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Review Article

Updates on *k13* mutant alleles for artemisinin resistance in *Plasmodium falciparum*

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Abstract *Background:* In the fight against malaria caused by *Plasmodium falciparum*, the successes achieved by artemisinin were endangered by resistance of the parasites to the drug. Whole genome sequencing approach on artemisinin resistant parasite line discovered *k13* gene associated with drug resistance. *In vitro* and *in vivo* studies indicated mutations in the *k13* gene were linked to the artemisinin resistance.
Methodology: The literatures published after April, 2015 up to December, 2016 on *k13* mutant alleles for artemisinin resistance in *Plasmodium falciparum* and relevant literatures were comprehensively reviewed.
Results: To date, 13 non-synonymous mutations of *k13* gene have been observed to have slow parasite clearance. Worldwide mapping of *k13* mutant alleles have shown mutants associated with artemisinin resistance were confined to southeast Asia and China and did not invade to African countries. Although *in vitro* ring stage survival assay of 0—3 h was a recently developed assay, it was useful for rapid detection of artemisinin resistance associated *k13* allelic marker in the parasite. Recently, dissemination of *k13* mutant alleles was recommended to be investigated by identity of haplotypes. Significant characteristics of well described alleles in the reports were mentioned in this review for the benefit of future studies.
Conclusion: According to the updates in the review, it can be concluded artemisinin resistance does not disseminate to India and African countries within short period whereas regular tracking of these mutants is necessary.

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Introduction

Artemisinins (ART) and ART combination therapy (ACT) reduced the global burden of malaria caused by *Plasmodium falciparum*.[1] However, the successes which have been achieved are threatened by emergence of resistance to ART.[2] ART are active against the stages of parasites in erythrocytes but their effectiveness is stopped by ring-stage resistance.[3,4] Although ART-resistance (ART-R) was first reported in western Cambodia,[5,6] it is now spread in southeast Asia and south China.[7–10] Serious consequences would occur due to disseminated ART-R, since most antimalarial drugs were resistant by *P. falciparum*.[11–13] ART-R in southeast Asia is a major threat to Africa and other malaria endemic countries throughout the world because chloroquine resistance (CQR) by the parasite has spread from this region to African countries causing high morbidity and mortality.[14,15]

With the whole genome sequencing approach of parasite line produced by ART pressure on sensitive parasite, Kelch protein which is encoded by the PF3D7_1343700 (*k13*) gene was discovered. *In vitro* and *in vivo* methods indicated mutations in the gene were linked to the ART-R.[14] WHO definition for confirmed resistance is the parasite harbouring the *k13* mutants associated with either persistent parasitaemia on day 3 or longer.[16]

Two of the *P. falciparum* isolates were started to be observed to have prolonged parasite clearance time in Cambodia during 2008. Parasites with prolonged clearance time were increasingly emerged in western Cambodia, Thailand and Vietnam in 2009.[15] It was observed non-synonymous (NS) mutations in *k13* gene such as Y493H, R539T, I543T and C580Y were correlated with prolonged *in vitro* and *in vivo* assessments.[14] Further studies indicated F446I mutant allele was highly prevalent in China-Myanmar border as well as in Myanmar especially northern region[17] although the mutant was not included in the list with prolonged *in vitro* and *in vivo* assessments.[18] C580Y mutant was predominant in Cambodia, Myanmar, eastern and western Thailand and observed to be molecular marker for ART-R in *P. falciparum* isolates.[14,17,19,20] In the previous studies, 13 NS mutations have been observed to have slow parasite clearance.[14,21–23] In this review, updates on information of *k13* mutant alleles in *P. falciparum* isolates in the literatures published after April, 2015 were revealed with significant characteristics of well described alleles in these reports. The information in this study will be beneficial for the tracking of ART-R in *P. falciparum* isolates.

