

Minireview Article

Molecular Determinants of Artemisinin Resistance in K13 Gene of *Plasmodium falciparum*

Abstract

Artemisinin-based combination therapy (ACT) is the first-line therapy in most malaria endemic countries. An impressive 47% reduction in mortality rate globally between 2000 and 2013 is achieved by ACT and artemisinin (ART) monotherapy. However, artemisinin resistance (AR) by *Plasmodium falciparum* is now prevalent across south-east Asia (SEA). AR is indicated by delayed parasite clearance more than 3 days after standard artemisinin treatment and reduced in-vitro insusceptibility. Recent works has shown association of AR with mutations in the propeller domain of a kelch gene on chromosome 13 (PF3D7_1343700, K13 gene) of *P. falciparum*. C580Y mutation of K13 gene was highly prevalent in Cambodia, Myanmar and eastern and western Thailand while F446I mutation is predominant in China-Myanmar border regions as well as Myanmar. AR is not reaching India and Africa where although non-synonymous mutations were present, these were not associated with delayed parasite clearance. Because the location of Myanmar is central between SEA and Africa, a country-specific strategy for Myanmar Artemisinin Resistance Containment (MARC) is necessary to be successfully achieved. Moreover, regular periodic tracking of prevalent molecular determinant like C580Y, F446I, etc. will be beneficial.

Keywords: Malaria, *Plasmodium falciparum*, artemisinin-based combination therapy, artemisinin resistance, K13 gene

Introduction

Artemisinin-based combination therapy (ACT) is the first-line therapy in most malaria-endemic countries [1]. However, AR is now prevalent across SEA [2-8]. It was evidenced by delayed parasite clearance after ART treatment. The first clinical ART resistant *P. falciparum* cases were observed in Cambodia in 2008 while reduced susceptibility to artesunate in vivo in the same location was reported in 2009 [1]. The World Health Organization (WHO) recommends that results of therapeutic efficacy studies to be complimented by molecular marker studies. A genome-wide association approach was finally able to pinpoint a specific gene associated with AR, the K13 gene on chromosome 13 of *P. falciparum* [9]. In vitro parasite survival rates and in vivo parasite clearance rates were strongly correlated with mutations in K13 propeller region (especially C580Y, R539T, and Y439H) in Cambodia indicating that these are important molecular determinants of AR [5,10].

AR in Greater Mekong Subregion (GMS) is a major threat to the malaria endemic countries throughout the world because chloroquine resistance (CQR) by *P. falciparum* has spread from GMS to Africa causing morbidity and high mortality especially in children [5, 11, 12]. Therefore ART Containment Project was initiated by WHO in GMS [9]. Out of these K13 mutations, C580Y is highly prevalent in Cambodia, eastern and western Thailand as well as Myanmar [5, 13]. In case of CQR,

39 K76T of *P.falciparum* chloroquine resistance transporter (*pfcr*t) gene was the important and
40 consistent molecular marker [14, 15]. In this review, prevalence and spread of K13 alleles in different
41 regions of SEA were mainly discussed. Furthermore, the information on comparison of C580Y of K13
42 propeller domain and K76T marker of *pfcr*t gene was provided in this article.

43 **The Starting of AR which is correlated with K13 gene mutations in Cambodia**

44 In 2008 in Cambodia, 2 out of 94 patients had prolonged parasite clearance time after a
45 monotherapy. In 2009, there was a confirmation trial in western Cambodia, Thailand and Vietnam in
46 which parasite clearance time was measured and prolonged parasite clearance time was observed
47 [11].

