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PHENOTYPIC AND GENOTYPIC CHARACTERISTICS TO TRACK ARTEMISININ RESISTANCE IN PLASMODIUM FALCIPARUM

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ABSTRACT

Artemisinin-based combination therapy (ACT) is the first-line therapy in most malaria endemic countries. However, artemisinin resistance (AR) by *Plasmodium falciparum* (*P. falciparum*) is now prevalent across southeast Asia (SEA). Clinical AR is defined as a reduced parasite clearance rate, or a persistence of microscopically detectable parasites on the third day of ACT. A reduced parasite clearance rate is expressed as an increased parasite clearance half-life. In turn, increased parasite clearance half-life parameter correlates strongly with results from the *in vitro* ring-stage survival assay ($RSA_{0–3\ h}$). Recent work has shown association of AR with mutations in the propeller domain of the *kelch* gene on chromosome 13 (*k13* gene) of *P. falciparum*. Four *k13* alleles with non-synonymous mutations were observed in the northeastern state in India and this state is the likely route for the AR *P. falciparum* to spread into the India in the near future because Myanmar, where AR *P. falciparum* is prevalent, is in the east. Monitoring of AR clinically by parasite clearance supported by molecular genotyping of signature gene is required to track the drug resistant *P. falciparum*.

Key Words: *Plasmodium falciparum*, artemisinin resistance, phenotypic characteristics, genotyping.

INTRODUCTION

Artemisinin-based combination therapy is the first-line therapy in most malaria endemic countries. However, AR by *Plasmodium falciparum* is now prevalent across SEA. AR parasites spreading from western Cambodia to the Greater Mekong Subregion (GMS) and through India to Africa, as previous experience with chloroquine- and sulphadoxine/pyrimethamine-resistant parasites are threatening the population staying in world's malaria endemic area [1-3]. Clinical AR is defined as a reduced parasite clearance rate [4-9], or a persistence of microscopically detectable parasites on the third day of ACT [10]. A reduced parasite clearance rate is expressed as an increased parasite clearance half-life [11, 12]. In turn, increased parasite clearance half-life parameter correlates strongly with results from the *in vitro* ring-stage survival assay ($RSA_{0–3\ h}$) [13].

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However, the present lack of a molecular marker hinders rapid detection of these parasites elsewhere, where ACTs remain the most affordable, effective antimalarial therapy. A molecular marker for widespread use is essential to detect and monitor the spread of AR [14].

The patients presented discordant data between parasite clearance half-life *in vivo* and $RSA_{0–3\ h}$ survival rate *in vitro* [13]. The parasite clearance half-life is not only determined by the intrinsic susceptibility of a parasite isolate to ART, but also by its developmental stage at the time of artemisinin (ART) treatment and host-drug and host-parasite parameters such as pharmacokinetics and immunity [15]. However, recent evidence for subpopulations of AR parasites in western Cambodia [16] suggests that there is distinct emergence of drug-resistant parasites. An alternative strategy to discover a molecular marker is to analyze mutations in laboratory-adapted parasite clones selected to survive high doses of ART *in vitro*. This information is useful to guide analysis of polymorphism in clinical parasite isolates from areas where AR is well documented.

This strategy was used to explore the molecular signatures of clinical AR in western Cambodia, where this phenotype was first reported [4,7].

This report introduced the information that the investigators have identified the molecular determinants of AR and these molecular signatures were associated with the phenotypic characteristics of AR. Recently, although the genotypic characteristics were confirmed for AR, these were not correlated with clinical parameters in India and phenotypic assays were indicated to confirm on these *P. falciparum* samples.

Discovery of K13-propeller domain

The ART-sensitive F32-Tanzania clone was cultured under a dose-escalating, 125-cycle regimen of ART for 5 years [17]. The parasite line was selected and named the ART-resistant F32-ART5. Whole-genome sequencing was undertaken for both F32-ART5 and F32-TEM (its sibling clone cultured without artemisinin). No deleted genes were identified in F32-ART5, compared to F32-TEM [14]. Acquired ART-resistance mutation(s) could be expected to be fixed in the sample after 5 years of continuous pressure in F32-ART5 strains. This genome wide analysis identified eight mutations in seven genes that were subsequently confirmed by sequencing of PCR products. Each gene harbors one mutant codon in F32-ART5 compared to sensitive strains [14]. The whole genome sequences of parasites were analyzed at various drug-pressure cycles to get the information when each mutation arose in the F32-ART5 lineage. This result indicated that the PF3D7_0110400 D56V and PF3D7_1343700 M476I mutations were acquired first, during the rapid increase of AR, and remained stable thereafter. PF3D7_1343700 M476I mutation is associated with an increase in the $RSA_{0–3\ h}$ to 12.8%. PCR analysis of the PF3D7_1343700 locus detected the M476I mutation at 30 drug-pressure cycles and it is consistent with the sharp increase in $RSA_{0–3\ h}$ survival rate [14].

