# *Pf*K13 Kelch Propeller Domain and *Pf*mdr1 Sequence Polymorphism in *Plasmodium falciparum* Field Isolates From Northeast Region, India

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**ABSTRACT:** Emergence of artemisinin-resistant *Plasmodium falciparum* in Southeast Asia has made an intense warning to global malaria control programme since Southeast Asia is playing a key role in the evolution of drug resistant malaria parasites. Several polymorphisms in K13 gene have been designated with developing resistance either *in vitro* or *in vivo*. In this study, polymorphisms within K13 kelch propeller domain and *Pf*mdr1 gene were investigated in a total of 134 *P. falciparum* field isolates circulating in 3 states of Northeast region of India, namely, Assam, Arunachal Pradesh, and Tripura because information regarding this is limited from this region. Six polymorphisms were detected, out of which 2 were new. A481***V*** mutation was found in 4.5% of the total field isolates. A675***V*** and D702***N*** new mutations were present in 3.7% and 1.5% isolates, respectively. Synonymous polymorphism at codon 703 was highly observed (6.7%) than other polymorphisms. N86***Y*** allele in *Pf*mdr1 gene was also noticed in isolates that contained mutation in K13 propeller domain, but N86 wild-type allele was found comparatively higher in frequency within overall isolates. Moreover, both nucleotide and haplotype diversities in K13 gene were high in Arunachal Pradesh isolates than other 2 states. This is the first report from Northeast region of India with evidence of A675***V*** candidate mutation along with 2 other new mutations which pays attention for further molecular surveillance in this region to document detailed scenario of K13 polymorphism pattern corresponding to artemisinin resistance.

**KEYWORDS:** *Plasmodium falciparum*, K13, polymorphism, Northeast region, India

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## Introduction

Southeast Asia (SEA) has a consistent role in the evolution of multidrug-resistant *Plasmodium falciparum* (*P. falciparum*) parasites.[1-5] After genome-wide association studies in concordance with artemisinin resistance, several polymorphisms in the kelch protein were designated with artemisinin resistance.[6-8] The propeller domain or propeller region located in the so-called highly conserved kelch protein gene within the chromosome 13 is involved in ubiquitin-based degradation and parasite's adaptation to oxidative stress.[9] Three domains have been ascribed in the predicted structure of K13 kelch protein, *i.e.*, a well-conserved *Plasmodium*-specific N-terminal domain, a BTB/POZ domain, and a C-terminal propeller domain which encode 6 canonical kelch motifs containing proteins that are evolutionary conserved across different species responsible for diverse cellular functions.[9,10] Changes in the primary amino acid sequence due to mutations in these motifs have been identified as determinants of artemisinin resistance in SEA.[11,12] Mutations in kelch motifs cause upregulation of unfolded protein response pathways.[13] Initially, a set of 20 different mutations (ranging from amino acid codons 440 to 623) was established *in vitro* as signatures of artemisinin resistance in western Cambodia.[8] But accumulation of data over time suggests that mutations in this gene vary geographically and mostly in accordance with the employed antimalarial regimens.[14-21] In a recent study, non-reference K13 mutations *viz.*, 584***V***, 580***Y***, 574***L***, 568***G***, 553***L***, 543***T***, 539***T***, 493***H***, 481***V***, 449***A***, 255***K***, and 189***T*** have been found involved in prolonged parasite clearance, and it was also stated that artemisinin resistance in SEA has emerged independently without sweeping from Cambodia.[22] In 2016, most of them became either validated or candidate K13 mutation for artemisinin resistance.[23] However, role of 584***V***, 481***V***, 255***K***, and 189***T*** mutations in artemisinin resistance are yet to establish.

The artemisinin resistance epicentre Myanmar shares its Northwestern boundaries with Northeastern states of India. More precisely, it has been explained that C580***Y***, F446***I***, and P574***L*** mutations along with other mutations in kelch propeller domain associated with artemisinin resistance are collectively spreading in a rapid manner towards upper Myanmar and extending up to regions near India.[16] Also, F446***I*** mutation has been considered as the most prevalent in China-Myanmar border than other mutations.[24] Recently, G533***A*** novel mutation has been reported in *P. falciparum* isolates of Northeast region, India along with other non-synonymous S549***Y***,

R561***H***, and A578***S*** mutations.[20] However, no polymorphism in K13 propeller domain has been found among *P. falciparum* clinical isolates in another part of India where artesunate-sulphadoxine/pyrimethamine has been used as artemisinin-based combination therapy (ACT) for malaria treatment.[25] Recently, it has been established that K13 polymorphisms associated with artemisinin resistance are not evident outside SEA and China, and moreover, mutant A578***S*** mutation has no role in artemisinin resistance manifestation.[26] Collectively, from different parts of the globe, more than 200 non-synonymous mutations in *Pf*K13 propeller domain have been reported, and mutation in this domain is still evolving.[23] However, not all non-synonymous mutations are indications of emergence of artemisinin resistance. Resistance to ACT cannot be stated easily in malaria endemic regions where ACT is the first-line treatment against malaria. It also depends on the concomitant resistance to partner drugs.

