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

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**Article Info**

**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 Next-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 found no 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 I72V 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).

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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; Amarantunga 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
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 resistance 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/endo-plasmic reticulum Ca²⁺-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-Madame 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, management. The launch of the Bioko Island Malaria Control Project (BIMCP) have had a marked impact on malaria transmission, management. The launch of the Bioko Island Malaria Control Project (BIMCP) have had a marked impact on malaria transmission.

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 Pf. 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 Lifiver 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 Mg²⁺), 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 H₂O (up to 40 μl).

PCR reaction conditions were listed in Table 1. All PCR products were analyzed using 1% 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.
There was a mutation at position 578. The frequency of A578S was 2.04% (2/98) (Table 2). No K13-propeller mutation was detected at positions 474, 476, 493, 508, 527, 537, 539, 543, 553, 561, 568, 574, and 580. Notably, the C580Y, R539T, and Y493H substitutions that were associated with ART resistance in vitro or delayed \textit{P. falciparum} parasite clearance in vivo in SE Asia were not detected on the samples from Bioko Island, EG.

3.2. \textit{PfATPase6} polymorphisms

Sequence of a total of 139 (91.45%, 139/152) \textit{PfATPase6} nested PCR products was obtained from 152 (88.37%, 152/172) PCR-positive samples out of the 172 isolates. The \textit{PfATPase6} SNPs were analyzed by comparing with the reference 3D7 strain (PF3D7_0106300) and listed in Table 2. The \textit{PfATPase6} SNPs were found at positions N569K and A630S with the mutation prevalence...
of 11 (7.91%, 11/139), and 2 (1.44%, 2/139), respectively (Table 2). In addition, a sample with the mixed type at position I723V was also discovered with the mutation prevalence of 1 (0.72%, 1/139) (Table 2). The remaining mutations of K561N, A623E, G639D, N683K, and S769N, all the investigated samples verified the wild-type genotype. Notably, the S769N substitution that was connected with ART resistance was not found from these samples.

### 4. Discussion

For EG, both the AS-AQ and DP are considered as the front-line treatment for uncomplicated falciparum malaria. It is essential to understand molecular mutation profiles of *P. falciparum* parasite for ART resistance and use this initial information for molecular assessment under antimalarial drug pressure. In current study, we only find limited mutation of K13-propeller and low frequency of PfATPas6 mutation of the clinical isolates from Bioko Island, EG. It is the initial report focusing on the molecular markers of K13-propeller and PfATPas6 for ART resistance on this Island.

The ART resistance considers as a major risk to public health, with the most rigorous potential effect in Sub-Saharan Africa, where the burden is seriously. Furthermore, the international investments and domestic investments, the prevention and elimination system for malaria are also insufficient in the region. The hazard of ART-resistant parasites scatters from Western Cambodia to the Greater Mekong Subregion and then to Africa particularly Sub-Saharan Africa, as happened previously with CQ and SP-resistant parasites (Wootton et al., 2002; Roper et al., 2004), is worrying (Ariey et al., 2014). A significant action of the WHO Global Plan for Artemisinin Resistance Containment is to increase monitoring and molecular surveillance (Talisuna et al., 2012). However, it is difficult to evaluate how long or when ART resistance mutations will appear in Africa including EG, and molecular detection can offer a profile to speedily discover for the appearance or importation of resistance alleles (Taylor et al., 2014). In SE Asia, the mutations of K13-propeller are found in both western Cambodia (Ariey et al., 2014; Straimer et al., 2015) and Bangladesh (Mohon et al., 2014). In Africa, only limited polymorphisms of K13-propeller are detected (Conrad et al., 2014; Taylor et al., 2014; Torrentino-Madamet et al., 2014). However, the K13-propeller polymorphism in returned migrant workers from Ghana is found in Shanglin of China (Feng et al., 2015). Thus, it is very necessary to strengthen *Plasmodium* parasites genotypic resistance surveillance with K13-propeller polymorphism. If there is no sufficient attention to long-term survey, it will be a disaster for human health.