PF3D7_1343700 gene has 4 Single Nucleotide Polymorphisms (SNPs) and all showed a significant association with $RSA_{0\text{-}3\ h}$ survival rates. Four mutant alleles are observed for PF3D7_1343700 gene, each harboring a single non-synonymous nucleotide polymorphism within a kelch repeat of the C-terminal K13-propeller domain. These four alleles are Y493H, R539T, I543T and C580Y located within repeats no. 2, 3, 3 and 4, respectively. Both the ART-susceptible parasite lines carry a wild-type K13-propeller. Based on these data and the acquisition of M476I in kelch repeat no. 2 by F32-ART5, it was assumed that K13-propeller polymorphism is a molecular signature of ART resistance in Cambodia [14].

What is Kelch protein and kelch repeats?

Kelch proteins are a widespread group of proteins with multiple kelch motifs. The kelch domain generally contains a set of five to seven kelch repeats that form a β-propeller tertiary structure. Kelch-repeat β-propellers are generally involved and important in protein-protein interactions. Kelch repeats are present in the C terminus of Kelch proteins. Each kelch repeat is a sequence of 44-55 amino acids in length, mostly occurring in clusters of 4 - 7 repeats [18].

K13-propeller polymorphic allele and AR

One hundred and sixty two patients from Cambodia, in whom parasite clearance half-lives have been already measured, were tested for SNPs to confirm that K13-propeller polymorphism is a molecular marker of clinical AR [14]. Those with mixed genotypes were excluded. Seventy-two patients of remaining 150 carried wild-type parasites while the rest of patients had parasites with only a single non-synonymous SNP in the K13-propeller region. These were C580Y, R539T and Y493H alleles. Patients with wild-type parasites had significantly shorter parasite clearance half-life (3.30 h) than those with SNPs. Parasites with C580Y, R539T, Y493H alleles had 7.19h, 6.64h and 6.28h, respectively. These data indicated that mutant alleles of K13 propeller Y493H identify slow-clearing parasites in malaria patients treated with ART [14].

***k13* mutant alleles in southeast Asia**

In Cambodia, it was reported that *k13* alleles (Y493H, R539T, I543T and C580Y) were correlated with prolonged *in vitro* parasite survival rates and *in vivo* parasite clearance rates [14]. Sequencing of the *k13*-propeller region of archived parasite isolates from Cambodian patients with malaria between 2001 and 2012 results in 17 mutant alleles, among which the three high-frequency alleles were C580Y, R539T and Y493H [14].

In the study on AR in Myanmar, 39% of the samples studied carried a *k13*-propeller mutation. 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 while the C580Y mutation was observed in over 90 samples of total 940 samples [19]. In the other study in Myanmar, 42 uncomplicated falciparum cases from the eastern border and 49 from the western border were tested for parasitemia after 3 days of artemether-lumefantrine treatment [20].

In the eastern border, 26.2% (11/42) of cases were positive in the test and K13 mutations were more frequent in the eastern border area. C580Y, M476I, A481V, N458Y, R539T, and R516Y were 68.9% of all K13 mutations and these alleles were significantly associated with day 3 parasitemia [20].

The study on 417 samples obtained in 2007 revealed a total of seven *k13* mutant alleles, with marked prevalence of C580Y, R539T and P574L in Thailand. The C580Y allele was the most common and widespread in Thailand including eastern and western regions [21].

In Vietnam, the study observed that 89 patients piperaquine (DHA-PPQ) and the median half-life was 6.2 h. The parasite clearance time ranged from 14.1 to 120.7 h, with a median of 61.7 h. One third of patients were positive with *P. falciparum* trophozoites at day 3 and all infections were cleared by day 5 [22]. The four SNPs of the K13 propeller domain were observed in a total of 83 samples. The mutant allele at positions 543 was 80.7% and at position 493 was 1.3% while no mutation at positions 539 and 580 which have common mutation in other southeast Asian countries [22].

