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

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Authors' contributions

This work was carried out in collaboration between both authors. Author ZL designed the study, reviewed the literatures and wrote the first draft of the manuscript. Author MTZ managed the literature searches, gave the opinion and checked the manuscript. Both authors read and approved the final manuscript.

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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 the global mortality rate between 2000 and 2013 has been achieved by ACT and artemisinin (ART) monotherapy. However, artemisinin resistance (AR) by *Plasmodium falciparum* (*P. falciparum*) is now prevalent across south-east Asia (SEA). AR is indicated by delayed parasite clearance of more than 3 days after standard ART treatment and reduced *in vitro* susceptibility. Recent work has shown association of AR with mutations in the propeller domain of the *kelch* gene on chromosome 13 (PF3D7_1343700, *k13* gene) of *P. falciparum*. The C580Y mutation of the *k13* gene is highly prevalent in Cambodia, Myanmar and eastern and western Thailand, while the F446I mutation is predominant in the China-Myanmar border regions as well as in Myanmar. AR has not reached India and Africa, where non-synonymous mutations not associated with delayed parasite clearance are present. Because the location of Myanmar is central between SEA and Africa, a country-specific strategy for Myanmar Artemisinin Resistance Containment (MARC) is necessary. Moreover, regular periodic tracking of prevalent molecular determinants such as C580Y and F446I mutations will be beneficial.

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1. INTRODUCTION

Artemisinin-based combination therapy (ACT) is used as first-line therapy in most malaria-endemic countries [1]. However, AR is now prevalent across SEA [2-8]. It is manifested 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* was reported in 2009 in the same location [1]. The World Health Organization (WHO) has recommended that therapeutic efficacy studies should be complemented by molecular marker studies. A genome-wide association approach was pinpointed 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 are strongly correlated with mutations in the *k13* propeller region (especially C580Y, R539T, and Y439H) in Cambodia, indicating that these are important molecular determinants of AR [5,10].

AR in the 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 the GMS to Africa causing morbidity and high mortality especially in children [5,11,12]. Therefore, the ART Containment Project was initiated by the WHO in the GMS [9]. Of these *k13* mutations, C580Y is highly prevalent in Cambodia, eastern and western Thailand and Myanmar [5,13]. In cases of CQR, K76T mutation of the *P. falciparum chloroquine resistance transporter* (*pfcrt*) gene was the important and consistent molecular marker [14, 15]. In this review, the prevalence and spread of K13 alleles in different regions of the SEA are discussed. Furthermore, information on comparison of the C580Y mutation of the *k13* propeller domain and the K76T marker of the *pfcrt* gene is provided in this article.

2. INITIATION OF AR IS CORRELATED WITH *K13* GENE MUTATIONS IN CAMBODIA

In 2008 in Cambodia, 2 out of 94 patients were found to have prolonged parasite clearance time after monotherapy. In 2009, a trial in western Cambodia, Thailand and Vietnam confirmed that the parasite clearance time was prolonged [11].

In 2014, Ariey et al. [5] indicated that the ART resistant phenotype was associated with mutations in the kelch propeller protein, K13, encoded by the *pf3d7_1343700* gene. In Cambodia, these polymorphisms were described and it was reported that *k13* mutants (Y493H, R539T, I543T and C580Y) correlated with prolonged *in vitro* parasite survival rates and *in vivo* parasite clearance rates. Sequencing of the *k13*-propeller region of archived parasite isolates from Cambodian patients with malaria between 2001 and 2012 was performed, and DNA sequences were analyzed. Overall, 17 mutant alleles were detected, among which the 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 WHO 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, and *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 recommendations. To date, more than 60 mutations in the *k13* gene have been described in SEA and Africa [5,16-20]. However, no resistance-associated mutations have been observed outside the GMS [2]. Multiple non-synonymous polymorphisms have been published, but there have been very few shared mutant alleles between African and SEA parasites until now [5,16,19]. *k13* resistance-associated mutants from SEA have not been found in Africa [17–20].