*Plasmodium falciparum* multidrug resistance gene is located in the chromosome 5 of *Plasmodium* genome and it encodes *Pf*mdr1 protein also known as Pgh-1.[27] This protein is basically localised in the digestive vacuole of the parasite.[28] Non-synonymous point mutations in this protein such as N86***Y***, Y184***F***, S1034***C***, N1042***D***, and D1246***Y*** alter accumulation of antimalarial drugs in the digestive vacuole and hence are associated with either manifesting true resistance phenotype or increased sensitivity against chloroquine, quinine, mefloquine, halofantrine, lumefantrine, and artesunate derivatives both *in vitro* and in clinical field isolates.[29-35]

Pertaining to artemisinin resistance, overall knowledge about polymorphism pattern in K13 kelch propeller domain against different ACTs is of utmost importance. As ACT-artemether lumefantrine (ACT-AL) is the first line of treatment against uncomplicated malaria in the Northeast region of India, so this study was conducted to document current *Pf*K13 kelch propeller polymorphisms as well as corresponding *Pf*mdr1 polymorphisms in circulating *P. falciparum* field isolates from this region. The findings of this study will throw light on polymorphism pattern in these genes because collective information regarding polymorphism in *Pf*K13 propeller domain and *Pf*mdr1 gene is scarce from this part of the country.

## Methodology

### *Ethics*

This study was reviewed and ethically approved by Institutional Ethics Committee, Regional Medical Research Centre, Northeast region (ICMR), Dibrugarh, Assam for enrolment of human individuals in this study.

### *Study site and sample collection*

This study was conducted in Assam, Arunachal Pradesh, and Tripura states of Northeast region, India. A total of 169 blood samples from malaria infected human subjects were collected in 2014 and 2015 during seasonal transmission. Individuals with symptomatic and uncomplicated malaria were enrolled only after obtaining written consent either from participated individuals or from their legal guardians. Infection with malaria parasite was first confirmed by rapid diagnostic test (SD Alere, Alere Medical Pvt. Ltd., Gurgaon, Haryana, India) from finger-pricked blood, and subsequently, venipunctured blood (maximum 3 mL), as a source of *P. falciparum* isolate, was collected in K3-EDTA Vacutainer for genotyping study.

### *Isolation of genomic DNA and screening of mono and mix parasite infection*

About 100 µL (final volume) genomic DNA was extracted from each samples using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and used for genotyping study. Before genotyping of target genes, samples were screened and verified through a highly sensitive nested polymerase chain reaction (PCR) protocol to determine whether they were mono-infected with *P. falciparum* parasite or mix-infected with other *Plasmodium* species.[36] Out of 169 malaria positive samples, a total of 134 samples were found to be mono-infected with *P. falciparum* parasite in the adopted nested PCR protocol (Figure 1). Samples with mix infection by other *Plasmodium* species were excluded, and only *P. falciparum* mono-infected samples were included for genotyping *Pf*K13 and *Pf*mdr1 gene.

### *PCR amplification, sequencing, and sequence alignment*

An 849-base pair (bp) Kelch propeller domain of K13 gene was amplified by a nested PCR protocol described recently.[37] Two separate partial fragments (534 and 864 bp, respectively) of *P. falciparum* multidrug resistance 1 (*Pf*mdr1) gene were also amplified by a nested PCR protocol with slight modifications[38] (Table 1). Consumables necessary for PCR were procured from Promega (Madison, WI, USA), and amplifications were performed in Veriti 96-Well Plate Thermal Cycler (Applied Biosystems, Foster City, CA, USA).

Polymerase chain reaction–amplified amplicons were purified by QIAEX II Gel Extraction Kit (Qiagen, Germany) after verifying in agarose gel (1.5%) electrophoresis. Purified PCR amplicons were outsourced to 1st Base DNA Sequencing Services, Malaysia, for bidirectional capillary sequencing using dideoxy chain termination chemistry.