A single mutation was predominated in the southern China

In the Yunnan Province of China near the border with Myanmar, artesunate and DHA-PPQ combination was used to treat *P. falciparum* malaria [23]. Therapeutic efficacy studies were undertaken on 329 samples from 2009 to 2012. Forty-four percent of infections have parasite clearance half-lives >5 hours (n = 109) after artemisinin treatment. Fourteen mutations in K13 were observed with a single mutation and F446I allele was predominated, with a prevalence of 36.5% (Table 1). *P. falciparum* infections in southern China have shown markedly delayed clearance of parasite after ACT [23].

Efficacy of artesunate-sulphadoxine-pyrimethamine (ART-SP) in northeastern India

The *k13* mutations findings in Myanmar have occurred in the north-west regions close to the Indian border [24]. 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-SP combination of ACT were used for malaria treatment. The efficacy of ART-SP was declining in northeastern India and exceeded the threshold for changing drug policy [24].

Based on the results of the study, the first subnational drug policy for India was changed to select artemether plus lumefantrine as the new first-line treatment in the northeastern region of India. Continued monitoring of antimalarial drug efficacy in north-eastern region is recommended for effective malaria control [24].

Phenotyping is necessary to be performed together with genotyping

In India, DNA sequence analysis of *k-13* gene from the 384 clinical isolates of *P. falciparum* showed six point mutations. The mutations were synonymous in two and, non-synonymous in four. Synonymous mutations were found at nucleotide positions 1377 and 1752 and non-synonymous (NS) mutations were observed at codon G533A, S549Y, R561H and A578S [26]. Only R561H and completed the 3 day course of dihydroartemisinin-A578S NS mutations have been reported previously in association with slow parasite clearance, but the G533A, S549Y have not (Table 1) [14,25]. The mutations were observed at very low frequencies revealing 1/384 proportion [26].

Although there is a report of presence of molecular determinants of AR in *k13* gene of *P. falciparum*, without phenotyping it is too early to conclude AR has reached India [26]. Now, Tracking Resistance to Artemisinin Collaboration (TRAC) is conducting detailed parasitic clearance assessments and genotyping because there is fore–going evidence that *k13* NS mutations were not associated with drug resistance. In the region where there is AR, there is an urgent need to do clinical and laboratory assessments on phenotyping together with genotyping of *k13* gene of the parasites [26].

Although there were NS mutations, treatment responses were good in the study. All these mutations were observed in northeastern states and this state was the gateway to India for the chloroquine resistant *P. falciparum* in the history. Therefore, this state is likely route for the AR *P. falciparum* to spread into the India in the near future [26] because the eastern country is Myanmar where AR *P. falciparum* is prevalent. Monitoring of AR by parasite clearance supported by molecular genotyping of signature gene is required to track the drug resistant *P. falciparum*.

A case of artesunate resistant *Plasmodium falciparum* in Kolkata

In October, 2012, a 57 years-old-female patient, a resident of central Kolkata, West Bengal, India, was suffering from fever with chills and rigor on alternate days. Microscopic observation of stained blood film suggested the presence of *P. falciparum* rings in her blood and *P. falciparum* antigen was also detected in rapid diagnostic test kit. She was treated by her physician with artesunate injection alone, 120 mg i.m. on the first day followed by 60mg i.m. daily for the next four days. She became afebrile and no malaria parasite was found in her blood [27].

After 13 days, the patient was attacked by another bout of fever. When her blood was examined, *P. falciparum* rings were present and falciparum antigen was also found to be positive. The membrane surface protein test suggests that the patient has recurrence of infection. This time, she was treated with six tablets of artemether 80 mg and lumefantrine 480 mg course. After treatment course, her blood was examined with the result of negative malaria parasite. She had no history of travelling to neighbouring countries in recent years. These findings demonstrated that the patient was clinically resistant to artesunate and it was a late treatment failure case. However, the combined therapy (artemether and lumefantrine) was effective in her case [27].