3. IS AR IN MYANMAR AN IMMEDIATE THREAT TO AFRICA?

In the study on AR in Myanmar, Tun et al. reported that 29 different mutations were observed, including nine mutations not described previously in SEA. It was noted 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 found distal to amino acid 440 of the K13 protein. The three previously described mutations F446I, P574L, C580Y were highly prevalent compared to the mutations unique to Myanmar (Table 1). Of these three common *k13* mutations, the C580Y mutation was observed in over 90 samples, F446I was found in 80 and P574L was found in 41. These three previously described highly prevalent mutations were present in more than 56% of the total number of drug-resistant samples. Most of the *k13* mutations in Myanmar were clustered in the first four blades of the propeller region. Synonymous mutations were found in fewer than 3 samples in this study. In the so-called stem section of the K13 protein, the E252Q mutation was commonly observed and was present in 17 samples [13].

AR may follow the historical paths of the spread of CQR from SEA, via Myanmar, through India to Africa, which led to loss of millions of lives in Africa from 1980 to 2004. Myanmar is an important part of the frontline in the battle to prevent the spread of AR to India and Africa [23]. However, direct spread of AR may take place due to increased international travel and migration [24]. The independent emergence of resistant parasite is an alternative scenario.

Myanmar has a high prevalence of malaria compared to other SEA countries [25]. Due to the wide- spread use of this drug, AR could reverse recent downward trends in morbidity and mortality from malaria in the country. Knowledge of the level of *k13* molecular determinants provides a snapshot of the extent of AR. Presently, ACTs are failing in areas where AR is spreading. The incidence of drug resistant *P. falciparum* will begin to rise again, constituting a true threat to the country [13].

The *k13* mutations findings in Myanmar have occurred in the northwest regions close to the Indian border. However, there is no evidence that AR has reached India in terms of *k13* mutations, although there has been a report of 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 suggest that *k13* mutations indicative of AR in Myanmar are not an immediate threat to African countries.

4. SPREAD OF AR ALLELES IN THAILAND

A total of 417 patient samples from 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, including marked prevalence of C580Y, R539T and P574L (Table 1) while the R575K and S621F mutations have not previously been reported in Thailand. AR alleles were present in 8 out of 10 Thai provinces sampled. The C580Y allele, which is associated with AR was 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, and many other mutations were observed in 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. This result is suggested by differences in the 8.6 kb locus downstream of the *k13* gene indicating that the C580Y mutations may have arisen independently at the Thai-Cambodia and Thai-Myanmar borders. C580Y alleles are less heterozygous with each other than with wild type, suggesting recent independent origins along the Thai-Cambodia and Thai-Myanmar borders. This hypothesis that two different haplotypes of C580Y emerged independently is supported by the association between geographic distance and genetic difference.

Miotto et al. [29] observed that parasites with the most common *k13* mutant alleles (C580Y, I543T, R539T, and Y493H) were found in countries in the SEA region, indicating that cross-border movement of *P. falciparum* may have already

occurred. Because of continuous drug pressure, it can be expected that resistant alleles, such as the C580Y mutation, may eventually become fixed in the population. Over 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 a 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 threaten effective antimalarial therapies. The study in Thailand indicated ART-resistant *k13* alleles were evolving along both the Thai-Cambodian border and the Thai-Myanmar border in 2007 before the ART containment project was implemented in 2009 [9].

5. F446I K13 MUTATION IS PREVALENT AT THE CHINA-MYANMAR BORDER

One hundred and eighty archived *P. falciparum* isolates from the China-Myanmar border, mainly collected between 2007 and 2012, were studied for mutations in the *k13* gene. Seventeen point mutations were identified in 46.1% of parasite clones, of which seven had not been previously described. The C580Y mutation which was demonstrated to correlate with AR in previous studies was observed in 1.6% of the parasite clones, while the F446I mutation was found in 27.2% of the clones (Table 1). In the kelch domain of the *k13* gene, point mutations were common and appeared in more than 40% of the samples. There was increase in the frequency of kelch domain mutations during the years of study [12].