Both forward and reverse DNA sequences were arranged in BioEdit sequence alignment editor to create consensus sequences.[39] Each sequence was checked for the presence of single-nucleotide polymorphisms (SNPs) by reading through both forward and reverse strands. K13 kelch propeller domain polymorpshism identified among studied isolates were further compared with sequences available in GenBank, National Center for Biotechnology Information (NCBI) by

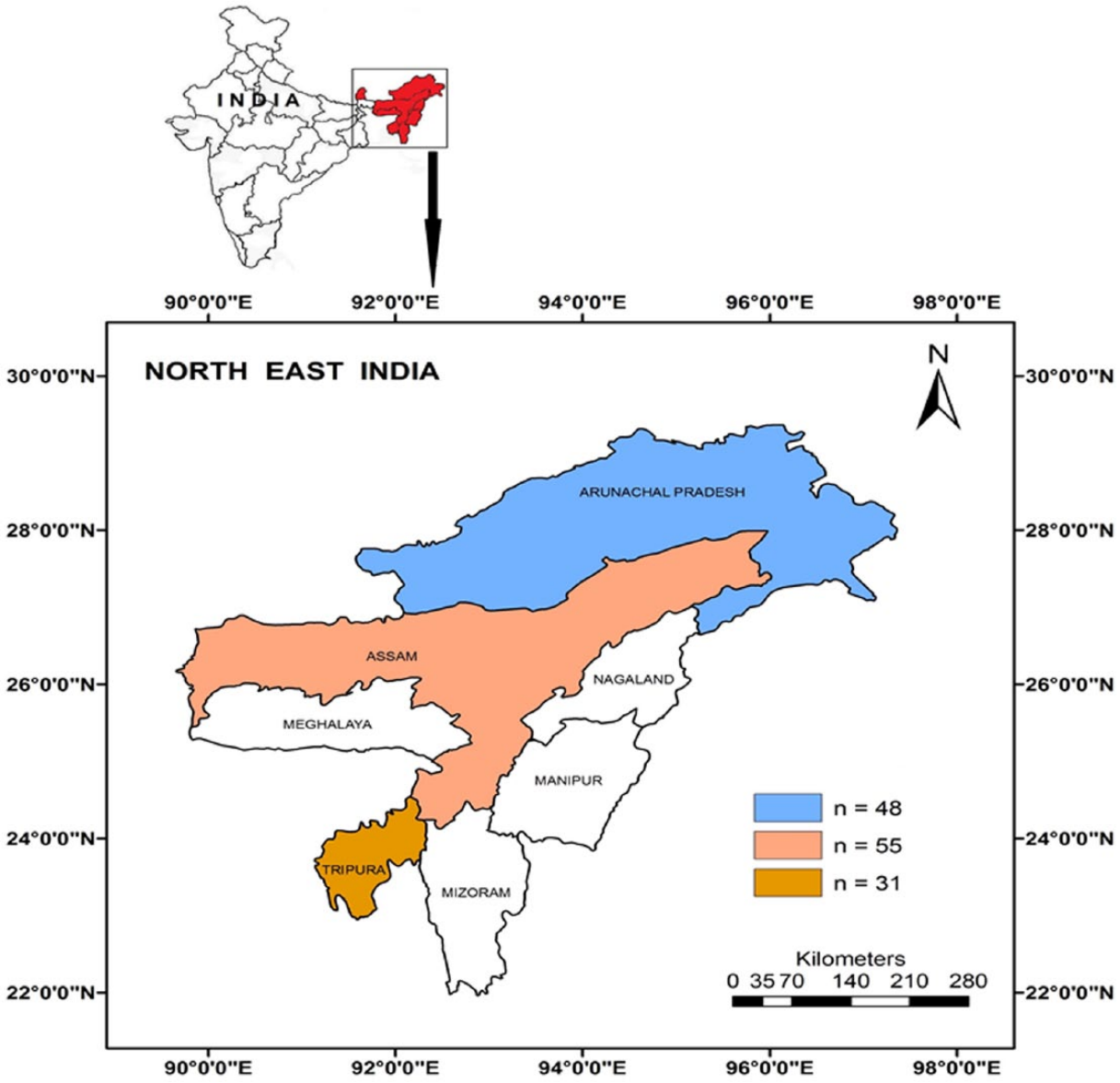


**Figure 1.** Map representing 3 states of Northeast India and number of *Plasmodium falciparum* isolates included from each site.

Basic Local Alignment Search Tool (BLAST).[40] The set of K13 gene sequences were further translated, and amino acid sequences were compared with *P. falciparum* 3D7 reference sequences (PF3D7_1343700 and XM_001350122) in Molecular Evolutionary Genetic Analysis version 6.06 (MEGA 6.06) in search of any mutation.[41] Similarly, *Pf*mdr1 polymorphisms in constituent codons were analysed in comparison with *P. falciparum* 3D7 reference sequence (XM_001351751).