48 In 2014, Arie et al. indicated that the ART resistant phenotype was associated with mutations on
49 the kelch propeller protein K13 encoded in PF3D7_1343700 gene. In Cambodia, these
50 polymorphisms were first described and reported that K13 mutants (mainly Y493H, R539T, I543T
51 and C580Y) had correlation with prolonged in vitro parasite survival rates and in vivo parasite
52 clearance rates. Sequencing of the K13-propeller of archived parasite isolates from Cambodian
53 patients with malaria between 2001–2012 was performed and DNA sequences were analysed.
54 Overall, 17 mutant alleles were observed, out of which three high-frequency alleles were C580Y,
55 R539T and Y493H (Table 1). The frequency of the C580Y allele increased significantly from 2001–
56 2002 to 2011–2012 in two western provinces, indicating rapid invasion of the mutant parasite
57 population and near fixation in these areas [5]. In August 2014, the World Health Organization
58 included K13 mutations in the new working definition for AR. The presence of >5% of patients
59 carrying K13 resistance-associated single nucleotide polymorphisms (SNPs) defines suspected AR
60 while K13 mutants are associated with either persistent parasitemia on day 3 or longer parasite
61 clearance defines confirmed resistance [2]. K13-propeller SNPs have been investigated in multiple
62 locations to detect the presence of resistant parasites before clinical resistance spreads, following
63 WHO recommendation. Up to now, more than 60 mutations in K13 gene have been described in
64 South East Asia and Africa [5, 16–20]. However, outside GMS no resistance-associated mutations
65 have been observed [1]. Multiple non-synonymous polymorphisms have been published, but there
66 were very few shared mutant alleles between African and SEA parasites up to now [5,16,19]. K13
67 resistance-associated mutants from Southeast Asia were not found in Africa [17–20].

68 **Is AR in Myanmar an immediate threat to Africa?**

69 In the study on AR in Myanmar, Tun et al. reported that 26 different mutations were observed,
70 including nine mutations not described previously in SEA. It was observed that 39% of the samples
71 studied carried a K13-propeller mutation while the combined K13-mutation prevalence was more
72 than 20%. AR extends across Myanmar as indicated by the information that *P. falciparum* parasites
73 carrying K13-propeller mutations were highly prevalent. Delayed parasite clearance after ART
74 treatment [5, 6, 8, 16] and reduced in-vitro insusceptibility [21, 22] were associated with the most
75 prevalent mutations. Normal rates of parasite clearance were associated with the uncommon
76 propeller mutations. Ninety percent of mutations were common after amino acid no. 440 of K13
77 protein. The three previously described mutations F446I, P574L, C580Y were highly prevalent than
78 the mutations unique to Myanmar (Table 1). Overall, C580Y mutation was observed in over 90
79 samples, F446I was 80 and P574L was 41, out of the three common K13 mutations. These three
80 previously described highly prevalent mutations constitute more than 56% of the total no. of drug

81 resistant samples. Most of the K13 mutations in Myanmar were clustered in first four blades of
82 propeller region. Synonymous mutations were rare with less than 3 samples in this study. In the so-
83 called stem section of the K13 protein, E252Q mutation was commonly observed and present in 17
84 samples [13].

85 AR can follow historical paths of the spread of CQR from SEA, via Myanmar, through India to Africa,
86 which leads to loss of millions of lives in Africa from 1980–2004. Myanmar is an important part of
87 the frontline in the battle to prevent AR not to spread to India and Africa [23]. However, a jumping
88 phenomenon, in which direct spread of AR due to increase in international travel and migration, can
89 occur [24]. Emergence of resistant parasite independently is an alternative scenario called popping.

90 Myanmar was highly prevalent with malaria when compared to other SEA countries [25]. AR, due to
91 its wide spread use of this drug, could reverse recent downward trends in morbidity and mortality
92 from malaria in the country. Knowledge of the level of K13 molecular determinants provides
93 snapshot information of the extent of AR. Present ACTs are failing in areas where AR is spreading.
94 The incidence of drug resistant *P. falciparum* will begin to rise again and this is the real treat to the
95 country [13].

96 The findings in Myanmar for K13 mutations indicated the northwest regions close to the Indian
97 border was extended by these mutations. However, there is no evidence that AR has reached India
98 in term of K13 mutations although there were 8 out of 169 patients who remained parasitemic on
99 day 3 after treatment. This delayed parasite clearance was observed in three different regions of
100 north-eastern India where ART and sulfadoxine–pyrimethamine (SP) combination of ACT were used
101 for malaria treatment.[26] There is no indication of presence of AR in Bangladesh [27] and relatively
102 few K13-mutant parasites at the western border of Myanmar with Bangladesh [13]. These data
103 suggests that K13 mutations indicative of AR in Myanmar is not immediate threat to African
104 countries.