Local evolution and distinctive origin of ART-R *K13* alleles in the China-Myanmar border

Ye et al.[24] had done analysis of 111 isolates of *P. falciparum* from China-Myanmar border for ART susceptibility using ring stage survival assay of 0–3 h ($RSA_{0-3\ h}$) and genotyping of *k13* genes. Furthermore, genotyping for *k13* mutations of 693 isolates collected from China and China-Myanmar/Thai-Cambodia/Thai-Myanmar (CM/TC/TM) borders was performed.[24] Twenty-four percent of the 111 isolates studied had higher $RSA_{0-3\ h}$. Eighteen isolates which harboured mutations previously associated with high ART resistant phenotype were reported from CM border (Table 1).[24]

To investigate the origin of ART resistance-associated mutants, 169 isolates from the CM border and Tengchong, TC border, TM border were genotyped at 11 neutral microsatellite loci and three SNPs identified to be associated with ART-R.[24] These isolates were grouped according to their geographic origins. Isolates from the CM border constitutes the first group and isolates from the TC/TM borders were in second group.[24] All the isolates having R539T mutation from the CM border clustered in the CM group whereas the isolates having this mutant from TM/TC border were grouped in the TM/TC one.[24] These data supported that R539T mutations had distinct origins and evolved locally (Table 1 and Fig. 1). This information was same for the other ART-R *k13* mutations associated with positive $RSA_{0-3\ h}$ identified in this study.[24]

A set of SNP markers which differentiated the Cambodian ART-resistant subpopulations from the other populations were termed KH2, KH3 and KH4. As R539T mutant isolates were included in the KH3, ten SNP markers from this subpopulation were selected to trace the origin of this mutant.[24] As a result, this combination of SNP markers clearly identified the origin of the R539T mutants from the CM border and TC/TM borders (Fig. 1).[24] Haplotypes 1–6 and haplotypes 7–10 were found in the isolates from the CM border and isolates from the TC/TM borders, respectively.[24] Of 15 isolates with R539T mutant from the CM border, nine had the wild type alleles for ten SNP markers. It is the interesting finding that the G6972A SNP in the PF3D7_0726400 gene was present in haplotypes 7–9 whereas it was absent in haplotypes 1–6 so that this SNP can differentiate R539T mutant isolates into CM and TC groups (Table 1).[24]

Of four study areas, Hainan, Tengchong, Xishuangbanna and CM border, more than 50% of the isolates in CM border carried the *k13* mutations whereas 12% and 4.9% of the isolates in Hainan and Xishuangbanna respectively had these mutations. Fifteen new NS mutations of the *k13* gene which were not present in southeast Asia regions were identified. The F446I mutation was the most common mutation in isolates of China whereas this mutation was rare in southeast Asia except Myanmar (Fig. 1). These data indicated that *k13* polymorphisms had different patterns between China and southeast Asia. This is the first study in which *k13* mutant alleles were reported from Hainan Island with the information ART-R associated A481V, P553L and H719N mutations were found. One isolate from CM border having a wild type allele of the *k13* gene showed a high survival rate. In some field isolates, no association of *k13* mutations and resistant phenotypes was observed which suggests there are additional molecules besides *k13* involved in ART-R.[24]

In vitro $RSA_{0-3\ h}$ assessment for ART-R mutants

The rapid and effective detection of resistant parasite is important to contain the ART-R.[24] ART-R is known to be slow parasite clearance rate in patients treated with an ART monotherapy or ACT.[3] To know the parasite clearance half-

Table 1 Description of five *k13* NS mutations well described in the current review.

k13 NS mutations	Significant characteristics
R539T	• It is characterized by both delayed *in vivo* parasite clearance and prolonged *in vitro* $RSA_{0-3\ h}$.[14,26] • Sixteen isolates with this mutation were found in China-Myanmar border.[24] • This allele was totally absent in in Myanmar.[42] • The mutant from the CM border evolved locally indicating the independent evolution of the mutant allele.[24] • This mutation is associated with high ART-resistant phenotype.[14,24]
C580Y	• The allele is one example of endemic mutations.[18] • The mutant is highly prevalent in Cambodia, Thailand and Myanmar.[42] • The frequency of the C580Y allele increased significantly in two western provinces of Cambodia and it became near fixation in these areas.[14] • Four C580Y haplotypes originate in Pailin, Western Cambodia, the epicentre of the emergence of ART-resistant *P. falciparum*.[5,6,18] • This mutation is associated with high ART-resistant phenotype.[14,24]
N458Y	• This mutant was observed to be associated with *in vivo* PHCL >5 h and *in vitro* $RSA_{0-3\ h}$ values > 1% in Thailand-Myanmar border.[30] • Association of N458Y mutation with ART-R was inconsistently reported.[33] • N458Y was confirmed by *in vitro*, and *in vivo* methods to be as a molecular marker of ART-R in the recent study.[30]
F446I	• The mutant was not different in the $RSA_{0-3\ h}$ rate with wild type alleles.[24] • Most patients with this mutant had a parasite clearance half-life below five hours.[24] • It is highly prevalent in China-Myanmar and Northern Myanmar[20,42] • This mutant is not consistently associated with ART-R.[25,26] • It will be necessary to clarify whether this mutant is an ART-resistant marker because there is association with prolonged parasite clearance half-life in the recent study.[7,24]
A578S	• The mutant has been commonly found in Africa.[18] • It was observed to be ART sensitive in $RSA_{0-3\ h}$ assessment.[18] • Independent emergence was the cause of its presence in African countries without evidence of dissemination.[18] • A578S mutant has been reported from North eastern region of India.[37] • It is not observed with confirmed ART-R.[37]