### *Statistical analysis*

Primary and raw data were put into Microsoft Office Excel, and frequencies were calculated using the same programme. Independent-sample *t* test was performed to compare between continuous variables. Sequence polymorphism analysis including synonymous and non-synonymous SNPs, polymorphic and monomorphic polymorphisms, haplotype diversity (Hd), and nucleotide diversity ($\pi$) was performed using DnaSP software version 5.10.[42] The ratio of non-synonymous to synonymous nucleotide substitution (dN/dS ratio) was assessed to determine departures from selective neutrality. For all performed tests, a $P$ value <0.05 was considered to be statistically significant (at the 5% level). Significance for probability of rejecting null hypothesis of strict neutrality (dN = dS) was determined in MEGA 6.06 (Nei-Gojobori method, bootstrap value 10,000 replication) using the two-tailed $Z$ test.[43]

### *Nucleotide submission and accession numbers*

Nucleotide sequences of *Pf*K13 gene in this study were deposited to GenBank, NCBI under accession numbers KX575512-KX575645, and nucleotide sequences of *Pf*mdr1 gene in this study were deposited to GenBank, NCBI

**Table 1.** PCR primers and thermal conditions for genotyping *Pf*K13 and *Pf*mdr1 gene.

| GENE | PRIMER | SEQUENCE (5′-3′) | SIZE, BP | PCR CONDITION | REFERENCES |
|---|---|---|---|---|---|
| K13 kelch propeller | Primary PCR | | | | Menard and Ariey[37] |
| | K13_PCR_F | CGGAGTGACCAAATCTGGGA | 2094 | 95°C for 15 min, 30 cycles (95°C for 30 s, 58°C for 2 min, 72°C for 2 min), then 72°C for 10 min; store at 4°C | |
| | K13_PCR_R | GGGAATCTGGTGGTAACAGC | | | |
| | Nested PCR | | | | |
| | K13_N1_F | GCCAAGCTGCCATTCATTTG | 849 | 95°C for 15 min, 40 cycles (95°C for 30 s, 60°C for 2 min, 72°C for 1 min), then 72°C for 10 min; store at 4°C | |
| | K13_N1_R | GCCTTGTTGAAAGAAGCAGA | | | |
| *Pf*mdr1 protein | | | | | |
| *Pf*mdr1 fragment I | Primary PCR | | | | Humphreys et al[38] |
| | FN1/1 | AGGTTGAAAAAGAGTTGAAC | 578 | 94°C for 3 min, 40 cycles (94°C for 1 min, 52°C for 2 min, 72°C for 1 min), then 72°C for 10 min; store at 4°C | |
| | REV/C1 | ATGACACCACAAACATAAAT | | | |
| | Nested PCR | | | | |
| | MDR2/1 | ACAAAAAGAGTACCGCTGAAT | 534 | 94°C for 3 min, 44 cycles (94°C for 1 min, 55°C for 2 min, 72°C for 1 min), then 72°C for 10 min; store at 4°C | |
| | NEWREV1 | AAACGCAAGTAATACATAAAGTC | | | |
| *Pf*mdr1 fragment II | Primary PCR | | | | |
| | MDRFR2F1 | GTGTATTTGCTGTAAGAGCT | 958 | 94°C for 3 min, 40 cycles (94°C for 45 s, 51°C for 2 min, 72°C for 1.5 min), then 72°C for 10 min; store at 4°C | |
| | MDRFR2R1 | GACATATTAAATAACATGGGTTC | | | |
| | Nested PCR | | | | |
| | MDRFR2F2 | CAGATGATGAAATGTTTAAAGATC | 864 | 94°C for 3 min, 40 cycles (94°C for 45 s, 55°C for 1.5 s, 72°C for 1 min), then 72°C for 10 min; store at 4°C | |
| | MDRFR2R2 | TAAATAACATGGGTTCTTGACT | | | |

Abbreviation: PCR, polymerase chain reaction.

under accession numbers KP998529-KP998534, KT315542-KT315553, and KX575026-KX575277.

## Results

Following the adopted nested PCR protocol, K13 kelch propeller domain from all 134 *P. falciparum* field isolates was successfully amplified (Figure 2), sequenced, and analysed for SNPs. We mostly gave importance about polymorphisms that might have occurred after codon 440 to correlate our findings with available data in SEA till date. However, we considered all polymorphisms (synonymous and non-synonymous) to evaluate the nucleotide diversity among the isolates.

### *Polymorphic sites in K13 kelch propeller domain*

Genotyping analysis of K13 kelch propeller domain revealed a total of 6 polymorphic sites among these field isolates.