105 **Spread of AR Alleles in Thailand**

106 A total of 417 patient samples from the year 2007 were investigated for AR alleles in the malaria
107 surveillance studies across ten provinces in Thailand. A total of seven K13 mutant alleles were found,
108 out of which C580Y, R539T, P574L were prevalent (Table 1) while the R575K and S621F mutations
109 have previously not been reported in Thailand. AR alleles were present in 8 out of 10 Thai provinces
110 sampled. C580Y alleles which are associated with AR were the most common and widespread in
111 Thailand. C580Y and R539T were the only two mutations observed in eastern Thailand [9]. C580Y
112 and R575K mutations have been reported near the Thai-Myanmar border while many other
113 mutations were observed in the western Thailand [19,28].

114 Analysis of microsatellite loci of two C580Y haplotype revealed that alleles circulating in the east and
115 west comprise two distinct lineages evidenced by differences in the 8.6kb locus downstream of the
116 K13 gene suggesting the C580Y mutations may have arisen independently in the Thai-Cambodia and
117 Thai-Myanmar borders. C580Y alleles are less heterozygous between each other when compared to
118 wild type suggests recent independent origins along the Thai-Cambodia and Thai-Myanmar borders.
119 The information which supports the hypothesis that two different haplotypes of C580Y emerged
120 independently was association between geographic distance and genetic difference.

Miotto et al. observed that parasites with the most common K13 mutant alleles (C580Y, I543T, R539T, and Y493H) were found in the countries in the SEA region, indicating that cross-border movement of *P. falciparum* may have already occurred [29]. Because of continuous drug pressure, it can be expected that resistant alleles, such as the C580Y mutation, may eventually become fixed in the population. During the 11 years (2001–2012), the C580Y allele prevalence increased from 40% to 90% in Pailin, Cambodia [5]. The C580Y mutation was nearing fixation in that population and occurrence of similar situation may be possible in Thailand. Because ACT is one of our last working treatment options for drug resistant *P. falciparum*, these AR data were threatening effective antimalarial therapies. The study in Thailand indicated ART-resistant K13 alleles have been evolving along both the Thai-Cambodian border and Thai-Myanmar border in 2007 before the ART containment project was implemented in 2009 [9].

F446I K13 mutation is prevalent in China – Myanmar border

One hundred and eighty archived *P. falciparum* isolates, mainly collected in 2007–2012 were studied for mutations in the K13 gene from the China-Myanmar border. Seventeen point mutations were identified in 46.1% of parasite clones, of which seven were not previously described before. The C580Y mutation that is correlated with AR in the previous studies was observed in 1.6 % of the parasite clones while F446I mutation was found in 27.2% of the clones (Table 1). In the kelch domain of the K13 gene, point mutations were common more than 40%. There was increase in the frequency of kelch domain mutations through the years of study [12].

Another outstanding phenomenon discovered in this study is 69.1% of parasites harboured eight N residues while wild type parasite has six N residues in the N-terminus of the K13 gene. Interestingly, the predominant mutation F446I was observed only in parasites with eight N repeats. In the previous study, this kind of micro-satellite variation in other gene is associated with altered sensitivities to quinines indicating this phenomenon was deserved to be investigated in the future study [30]. Clinical efficacy studies in this region detected day-3 parasite positivity rate to >10% after artesunate treatment. As a result, increased parasite surveillance with the genetic determinants is needed to continue to determine further spread of AR [12].

Mechanisms of Pf kelch 13 mutation in artemisinin resistance

Artemisinins are potent inhibitors of *Plasmodium falciparum* phosphatidylinositol-3-kinase (PfPI3K). When there is mutation in PfKelch 13, polyubiquitination of PfP13K is reduced and there is increased level of this kinase, resulting in normal metabolism of the parasite. Consequently, *P. falciparum* erythrocytic stage is continuous and clinical malaria is progressing. This indicates mutations in PfKelch 13 gene or increased PfP13K is the marker of AR [31].

