BRIEF REPORT

K13-Propeller Polymorphisms in *Plasmodium falciparum* Parasites From Sub-Saharan Africa

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Mutations in the *Plasmodium falciparum* K13-propeller domain have recently been shown to be important determinants of artemisinin resistance in Southeast Asia. This study investigated the prevalence of K13-propeller polymorphisms across sub-Saharan Africa. A total of 1212 *P. falciparum* samples collected from 12 countries were sequenced. None of the K13-propeller mutations previously reported in Southeast Asia were found, but 22 unique mutations were detected, of which 7 were nonsynonymous. Allele frequencies ranged between 1% and 3%. Three mutations were observed in >1 country, and the A578S was present in parasites from 5 countries. This study provides the baseline prevalence of K13-propeller mutations in sub-Saharan Africa.

Keywords. K13-propeller; artemisinin resistance; sub-Saharan Africa.

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The emergence of *Plasmodium falciparum* parasites resistant to artemisinin and its derivatives, recently documented in Southeast Asia [1–3], threatens to reverse recent gains made in malaria control worldwide and presents a major roadblock to eliminating malaria. Although in vivo efficacy studies are widely used for tracking artemisinin resistance in Southeast Asia, in Africa and other malaria-endemic regions, there is a need for concerted efforts to develop in vitro or ex vivo assays and to identify genetic markers of artemisinin resistance. Toward this effort, Witkowski et al recently described novel phenotypic assay for detection of artemisinin-resistant *P. falciparum* parasites, the ring-stage survival assay ($RSA_{0-3\ hours}$) [4]. Furthermore, evaluation of the *P. falciparum* genome for regions of recent strong evolutionary selection and genome-wide association studies revealed regions on chromosomes 10 and 13 that are potential loci involved in artemisinin resistance [3, 5].

In a recent study, Ariey et al showed that $RSA_{0-3\ hours}$ survival rates and slow parasite clearance were associated with single-nucleotide polymorphisms (SNPs) in the PF3D7_1343700 kelch propeller domain on chromosome 13 (K13-propeller) [6]. These authors demonstrated that the mutant alleles Y493H, R539T, I543T, and C580Y within the kelch repeat motif of the C-terminal K13-propeller domain conferred significantly higher $RSA_{0-3\ hours}$ survival rates. Further analysis of geographic diversity of K13-propoller polymorphisms across 10 provinces in Cambodia revealed a large number of mutations, with the C580Y allele accounting for 85% of all mutations. The majority (74%) of the parasites harbored a single nonsynonymous mutation in the K13-propeller with geographic disparity in their distribution; K13-propeller mutations were more prevalent in provinces with documented artemisinin resistance.

For K13-propeller polymorphisms to be used universally as a tool for tracking artemisinin resistance and translated into a public health tool, global validation of these markers must be conducted. Toward this effort, we sought to assess the prevalence of polymorphisms in the K13-propeller gene in samples collected across sub-Saharan Africa in regions where artemisinin-combination therapies (ACTs) are routinely used for treatment of malaria. This study provides baseline prevalence of K13-propeller polymorphism in sub-Saharan Africa.

METHODS

Study Samples and Processing

Ethical approval was obtained from relevant national and/or institutional review boards for all participating institutions, and all

Table 1. Distribution and Prevalence of K13-Propeller Polymorphisms in 12 Sub-Saharan African Countries

