Malaria treatment in Southeast Asia is threatened with the emergence of artemisinin-resistant *Plasmodium falciparum*. Genome association studies have strongly linked a locus on *P. falciparum* chromosome 13 to artemisinin resistance, and recently, mutations in the kelch13 propeller region (*Pfk-13*) were strongly linked to resistance. To date, this information has not been shown in Indian samples. *Pfk-13* mutations were assessed in samples from efficacy studies of artemisinin combination treatments in India. Samples were PCR amplified and sequenced from codon 427 to 727. Out of 384 samples, nonsynonymous mutations in the propeller region were found in four patients from the northeastern states, but their presence did not correlate with ACT treatment failures. This is the first report of *Pfk-13* point mutations from India. Further phenotyping and genotyping studies are required to assess the status of artemisinin resistance in this region.

The emergence and spread of drug resistance is a major obstacle to combating malaria. Resistance in *Plasmodium falciparum* to chloroquine and antifolates (1), which arose in Southeast Asia, spread across India and Africa and resulted in substantial increases in global malaria morbidity and mortality. This eventually led to the introduction of artemisinin (ART)-based combination therapy (ACT) (2) as first-line treatment for uncomplicated falciparum malaria. Until 2005, chloroquine (CQ) was the drug of choice for treatment of malaria in India, except in CQ-resistant cases, where sulfadoxine-pyrimethamine (SP) was recommended. However, with the increasing cases of resistance to SP during 2003 to 2004 (3) and the WHO recommendations, artemisinin-based combination therapy (ACT) of artemesunate with sulfadoxine-pyrimethamine (AS+SP) was introduced in 2007 as the first-line antimalarial for the treatment of confirmed falciparum malaria cases in chloroquine-resistant areas in the country. In 2010, this ACT was used universally across the country for treating falciparum malaria cases (4).

ACTs have been well tolerated, safe, and highly effective. Unfortunately, artemisinin resistance in *P. falciparum* has emerged in Southeast Asia (Myanmar, Thailand, Cambodia, Vietnam, and Laos), threatening the recent gains in malaria control and elimination. Westward spread to India and Africa is a major concern (5–10). The mechanism of action of the artemisinin drugs remains unclear. Although artemisinin resistance is characterized by slow parasite clearance, several molecular markers have been proposed (11–13). Genome association studies strongly linked a locus on *P. falciparum* chromosome 13 to artemisinin resistance, and this was recently explained by the discovery that mutations in the kelch13 propeller region (*Pfk-13*) are strongly linked to resistance (14). *k-13* is a 1-exon gene that codes for a putative kelch protein and has three domains: a plasmodium-specific domain, a BTB/POZ, and a C-terminal six-blade propeller (15). Mutations in the propeller region are linked to resistance. *Pfk-13* is well conserved across *Plasmodium* species and is thought to mediate protein-protein interactions (14).

Three point mutations (G533A, R539T, and C580Y) in the kelch motif of *Pfk-13* were correlated with the artemisinin resistance phenotype in the original study (14). A protein structure-modeling study showed that these mutations can alter the biological function of this putative protein (14). More than 30 single nucleotide polymorphisms in the propeller region of *Pfk-13* have since been associated with artemisinin resistance, although each resistant isolate has only one of these mutations.

Given that CQ-resistant *P. falciparum* strains spread to India from Southeast Asian countries through the northeastern states, a similar scenario may be expected for the spread of artemisinin-resistant *P. falciparum* from the epicenter in Southeast Asia (5–10). If artemisinin resistance does spread to or emerge in India, the public health consequences will be immense. India is also experiencing declining efficacy of its currently recommended first-line ACT (artesunate plus sulfadoxine-pyrimethamine [AS+SP]) in the northeastern states of the country (16, 17). In the present study, point mutations in *Pfk-13* were studied in *P. falciparum* isolates collected from patients enrolled in ACT efficacy studies from different sites in India. Although artemisinin resistance is defined by slow parasite clearance, these studies did not include detailed assessments of parasite dynamics.

