Original Research Article

Evaluating the Genetic Landscape of *Plasmodium falciparum PfKelch13* Gene Polymorphisms in Côte d'Ivoire Following a Decade of Artemisinin-based Combination Therapy.

ABSTRACT

Introduction: Artemisinin-based combination therapies (ACTs) are the mainstay of malaria treatment globally. However, their effectiveness is threatened by the emergence of resistance in *Plasmodium falciparum* (*P.f*), particularly in Southeast Asia (SEA). Specific mutations within the *pfKelch13* gene, such as Cys-580-Tyr, Arg-539-Thr, Tyr-493-His, and Ile-543-Thr, have been strongly linked to delayed parasite clearance following ACT treatment. This study aimed to investigate polymorphisms within the *pfKelch13* gene (also known as the *K13-propeller* or *K13* gene) in four regions of Côte d'Ivoire. Côte d'Ivoire experiences year-round malaria transmission and has utilized ACTs for over a decade.

Methods: From September 2013 to July 2014, samples were collected from patients residing in Abidjan (south), Ayamé (southeast), Man (west), and Korhogo (north) who presented with microscopically confirmed uncomplicated malaria. Following *P.falciparum* DNA extraction, nested PCR was employed to amplify an 849 bp fragment of the *pfKelch13* gene. Amplicons were subsequently sequenced and analyzed using BioEdit software.

Results: 531 DNA sequences were analyzed including 301 (58.7 %) from Abidjan, 61(11.5 %) from Ayamé, 93 (17.5 %) from Man and 76 (13.4 %) from Korhogo. Only 20 isolates carrying 22 mutations were observed including 6 non-synonymous single-nucleotide polymorphisms (nsSNP): 4 in Abidjan (Asp-535-Met; Ala-578-Ser; Phe-583-Ser and Ile-601-Thr) and 2 in Korhogo (Asp-559-Asn and Val-510-Met). Only synonymous SNP (sSNP) were identified in the two other Towns. The proportion of mutated pfK13 sequences is 3.8 % (20/531).

Conclusion: The identification of non-synonymous mutations in this study underscores the importance of heightened surveillance for potential ACT resistance in *P. falciparum* within Côte d'Ivoire. Combining in vitro assays, such as the Ring-stage Survival Assay, with molecular testing will be crucial for definitively determining the phenotypic impact of these mutations on parasite susceptibility to ACTs

Keywords: *PfKelch13*, *P. falciparum*, Resistance, ACTs, Côte d'Ivoire

1. INTRODUCTION

Malaria, transmitted by female Anopheles mosquitoes, is the most prevalent and critical parasitic disease in tropical regions. Concerted efforts against both malaria vectors (*Anopheles*) and parasites (*Plasmodium*) have significantly reduced its incidence (29 %) and mortality (60 %) over the past 15 years [1]. However, malaria still caused an estimated 234 million cases and 593,000 deaths worldwide in 2022, with children under 5 disproportionately affected [2].The emergence of parasite resistance to antimalarial drugs, particularly artemisinins, poses a significant challenge to malaria elimination. This resistance initially manifests as delayed parasite clearance, progressing to complete therapeutic failure [3-5]. To combat resistance, the World Health Organization (WHO) recommends ACTs, which combine an artemisinin derivative (artemether, artesunate, or dihydroartemisinin) with a longer-acting partner drug (mefloquine, amodiaquine, lumefantrine, piperaquine, etc.) [6].

However, the emergence of ACT resistance in Southeast Asia, particularly Cambodia, threatens this strategy.

The first reports of *P.falciparum* resistance to artemisinin derivatives originated near the Thai-Cambodian border, following artesunate-mefloquine administration [7]. Studies in western Cambodia and northwestern Thailand confirmed tolerance to artesunate, characterized by delayed parasite clearance, after monotherapy [4-5, 8]. Research suggests a parasite genetic basis for ACT resistance [9]. Several genomic studies have implicated chromosomes 10, 13, and 14 in the resistance phenotype. On chromosome 13, mutations within a region harboring seven candidate genes were observed [10-11]. The *pfKelch13* gene, located on chromosome 13, has been identified as a key player in artemisinin resistance. Mutations in this gene, including Thr-493-His, Arg-539-Thr, Cys-580-Tyr, and Ile-543-Thr, have been demonstrably linked to delayed parasite clearance in both in vivo and in vitro studies [12]. These findings were further corroborated by a survival test involving genetically modified parasites [13].

