

Minireview : Molecular Determinants of Artemisinin Resistance in **k13** Gene of *Plasmodium falciparum*

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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* susceptibility. 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, non-synonymous mutations, south-east Asia, Africa

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, K76T of *P.falciparum chloroquine resistance transporter (pfcr)* gene was the important and consistent molecular marker [14, 15]. In this review, prevalence and spread of K13 alleles in different regions of SEA were mainly discussed. Furthermore, the information on comparison of C580Y of *k13* propeller domain and K76T marker of *pfcr* gene was provided in this article.

The Starting of AR which is correlated with *k13* gene mutations in Cambodia

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

In 2014, Arie et al. indicated that the ART resistant phenotype was associated with mutations on the kelch propeller protein K13 encoded in *pf3d7_1343700* gene. In Cambodia, these polymorphisms were first described and reported that *k13* mutants (mainly Y493H, R539T, I543T and C580Y) had correlation with prolonged in vitro parasite survival rates and in vivo parasite clearance rates. Sequencing of the *k13*-propeller of archived parasite isolates from Cambodian patients with malaria between 2001–2012 was performed and DNA sequences were analysed. Overall, 17 mutant alleles were observed, out of which three high-frequency alleles were C580Y, R539T and Y493H (Table 1). The frequency of the C580Y allele increased significantly from 2001–2002 to 2011–2012 in two western provinces, indicating rapid invasion of the mutant parasite population and near fixation in these areas [5]. In August 2014, the World Health Organization included *k13* mutations in the new working definition for AR. The presence of >5% of patients carrying *k13* resistance-associated single nucleotide polymorphisms (SNPs) defines suspected AR while *k13* mutants are associated with either persistent parasitemia on day 3 or longer parasite clearance defines confirmed resistance [2]. *k13*-propeller SNPs have been investigated in multiple locations to detect the presence of resistant parasites before clinical resistance spreads, following WHO recommendation. Up to now, more than 60 mutations in *k13* gene have been described in South East Asia and Africa [5, 16-20]. However, outside GMS no resistance-associated mutations have been observed [2]. Multiple non-synonymous polymorphisms have been published, but there were very few shared mutant alleles between African and SEA parasites up to now [5,16,19]. *k13* resistance-associated mutants from Southeast Asia were not found in Africa [17–20].

Is AR in Myanmar an immediate threat to Africa?

In the study on AR in Myanmar, Tun et al. reported that 26 different mutations were observed, including nine mutations not described previously in SEA. It was observed that 39% of the samples studied carried a *k13*-propeller mutation while the combined *k13*-mutation prevalence was more

than 20%. AR extends across Myanmar as indicated by the information that *P. falciparum* parasites carrying **k13**-propeller mutations were highly prevalent. Delayed parasite clearance after ART treatment [5, 6, 8, 16] and reduced in-vitro insusceptibility [21, 22] were associated with the most prevalent mutations. Normal rates of parasite clearance were associated with the uncommon propeller mutations. Ninety percent of mutations were common after amino acid no. 440 of K13 protein. The three previously described mutations F446I, P574L, C580Y were highly prevalent than the mutations unique to Myanmar (Table 1). Overall, C580Y mutation was observed in over 90 samples, F446I was 80 and P574L was 41, out of the three common **k13** mutations. These three previously described highly prevalent mutations constitute more than 56% of the total no. of drug resistant samples. Most of the **k13** mutations in Myanmar were clustered in first four blades of propeller region. Synonymous mutations were rare with less than 3 samples in this study. In the so-called stem section of the K13 protein, E252Q mutation was commonly observed and present in 17 samples [13].

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

Myanmar was highly prevalent with malaria when compared to other SEA countries [25]. AR, due to its wide spread use of this drug, could reverse recent downward trends in morbidity and mortality from malaria in the country. Knowledge of the level of **k13** molecular determinants provides snapshot information of the extent of AR. Present ACTs are failing in areas where AR is spreading. The incidence of drug resistant *P. falciparum* will begin to rise again and this is the real treat to the country [13].