life, it needs frequent parasite density counts in malaria cases.[3] Such *in vivo* studies are financially demanding and inconvenient for patients as well as their families because hospitalisation is necessary.[3] *In vitro* assay that correlates with parasite clearance half-life would be convenient. There is strong correlation between ring stage survival rates and *in vivo* parasite clearance rates.[24] Furthermore, there is no influence of confounding factors, like dosage, absorption and metabolism of drugs and host immunity.[24]

In the study in China-Myanmar border, susceptibility to ART in *P. falciparum* isolates was assessed by $RSA_{0-3\ h}$.[24] Among the *P. falciparum* isolates from CM border, $RSA_{0-3\ h}$ has been ranged from 0.01%—16.6%. The isolates harbouring the wild type *k13* gene exhibited median survival rate of 0.26%.[24] Isolates carrying mutation in the *k13* propeller has shown median survival rate of 2.95% whereas four isolates had >10%. The high level of survival rates (>10 up to 16.6%) were observed in three isolates with the R539T mutation strongly associated with *in vivo* delayed parasite clearance.[24] Ring stage survival rates were significantly higher in parasites with mutations in the *k13* propeller region. However, there were two exceptional mutants which were F446I and P441L.[24] Twenty seven isolates with F446I had median survival rates of 0.62% suggesting that isolates with this mutant were not different with the wild type isolates (Table 1).[24] The study support that rapid detection of ART resistance in the parasite is effective when $RSA_{0-3\ h}$ and *k13* allelic marker is used together.[24]

A worldwide mapping of *k13* mutant alleles

Because of the widespread study including *k13* allelic mapping in 163 sites and therapeutic efficacy assessment in 36 countries, a lot of information were learnt in the study by Menard et al.[18] Of 1250 *P. falciparum* isolates, 108 NS mutations were identified. Seventy of 108 NS mutations were newly reported in this study whereas 38 were the mutations which had been previously identified.[7,10,14,22,23,26,37,39] *k13* nonsynonymous mutations were highly heterogeneous in western Cambodia up to >90%, intermediate in Myanmar and Vietnam and moderately diverse in Thailand, China, eastern Cambodia and Laos.[18]

ART-R is prevalent in southeast Asia and China while South America, Central and South Asia, Oceania and the Philippines are free of NS *k13* mutations indicating that ART-R does not spread to these regions. There was no evidence of invasion to Africa by Asian artemisinin resistant alleles while NS mutations were observed with low-frequency. Furthermore, these mutations were not associated with clinical ART-R.[18]