While these mutations were initially confined to Asia, human movement raises concerns about their potential spread to other endemic regions, especially sub-Saharan Africa [14]. Although nine mutations associated with artemisinin resistance have been detected in African isolates, their prevalence remains relatively low [15]. In Côte d'Ivoire, artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) were the first- line and second-line treatments for uncomplicated malaria, respectively. However, only therapeutic efficacy studies have been routinely conducted to monitor their effectiveness [16-23]. Notably, no investigations have been undertaken to analyze the *pfKelch13* gene in parasites circulating within the country's diverse epidemiological zones. This study aims to address this critical gap by analyzing the polymorphism of the *pfKelch13* gene to identify potential mutations conferring resistance to ACTs in Ivorian *P.falciparum* isolates.

2. METHODOLOGY

2.1. Site and study period

This study was conducted from September 2013 to July 2014 in four sentinel sites for monitoring antimalarial resistance in Côte d'Ivoire: Abidjan, Ayamé, Man, and Korhogo. Abidjan, the economic capital, located in southern Côte d'Ivoire. The study was conducted in Abobo, specifically at the community-based health facility of Anonkoua-Kouté (5°25'55.90"N, 4°02'45.27"W). This area experiences high annual rainfall exceeding 1700 mm, supporting year-round malaria transmission. Ayamé, located in the southeast, approximately 100 km from Abidjan (5°36'12.43"N, 3°09'19.36"W). Ayamé benefits from similar rainfall patterns (1700 mm/year) to Abidjan, resulting in persistent malaria transmission. Man is situated in the western mountainous and forested region (7°24'N, 7°33'W). This area receives abundant average annual rainfall (1800 mm/year), with malaria transmission occurring for 8 to 12 months of the year. Korhogo is the capital of the northern savannas health district (9°59'N, 6°49'W). Korhogo experiences a sub-Sahelian climate with less intense malaria transmission compared to other regions. Average annual rainfall is lower here at 1289 mm.

2.2. Study population and Sampling

This study enrolled patients with symptoms suggestive of malaria who presented at the study sites across all age groups. Isolates from Abidjan, Man, and Korhogo were collected from patients participating in clinical trials evaluating the therapeutic efficacy of artemisinin-combination therapies (ACTs) under the supervision of the National Malaria Control Program (PNLP). Only isolates obtained on the day of enrollment (Day 0) were included in this study.

Additionally, in Abidjan, isolates collected during routine clinical care from March to July 2014 were also included. Isolates from Ayamé were collected from patients presenting to the general hospital with suspected malaria during routine consultations.

For sample processing, the malaria diagnosis was initially performed using a rapid diagnostic test (RDT), specifically the SD BIOLINE Malaria Antigen P.f® test. This was followed by microscopic confirmation through examination of Giemsa-stained thick and thin blood smears. For patients with confirmed malaria, blood were collected from a vein at the elbow crease into an EDTA tube. Three (100 µl each) drops of samples were then spotted onto Whatman 3MM filter paper. The filter papers were dried in a dust-protected environment before storage at room temperature. Patients were included in the clinical trials if their parasitemia ranged from 1,000 to 200,000 trophozoites/µL of blood. Patients presenting for routine care were included regardless of their parasite density. Written informed consent was obtained from all adult participants or their legal guardians. In the case of minor children, assent was obtained in addition to parental consent. The study protocol was approved by the Ivory Coast National Ethics Committee during the session of August 14, 2013 (N° 56/MSLS/CNER-dkn).

2.3. DNA isolation and genotyping

QiaAmp DNA Blood Mini Kit (250 tests/kit; Qiagen # 51306) was used to isolate *P.falciparun* DNA according to the manufacturer's recommendations. Polymorphic domain of *pfKelch13* gene has been amplified by nested-PCR [12]. The expected final size of the fragment corresponding to the amplified region was 849 bp. The pairs of primers K13_PCR_F-CGGAGTGACCAAATCTGGGA / K13_PCR_R-GGGAATCTGGTGGTAACAGC were used for the first amplification and K13_N1_F-GCCAAGCTGCCATTCATTTG/K13_N1_R-GCCTTGPCRAGAAAGA for the second.