The findings in Myanmar for **k13** mutations indicated the northwest regions close to the Indian border was extended by these mutations. However, there is no evidence that AR has reached India in term of **k13** mutations although there were 8 out of 169 patients who remained parasitemic on day 3 after treatment. This delayed parasite clearance was observed in three different regions of north-eastern India where ART and sulfadoxine–pyrimethamine (SP) combination of ACT were used for malaria treatment.[26] There is no indication of presence of AR in Bangladesh [27] and relatively few **k13**-mutant parasites at the western border of Myanmar with Bangladesh [13]. These data suggests that **k13** mutations indicative of AR in Myanmar is not immediate threat to African countries.

Spread of AR Alleles in Thailand

A total of 417 patient samples from the year 2007 were investigated for AR alleles in the malaria surveillance studies across ten provinces in Thailand. A total of seven **k13** mutant alleles were found, out of which C580Y, R539T, P574L were prevalent (Table 1) while the R575K and S621F mutations have previously not been reported in Thailand. AR alleles were present in 8 out of 10 Thai provinces sampled. C580Y alleles which are associated with AR were the most common and widespread in Thailand. C580Y and R539T were the only two mutations observed in eastern Thailand [9]. C580Y and R575K mutations have been reported near the Thai-Myanmar border while many other mutations were observed in the western Thailand [19,28].

Analysis of microsatellite loci of two C580Y haplotype revealed that alleles circulating in the east and west comprise two distinct lineages evidenced by differences in the 8.6kb locus downstream of the **k13** gene suggesting the C580Y mutations may have arisen independently in the Thai-Cambodia and Thai-Myanmar borders. C580Y alleles are less heterozygous between each other when compared to wild type suggests recent independent origins along the Thai-Cambodia and Thai-Myanmar borders. The information which supports the hypothesis that two different haplotypes of C580Y emerged 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

Although there are many proposed mechanisms of artemisinins in killing parasites, there was strong evidence that these are potent inhibitors of *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K). When there is C580Y mutation in *P. falciparum* **kelch13** (**Pfkelch13**), polyubiquitination of PfPI3K and its binding to PfKelch13 were reduced resulting in normal level of PfPI3K with consequent normal metabolism of parasite. This information points out that mutation in **kelch 13** gene gives rise to loss

of effect of ART. Therefore it is concluded that mutations in *k13* gene is the possible molecular determinants for 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 Kenya out of four non-synonymous mutants, the mutant allele A578S in the *k13* propeller gene was detected in 2 consecutive seasons on Mfangano Island. This mutation is close to the highly prevalent SNP C580Y from Cambodia and modifies amino acid from being hydrophobic to hydrophilic one. This change is thought to be necessary in protein-protein interactions. The investigation of genotypes of the parasites from this island is critical to understand the ACT efficiency in this geographic area. However, there was no information on in vitro parasite survival rates, and in vivo parasite clearance rates for this mutant in this study [1].

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 countries or regions	Samples of years and Sample size	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
Myanmar (including Tak province of Thailand)[13]	2013-2014 940 samples	C580Y, F446I, P574L**, A676D, G449A, N458I,N537I, E252Q, M476I, R561H ^b .
Thailand (Thai-Cambodia and Thai- Myanmar border regions) [9]	2007 417 samples	C580Y, R539T, P574L**, S621F, R575K, E556D, N458Y.
China-Myanmar border [12]	2007-2012 180 samples	F446I, P574L**, C580Y, K189T, E252Q, R255K, P441L, N458Y, A676D, H719N, N11Y, I352T, I376V, P443S, C469Y, L492S, F495L
African countries^a		
Angola [33]	Before ACT –2003 After ACT - 2010 100 samples	Synonymous R471R, R575R
Mozambique[33]	Before ACT – 2003-2005 After ACT – 2010 100 samples	Non-synonymous V494I
Kenya [1]	2012-2013 539 samples	Non-synonymous M442V, N554S, A569S, A578S

		Synonymous C439C, S477S, Y500Y, N531N, G538G
Senegal [34]	2012-2013 138 samples	Non-synonymous T149S, K189T

^a There were other studies in African countries in which non-synonymous mutations were observed [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 *pfprt* gene

In this section, the importance of C580Y marker in **k13** gene for AR was discussed in comparison with K76T marker of *pfprt* gene which is the major molecular determinant of CQR in three characteristics which was also mentioned in brief in Table 2.

