Biomedical Sciences
2020; 6(2): 38-43
http://www.sciencepublishinggroup.com/j/bs
doi: 10.11648/j.bs.20200602.14
ISSN: 2575-3924 (Print); ISSN: 2575-3932 (Online)

Review Article

Distribution and Contribution of K13-propeller Gene to Artemisinin Resistance in sub-Saharan Africa: A Systematic Review

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To cite this article:

Laura Nyawira Wangai, Kenny Kimani Kamau, Immaculate Marwa, Elly OMunde, Samuel Mburu, John Mwangi, Mark Webale, Dennis Butto, Lucy Kamau, John Hiuhu. Distribution and Contribution of K13-propeller Gene to Artemisinin Resistance in sub-Saharan Africa: A Systematic Review. *Biomedical Sciences*. Vol. 6, No. 2, 2020, pp. 38-43. doi: 10.11648/j.bs.20200602.14

Received: May 26, 2020; **Accepted**: June 10, 2020; **Published**: June 20, 2020

Abstract: The observed clinical failure after treatment with artemisinin combination therapy (ACT) has recently been confirmed in western Cambodia. Evidence of declining ACT efficacy has also been reported in Africa. Molecular markers for artemisinin resistance have played an essential role in monitoring the spread of the resistant phenotype and identifying the mechanisms of resistance. Several candidate genes, including the *P. falciparum kelch* propeller region (*K13*). However, in sub-Saharan Africa, despite the observed delayed clearance after treatment, the association between ART resistance and *K13* gene is questionable as studies have not found significant mutations or an association with the delayed parasite clearance rate following ACT treatment. There is need for more data to clarify the significance of *K13*-propeller mutations as markers of artemisinin resistance in Africa. An electronic search of studies in sub-Saharan Africa from 2014 to date was done via PubMED, SCOPUS, and EMBASE databases. The search was conducted independently by two librarians. The articles were screened for selection using a priori criteria set following PRISMAP and STREGA guidelines. Data analysis was performed in R-statistics software. A total of 197 articles were identified from Pubmed= 139, Research gate=40, Bibliography/other searches=18, of which 102 did not meet the selection criteria. A total of 74 independent *K13* mutations were identified across malaria-affected African countries. Only 7 unconfirmed K13 mutations were associated with delayed parasite clearance half-life (t1/2>3 h). The majority, 47.5% (35/74), of the mutations were reported in single *P. falciparum* parasite isolate. Of the 74 *K13*-mutations, nearly two-thirds were reported as new alleles. Twenty-seven (27) non-synonymous mutations in the *Pfkelch13* gene were identified. Although artemisinin resistance in South-East Asia seems to be a heritable genetic trait, none of the candidate genes suggested by earlier studies confer artemisinin resistance to the observed clinical failure in Africa. Mutations outside the *Pfkelch13* propeller region associated with increased ART parasite clearance half-life occur in malaria-affected regions in Africa. The use of a genome-wide approach by whole genome sequencing and gene expression transcriptome studies to identify the molecular basis of artemisinin resistance is warranted to aid in identification potential markers for ACT resistance in Africa.

Keywords: Malaria, Artemisinin Resistance, *K13-propeller* Gene Polymorphism, Sub-Saharan Africa, *Plasmodium falciparum*

1. Introduction

Globally, the control of malaria relies upon the high efficacy of artemisinin combination therapies (ACTs). ACTs are the currently used drugs nearly in all malaria-endemic countries [1]. In Southeast Asia, the efficacy of ACT has already been threatened by the declining *Plasmodium falciparum* susceptibility to artemisinin, which clinically manifests as delayed parasite clearance [1, 2]. While thus far limited to SEA, this delayed-clearance phenotype is increasingly detected and correlates with increases in ACT failures. Delayed clearance is therefore widely considered to be associated with clinically significant *P. falciparum* artemisinin resistance [2, 3]. The spread of these artemisinin resistant parasites could undermine artemisinin-based therapies and imperil global malaria control. An increase of 2 million malaria cases was observed in 2019 when 218 million cases were reported compared to 216 million in 2018 [4]. With malaria being one of the leading causes of morbidity and mortality especially in tropical countries, a further increase of an already high burden of malaria is a cause for concern. It is an indication of the need to rethink current malaria control efforts. To date ART agents and their derivatives remain the only effective agents currently used in malaria control efforts. However, decreased *P. falciparum* parasite sensitivity that has emerged in Southeast Asia presents a threat to future efficacy of ACT.