The first PCR was done in 25 µl containing 0.625 µL of each primer (10 µM), 5µL of premix (5x HOT FIREPol® Master Mix Ready to Load with 12,5 mM MgCl2; Solis Biodyne), 13.75 µL of molecular biology water and 5 µL of DNA. As to second PCR, 50 µl containing 1.25 µL of each primer (10 µM), 10 µL of premix (5x HOT FIREPol® Master Mix Ready to Load with 12,5 mM MgCl2; Solis Biodyne), 32.5 µL of molecular biology water and 5 µL of DNA were prepared. The PCR reactions were carried out in GenAmp700® thermal cycler (Applied Biosystems™) in following conditions: 15 mn at 95 °C (initial denaturation); 30 s at 95 °C (denaturation), 2 mn at 58 °C (annealing) and 2 mn at 72 °C (extension) for 40 cycles and 10 mn at 72 °C (final extension). Except annealing and extension time (1mn each), both PCR performed under the same conditions. PCR products were detected using 2 % agarose gel electrophoresis and SyberGreen® staining. Extraction and genotyping took place within molecular biology platform (pfbm) of Institute Pasteur de Côte d'Ivoire (IPCI). Positive controls (K1 to K6), in the form of DBS, were provided by the Pasteur Institute of Cambodia (IPC). DNA of these parasites was extracted and amplified simultaneously with those of the present work.

2.4. Sequencing

Following amplification, the 849 bp secondary PCR products were dispensed into 96-well plates. These plates were then shipped to Macrogen Institute (Seoul, South Korea) for Sanger sequencing of both strands. The reference sequence for polymorphism identification was the 3D7 strain of *P.falciparum* (PF3D7_1343700), analyzed using BioEdit software [24].

2.5. Statistical analyses

Data were compiled in Microsoft Excel (version 2003) and analyzed using GraphPad Prism 6 software. Mean parasite densities between study sites were compared using the Student's t-test. The Chi-square test was used to compare proportions between groups. A significance level of alpha = 0.05 was employed for all statistical tests.

3. RESULTS

3.1. Patient demographic characteristics

A total of 593 isolates were collected, with 309 from women and 284 from men (sex ratio: 1.1 females to males). The average patient age was 14 years old (range: 1-80 years). The mean parasite density across all four study sites was 49,591 trophozoites/µL of blood (ranging from 1,000 to 587,000). However, parasite densities varied significantly between locations. In Ayamé, it is 82,937 trophozoites/µL of blood (1,340-397,730) and 41,089 trophozoites/µL of blood (2,005-200,000) in Man. At Korhogo, 23,939 trophozoites/µL of blood (lowest density). And in Abidjan, 52,281 trophozoites/µL of blood (1,000-587,000). Statistical analysis revealed a significantly higher parasite density in Ayamé compared to all other sites ($p < 0.0001$). Additionally, parasite densities in Man were significantly higher than those in Korhogo ($p = 0.012$). However, no significant difference was observed between parasite densities in Abidjan and Man ($p = 0.107$) (Table 1).

Table 1: Demographic and parasitological characteristics of patients

Characteristics	Abidjan	Man	Korhogo	Ayamé	Total
Number	319	119	85	70	593
Gender (F/M)	176/143	55/64	47/32	31/39	309/284
Ages Mean (min-max)	13.9 ±0.6 (1- 68)	14.4 ± 1.2 (1- 62)	13.1 ±1.5 (1- 80)	18.2 ±1.5 (1- 53)	14.0 ±0.5 (1-80)
Age groups					
< 5 years (%)	48 (15)	26 (21.8)	19 (22.4)	15 (41.4)	108 (18.2)
≥ 5 years (%)	271 (85)	93 (78.2)	66 (77.6)	55 (78.6)	485 (81.8)
Parasite densities (PD) Mean	52281 ±3875	41089 ±4614	23939 ±4527	82937 ±10424	49591 ±2741