Country (Site[s]; Samples, No.), Codon Position	Reference aa (nt)	Mutant aa (nt)	Samples With Mutant Allele, No.	Prevalence, %
Cameroon (Buea; n = 11)				
No SNPs	. . .	. . .	. . .	. . .
Côte d'Ivoire (Koumassi, Abobo, Yopougon; n = 98)				
478	Thr (acc)	Thr (acA)	1	1.02
496	Gly (ggt)	Gly (ggC)	1	1.02
557	Ala (gca)	Ser (Tca)	1	1.02
567	Glu (gag)	Glu (gaA)	1	1.02
592	Gly (gga)	Gly (ggG)	1	1.02
DRC (Kinshasa; n = 82)				
493	Tyr (tac)	Tyr (taT)	1	1.22
578	Ala (gct)	Ser (Tct)	1	1.22
Ethiopia (Nazareth/Adama, Gambela, West Arsi; n = 82)				
No SNPs	. . .	. . .	. . .	. . .
Gabon (Libreville; n = 93)				
471	Arg (cgt)	Arg (cgC)	1	1.08
578	Ala (gct)	Ser (Tct)	1	1.08
589	Leu (gtc)	Ile (Atc)	1	1.08
Ghana (Cape Coast; n = 92)				
469	Cys (tgc)	Cys (tgT)	2	2.17
566	Val (gta)	Ile (Ata)	3	3.26
610	Lys (aaa)	Lys (aaG)	1	1.09
Ghana (Navrongo; n = 99)				
459	Ser (tcg)	Ser (tcA)	1	1.01
468	Glu (caa)	Glu (caG)	1	1.01
469	Cys (tgc)	Cys (tgT)	1	1.01
493	Tyr (tac)	Tyr (taT)	1	1.01
535	Thr (acg)	Thr (acA)	1	1.01
578	Ala (gct)	Ser (Tct)	1	1.01
Kenya (Kisumu; n = 108)				
478	Thr (acc)	Thr (acG)	1	0.93
509	Glu (gag)	Glu (gaA)	1	0.93
569	Ala (gca)	Thr (Aca)	1	0.93
578	Ala (gct)	Ser (Tct)	3	2.78
630	Tyr (tac)	Phe (tTc)	1	0.93
Madagascar (Antananarivo; n = 97)				
No SNPs	. . .	. . .	. . .	. . .
Mali (Faladje; n = 91)				
503	Lys (aag)	Lys (aaA)	1	1.10
578	Ala (gct)	Ser (Tct)	1	1.10
Nigeria (Lagos; n = 89)				
No SNPs	. . .	. . .	. . .	. . .
Tanzania (Tanga; n = 92)				
493	Tyr (tac)	Tyr (taT)	1	1.09
576	Ser (tca)	Leu (tTa)	1	1.09
Gambia (Banjul; n = 77)				
489	Asn (aat)	Asn (aaC)	1	1.30

Abbreviations: aa, amino acid; DRC, Democratic Republic of the Congo; SNP, single-nucleotide polymorphism; nt, nucleotide.

relevant guidelines for conducting human studies per national and/or institutional requirements were followed. Informed consent was obtained from patients or their parents/guardian. *P. falciparum*–positive blood samples were collected in 2013–2014 from 12 sub-Saharan African countries, from which members of the Plasmodium Diversity Network Africa consortium are

drawn (Table 1) [7]. Samples from each institution were collected per site(s) specific protocol and processed using more harmonized protocols across the network. Individuals aged ≥6 months presenting at study sites with falciparum malaria, confirmed with light microscopy and/or malaria rapid diagnostic tests, were enrolled into the study. Venous blood or dried blood spots (DBS) were collected for analysis. A 2–5-mL sample of venous blood was depleted of leukocytes within 6 hours of collection, using Whatman CF11 cellulose powder filtration columns (GE Healthcare Bio-Sciences, Piscataway, NJ), following a slightly modified WWARN protocol (http://www.wwarn.org). Approximately 100 μL of blood was blotted onto various supports, including 3MM Whatman FTA cards (Kent, WA) and 903 Protein Saver Cards (Pittsburgh, PA). DNA was extracted from whole blood or leukocyte-depleted blood, using the QIAamp DNA Blood kit, and from DBS, using the QIAamp DNA investigator kit (Qiagen, Valencia, CA) per the manufacturer's recommendation. DNA was stored and shipped at appropriate temperatures.

K13-Propeller Sequencing

Sequencing was done at the Wellcome Trust Sanger Institute (Hinxton, United Kingdom). The K13-propeller was amplified by polymerase chain reaction (PCR), using the AccuPrime *Pfx* SuperMix (Invitrogen, Carlsbad, CA), using previously published primers [6]. An aliquot of the PCR products was analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide to confirm amplification. Double-strand capillary sequencing of PCR products was performed by means of Sanger sequencer standard methods, using the Applied Biosystems 3730XL system. Sequence data were analyzed using the Genome Assembly Program GAP 4 to identify SNP combinations. All SNPs were further assessed by an investigator and called using 3D7 as the reference genome. Sequences were deposited in GenBank under accession numbers KM882612–KM882639.

RESULTS

Distribution and Prevalence of K13-Propeller Polymorphisms in *P. falciparum* Samples

A total of 1212 DNA samples were successfully sequenced and analyzed. The table shows number of samples analyzed from each country and the distribution of the K13-propeller polymorphisms. A total of 22 unique mutations were detected, of which 7 were nonsynonymous. Ghana had the largest number of samples analyzed, as well as the largest number of SNPs. A single synonymous mutation in codon C469C was present in both regions of Ghana where samples were collected. Côte d'Ivoire and Kenya had the second largest number of SNPs, with 5 different mutations each. Kenyan samples had the largest number of nonsynonymous mutations, where 3 of 5 SNPs were nonsynonymous.