The artemisinin resistance reported in Southeast Asia was attributed to mutations in the *Kelch* propeller domains of the wild-type parasite gene [5]. *K13*-propeller polymorphisms are associated with in-vitro parasite survival in the presence of artemisinin and with delayed clearance in vivo after artemisinin therapy [6]. In western Cambodia, where artemisinin-resistant phenotype was initially reported, 3 mutations—C580Y, R539T, and Y493H—were reported and associated with delayed parasite clearance after treatment [7, 8]. Across sub-Saharan Africa, ACT for uncomplicated malaria has been highly efficacious, and parasite clearance is rapid in vivo studies [9, 10]. However, recently evidence of declining ACT efficacy was in reported in Africa where delayed parasite clearance was observed [11, 12].

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. Towards this effort, we sought to assess the findings of the previous work on mutations in the *K13*-propeller gene in samples collected across sub-Saharan Africa from 2014 to date and its contribution to the observed delayed parasite clearance in regions where artemisinin-combination therapies (ACTs) are routinely used for treatment of malaria. This systematic review provides an overview of the distribution of mutations in *K13*-propeller gene in sub-Saharan Africa and their contribution to the observed delayed parasite clearance after treatment with ACT.

2. Methods

2.1. Selection Criteria

An independent database search of MEDLINE via PubMED, SCOPUS, EMBASE, and LILACS/VHL databases search was performed to identify studies that investigated K13 gene polymorphisms among *P. falciparum* parasites in malaria-affected countries in sub-Saharan Africa. Search terms such as "*K13*-propeller gene polymorphisms", "*K13*-polymorphisms", "K13-gene polymorphisms", "K13-propeller gene mutations", "K13-mutation", "Artemisinin resistance genes", "Artemisinin resistance alleles" "Artemisinin resistance mutations", "Artemisinin resistance mutations", "*Plasmodium falciparum*", "Artemisinin", "Artemether", "Artemether", "Dihydroartemisinin", "Artemisinin derivatives", ACT, ART, "malaria-affected African countries". Searching for articles in selected databases using the stated search terms was restricted to title or objectives.

2.2. Secondary Data Extraction

Data extraction criteria was designed in Microsoft Excel 2016 (Microsoft Corp, Washington, USA) and extracted the relevant information from papers including, author, year of publication, country/region, number of samples collected, allele calling algorithm, source of DNA, years covered by study, number of parasite DNA where genotyping was attempted, number of parasite DNA samples where genotyping was successful, *K13* mutations confirmed to cause delayed ART parasite clearance (validated SNPs in K13 propeller mutations), non-validated SNPs in K13 gene (K13 mutations that have not been confirmed to cause delayed ART parasite clearance), non-propeller K13 mutations, non-validated K13 mutations associated with delayed ART parasite clearance, nucleotide changes in K13 mutations, genotyping method used, duration of ART use, nature of ART use (monotherapy/combination) and source of K13 mutation.

2.3. Risk of Bias

Minimizing bias in article retrieval, inclusion and data extraction, we validated electronic search in PubMed by conducting an independent and similar search. Extracted data was transferred to R statistic software for analysis. In the structured data synthesis summaries (proportions and percentages) of the variables of interest were extracted. These included: non-synonymous SNPs in *Pfkelch13* gene, duration of ART use prior to data collection, source of mutations (inherited/novel), status of ART use (monotherapy or combination therapy), efficacy of ACT nucleotide variation in K13 gene, spread of K13 mutations, *Pfkelch1*3 mutations not confirmed to result to ART treatment failure (non-validated) but associated with delayed *P. falciparum* clearance, prevalence of K13 mutations. Heterogeneity in selected full-text articles was inferred from the summary estimates of statistics. The risk of biasness in publications was assessed using indirect assessment of rank correlation between effect size and sample

size used as described by using Kendall's tau method.

2.4. Statistical Analysis

Statistical analysis was done using R statistic version 3.5.1 and STATA software version 16 (Stata Corporation, Texas, USA). Comparative analysis of genetic differences in parasites between different geographical regions (countries) was done. Moreover, analysis between parasites obtained from patients with fast versus slow clearance rates was done. Comparisons between sites were conducted for each genotype using the chi-square test or Fisher's exact test, as appropriate. To compare the fast and slow responders, the clearance rate was estimated for the slow responders.