Another outstanding phenomenon discovered in this study is that 69.1% of parasites harboured eight N residues while the 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 type of micro-satellite variation in other genes is associated with altered sensitivity to quinine, indicating that this phenomenon merits investigation in future studies [30]. Clinical efficacy studies in this region detected a day-3 parasite positivity rate of >10% after artesunate treatment. As a result, increased parasite surveillance of genetic determinants is needed to continue to determine the further spread of AR [12].

6. MECHANISMS OF THE *PF KELCH 13* MUTATION IN ARTEMISININ RESISTANCE

Although there are many proposed mechanisms of the role of artemisinin in killing parasites, there is strong evidence that it is a potent inhibitor of *P. falciparum* phosphatidylinositol-3-kinase (PfPI3K). A C580Y mutation in *P. falciparum kelch13* (*Pfkelch13*) is responsible for reduction in polyubiquitination of PfPI3K and its binding to PfKelch13 resulting in a normal level of PfPI3K with consequent normal metabolism of the parasite. This information notes that mutations in the *kelch 13* gene give rise to the loss of the effect of ART. Therefore it is hypothesized that mutations in the *k13* gene are a possible molecular determinant of AR [31].

7. A GAIN TO LOSS: ARTEMISININ RESISTANCE

As a consequence of resistance of *P. falciparum* to widely used antimalarial drugs such as chloroquine (CQ) and SP, mortality and morbidity rates due to malaria have increased. In 2005, the WHO recommended ACT as a first-line treatment for falciparum malaria in all malaria endemic countries. ART derivatives are highly potent, rapidly eliminated antimalarial drugs, which clear parasitemia more rapidly than do other antimalarial agents [6]. The use of ACT and ITN reduced the morbidity and mortality to a certain extent [32]. A slow parasite clearance rate has been observed in SEA countries recently, threatening the gains achieved by ACT. A slow parasite clearance rate is due to a reduced susceptibility of the ring stage parasite. There is marked variation in parasite clearance rates in various regions of SEA. Median parasite clearance half-life values ranged from 2 hours in Laos to 7 hours in Thailand. A widely used criterion for AR is the percentage of patients with parasitemia detectable by microscopy at 72 hours of treatment [6].

8. EFFICACY OF ART IN AFRICA

In African countries, it has been clearly observed that ACTs can clear parasites efficiently. Notably, *k13* propeller mutations are not common. Synonymous mutations occur more frequently than non-synonymous mutations (Table 1) [1,20,33,34]. Out of four non-synonymous mutants, the mutant allele A578S in the *k13*

propeller gene was detected during two consecutive seasons on Mfangano Island, Kenya. This mutation is close to the highly prevalent C580Y SNP from Cambodia and modifies an amino acid from being hydrophobic to hydrophilic. This change is thought to be necessary in protein-protein interactions. The investigation of genotypes of the parasites from this island is critical to understanding ACT efficiency in this geographic area. However, there has been no information on *in vitro* parasite survival rates and *in vivo* parasite clearance rates for the mutant in this study [1].

In addition, because ART was given as an ACT, no early treatment failure and no deaths due to AR were observed. AR can arise independently as millions of treatment courses have been administered across populations. According to the SEA experience, AR has been independently detected three times. The chance of AR emerging in Africa seems to be greater from independent development rather than through the spread from SEA. The *k13* SNPs specific to Africa are completely different to the ones prevalent in SEA [12].

9. C580Y MARKER IN THE *K13* GENE IN COMPARISON WITH THE K76T MARKER OF THE *PFCRT* GENE

In this section, three characteristics related to the importance of the C580Y marker in the *k13* gene for AR are discussed in comparison with the K76T marker of the *pfcrt* gene which is the major molecular determinant of CQR (Table 2).

