DNA sequencing using a ABI 3730×L automated sequencer (PE Biosystems, CT, USA). The data was analyzed using the DNAstar (DNASTAR Inc., Madison, WI, USA). The 3D7 *K13*-propeller and *PfATPase6* sequences were used as the references.

2.5. Data analysis

The data was analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL). The mutant and wild-type alleles of the collected clinical samples were used to generate the prevalence of the alleles. A two-tailed *P*-value is less than 0.05 was considered statistically significant. The 95% confidence intervals (95% CI) was calculated as described previously (Li et al., 2014).

3. Results

3.1. K13-propeller polymorphisms

Sequence of a total of 98 (90.74%, 98/108) *K13-propeller* nested PCR products was obtained from 108 (62.79%, 108/172) PCR-positive samples out of the 172 isolates. The *K13-propeller* SNPs were analyzed by comparing with the reference 3D7 strain