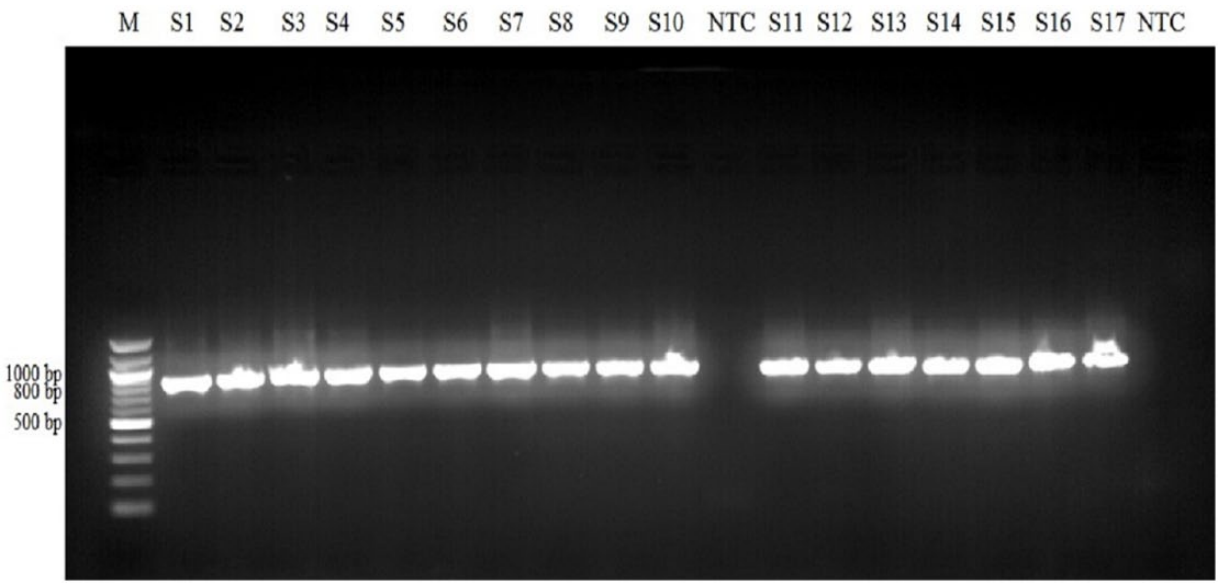


**Figure 2.** 849 bp *Pf*K13 gene partial coding sequence covering kelch protein codons. Lane M – 100 bp DNA ladder, lane S1 to S17 – amplicons from sample DNA, lane NTC – no template control.

**Table 2.** Polymorphism observed in *Pf*K13 kelch propeller domain of *Plasmodium falciparum* isolates in Northeast region, India.

| AMINO ACID CODON | NUCLEOTIDE SITE | REFERENCE AMINO ACID | MUTANT AMINO ACID | REFERENCE TRIPLET CODON | SNP IN TRIPLET CODON | FREQUENCY, n/N (%) |
|---|---|---|---|---|---|---|
| 481[a] | 1442 | A | V | GCT | G*T*T | 6/134 (4.5) |
| 675[a] | 2024 | A | V | GCT | G*T*T | 5/134 (3.7) |
| 700[b] | 2100 | S | — | TCA | TC*G* | 2/134 (1.5) |
| 701[b] | 2103 | P | — | CCA | CC*G* | 2/134 (1.5) |
| 702[a] | 2104 | D | N | GAT | *A*AT | 2/134 (1.5) |
| 703[b] | 2109 | T | — | ACA | AC*C* | 9/134 (6.7) |

[a]Non-synonymous mutations resulting mutant alleles.
[b]Synonymous polymorphisms. Mutations in codons 700, 701, and 702 were observed collectively in 2 isolates of Arunachal Pradesh.

Isolates from Arunachal Pradesh showed the highest number of polymorphic sites, whereas isolates from Assam and Tripura contained only 1 polymorphic site within this domain.

### *Synonymous SNPs in K13 kelch propeller domain*

In total, 3 synonymous SNPs were detected in this study (Table 2). Isolates from Assam were not found with any synonymous SNP. Only 1.5% isolates (2/134) particularly from Arunachal Pradesh were found to possess 2 synonymous SNPs in codons 700 and 701. In both codons, substitutions of adenine by guanine were observed at corresponding nucleotide sites 2100 and 2103, respectively. A set of 9 isolates (6.7%, 9/134) collectively from Arunachal Pradesh and Tripura contained another synonymous SNP at codon 703 in which substitution of adenine by cytosine was observed at its corresponding nucleotide site, *i.e.*, 2109.