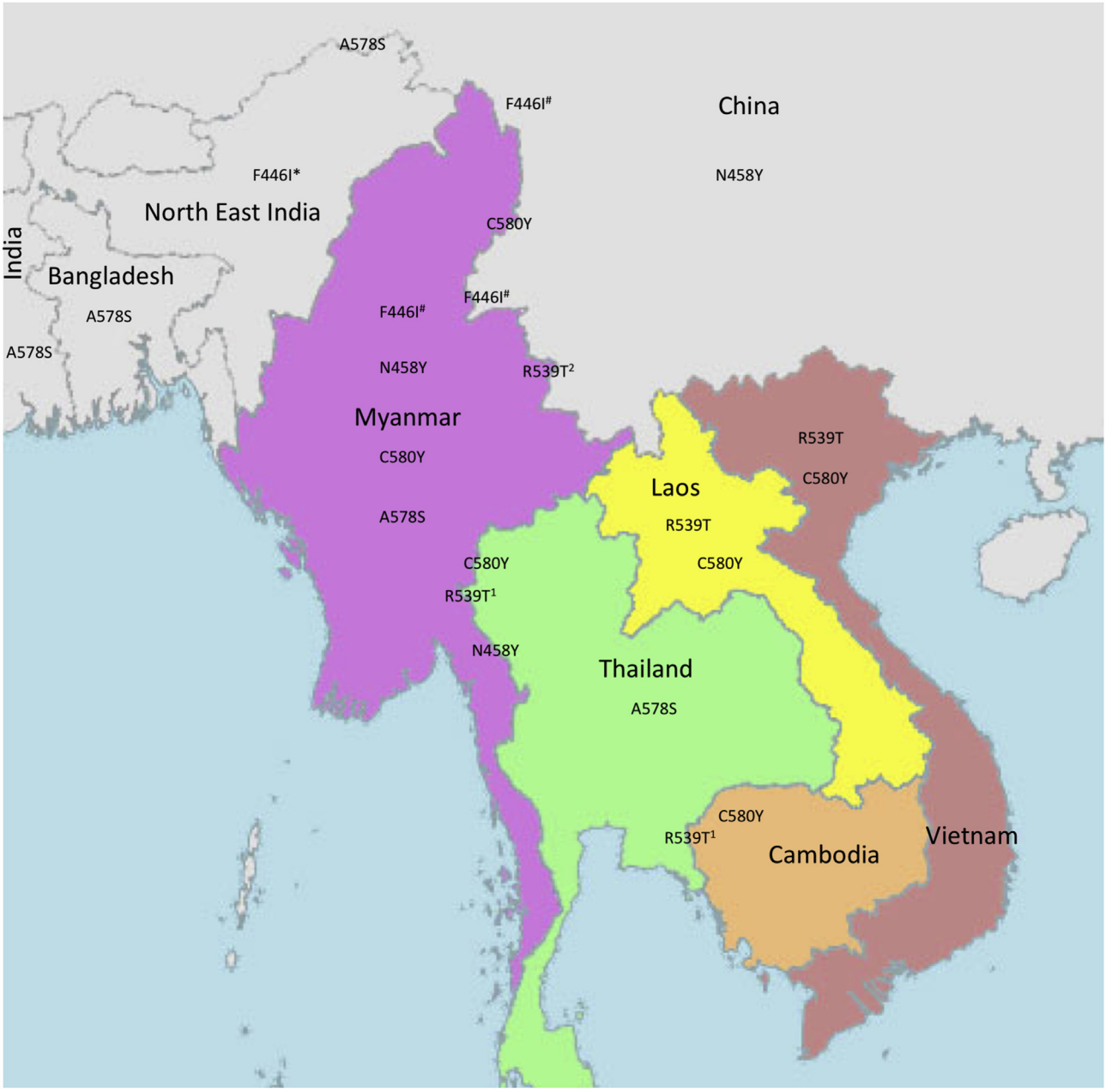

Figure 1. Distribution of five *k13* mutants described in southeast Asia and Asia regions. R539T[1] and R539T[2] indicate that the mutant evolved locally and had distinct origin in TC/TM borders and CM border respectively. The data was observed in investigation of dissemination of *k13* mutations by haplotypes identity and microsatellite typing. # indicates F446I mutant was prevalent in CM border, Myanmar and China and absent in other southeast Asian countries. * indicates only one isolate with F446I mutant was found in northeast India.

In southeast Asia and China, there were two independent foci of ART-R namely the region that includes Cambodia, Vietnam, and Laos and the other that includes western Thailand, Myanmar, and China. Many new resistance-associated mutations other than that had been reported previously were observed in these two regions.[19,22,25]

For study of dissemination of *k13* mutant alleles, the genetic relatedness of isolates having the same *k13* mutation were assessed for the SNP in two neighbouring PF3D7_1337500 (*k13*_151) and PF3D7_1339700 (*k13*_159) loci.[18] DNA sequences were analysed to identify individual alleles for each locus. Ten alleles for *k13*_151 and 42 alleles for *k13*_159 were observed to generate haplotypes for investigation of dissemination.[18]

Three of 17 C580Y haplotypes were commonly distributed in Cambodia, Vietnam, and Laos whereas the rest of 14 haplotypes were located in Cambodia, Thailand, and Vietnam.[18] Similar investigations were done for the Y493H and R539T and probable west-to-east dissemination was observed.[18] While 8 haplotypes of F446I were located in China or Myanmar, 3 were distributed in Myanmar. No overlapping was observed between the sets of haplotypes

in the two main regions of ART-R in Asia. This indicated the selective pressures in the two areas were different.[18] There was dissemination of endemic mutations in these regions (Table 1).[18]