Table 1. Mutations in the *kelch 13* gene observed in SEA and Africa

SEA countries or regions	Samples 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 additional studies in African countries in which non-synonymous mutations were detected [20}.*
[b] *There were a total of 29 non-synonymous mutations in Myanmar. Only the common mutations are shown in this table*
*** Mutations in bold were the most prevalent ones in four SEA regions. Mutations highlighted in yellow and blue were present in all four regions of SEA. Mutations highlighted in red and green were present in two regions of SEA*

9.1 First Identification of Markers

In the search for the gene responsible for AR, genome-wide association studies (GWAS) were applied (Table 2). *P. falciparum* lines resistant to dihydroartemsinin (DHA) were developed *in vitro* in the laboratory. By analyzing 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, the C580Y mutation was engineered into the propeller domain of the *k13* gene of a sensitive *P. falciparum* strain. An *in vitro* ART sensitivity assay was performed afterwards, and it was observed that two clones had showed resistance during the ring stages [11]. In this way, the C580Y mutation was discovered in the laboratory. In the western Cambodian study, this mutation was identified at a high prevalence compared with that of other point mutations [5]. Also in a very recent study in Myanmar, the C580Y mutation was detected in more than 90 isolates, and it was more prevalent than two other prevalent mutations [13].

The *P. falciparum* chloroquine resistance (CQR) transporter (PfCRT) protein is encoded by the *pfcrt* gene. Mutations in the *pfcrt* gene are the central determinant for CQR. An amino acid substitution, which is caused by a single nucleotide change at residue 76 of PfCRT, causes CQR. The K76T mutation was first observed in a genetic cross experiment between Dd2 CQR- and HB3 chloroquine-sensitive (CQS) clones together with seven other point mutations (Table 2). Out of 8 mutations, seven mutations were found in CQR parasite lines from Asia, Africa, and South America. All *pfcrt* alleles in CQR parasites consistently include the K76T and A220S mutations accompanied by changes in two to six other loci [14].

9.2 Consistency and Importance of Markers

Although C580Y is highly prevalent in ART-resistant regions and is associated with delayed parasite clearance after a standard ACT course, it cannot be used as a consistent molecular marker for AR. While the C580Y mutation was prevalent in one region, other regions had high prevalence of the F446I and P574L mutations in Myanmar [13]. In western Cambodia, seven individual mutations seem to have arisen independently. These mutations include C580Y, which is reaching near fixation level in various regions [13]. The most prevalent *k13* mutation in eastern and western Thailand and Myanmar was the C580Y mutation (Table 2). Other mutations are outcompeted by better alternatives such as C580Y although the widespread P574L mutation seems to be associated with parasite clearance that is at least as slow as that associated with C580Y [6,16]. In conclusion, the C580Y mutation is associated with very slow parasite clearance when compared with that of other point mutations.

In cases in which the amino acid lysine is replaced at codon 76 by threonine (K76T mutation), the CQR phenotype is detected. Therefore, this mutation was proposed as a molecular marker for the detection of CQR *P. falciparum* malaria [15]. The presence of the K76T mutation in CQR *P. falciparum* isolates was confirmed in field isolates from different countries [35-45]. Some studies strongly indicate that the point mutation K76T in the Pfcrt protein is a molecular marker for faster detection of CQR *P. falciparum*.

9.3 Molecular Markers as Epidemiologically Applicable Haplotypes

More than 60 mutations in the *k13* gene have been reported in SEA and Africa. However, those mutations in Africa were rarely the same as the prevalent mutations described in SEA and were not associated with AR [2,33]. In Myanmar, there was evidence of combined mutations in 20% of the samples. However, the authors did not mention any specific association [13]. In Western Cambodia and Myanmar, there were mutations in three adjacent amino acid residues, namely R539T, G538V and N537I, while pairs of adjacent amino acid mutations were observed in Cambodia and Myanmar [5,13]. However, there was no association of these mutations in only one isolate. Because the association of three or more point mutations is relatively rare in isolates, the haplotype terminology for epidemiological purposes cannot be applied in the way as K76T and its associated mutations in CQR. However, in terms of microsatellite diversity, and differences in the 8.6 kb locus downstream of the *k13* gene, two haplotypes of C580Y have been described (Table 2) [9].