### *Non-synonymous SNPs: A481V, A675V, and D702N mutation in K13 kelch propeller domain*

No isolate from Tripura was found with any non-synonymous SNP, showing a conservative wild-type kelch propeller protein in circulating parasite population. 4.5% isolates in Arunachal Pradesh (6/134) had a non-synonymous SNP at nucleotide site 1442 of codon 481 (GCT to G***T***T, substituting cytosine by thymine) which resulted in A481***V*** mutation in the protein (Table 2). Apart from 20 earlier known site mutations, 2 new mutations A675***V*** and D702***N*** (having non-synonymous SNPs at nucleotide sites 2024 and 2104, respectively) were observed. A675***V*** mutation was found in 3.7% isolates (5/134; 1 in Arunachal Pradesh and other 4 in Assam). Similarly, 1.5% isolates (2/134) exclusively from Arunachal Pradesh were also found with D702***N*** mutation. Two isolates from Arunachal Pradesh were collectively found with mutations in 3 consecutive codons, *i.e.*, 700, 701, and

**Table 3.** *Pf*mdr1 mutation observed in *Plasmodium falciparum* isolates in Northeast region, India.

| VALUE OF MUTATIONS | ARUNACHAL PRADESH (N = 48) | ASSAM (N = 55) | TRIPURA (N = 31) | TOTAL (N = 134) |
|---|---|---|---|---|
| N86***Y*** | 14 (29.2%) | 16 (29.1%) | 11(35.5%) | 41 (30.6%) |
| Y184***F*** | — | 26 (47.3%) | 2 (6.5%) | 28 (20.9%) |
| Haplotypes | | | | |
| NYSND | 34 (70.83%) | 18 (32.73%) | 20 (64.52%) | 72 (53.73%) |
| ***Y***YSND | 14 (29.2%) | 11 (20.0%) | 9 (29.03%) | 34 (25.4%) |
| N***F***SND | — | 21 (38.2%) | — | 21 (15.7%) |
| ***YF***SND | | 5 (9.1%) | 2 (6.5%) | 7 (5.22%) |

702. In total, 9.7% (13/134) isolates were observed with non-synonymous mutation in their K13 kelch propeller domain gene.

### *Pfmdr1 mutant alleles*

Sequence polymorphism analysis (in codons 86, 184, 1034, 1042, and 1246) of *Pf*mdr1 protein gene revealed that codons 86 and 184 of *Pf*mdr1 protein were having mutant as well as wild-type alleles; rest of the codons were found to contain wild-type alleles only. The 86***Y*** allele was found in 29.2% (14/48), 29.1% (16/55), and 35.5% (11/31) isolates from Arunachal Pradesh, Assam, and Tripura, respectively. The 184***F*** mutant allele was found in 47.3% (26/55) and 6.5% (2/31) isolates from Assam and Tripura, respectively, and this mutant allele was not found in any isolates from Arunachal Pradesh (Table 3). Considering these 2 mutations, isolates were observed with 2 single mutants (*i.e.*, ***Y***YSND and N***F***SND) and 1 double mutant (*i.e.*, ***YF***SND) *Pf*mdr1 protein haplotypes. Among all mutant protein haplotypes, single mutant ***Y***YSND haplotype was found in 25.4% (34/134) isolates followed by N***F***SND and ***YF***SND in 15.7% (21/134) and 5.22% (7/134) isolates, respectively. However, wild NYSND haplotype was observed in 53.73% (72/134) isolates.

Moreover, 22 isolates that exhibited polymorphisms in K13 propeller domain were found to contain both wild-type and mutant alleles in *Pf*mdr1 codon 86. Among these, 9 isolates were presented with N86***Y*** mutant allele, *i.e.*, A481***V*** in *Pf*K13 with N86***Y*** in *Pf*mdr1 (1/6), A675***V*** in *Pf*K13 with N86***Y*** in *Pf*mdr1 (3/5), S700 + P701 + D702***N*** in *Pf*K13 with N86***Y*** in *Pf*mdr1 (1/2), and T703 in *Pf*K13 with N86***Y*** in *Pf*mdr1 (4/9). Remaining 13 isolates, though, possessed polymorphisms in K13 propeller domain but were found to have wild-type allele in *Pf*mdr1 codon 86 (Table 4). No other codons in *Pf*mdr1 were found with mutant allele among these isolates.

**Table 4.** Corresponding *Pf*mdr1 mutation found in isolates with *Pf*K13 kelch propeller mutations.

| K13 CODON WITH MUTATION | MDR1 CODON | TOTAL |
|---|---|---|
| A481***V*** (n = 6) | NYSND | 5 |
| | ***Y***YSND | 1 |
| A675***V*** (n = 5) | NYSND | 2 |
| | ***Y***YSND | 3 |
| S700 + P701 + D702***N*** (n = 2) | NYSND | 1 |
| | ***Y***YSND | 1 |
| T703 (n = 9) | NYSND | 5 |
| | ***Y***YSND | 4 |

### *Molecular diversity of K13 kelch propeller domain gene among isolates*

The overall nucleotide diversity and mean haplotype diversity observed among these field isolates were 0.0004 and 0.295, respectively. Nucleotide and haplotype diversities were observed significantly higher in isolates of Arunachal Pradesh than Assam and Tripura ($P = 0.04$ and 0.038, respectively) (Table 5). The ratio of non-synonymous to synonymous mutation (dN/dS) among Arunachal Pradesh isolates was found to be greater than 1 (1.022), indicating positive selection of K13 polymorphisms. The probability of rejecting null hypothesis of strict neutrality (dN = dS) was found nonsignificant in all 3 studied parasite populations.