Eight NS mutations namely C580Y, F446I, R539T, N458Y, N537D, I543T, P553L, and P574L were associated with positive results on day 3 in Southeast Asia and China. Previously used parasite-clearance half-lives to identify ART-R of these 8 mutations were consistently positive with day 3 assessment (Table 1).[7,14,22,26–28]

Ménard et al.[18] mentioned the weakness of their study although the coverage of their study is widespread. The isolates represented only samples convenient to take in the areas surveyed. There was lack of information from the malaria endemic area like India. India is important to be studied because it is the western boundary of the region where ART-R is currently prevalent. In some regions where malaria is present, only small numbers of samples were available.[18]

The study recommended investigating the identity of flanking haplotypes to get the information about the dissemination of *k13* mutations.[18] Molecular surveillance in resistance-free areas will be necessary to include detection of invasion by known resistant *k13* alleles and tracking of dissemination of new NS mutations.[18]

Confirmation of molecular markers for ART-R by *in vitro*, and *in vivo* methods

After it has been emerged in western Cambodia in 2008, ART-R was observed in neighbouring countries including Thailand-Myanmar border.[9,26] In cases treated by ART or ACT, resistance was partial and shown as an increased parasite clearance half-life (PCHL) of >5 h.[29] However, ART-R has been also characterized by *in vitro* $RSA_{0-3\ h}$, cut off 1%[3,30,31] and mutations in the *k13* propeller domain because of inconvenience of PCHL assay.[14,21] In the study of $RSA_{0-3\ h}$ among culture-adapted parasites from 13 resistant and 13 sensitive infections in Cambodia, the survival rates differed significantly showing 10·88% and 0·23% respectively with the consequence of taking 1% as cut off value.[3] However, marked variability in *k13* mutations requires confirmation of specific mutant as ART-R marker by *in vivo* and *in vitro* assays.[2,32]

Correlation of *in vivo* PCHL, *in vitro* $RSA_{0-3\ h}$ and *k13* alleles were performed in the study in Thailand-Myanmar border.[30] On the basis of PCHL, 33 cases were selected at the Mae Sot, Thailand during 2011—2013 after treated with ART followed by ART/Mefloquine and were assessed by *in vitro* assay for Art-R phenotypes.[26] Cases associated with *in vivo* PHCL >5 h and *in vitro* RSA values > 1% harboured C580Y. Confirmation of C580Y allele as a molecular marker of ART-R was done in the previous studies (Table 1)[3,14,21,26] whereas the other common mutation N458Y in the study was inconsistently correlated with ART-R because one drug sensitive case was reported at the China-Myanmar border.[33] World Health Organization defined that confirmation by *in vitro* and *in vivo* methods were necessary for a molecular marker to be ART-R (Table 1).[2] Boullé et al. observed that N458Y mutant was found to have association with PHCL >5 h and *in vitro* RSA values > 1%.[30]

k13 mutations in five study sites of Myanmar

One year study from October 2013 to September 2014 was performed in five malaria-endemic areas of Myanmar namely Ann, Homemalin (western Myanmar border), Myit Kyi Nar (northern Myanmar) Kyauk Me and Phruso (eastern Myanmar).[34]

In Kyauk Me and Phruso the samples were analysed for *k13* mutations and *pfmdr1* copy number variation. After a standard course of artemether-lumefantrine, day-3 parasite clearance rates were used to assess a portion of patients in two study sites, Homemalin and Kyauk Me.[34]

Of 250 samples, 206 samples had been successfully sequenced for *k13* gene. About one third of the samples had NS mutations in *k13* propeller region with three-quarter of mutations previously associated with ART-R. Ann in Rhakhine State had less than 10% of propeller mutation.[34] The finding was consistent with previous data indicating that ART-R was absent in western Myanmar.[35,36] The high prevalence of *k13* mutants in Myit Kyi Nar (Kachin State) was consistent with data from CM border where the F446I mutation is the most prevalent type of *k13* mutation and associated with reduced *in vivo*[7,33] and *in vitro* susceptibility.[33]