Table 2: PCR and sequencing results

Sites	Isolates	PCR K13 N (%)	Sequences obtained N (%)	Sequences analyzed N (%)	Mutated sequences (N=20)		
					S N (%)	NS N (%)	NS + S N (%)
Abidjan	319	309 (96.8)	308 (100)	301 (97.4)	8 (1.5)	2 (0.4)	1 (0.2)
Ayamé	70	64 (91.4)	62 (96.9)	61 (98.4)	1 (0.2)	0 (0.0)	0 (0.0)
Korhogo	85	78 (91.8)	78 (100)	76 (97.4)	4 (0.7)	2 (0.4)	0 (0.0)
Man	119	108 (90.7)	108 (100)	93 (86.1)	2 (0.4)	0 (0.0)	0 (0.0)
Total	**593**	**559 (94.3)**	**557 (99.6)**	**531 (95.3)**	**15 (2.8)**	**4 (0.8)**	**1 (0.2)**

S= Synonymous; NS= Non-synonymous; N= Number

3.2. Genotyping and detection of pfK13 mutants

Out of the 593 collected isolates, DNA amplification and subsequent sequencing were successful for 559 (94.3 %) and 557 (99.6 %) samples, respectively. Following sequence sorting, 531 sequences (95.3 %) were successfully aligned and analyzed. Overall, sequence analysis revealed mutations in 20 isolates (3.8 %). Among these mutated parasites, 15 harbored only synonymous mutations, while 4 exhibited only non-synonymous mutations. Notably, only one isolate contained a parasite with both a synonymous mutation and two non-synonymous mutations, representing a triple mutation (Tables 2 and 3). In terms of alleles, 17 types of mutations have been identified, including 11 synonymous (Cys-469-Cys, Thr-478-Thr, Tyr-493-Tyr, Gly-496-Gly, Val-510-Val, Tyr-519-Tyr, Thr-535-Thr, Asn-537-Asn, Val-589-Val, Arg-597-Arg and Ile-601-Thr) and 6 non-synonymous (Val-510-Met, Thr-535-Met, Asp-559-Asn, Ala-578-Ser, Phe-583-Leu and Pro-655-Pro). The Cys-469-Cys, Val-510-Val and Asn-537-Asn alleles were each identified in two different isolates while Thr-535-Thr was detected in three isolates. Hence, 22 point mutations identified in 20 isolates (Table 3). As to proportions of mutations identified, Thr-535-Thr mutation (0.6 %) is the most mutation represented followed by Cys-469-Cys (0.4 %), Val-510-Val (0.4 %) and Asn- 537-Asn (0.4 %) and the others 0, 2 % each. Overall, the prevalence of mutations is 4.4 % (Table 3). The distribution of mutated sequences according to study sites indicates 3.6 % (11/301), 2.1 % (2/93), 7.9 % (6/76) and 1.6 % (1 / 61) respectively in Abidjan, Man, Korhogo and Ayamé. The comparison of these proportions indicates a significant difference between those of Korhogo and Abidjan (P = 0.002) and between those of Korhogo and Man (P = 0.047).

Table 3: Polymorphism observed in the *PfKelch13* gene in the different sites

Sites	Sample ID	Reference	Isolat	SNP position	n (%)	Types of SNP	Alleles
Abidjan	099-14	gct	**T**ct	**Ala - 578 - Ser**	1 (0.2)	nsSNP	4
	04-023*	att	**C**tt	**Ile - 601 - Thr**	1 (0.2)		
	04-023*	ttt	**C**tt	**Phe -583 - Leu**	1 (0.2)		
	53 PK18	acg	a**T**g	**Thr - 535 - Met**	1 (0.2)		
	348 ANK	ggt	gg**C**	Gly - 496 - Gly	1 (0.2)	sSNP	7
	01-005	tat	ta**C**	Tyr - 519 - Tyr	1 (0.2)		
	01-035	aga	**C**ga	Arg - 597 - Arg	1 (0.2)		
	101-14	cca	cc**C**	Pro - 655 - Pro	1 (0.2)		
	04-023*/054-14	aat	aa**C**	Asn - 537 - Asn	2 (0.4)		
	207 HGA/01-111	gtg	gt**A**	Val - 510 - Val	2 (0.4)		
Abidjan and Man	148-14/03-046	tgc	tg**T**	Cys - 469 - Cys	2 (0.4)		
Korhogo	02-062	gat	**A**at	**Asp - 559 -Asn**	1 (0.2)	nsSNP	2
	02-133	gtg	**A**tg	**Val - 510 - Met**	1 (0.2)		
	02-008	acc	ac**A**	Thr - 478 - Thr	1 (0.2)	sSNP	3
	02-009	gtc	gt**G**	Val - 589- Val	1 (0.2)		
Ayamé and Korhogo	02-058/02-092/286 AYA	acg	ac**A**	Thr - 535 - Thr	3 (0.6)		
Man	03-097	tac	ta**T**	Tyr - 493 - Tyr	1 (0.2)	sSNP	1
Total	**20/531 (3.8 %)**				**22 (4.4)**		**17**