## Discussion

Northeast region of India has remained as an evolutionary setback for evolving drug-resistant *P. falciparum*, and introduction of new mutations in key drug-metabolising enzymes is continuing. Recently, a triple mutant sulfadoxine-resistant haplotype (*i.e.*, IS***GNG***A) also became evident in a constituting state of Northeast India[44] which was only evident earlier in western

**Table 5.** Molecular diversity indices observed in *Pf*K13 kelch propeller domain of *Plasmodium falciparum* isolates from Northeast region, India.

| PARAMETERS AND CHARACTERISTICS | VALUE OF | | | |
|---|---|---|---|---|
| | ARUNACHAL PRADESH | ASSAM | TRIPURA | ALL SITES |
| Sample size | 48 | 55 | 31 | 134 |
| Total nucleotide sites observed | 849 | 849 | 849 | 849 |
| Monomorphic | 843 | 848 | 848 | 843 |
| Polymorphic | 6 | 1 | 1 | 6 |
| No. of segregating sites | 6 | 1 | 1 | 6 |
| No. of changes | | | | |
| Synonymous | 3 | 0 | 1 | 3 |
| Non-synonymous | 3 | 1 | 0 | 3 |
| No. of haplotypes | 5 | 2 | 2 | 5 |
| Nucleotide diversity, π (SD) | 0.00082 (0.00159) | 0.00016 (0.00026) | 0.00027 (0.00029) | 0.00044 (0.00129) |
| Haplotype diversity (Hd) | 0.480 (0.081) | 0.137 (0.060) | 0.232 (0.090) | 0.295 (0.050) |
| Tajima's value D (dN/dS) | 1.022 | NA | NA | 0.993 |

Abbreviation: NA, not applicable.

Cambodia.[45] A unique finding in this study is the documentation of A481***V*** mutation in circulating *P. falciparum* isolates from Arunachal Pradesh. To the best of our knowledge, no report with such finding is available from this region till date. Although we observed this mutation in low frequency within this region, it has been found to be present comparatively higher than that of the earlier findings in Myanmar.[14] Two new mutations, *i.e.*, A675***V*** and D702***N***, observed in this study have given our finding a distinct importance. These 2 mutations have also not been reported earlier from this region. Amino acid mutations, *i.e.*, A676***D*** and H719***N*** (near 675 and 720 codons, respectively), of K13 propeller blade VI have been described recently from China-Myanmar border in low frequencies.[16] Despite the actual role, we assume that D702***N*** mutation might have emerged independently in this region and further evidence in future will unlock an approach for validation of its role in artemisinin resistance. However, A675***V*** allele has been observed in frequency next to A481***V*** allele. In a recent report by World Health Organization on Global Malaria Programme, A675***V*** mutation has been confirmed as a candidate mutation in *Pf*K13 propeller domain involved in conferring artemisinin resistance.[23] Hence, evidence of this mutation in Assam and Arunachal Pradesh of Northeast region would incur primary importance in near future. Among all mutations, codon 703 with synonymous mutation has been recorded comparatively high in this set of field isolates. However, presence of this mutation is also consistent with the recent study in Myanmar.[14] It is important to note that although these mutations are documented with low frequencies in this region, further surveillance would confirm the presence of these mutants in other areas of Northeast region. We further note that none of the studied isolates have found to be present with K13 mutations (*viz.*, G533A and R561H) that were found earlier from this region.[20] Most recently, an important non-synonymous candidate mutation, *i.e.*, F446***I*** within K13 propeller domain is reported in a Myanmar bordering area of Arunachal Pradesh.[9] Presence of F446***I*** mutation in this region, though, is notable, but it is very low in Arunachal Pradesh. Contrary to this, frequencies of A481***V*** and A675***V*** non-synonymous mutation found in this study are higher than that of F446***I***. However, A481***V*** mutation has been described as non-reference allele responsible for prolonged parasite clearance half-life[22] and its role is yet to evaluate.

Day-to-day progress in molecular surveillance of drug-resistant malaria parasites has opened new paradigm for proper understanding of its kind, nature, and spread. Different cross-sectional studies have described the scenario of prevailing and predominating K13 mutations in SEA, particularly, C580***Y*** has reached fixation at the Myanmar-Thailand border and M476***I*** prevailing in eastern border of Myanmar.[8,14,19,22,24,46,47] In this study, neither C580***Y*** nor M476***I*** allele has been recorded in circulating isolates within this region. Moreover, evidence of F446***I*** mutation in recent study indicates that it has either emerged in this region or spread from near most foci.