The prevalence of the majority of the mutant alleles was low. The highest prevalence of SNPs was observed in Ghana (approximately 3% for the nonsynonymous V566I and synonymous C469C; Table 1). Only 3 SNPs appeared in >1 country: the nonsynonymous mutant allele A578S and the synonymous Y493Y and T478T mutant alleles. A578S was present in parasites from the Democratic Republic of the Congo, Gabon, Ghana, Kenya, and Mali. The prevalence of the A578S mutant allele was highest in parasites from Kenya, at 2.7%, compared with approximately 1% in the other 4 countries. Samples from Cameroon, Ethiopia, Madagascar, and Nigeria did not have parasites with K13-propeller mutant allele (Table 1). Three of 190 samples (1.6%) from Ghana and 3 of 108 (2.8%) from Kenya showed polyclonal infections, as assessed by the presence of minor peaks or >1 peak on the sequencing graphs.

DISCUSSION

We present the prevalence of SNPs in the PF3D7_1343700 kelch propeller domain assessed in >1200 *P. falciparum* parasite isolates from 12 countries across sub-Saharan Africa. We detected 7 nonsynonymous mutations, but none of them were among the 18 SNPs recently described in Southeast Asia [6]. This is in line with recent studies, which did not detect any of the SNPs associated with artemisinin resistance in Southeast Asia in sub-Saharan African parasites [8, 9]. Nevertheless, 2 of the African nonsynonymous SNPs, A578S and V566I may be of interest. First, the A578S SNP was present in 5 countries spanning from East to Central and West Africa (Kenya, Democratic Republic of the Congo, Gabon, Ghana, and Mali). This SNP was also found in recent studies in samples from Uganda and Kenya [8, 9]. Besides, both A578S and V566I SNPs were present at a prevalence of >1% in at least 1 location. Second, these 2 SNPs are located close to the C580Y mutation, which was found to be the most critical genetic determinant of artemisinin resistance in Southeast Asia [6]. The C580Y SNP is located within a kelch repeat of the C-terminal K13-propeller domain, located within repeat number 4, antiparallel β1 sheet, which begins and ends at amino acids 579–583. The A578S is located within repeat number 4 but not in the antiparallel β sheet, whereas the V566I mutation is located in the C-terminal K13-propeller domain in repeat number 3, within the β4 sheet. On codon 578, a neutral nonpolar amino acid (A) is replaced with a neutral but polar amino acid (S), while both the wild-type and the mutant alleles at codon 566 present neutral nonpolar amino acids. Additional molecular and biochemical studies should investigate whether these mutations destabilize the domain scaffold and alter the functions of this protein, resulting in altered artemisinin sensitivity.

In Southeast Asia, there is a strong relationship between K13-propeller mutations and delayed parasite clearance following artemisinin treatment [6, 10]. However, with the exception of 1 case in Nigeria and 2 cases in the Democratic Republic of the Congo, there have been no reported cases of delayed parasite

clearance or of prolonged $RSA_{0–3\ hours}$ in sub-Saharan Africa to date [10, 11], and a key question is whether K13-propeller mutations observed in Africa are also associated with artemisinin resistance [12]. This raises the possibility that K13-propeller mutations do not cause artemisinin resistance in isolation but act in combination with other genetic or nongenetic factors that differ in African and Southeast Asia parasite populations. Indeed, subpopulations of parasites can already be categorized as susceptible or resistant, based on their genetic profile [13]. Parasite ancestry, geographical, clinical, epidemiological, and genetic diversities are some of the critical parasite characteristics that must be considered when validating K13-propoller polymorphisms for tracking ACTs resistance beyond Southeast Asia. The actual SNP(s) that confer resistance might differ from one location to another, depending on the parasite genetics. Unlike in Southeast Asia, parasites in malaria-endemic regions of sub-Saharan Africa are less likely to be clonal because of the high transmission intensity and are thus likely to lose potential resistance-conferring alleles to outcrossing. Since African K13-propeller mutations were shown to be different from those seen in Southeast Asia, these results are a strong evidence for the need of further studies to assess whether K13-propeller mutations are relevant in determining artemisinin resistance in parasite isolates in sub-Saharan Africa.

Genetic determinants of resistance to other antimalarial drugs have already been validated as tools for detecting and tracking resistance. These markers have been used by policy makers to make decisions, guide national malaria treatment policies, and monitor changes in parasite drug susceptibility following changes in drug treatment policies [14]. If the K13-propeller SNPs are validated as molecular marker for artemisinin resistance globally, this will be a critical benchmark in malaria control. This study provides baseline prevalence of K13-propeller mutations in sub-Saharan Africa with samples collected in the past few months. The baseline information will be critical in tracking the emergence and/or spread of *P. falciparum* resistance to artemisinin in sub-Saharan Africa [12]. There is now an important need for local studies of clinical resistance to artemisinin and for in vitro and ex vivo $RSA_{0–3\ hours}$ data to clarify the significance of K13-propeller mutations as markers of artemisinin resistance Africa.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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