1. First identification of the markers

1.1. 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].

1.2. *P.falciparum* chloroquine resistance (CQR) transporter (PfCRT) protein was encoded by *pfcr*t gene. Mutation in *pfcr*t gene is the central determinant for CQR. The amino acid substitution, which is caused by one nucleotide change, at residue 76 of PfCRT causes CQR. 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 (Table 2). Out of 8 mutations, seven of the mutations were found in CQR parasite lines from Asia, Africa, and South America. All *pfcr*t alleles in CQR parasites consistently include mutations for K76T and A220S accompanied by changes in two to six other mutations [14].

2. Consistency and Importance of the markers

2.1. 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.

2.2. In case when amino acid residue lysine is replaced at codon 76 by threonine that means K76T mutation, CQR phenotype is appeared. 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 Pfcrt protein.

3. Molecular markers as epidemiologically applicable haplotypes

3.1. More than 60 mutations in **k13** gene was reported in SEA and Africa. **However, those mutations in Africa were not associated with AR [2,33].** 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.

3.2. 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 susceptibility. 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].

Table 2. Three different characteristics of C580Y marker of *k13* gene and K76T marker of *pfcr*t gene

Characteristics	C580Y marker of <i>k13</i> gene	K76T marker of <i>pfcr</i> t gene
First identification of the marker	Genome-wide association studies (GWAS) 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	C580Y was higher prevalent than other markers in artemisinin resistant regions It is not a consistent molecular marker for AR [5,9,12,13].	It is a molecular marker for the faster detection of CQR <i>falciparum</i> malaria [37,39, 40,42,44].
Haplotypes	Microsatellite diversity flanking <i>k13</i> gene determines two haplotypes of C580Y [9].	Based on amino acids at codon 72–76 including K76T, a number of <i>pfcr</i> t haplotypes have been identified for epidemiological purpose [46].

The future of ACT or ART monotherapy

The introduction of new antimalarial drug is followed by resistance to the drug in the following years. One of the antimalarial drug **atovaquone** was resistant by *P. falciparum* in the same year in which it was started to use. After resistance to CQ, S-P, mefloquine and **atovaquone** have acquired, the new antimalarial class of new drug can be extracted from the plant *Artemesia annua*. ART based drug are combined with the other antimalarial drug to be used as combined therapy ACT. WHO recommended that ART to be used together with other drug because of the reason to delay the appearance of the resistance. In areas where other drugs are resistant by the parasite, ACTs are the most effective treatment and parasite clearance are very rapid until recently. To date, parasites have acquired resistance to ART monotherapy as well as some ACTs [11]. After widespread introduction of dihydroartemisinin–piperaquine in Cambodia, there is rapidly emerging resistance to both the ART and the piperazine components within 3 years [47].

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

Conclusions and Recommendations

AR is the threat to African countries to fight against malaria as previous experience with CQR causing high morbidity and mortality in Africa in the later part of 20th century. Myanmar is at the central location of spread of drug resistance from SEA to India and ultimately Africa. AR was spreading in Myanmar. Although elimination of *P. falciparum* parasites in the Mekong region is a top priority [48], this task will not be easily achieved before clinical efficacy of ACTs wanes. However, the success of malaria research in defining the molecular determinants of AR is the gaining point in the war against malaria. Because of these molecular markers, the extent of AR in Myanmar could be well studied and containment strategy could be laid down. The results up to now indicated that AR associated mutations of **k13** gene were still absent out of GMS. However, subclinical infections can be the source of drug resistant malaria which can be determined by *pfmdr1* copy number (CN) [49]. Therefore, tracking of AR by molecular determinants of **k13** genes and detection of the *pfmdr1* CN should be included as strategic plan for the MARC program in Myanmar. Screening of **k13** mutations together with other markers and other anti-mosquito measures should be intensively done in all the regions including remote areas in GMS including Myanmar.

DISCLOSURE

The authors have no conflict of interest to disclose.

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