A gain to loss: Artemisinin resistance

As a consequence of resistance of *P. falciparum* to widely used antimalarial drugs like chloroquine (CQ) and SP, mortality and morbidity rates due to malaria were increased. In 2005, WHO recommended ACT to be used as first-line treatments for falciparum malaria in all malaria endemic countries. ART derivatives are highly potent, rapidly eliminated antimalarial drugs, which clear parasitemia more rapidly than other antimalarial agents [6]. Use of ACT and ITN reduced the

morbidity and mortality to a certain extent [32]. However, recently slow parasite clearance rate was observed in SEA countries and this information endangered the gains achieved by ACT. Slow parasite clearance rate was due to reduced susceptibility of ring stage parasite. There was marked variation in parasite clearance rate in various regions of SEA. Median parasite clearance half-life values ranged from 2 hours in Laos to 7 hrs in Thailand. A widely used criterion for AR is the percentage of patients with parasitemia detectable on microscopy at 72 hours of treatment [6].

Efficacy of ART in Africa

In African countries, it was well observed that ACTs can clear parasites efficiently. Besides that K13 propeller mutations are not at high level. Synonymous mutations were more common than non-synonymous mutations (Table 1) [1] [20] [33] [34]. In addition, because ART was given definitely as ACTs, there are no early treatment failure as well as no deaths due to AR. AR can be arose independently because millions of courses were given to the populations. According to SEA experience, AR has been developed independently three times. The chance of emerging of AR in Africa seems to be independently rather than spread from SEA. The K13 SNPs specific in Africa are totally different to the ones prevalent in SEA [12].

Table 1. Mutations in Kelch 13 gene observed in SEA countries and African countries

SEA country or region	Samples of years and Sample size	Mutations observed	African country ^a	Study Period	Mutations observed
Cambodia [5]	2001 – 2012 1091 samples	C580Y, R539T Y493H**, G449A I543T, N537I T474I, P574L R561H, A481V D584V, S623C P553L, V568G N458Y, G533S T508N	Angola [33]	Before ACT –2003 After ACT- 2010. 100 samples	Synonymous R471R R575R
Myanmar	2013-2014	C580Y, F446I,	Mozambique[33]	Before ACT	Nonsynonymous

(including	940	P574L** ,		– 2003-2005	V494I
Tak province	samples	A676D, G449A,		After ACT –	
of		N458I,		2010	
Thailand)[13]		N537I, E252Q,		100 samples	
		M476I, R561H,			
		G538V ^b			
Thailand	2007	C580Y , R539T ,	Kenya [1]	2012-2013	Nonsynonymous
(Thai-	417	P574L** ,		539 samples	M442V, N554S,
Cambodia	samples	S621F, R575K,			A569S, A578S
and Thai-		E556D, N458Y.			Synonymous
Myanmar					C439C, S477S
border					Y500Y, N531N
regions) [9]					G538G
China-	2007-2012	F446I , P574L** ,	Senegal [34]	2012-2013	T149S, K189T
Myanmar	180	C580Y , K189T,		138 samples	
border [12]	samples	E252Q, R255K,			
		P441L, N458Y,			
		A676D, H719N,			
		N11Y, I352T,			
		I376V, P443S,			
		C469Y, L492S,			
		F495L			

177

178 ^a There were other studies in African countries in which non-synonymous mutations were observed

179 [20}.

^b There were totally 29 non-synonymous mutations in Myanmar. Only the common mutations were shown in this table.

** Bolded mutations were the most prevalent mutation in SEA 4 regions. Yellow and blue highlighted mutations were present in all four regions of SEA. Red and Green highlighted mutations were present in two regions of SEA.

C580Y marker in K13 gene in comparison with K76T marker of *pfcr* gene

First identification of the markers

In search of the gene responsible for AR, genome-wide association studies (GWAS) were applied (Table 2). In vitro resistant lines to dihydroartemisinin (DHA) could be developed in *P. falciparum* in the laboratory. By simply analysing the complete genome of the laboratory-adapted resistant clones, the molecular marker was discovered. Tucker and colleagues showed that resistant lines acquired few non-synonymous mutations, including one in an uncharacterized protein on chromosome 13, PF3D7_1343700, which would later come to be known as kelch13 [5]. The K13 gene mutation was associated with AR although it was not the cause. For confirmation, a genome modification method which was well applied in other eukaryotes was used to study the K13 alleles and it was observed that these K13 alleles gave rise to AR. C580Y mutation was engineered into propeller domain of K13 gene of a sensitive *P. falciparum* strain. In vitro drug sensitivity assay was done afterwards with ART and it was observed that two clones had showed resistance of ring stages [11]. In this way, C580Y mutation was found out in the laboratory. In the study in western Cambodia, this mutation was identified at high prevalence when compared with other point mutations [5]. Also in the very recent study in Myanmar, C580Y mutation was observed in more than 90 isolates and it was more prevalent than other two prevalent mutations [13].