Table 1. The change of amino acid residue in different alleles of *k13* gene *in P. falciparum* isolates from Two Myanmar States, China and India

Location of isolates from which *k13* gene is investigated, type of allele and the repeat no. this mutation is present	**Amino acid sequence starting from 441 residue to 580 residue of Kelch protein**
Wild Type	PFPLVFCIGGFDGVEYLNSM ELLDISQQCWRMCTPMSTKK AYFGSAVLNNFLYVFGGNNY DYKALFETEVYDRLRDVWYV SSNLNIPRRNNCGVTSNGRI YCIGGYDGSSIIPNVEAYDH RMKAWVEVAPLNTPRSSAMC
Myanmar (Kayin State) **R575K allele *** **Present in repeat no. 4 of propeller domain [20]**	PFPLVFCIGGFDGVEYLNSM ELLDISQQCWRMCTPMSTKK AYFGSAVLNNFLYVFGGNNY DYKALFETEVYDRLRDVWYV SSNLNIPRRNNCGVTSNGRI YCIGGYDGSSIIPNVEAYDH RMKAWVEVAPLNTPKSSAMC
Myanmar (Chin State) **G449A allele*** **Present in repeat no. 1 of propeller domain[20]**	PFPLVFCIAGFDGVEYLNSM ELLDISQQCWRMCTPMSTKK AYFGSAVLNNFLYVFGGNNY DYKALFETEVYDRLRDVWYV SSNLNIPRRNNCGVTSNGRI YCIGGYDGSSIIPNVEAYDH RMKAWVEVAPLNTPRSSAMC
Southern China **F446I allele**** **Present in repeat no. 1 of propeller domain [23]**	PFPLVICIGGFDGVEYLNSM ELLDISQQCWRMCTPMSTKK AYFGSAVLNNFLYVFGGNNY DYKALFETEVYDRLRDVWYV SSNLNIPRRNNCGVTSNGRI YCIGGYDGSSIIPNVEAYDH RMKAWVEVAPLNTPRSSAMC
Northeastern India **S549Y allele***** **Present in repeat no. 3 of propeller domain [26]**	PFPLVFCIGGFDGVEYLNSM ELLDISQQCWRMCTPMSTKK AYFGSAVLNNFLYVFGGNNY DYKALFETEVYDRLRDVWYV SSNLNIPRRNNCGVTSNGRI YCIGGYDGYSIIPNVEAYDH RMKAWVEVAPLNTPRSSAMC

*One of the alleles observed in these states of Myanmar
**The commonest allele in southern China
***This allele is not associated with slow parasite clearance. The mutated amino acids were highlighted with green.

CONCLUSIONS

Because chloroquine resistance has been spread from Myanmar, the western part of GMS through India to Africa where there was high mortality rate, AR in Myanmar was an important phenomenon for the malaria endemic area of the world including India and Africa [19]. The question whether AR has reached India or not is worrisome for the investigators of this drug resistant deadly parasite, *P. falciparum*.

Although AR molecular determinants were started to be present in *k13* gene of *P. falciparum* isolates of India, there were no strong phenotypic characteristics [26]. Although there was one case report of artesunate resistant falciparum malaria [27], there were no many cases spreading throughout India. In addition, *k13* amino acid point mutations with slow parasite clearance were not highly prevalent even in northeastern India which is the region adjacent to Myanmar where there was 39% prevalence rate of total *P. falciparum* isolates [19]. Therefore it cannot be concluded that AR *P. falciparum* is arriving at India and going to be spread to the whole country. However, continued monitoring of genotypic and phenotypic characteristics is necessary to track AR *P. falciparum*, especially in northeastern India. In the study in Myanmar, 326 of 371 samples (88%) carried mutations known to be associated with phenotypic resistance, including the F446I mutation. AR is associated with resistance to partner drug. Reliance on single partner drugs in artemisinin-based combination therapy is ineffective on long term [28]. This lesson was provided by the study in northeast India [24]. In addition, the investigator should not neglect the phenotypic laboratory studies.

Cambodian parasites with mutant K13-alleles display a wide range of $RSA_{0–3\ h}$ survival rates (3.8–58%) and parasite clearance half-lives (4.5-11.5 h). Therefore, further studies need to be targeted at additional genetic determinants of AR, which may reside in the recently identified selected regions [29, 30]. In this context, analyzing the $RSA_{0–3\ h}$ survival rates as a quantitative phenotypic trait among parasites to identify additional genetic loci involved in AR.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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