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**Citation**


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**Supplemental material**

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MATERIALS AND METHODS

A total of 389 *P. falciparum* samples were collected from patients enrolled in prospective ACT (AS+SP) therapeutic efficacy studies conducted between 2009 and 2013 as part of the nationwide sentinel site antimalarial drug therapeutic assessment system (16, 17). Directly observed treatments with quality-assured drugs from standard Indian manufacturers were given to the enrolled patients. These drugs were supplied by the state health departments and were used within their expiry period. We obtained informed, written consent from each enrolled adult and from a legal guardian of each enrolled child. The study protocol was approved by the Government of India, Ministry of Health and Family Welfare, and the scientific advisory committee of the National Institute of Malaria Research (NIMR). The institutional ethics committee (IEC) approved the secondary study.

During 2008, NIMR and the National Vector Borne Disease Control Programme (NVBDCP) selected 25 sentinel sites for routine monitoring of antimalarial drug resistance. These sites were purposely selected to provide a representative cross-section of malaria ecotypes, transmission intensities, and geographic regions. The blood samples used for the study were from 15 sentinel sites spread across different periods and geographic regions. Open-label, single-arm prospective studies were conducted (16) as per WHO protocol and patients were followed up to day 28 during 2011 and 2013 and up to day 42 during 2012. The studies included five districts from the northeastern region (Gomati in Tripura, Lunglei in Mizoram, West Garo Hill in Meghalaya, Karbi Anglong in Assam, and Changlang in Arunachal Pradesh); two from West Bengal (Jalpaiguri and Kolkata); and one each in Odisha (Sundergarh), Jharkhand (Simdega), Chhattisgarh (Kanker), Maharashtra (Gadchiroli), Rajasthan (Paratapgarh), Madhya Pradesh (Betul), Gujarat (Surat), and Andhra Pradesh (Vishakhapatnam). Details about each study site, such as the prevalence of malaria, introduction of ACT as first-line treatment, and relevant geographic information, as well as the sample details, are provided in the supplemental material.

Genomic DNA was extracted using the QIAamp mini DNA kit (Qiagen, Germany) from microscopy-diagnosed *P. falciparum*-positive blood spotted on Whatman filter paper (3-mm) strips. Paired blood samples, i.e., from day 0 and the day of reappearance of parasitemia, were analyzed using three well-established molecular markers (*msp-1*, *msp-2*, and *glurp*) for differentiating recrudescence from reinfection. *Pfk-13* genes from all samples were amplified by PCR and DNA sequenced (Macrogen, South Korea) per protocols reported previously (14). This primer set covered all mutations reported to be associated with artemisinin resistance, which were from codon 427 to 727 of *Pfk-13* (see Fig. S1 in the supplemental material) (10). High-fidelity Taq DNA polymerase (Kapa Hi-Fidelity Hot Start master mix) was used to minimize PCR-incorporated nucleotide errors. All mutations observed in the study from the northeastern region (Gomati in Tripura, Lunglei in Mizoram, West Garo Hill in Meghalaya, Karbi Anglong in Assam, and Changlang in Arunachal Pradesh); two from West Bengal (Jalpaiguri and Kolkata); and one each in Odisha (Sundergarh), Jharkhand (Simdega), Chhattisgarh (Kanker), Maharashtra (Gadchiroli), Rajasthan (Paratapgarh), Madhya Pradesh (Betul), Gujarat (Surat), and Andhra Pradesh (Vishakhapatnam) (Fig. 1). Details about each study site, such as the prevalence of malaria, introduction of ACT as first-line treatment, and relevant geographic information, as well as the sample details, are provided in the supplemental material.