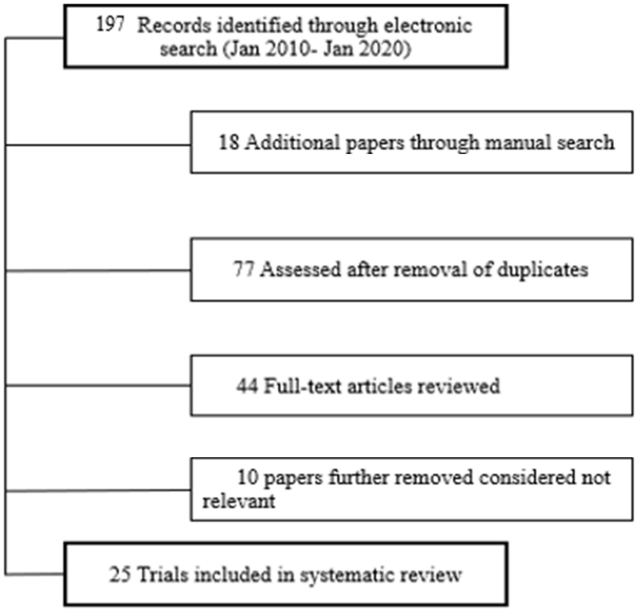

Figure 1. *Flow chart showing article selection for the review.*

3. Results

3.1. Distribution of K13-Propeller Polymorphisms in P. falciparum across sub-Saharan Africa

A total of 25 published trials were successfully reviewed and analyzed. The table 1 below presents a summary of samples analyzed per study from each country and the distribution of the *K13*-propeller polymorphisms. A total of 74 independent k13 mutations were identified across malaria-affected African countries where 7 unconfirmed k13 mutations were associated with delayed parasite clearance. Out of all the trials reviewed Kenya had the largest number of samples analyzed, as well as higher prevalence of SNPs.

A single synonymous mutation in codon C469C was found to be reported in a number of West African countries i.e. Ghana, Nigeria and Kenya. Among the three countries samples from Kenya had the second largest number of SNPs, with 5 different mutations each. Overall, majority of the reported mutations were non-synonymous. Only 3 SNPs appeared in >1 country: the non-synonymous 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 countries. Studies from countries like Cameroon, Ethiopia, Madagascar, and Nigeria have not reported parasites with *K13*-propeller mutant allele.

In another study in Mali mutations were found within six kelch domains of the protein (K13 propeller), which is hypothesized to be the region of the protein associated with prolonged parasite clearance. Interestingly, two haplotypes contained mutations in the same position as mutations observed in Cambodia but had different amino acids present (i.e., G449S and D584N). In addition, mutations at positions 578 and 581 were also observed in Mali and were very close to the C580Y mutation, a key mutation associated with artemisinin resistance in Cambodia.

A total of three different mutations (R471R, R575R and V494I) were identified in five samples, all collected after the introduction of ACT in Angola and Mozambique. The R471R mutation detected in Angola has already been reported in other African countries such as DR-Congo and Gabon. However, the mutations R575R (Angola) and V494I (Mozambique), remains to be validated.

Polymorphism of K13-propeller by sequencing 602 *P. falciparum* isolates collected from patients with uncomplicated malaria in Niger, during a rainy season. Thirteen single-nucleotide polymorphisms (SNPs) including eight specific to Niger at a low frequency from 0.02% to 2.7% was recorded.

Two mutations, R539T and P574L which have also been associated with ART resistance, were observed in two samples from Angola and Equatorial Guinea. The key mutations associated with delayed parasite clearance time in Southeast Asia and with high survival rates in *invitro* (C580Y, I543T, R539T, N458Y and Y493H) has not been reported.

Table 1. *Summary of the major findings in the articles reviewed.*

Author and year of publication	Country	Year	*PfK13* (*kelch* propeller region) codon position	No of corresponding genes (Number of patients)	Highest reported Prevalence (%)	P value
Kamau et al.,[13]	Democratic Republic of the Congo, Gabon, Ghana, Kenya, Mali	2013-2014	A578S	95 ± 9.0	2.3 ± 0.7	0.074
	Democratic Republic of the Congo		Y493Y	82	1.2	0.812
	Kenya		T478T	108	1	0.321
Torrentino-Madamet et al.,[14]	Senegal	2012-2014	T149S and K189T	138	6.3	

Author and year of publication	Country	Year	*PfK13* (*kelch* propeller region) codon position	No of corresponding genes (Number of patients)	Highest reported Prevalence (%)	P value
Amed Ouattara et al., [15]	Mali	2014-2015	G449S and D584N	87	1	0.263
Escobar et al., [5]	Mozambique	2013-2015	V4941	50		0.801
	Angola		R471R, R575R	50		0.121
Laurent et al., [16]	Kenya	2016-2017	A578S, V568G, D584G, and R539K	251	5.2%	0.549
Gupta et al., [17]	Mozambique	2016-2018	L619L, F656I, V666V and G690G	351		0.886
Laminou et al.,[18]	Niger	2015-2016	C580Y, R539T, Y493H, I543T and N458Y	602	2.7	0.533
Musyoka et al [19]	Kenya	2015-2017	W611S	94		0.756