The survey of *P. falciparum* K13-propeller polymorphisms primarily explore a diversity of mutations across on Bioko Island, EG. Limited polymorphisms associated with ART resistance from SE Asia are observed in the clinical isolates. Recent study reports that the SNP mutations at Y493H, I543T, R539T, and C580Y are powerfully connected with prolonging *P. falciparum* parasite survival time *ex vivo*; ART resistance *in vitro* has to be M476I mutation-related, which indicates that mutations of K13-propeller can generate an ART-resistance phenotype *in vitro* with genetic background from African parasite (Ariey et al., 2014). Although the five mutations play crucial role during ART-resistance to *P. falciparum* parasite *in vitro* and *in vivo*, we observe none of these mutations in the survey parasite samples. Only one K13-propeller A578S mutation (2.04%, 2/98) that previously reported from Cambodia is discovered in the six K13-propeller blades. This mutation is previously found in 0.75% (1/133) of the isolates tested in Uganda and also presents in parasites from DRC, Gabon, Ghana, Kenya, and Mali (Ariey et al., 2014; Conrad et al., 2014; Kamau et al., 2015). Although the prevalence of A578S mutant allele from Bioko Island is lower than Kenya at 2.7%, it is still higher in parasites compared to 1% in the other four countries of Sub-Saharan Africa. This unusual polymorphism also merits further characterization (Taylor et al., 2014). Mutations of A481V, G533C and A578S are confirmed and adjacent to the Y493H, R539T, C580Y mutation, and propose the mutations may have a significant effect on three-dimensional structure of the K13-propeller. Furthermore, the mutations of S522S, Y588H report in Ugandan children and A557S in Congo have not detected on Bioko Island (Conrad et al., 2014; Taylor et al., 2014). The V520A mutation identified from West, Central and East Africa is also not found on the Island, Dakar and Uganda (Sylla et al., 2013; Taylor et al., 2014). These results encourage and suggest ART resistance is not yet established in Africa particular on Bioko Island, EG. Although none of the mutations associated with ART resistance in SE Asia are detected in Africa (Conrad et al., 2014; Taylor et al., 2014), numerous novel K13-propeller coding substitutions bothers in the whole continent of Africa. The phenotypes of these coding polymorphisms remain unclear and will require further characterization to better characterize the clinical impact on ART resistance in Africa. Further analysis of phylogenetic tree or haplotype network is needed to trace the origin of the K13-propeller mutations and to determine whether the ART resistance is widely spreaded from SE Asia or emerged independently in Bioko Island, EG (Nyunt et al., 2014).

Polymorphisms evaluation of PfATPas6 in Africa have occurred rarely (Mugittu et al., 2006; Legrand et al., 2008; Happi et al., 2009; Menegon et al., 2010; Kamugisha et al., 2011; Zatra et al., 2012). The current study initial describes for the PfATPas6 polymorphism on Bioko Island, EG. Among the three different observed mutations, only one, the N569K mutation, is relatively frequent 7.91% of isolates. The previous studies have also found a high prevalence of this mutation in Zanzibar (36%) and Tanzania (29%) (Dahlstrom et al., 2008), Niger (17.2%) (Ibrahim et al., 2009). The PfATPas6 S769N mutation is absent in all Bioko samples and consistent with previous results in Africa countries (Mugittu et al., 2006; Legrand et al., 2008; Happi et al., 2009; Menegon et al., 2010; Kamugisha et al., 2011; Zatra et al., 2012) and South America (Adhin et al., 2012). It demonstrates that the Africa and South America share the similar molecular pattern of PfATPas6 for ART resistance.

### 5. Conclusions

The present study shows that the low prevalence polymorphism mutations of PfATPas6 and limited mutations of K13-propeller,
potentially associated with ART resistance, are obviously observed on Bioko Island, EG. Continuous molecular surveillance with K13-propeller gene as ART resistance marker is exceedingly recommended on Bioko Island, EG. Furthermore, it might be helpful for developing and updating guidance for the use of ACTs.

Conflicts of interest

We declare that we have no conflict of interest.

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