In this study in Myanmar, most of the *k13* mutations known to be associated with slow parasite clearance were not observed. The reason may be possibly due to different levels of drug pressure although mutations observed in different locations in Myanmar with particular drug usage histories cannot be related.[34]

Of 36 patients assessed in two sites for day-3 parasitaemia after ACT, all patients were negative except one from Homemalin. It is surprising that in spite of presence of *k13* propeller mutations, day-3 positivity rate was lower than the previous clinical study.[34]

Emergence of *k13* mutant alleles in northeast region of India

The whole *k13* gene sequences were available in 254 samples collected from Tripura, Mizoram and Arunachal Pradesh states during 2014—2015. These three states are present in northeast region of India where there are borders with Myanmar and Bangladesh.[37] Three NS mutations F446I, A578S and K189T were found in these states. As propeller region starts from amino acid no. 440, two of these are in *k13* propeller region whereas K189T is outside this region.[37]

F446I NS mutation was identified in the India-Myanmar border region. This was the first observation of F446I in northeast India (Fig. 1). The mutant has been observed to be associated with delayed parasite clearance in the northern Myanmar, as well as in China (Table 1).[38] In the study in India—Myanmar border, treatment outcome was not correlated with this mutation. The mutant was found to have intermediate rate of parasite clearance in Upper Myanmar region.[17] The interesting fact is this mutant was observed in only one sample in Arunachal Pradesh State, the border with Myanmar (Table 1).[37] This mutant was reported to be highly prevalent in Myanmar. This mutant was absent across the India—Bangladesh border, an information consistent with the absence of *k13* mutant parasites in Bangladesh.[37]

A578S mutant has been reported from Mizoram adjacent to Bangladesh.[39] However, this mutation has been no correlation with treatment outcome in the previous studies. A578S mutant has been reported with no available data on clinical outcome in Bangladesh.[40] A578S mutation is not associated with ART-R whereas it was commonly reported in African countries in a recent study (Table 1).[18]

K189T was observed from two study sites, Mizoram and Tripura, the Bangladesh border at higher frequency. A recent study at the China-Myanmar border revealed prevalence of K189T mutation at lower frequency compared to a higher prevalence found in Dakar, Senegal.[38,41] Although the K189T mutation was common in these two sites, there is no correlation with clinical phenotype.

Significant characteristics of *k13* NS mutations well described in the reviewed literatures

Of the studies in this review, five NS mutations have been observed for special characteristics to be noted for ART-R *in P. falciparum* and were mentioned in Table 1. The facts were tried to be comprehensive and beneficial for the future studies. Distribution of these *k13* mutants in southeast Asia and Asia regions was shown in Fig. 1.

Conclusions

Two successes in the malaria research in the fight against falciparum malaria were highlighted in this review. These were a novel *in vitro* $RSA_{0-3\ h}$ assessment and a new method of investigation of dissemination of *k13* mutations by haplotypes identity.[3,18,24] This review indicated that *in vitro* $RSA_{0-3\ h}$ superseded *in vivo* PCHL.[24] However, World Health Organization recommended ART-R *k13* mutant alleles should be confirmed by *in vitro*, and *in vivo* methods to be used as a molecular marker of ART-R.[2] Investigating the identity of flanking haplotypes to find out the dissemination of *k13* mutations was recommended[18] and better approach than microsatellite typing[19] or whole-genome sequencing.[25] In the study by Ye et al.[24] some *k13* NS mutations have evolved locally and had distinctive origins.[24]

In southern Myanmar in 2012, a portion of *P. falciparum* isolates have been shown markedly delayed parasite clearance after artesunate monotherapy.[8] In the current study in Myanmar, most of the *k13* mutant alleles known to be associated with slow parasite clearance were not reported.[34] Finding of the F446I mutant in Northeast India was taken seriously and should be tracked together with *in vitro* $RSA_{0-3\ h}$ because of the reasons it was highly prevalent in the neighbouring country, Myanmar and associated with delayed parasite clearance.[36]

According this review, a conclusion is drawn in which ART-R does not disseminate to India and African countries within short period. The reason is that within 2—3 years of studies, ART resistant mutant alleles associated with delayed parasite clearance were not observed in these regions. However, tracking of these mutant alleles in *P falciparum* isolates with simultaneous assessment of clinical outcome should be regularly performed in India which is the gateway to African countries.

Conflicts of interest

Authors in this study do not have any conflict of interest to declare.

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