Presence of wild-type or mutant allele in codons 86, 184, and 1246 of *Pf*mdr1 protein collectively determine susceptibility and resistance against ACT-AL treatment policy.[48] In this study, we have found only wild-type alleles in codons 184 and 1246 in isolates which are with K13 polymorphism, but a few are with mutant N86***Y*** allele indicating about level of tolerance either against artemether or against lumefantrine, or both. N86 allele in *Pf*mdr1 has been shown *in vitro* in conferring less susceptibility to artesunate.[49] Few isolates from Arunachal Pradesh have been

found to contain only N86***Y*** allele, but 42 isolates from Assam and 13 isolates from Tripura have both N86***Y*** and Y184***F*** alleles. However, these isolates containing *Pf*mdr1 mutations in both 86 and 184 codons have been found to possess no polymorphism in their K13 kelch propeller domain. Mutation in codon 184 has been described previously responsible to mediate resistance against some antimalarials.[50] But it is unclear that both N86 and Y184***F*** mutations are involved either in lumefantrine resistance or implicated with compensating other mechanisms in parasite fitness against antimalarials.[51] Exact association of *Pf*mdr1 and *Pf*K13 mutations in resistance development cannot be ascertained as study reports including these 2 genes are not sufficiently available. Wild-type N86 allele in *Pf*mdr1 has been found with increased tolerance to both artemether and lumefantrine separately.[52-54] Moreover, the presence of N86 wild-type allele is a probable factor in the development of resistance against lumefantrine, and frequency of this allele varies according to different malarial transmission settings.[55,56] *Pf*mdr1 Y184***F*** allele has either minor impact on drug response or weaker association with antimalarial effectiveness as compared with N86***Y*** mutation, and expression of wild-type or mutant allele in codon 184 primarily depends on codon 86 status, class of drug to which parasite expose and genetic background of *Pf*crt gene.[29,57,58] Based on these, it can be said that parasites in this region with N86 wild-type allele in *Pf*mdr1 have acquired increased tolerance to artemether and lumefantrine partner drug. And mutant N86***Y*** allele has effect on high resistance development against chloroquine. In this study, N86 has been found in higher frequency (*i.e.*, 69.4%) indicating most of the isolates undergoing increased tolerance to lumefantrine. It is also noteworthy to state that presence of Y184***F*** mutation in considerable amount of *P. falciparum* isolates in this region may be due to increased artemether-lumefantrine drug pressure on parasite population. However, the presence of both wild-type and mutant alleles in *Pf*mdr1 codon 86 along with K13 A675***V*** mutation is notable because A675***V*** is a candidate mutation for artemisinin resistance. In addition, we want to note that evidence of higher nucleotide and haplotype diversity in Arunachal Pradesh field isolates compared with isolates of other 2 states are due to the presence high segregating sites in K13 gene.

## Conclusions

It can be said that K13 mutations involved in artemisinin resistance differ geographically. The evidence of A481***V***, A675***V***, and D702***N*** non-synonymous mutations in this region is a quite important finding, suggesting emergence of other more K13 polymorphisms in the future. Screening of mutations in *Pf*mdr1 gene along with K13 propeller domain should also be taken into account to establish how mutations in this gene are associated with K13 mutations in different malaria endemic settings and correlated with resistance development because mutations in *Pf*mdr1 gene are involved in modulating tolerance against a group of antimalarial drugs. Most importantly, this study has confirmed the presence of A675***V*** mutation for the first time in this region, indicating emergence of artemisinin resistance *P. falciparum* parasites. Higher frequency of *Pf*mdr1 N86 wild-type allele also point towards parasites' increased tolerance to the partner drug of currently employed ACT. In short, it can be concluded that further molecular surveillance to document K13 and *Pf*mdr1 mutation patterns in details is of utmost importance in this part of India. Moreover, study in larger samples set will deliberately give a clear picture about prevalent and predominant K13 and *Pf*mdr1 mutation patterns in this region and their role against currently employed ACT-AL drug policy. These will help in understanding whether artemisinin resistance has been evolved in this region, and if so, it will further assist in implementing effective control programmes to avoid spread of resistant *P. falciparum* population from this region to other areas.

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## Author Contributions

MKD, MCK, and PD conceived and designed the experiments. MKD and SC analysed the data and wrote the first draft of the manuscript. PD and MCK contributed to the writing of the manuscript. MCK, PD, MKD, and SC agree with manuscript results and conclusions. PD. MCK, and SC jointly developed the structure and arguments for the paper. PD, MCK, MKD, and SC made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

## Disclosures and Ethics

As a requirement of publication, author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality, and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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