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

Characteristics	C580Y marker of *k13* gene	K76T marker of *pfcrt* gene
First identification of the marker	Genome-wide association studies (GWAS) followed by genome modification method enabled allele discovery (C580Y) [5,11].	Genetic cross between Dd2 CQR and HB3 CQS clones [14].
Consistency and importance of the marker	C580Y is more prevalent than other markers in artemisinin resistant regions, though it is not a consistent molecular marker for AR [5,9,12,13].	It is a molecular marker that enables faster detection of CQR falciparum malaria [37,39,40,42,44].
Haplotypes	Microsatellite diversity flanking *k13* gene characterizes two haplotypes of C580Y [9].	Based on amino acids at codon 72–76 including K76T, a number of *pfcrt* haplotypes have been identified for epidemiological purpose [46].

These two haplotypes arose independently in distant geographical areas, which have epidemiological significance.

Fifteen point mutations in the *pfcrt* gene resulting in amino acid changes have been reported [Ref]. Based on amino acids at codons 72–76, a number of *pfcrt* haplotypes have been identified for epidemiological purposes (Table 2). These haplotypes vary with geographic origin and their CQ susceptibility. In CQR isolates from Asia/Africa and South America, CVIET and SVMNT haplotypes were described, respectively, while the CVMNK haplotype is universally identified in CQS parasites [46].

10. THE FUTURE OF ACT OR ART MONOTHERAPY

Introduction of new antimalarial drugs can lead to drug resistance in subsequent years. *P. falciparum* developed resistance to the antimalarial drug atovaquone in the same year as it was first used. After resistance to CQ, S-P, mefloquine and atovaquone had developed, a new class of antimalarial drug was extracted from the plant *Artemisia annua*. ART based drugs are combined with other antimalarial drugs to be used as combined therapy ACT. The WHO recommended that ART be used together with other drugs to delay the appearance of resistance. In areas where the parasite shows high resistance, ACT treatment is the most effective and parasite clearance was 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 was rapidly emerging resistance to both the ART and the piperaquine components within 3 years [47].

AR has had a large impact in the treatment of malaria patients. Health authorities have tried to focus on intensive planning to prevent the spread of AR. However, the efficacy of this drug is decreasing in comparison with the previous decade when the WHO recommended it to treat falciparum malaria. It is assumed that in the future ART will have the same path as CQ, which will threaten the endemic malaria regions of the world [11].

11. CONCLUSION AND RECOMMENDATIONS

AR is a threat to African countries in the fight against malaria, as previous experience with CQR caused high morbidity and mortality in Africa in the later part of 20th century. Myanmar is at a central location in the spread of drug resistance from SEA to India and ultimately Africa. AR is 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 the clinical efficacy of ACTs wanes. However, the success of malaria research in defining the molecular determinants

of AR may be a turning point in the war against malaria. As a result of these molecular markers, the extent of AR in Myanmar can be studied, and containment strategies can be developed. The results to date indicate that AR associated mutations of the *k13* gene have not been detected outside of GMS. However, subclinical infections can be the source of drug-resistant malaria, as has been shown by *pfmdr1* copy number (CN) [49]. Therefore, tracking AR by molecular determinants of *k13* genes and detection of the *pfmdr1* CN should be included as a strategic plan for the MARC program in Myanmar. Screening of *k13* mutations together with other markers and other anti-mosquito measures should be intensively undertaken in all regions of Myanmar and other remote areas in GMS.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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