*Triple mutant sequence (04-023); nsSNP= non-synonymous single nucleotide polymorphism; sSNP= synonymous single nucleotide polymorphism

4. DISCUSSION

The emergence of artemisinin-resistant *P.falciparum* strains in Southeast Asia [4, 12] poses a significant public health threat. Human movement [25-28] or local parasite evolution [29] could further disseminate these resistant strains to other endemic regions. Initially, four specific mutations (Cys-580-Tyr, Arg-539-Thr, Tyr-493-His and Ile-543-Thr) were associated with artemisinin resistance. However, the number of reported mutations has grown to over 15, with additional candidate mutations identified in both Southeast Asia, the initial epicenter, and in countries using artemisinin-combination therapy (ACTs) [30]. These findings suggest the possibility of mutations arising independently or persisting despite ACT use. In African countries, a significant number of single nucleotide polymorphisms (SNPs) in the *pfKelch13* gene have been reported [15, 30-32]. Notably, three mutations (Cys-469-Phe, Pro-574-Leu, and Ala-675-Val) associated with delayed parasite clearance in Southeast Asia were detected in Uganda [33]. Similarly, Rwanda has witnessed an increase in the pfKelch13 polymorphism rate (0 % in 2010 to 12.1 % in 2019), including the validated mutation Arg-561-His [34-35].

However, most African polymorphisms differ from those observed in Southeast Asia [36]. Additionally, some genetic changes might potentially reduce *P.falciparum* sensitivity to artemisinin derivatives [37-38]. These findings highlight the need for continued monitoring and potentially alternative genetic surveillance strategies.

Since the introduction of artemisinin derivatives in Côte d'Ivoire, several studies have consistently evaluated the effectiveness of artemisinin-based combination therapies (ACTs), particularly artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ) [16, 18-20, 22-23]. The results of these studies showed high efficacy (>90 %), although some cases of parasitological failure due to re-infection were observed. Despite this encouraging data, the history of resistance development to previous antimalarial drugs, such as chloroquine [39-41] and sulfadoxine-pyrimethamine [40, 42-43], raises concerns about the possible ineffectiveness of artemisinin derivatives. Therefore, the analysis of Kelch13 gene polymorphism in four health districts of the country to determine the prevalence of possible pfK13 mutants is imperative. While our analysis did not reveal any of the major mutations in the *pfKelch13* gene associated with delayed parasite clearance and reduced susceptibility to artemisinin in young parasites, several non-synonymous single nucleotide polymorphisms (SNPs) were identified. These include Val-510-Met, Thr-535-Met, Asp-559-Asn, Ala-578-Ser, Phe-583-Leu, and Ile-601-Thr. Importantly, none of these SNPs are included among the 16 structurally related mutations linked to *P.falciparum* resistance to artemisinin [15].