P. falciparum chloroquine resistance (CQR) transporter (PfCRT) protein consists of 424 amino acids and was encoded by *pfcr* gene. Mutation in *pfcr* gene is the central determinant for CQR. It was suggested that this protein transports haemoglobin digestion products as well as ions. The amino acid substitution, which is caused by one nucleotide change, at residue 76 of PfCRT causes CQR. The 36kb segment of chromosome 7 carries a 13-exon gene, *pfcr*. K76T mutation was first observed in the genetic cross experiment between Dd2 CQR and HB3 chloroquine sensitive (CQS) clones together with other seven point mutations at the amino acid positions 74, 75, 220, 271, 326, 356, and 371 (Table 2). Out of 8 mutations, seven of the mutations were found in CQR parasite lines from Asia, Africa, and South America. On exon no. 2, three amino acids 74, 75, 76 were present. All *pfcr* alleles in CQR parasites consistently include mutations for K76T and A220S accompanied by changes in two to six other mutations. The significance of K76T and A220S in CQR was proved by a CQ trial in West Africa [14].

Consistency and Importance of the markers

Although C580Y was highly prevalent in ART resistant regions and it was associated with delay parasite clearance after standard ACT course, it cannot be used as a consistent molecular marker for AR. While C580Y mutation was prevalent in one region, other regions were specific with F446I mutation and P574L in Myanmar [13]. In western Cambodia, seven individual mutations seem to have arisen independently. These mutations include C580Y, which is reaching near fixation in various regions [13]. The most prevalent K13 mutation in eastern and western Thailand and Myanmar was C580Y mutation (Table 2). Other mutations are outcompeted by fitter alternatives such as C580Y although the widespread P574L mutation seems to be associated with parasite clearance that is at least as slow as C580Y [6,16]. In conclusion, C580Y mutation is associated with very slow parasite clearance when compared with other point mutations.

In case when amino acid residue lysine is replaced at codon 76 by threonine that means K76T mutation, CQR phenotype is appeared. Fidock et al. have shown there is association of K76T point mutation in *Pfcr*t protein and the CQR in their study [14]. Therefore, the possibility of this marker to be molecular marker to detect CQR *P. falciparum* malaria was proposed [15]. Studies from different countries, carried out on field isolates confirmed the presence of K76T mutation in CQR *P. falciparum* isolates [35-45]. Some literatures strongly indicate that a molecular marker for the faster detection of CQR *P. falciparum* is a point mutation K76T in the *Pfcr*t protein (Table 2).

Molecular markers as epidemiologically applicable haplotypes

More than 60 mutations in K13 gene was reported in SEA and Africa. However, those mutations in Africa were not associated with AR. The mutations observed in Africa rarely shared with prevalent mutations described in SEA. In Myanmar, there was evidence of combined mutations in 20% of samples. However the authors did not mention the associated mutations [13]. In Western Cambodia and Myanmar, there were mutations in three continuous amino acid residues namely R539T, G538V and N537I with two in Cambodia and two in Myanmar [5, 13]. However, there was no association of these mutations in only one isolate. Up to now, because of association of three or more point mutations in isolates is relatively rare, the haplotype terminology for epidemiological purpose cannot be applied in the way as K76T and its associated mutations in CQR. However, in terms of microsatellite diversity and differences in the 8.6kb locus downstream of the K13 gene two haplotypes of C580Y was described (Table 2.) [9]. These two haplotypes arose independently in distant geographical areas, which were epidemiological significance.

Fifteen point mutations in the *pfcr*t gene resulting in amino acid changes have been reported. Based on amino acids at codon 72–76, a number of *pfcr*t haplotypes have been identified for epidemiological purpose (Table 2). These haplotypes varied with geographic origins and their CQ susceptibilities. In CQR isolates from Asia/Africa and South America, CVIET and SVMNT haplotypes were described respectively while CVMNK haplotype is universally identified in CQS parasites [46]. Sutar et al. analysed DNA sequence of the second exon of the *pfcr*t gene and the 5' end of the *P. falciparum* multidrug-resistance 1 (*pfmdr*-1) gene. The two mutations primarily implicated in CQ-resistance are *pfcr*t K76T and *pfmdr*-1 N86Y mutations in these two respective genes. Most of the previous works on CQR in *P. falciparum* isolates mentioned that both *pfcr*t K76T and *pfmdr*-1 N86Y mutations are commonly used genetic markers for determination of CQR [47].