3.2. Prevalence of Non-synonymous SNPs in Pfkelch13 Gene

The overall prevalence of non-synonymous SNPs in pfkelch13 gene (confirmed and unconfirmed) in reviewed articles from malaria-endemic countries was 27.6% (194/827) (95% CI 17.9–35.3%). Of 165 non-synonymous SNPs in pfkelch13 gene reported in this review, 75 (45.5%) were found in single *P. falciparum* parasite infections. In Africa, 47 of 76 (61.8%) reported non-synonymous k13 mutations were found in single *P. falciparum* parasite infections. The most prevalent k13 mutation, 40.8% (31/76) in Africa is A578S. The other non-synonymous SNPs in pfkelch13 gene frequently reported among African parasite infections include T149S, D464H, D584V, K189T, N554L and V494I. The k13 mutation P574L that occurs in SEA was reported in Rwanda and Uganda and in a study on immigrants from Africa to China. The other mutations reported in low frequency in both Africa and SEA include A675V, P584L, R575K, 522C 189T, R561H, M476I, F446I, N554S, and A578S. Twenty of the seventy-four (27%) non-synonymous K13 mutations in Africa had also been reported in Southeast Asia.

4. Discussion

In this study, we have attempted to profile the distribution of *K13* polymorphisms in Africa. The breadth of this survey, with 197 articles that were sampled for molecular mapping in malaria-affected African countries with data regarding therapeutic efficacy of ACT. In this systematic review we report 7 k13 mutations that are not confirmed (not validated) despite being associated with delayed parasite clearance. Despite the current association of some of these alleles with increased parasite clearance half-life, there is a need to validate all frequently reported point mutations in the K13 gene among *P. falciparum* isolates in different regions, especially in sub-Saharan Africa, which bears over 90% of malaria burden. A recent study by Mukherjee et al. [20] showed that parasites carrying K13 mutation D584V had higher *in vitro* survival rates. Mutations (D584 V) have been reported in SEA and among African migrants to China [21]. In the present review, 25 of 74 K13 mutations observed had occurred outside the propeller region of pfkelch13 gene. Seven of these mutations have been associated with delayed parasite clearance rates in Africa. Molecular screening for ART-resistance currently is based on sequencing propeller region of pfkelch13 gene (1, 725, 980–1726, 940 bp; amino acid positions 419–707). This review indicate that studies have recently established existence of non-synonymous mutations outside the propeller region of *Pfkelch13* gene that are associated with increased ART parasite clearance half-life. A mutation in K189T was reported by Torrentino-Madamet et al. [14] in Senegal and Ikeda et al.[11] in Uganda.

In Southeast Asia, findings have shown a strong relationship between K13- propeller mutations and the reduced parasite clearance after artemisinin combination treatment [22, 23]. However, in sub-Saharan Africa, studies indicate no clear association between the observed mutations in K13 gene and the reported cases of delayed parasite clearance or of prolonged [24, 25] and a key question is whether K13-propeller mutations observed in Africa are also associated with artemisinin resistance. This raises the possibility that K13-propeller mutations do not cause artemisinin resistance in isolation but act in combination with other genetic or non-genetic factors that differ in African and Southeast Asia parasite populations.

Since mutations in *Pfk13*, the candidate gene investigated here do not account for the reported cases of drug failure after treatment with ACT in Africa, other approaches should be followed. These include whole-genome sequencing of resistant parasite genes for comparison with the sequences of sensitive strains [26, 27], analysis of gene expression profiles using microarrays, microsatellite mapping, genome wide hybridization and single nucleotide polymorphism mapping [27]. These findings do not exclude the possibility that the gene products of the genes examined play a role in the mechanism of artemisinin's action, since resistance can be conferred through a variety of mechanisms, such as changes in influx and efflux mechanisms. In addition, the review identifies that artemisinin resistance is conferred by multiple gene mutations, with each thought to contribute differently to resistance.

5. Conclusion

Although artemisinin resistance in South-East Asia seems to be a heritable genetic trait, none of the candidate genes suggested by earlier studies confer artemisinin resistance to the observed clinical failure in Africa. The use of a genome-wide approach by whole genome sequencing and gene expression transcriptome studies to identify the

molecular basis of artemisinin resistance is now indicated.

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