One isolate from Abidjan exhibited a triple mutation (Asn-537-Asn/Phe-583-Leu/Ile-601-Thr). Although these specific mutations haven't been linked to reduced ACT efficacy, the presence of multiple mutations in a single parasite warrants further investigation regarding their potential impact on future drug resistance. Interestingly, the Ala-578-Ser mutation, frequently observed in African isolates [32, 44], was also detected in our study. Unlike the nearby Cys-580-Tyr mutation, which is strongly associated with delayed parasite clearance, Ala-578-Ser is not typically linked to this phenotype. However, a study by [45] reported an association between Ala-578-Ser and prolonged parasite clearance in Ugandan children with severe malaria treated with injectable artesunate. This finding suggests a potential role for Ala-578-Ser in severe malaria, at least in Africa, requiring further research. It is noteworthy that a recent study in neighboring Ghana identified a 3.6 % prevalence of the C580Y mutation in blood donors (Aninagyei et al., 2020), raising concerns about potential spread to Côte d'Ivoire. Additionally, the proportion of Ala-578-Ser mutations in Ghana has increased significantly, from 0.23 % (2007-2016) to 4.8 % in 2020 [46-47].

While the absence of major resistance-associated mutations in the *pfKelch13* gene suggests no current artemisinin resistance in Côte d'Ivoire, the observed diversity of pfKelch13 mutants raises concerns about the potential for de novo emergence. This concern aligns with previous studies highlighting the possibility of new resistance arising through

spontaneous mutations within the parasite population [29, 48]. Indeed, the emergence and spread of antimalarial drug resistance are complex processes influenced by several factors, including parasite biology. Spontaneous mutations in the parasite genome can lead to the development of new resistant strains. This resistance arises when these mutations, conferring reduced drug susceptibility, are selected for and then transmitted to future generations of parasites.

Malaria transmission intensity can also influence the spread of drug resistance, although the exact relationship remains unclear. Some studies suggest that high transmission areas favor the emergence of resistant strains [49]. This is because resistant mutants have a higher chance of transmitting their mutations to the next generation in high-transmission settings. Conversely, others propose that low transmission areas might see a higher frequency of mutations due to a greater likelihood of fusion between gametes carrying mutated genes [50].

Our study observed a higher prevalence of mutations (7.9 %) in Korhogo, a region with reportedly low malaria transmission, compared to Abidjan (3.6 %) and Man (2.1 %). While seemingly contradictory, this finding could be explained by the "fusion hypothesis" mentioned above. Low transmission areas often have a higher proportion of monoclonal infections (infections caused by a single parasite clone). In such cases, the chance of two gametes carrying different mutations fusing is increased, potentially leading to a higher frequency of observed mutations despite lower transmission rates. Supporting this hypothesis, studies in northern Togo have shown a strong correlation between rainfall (associated with higher transmission) and malaria prevalence [51]. Additionally, the significant difference in mean parasitemia (parasite burden) observed between Korhogo and Man in our study further suggests potential differences in transmission intensity between these locations.

Our study identified several non-synonymous single nucleotide polymorphisms (SNPs) in the *pfKelch13* gene in Côte d'Ivoire. Notably, none of these mutations were previously reported in neighboring West African countries [44, 52-58]. Furthermore, these SNPs are not associated with artemisinin resistance in Southeast Asia (SEA) and are not known to be prevalent in migrants from neighboring countries. These observations suggest that the observed pfKelch13 SNPs might be unique to parasite populations in Côte d'Ivoire and potentially represent the result of purifying selection (selection favoring beneficial mutations). Further in vitro studies are necessary to determine whether these specific mutations have any impact on the effectiveness of ACTs in Côte d'Ivoire.

The delayed emergence of artemisinin resistance in Africa compared to SEA might be attributed to several factors. First, artemisinin was introduced much later in Africa (2000-2005) compared to SEA (1970s) [59]. This reduced selection pressure for resistance-conferring mutations in African parasites. Second, genetic differences between parasite populations in SEA might make them inherently more susceptible to developing artemisinin resistance compared to African parasites [60-61]. Additionally, the majority of reported artemisinin-resistant parasites originate from the Greater Mekong Subregion [62-63], suggesting potential regional influences on resistance emergence.

5. CONCLUSION

Our analysis revealed that 3.8 % of the isolates harbored mutated parasites containing non-synonymous SNPs. Importantly, these mutations were not associated with delayed parasite clearance. Nevertheless, the presence of these mutations underscores the importance of heightened surveillance for potential artemisinin resistance in *P.falciparum*. Combining in vitro Ring-stage Survival Assays with molecular testing will be crucial for definitively determining the phenotypic impact of these mutations on parasite susceptibility to ACTs.

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