256 Table 2. Comparison of different characteristics of C580Y marker of K13 gene and K76T marker of
257 *pfcr* gene

Characteristics	C580Y marker of K13 gene	K76T marker of <i>pfcr</i> gene
First identification of the marker	Genome-wide association studies (GWAS) - analysis of mutations acquired specifically by laboratory-adapted parasite clones which survive high doses of ART in vitro, followed by genome modification method with discovered allele (C580Y) [5,11]	Genetic cross between Dd2 CQR and HB3 CQS clones [14]
Consistency and Importance of the marker	1. C580Y was higher prevalent than other markers in artemisinin resistant regions [5,9,13]. 2. It was associated with delayed and longer parasite clearance than other markers after standard ACT course [13]. 3. It is not a consistent molecular marker for AR [12].	1. Field isolates confirmed the presence of K76T mutation in CQR <i>P. falciparum</i> isolates in different malaria endemic countries [40-42]. 2. It is a molecular marker for the faster detection of CQR <i>falciparum</i> malaria [37,39, 40,42,44].
Haplotypes	Microsatellite diversity flanking K13 gene determines two	Based on amino acids at codon 72-76 including K76T, a

haplotypes of C580Y [9].	number of <i>pfcr</i> haplotypes
	have been identified for
	epidemiological purpose [46].

258

259 The future of ACT or ARTmonotherapy

260 The introduction of new antimalarial drug is followed by resistance to the drug in the following
 261 years. One of the antimalarial drug atovacone was resistant by *P. falciparum* in the same year in
 262 which it was started to use. After resistance to CQ, S-P, mefloquine and atovacone have acquired,
 263 the new antimalarial class of new drug can be extracted from the plant *Artemisia annua*. ART based
 264 drug are combined with the other antimalarial drug to be used as combined therapy ACT. WHO
 265 recommended that ART to be used together with other drug because of the reason to delay the
 266 appearance of the resistance. In areas where other drugs are resistant by the parasite, ACTs are the
 267 most effective treatment and parasite clearance are very rapid until recently. To date, parasites have
 268 acquired resistance to ART monotherapy as well as some ACTs [11]. After widespread introduction
 269 of dihydroartemisinin–piperaquine in Cambodia, there is rapidly emerging resistance to both the
 270 ART and the piperaquine components within 3 years [48].

271 AR has impact in the treatment of malaria patient. Health authorities tried to focus on intensive
 272 planning to prevent AR from spreading. However, the efficacy of this drug loses reliance when
 273 compared with previous half decade. It is assumed that in the future ART will have the same
 274 situation as CQ and this will threaten the malaria endemic regions of the world [11].

275 Conclusions and Recommendations

276 AR is the threat to African countries to fight against malaria as previous experience with CQR
 277 causing high morbidity and mortality in Africa in the later part of 20th century. Myanmar is at the
 278 central location of spread of drug resistance from SEA to India and ultimately Africa. AR was
 279 spreading in Myanmar. Although elimination of *P. falciparum* parasites in the Mekong region is a top
 280 priority [49], this task will not be easily achieved before clinical efficacy of ACTs wanes. However,
 281 the success of malaria research in defining the molecular determinants of AR is the gaining point in
 282 the war against malaria. Because of these molecular markers, the extent of AR in Myanmar could be
 283 well studied and containment strategy could be laid down. The results up to now indicated that AR
 284 associated mutations of K13 gene were still absent out of GMS. However, subclinical infections can
 285 be the source of drug resistant malaria which can be determined by *pfmdr1* copy number (CN) [50].
 286 Therefore, tracking of AR by molecular determinants of K13 genes and detection of the *pfmdr1* CN
 287 should be included as strategic plan for the MARC program in Myanmar. Screening of K13 mutations
 288 together with other markers and other anti-mosquito measures should be intensively done in all the
 289 regions including remote areas in GMS including Myanmar.

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