![FIG 1 Details of *Plasmodium falciparum* ACT efficacy study sites in India.](image-url)

<table>
<thead>
<tr>
<th>Study Site; Year (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Assam (Karbi Anglong); 2012 (10)</td>
</tr>
<tr>
<td>2. Arunachal Pradesh (Changlang); 2012 (24)</td>
</tr>
<tr>
<td>3. Andhra Pradesh (Visakhapatnam); 2010 (20)</td>
</tr>
<tr>
<td>4. Chhattisgarh (Kanker); 2011 (20)</td>
</tr>
<tr>
<td>5. Gujarat (Surat); 2010 (23)</td>
</tr>
<tr>
<td>6. Jharkhand (Simdega); 2011 (20)</td>
</tr>
<tr>
<td>7. Maharashtra (Gadchiroli); 2010 (24)</td>
</tr>
<tr>
<td>8. Madhya Pradesh (Betul); 2010 (22)</td>
</tr>
<tr>
<td>9. Mizoram (Lunglei); 2011 (20), 2012(32), 2013 (20)</td>
</tr>
<tr>
<td>10. Meghalaya (West Garo Hills); 2010 (13)</td>
</tr>
<tr>
<td>11. Odisha (Sundergarh); 2012 (22)</td>
</tr>
<tr>
<td>12. Rajasthan (Pratapgarh); 2010 (20)</td>
</tr>
<tr>
<td>13. Tripura (Gomati); 2012 (38), 2013 (20)</td>
</tr>
<tr>
<td>14. West Bengal (Kolkata); 2010 (20)</td>
</tr>
<tr>
<td>15. West Bengal (Jalpaiguri); 2011 (16)</td>
</tr>
</tbody>
</table>
were validated with another independent PCR and by resequencing
Pfk-13.

**Nucleotide sequence accession numbers.** DNA sequences of represen-
tative samples showing wild and mutant types were submitted to Gen-
Bank and assigned accession numbers KP780808, KP790255, KP790256,
KP790257, KP790258, KP790259, KP790260, and KP790261.

**RESULTS**

Samples from patients who responded (adequate clinical and para-
sitological response [ACPR]) to ACTs were obtained from all
study sites, while PCR-corrected confirmed recrudescences (n =
42) were obtained from seven sites: Arunachal Pradesh (n = 6),
Tripura (n = 15), Mizoram (n = 11), Gujarat (n = 3), Maharas-
hra (n = 4), Madhya Pradesh (n = 2), and West Bengal (n = 1) (see
Fig. S2 in the supplemental material) (16, 17). Pfk-13 was PCR
amplified successfully from all responders, but five samples from
nonresponders could not be amplified. DNA sequence analysis of
Pfk-13 from the 384 clinical isolates of *P. falciparum* showed six
point mutations and one deletion. The mutations were synony-
maous in two and nonsynonymous (NS) in four, and there was one
deletion causing frameshift. Synonymous substitutions were
found at nucleotide positions 1377 (G-A) and 1752 (T-C), and NS
substitutions were observed at codons 533 (G-A), 549 (S-Y), 561
(R-H), and 578 (A-S) (Table 1). The frameshift mutation was
observed at nucleotide position 1991 (deletion of A nucleotide).
Only NS mutations were used for further analysis. The 561 (R-H)
and 578 (A-S) mutations were reported previously in association
with slow parasite clearance, but the 533 (G-A) and 549 (S-Y)
mutations have not been reported to date (10, 14). The mutations
were observed at very low frequencies (0.26%, 1/384).

Among the four NS substitutions, three were observed in
northeastern states and one was observed in Jalpaiguri, which is
the gateway to northeastern states (Assam) (Fig. 1). Thus, no NS
mutations were observed in isolates collected from other parts of
India (Table 2).

In addition, a higher prevalence of point mutations in the *P.
falciparum* dihydropteroate synthase (*dhps*) and dihydrofolate
reductase (*dhfr*) genes was also observed in the northeastern states
compared with isolates from other parts of India (Table 3) (16, 17).

**DISCUSSION**

This is the first report of *Pfk-13* point mutations from India. Does
this mean that artemisinin resistance has already spread to India?
Without corresponding phenotyping, it is premature to conclude
that artemisinin resistance has arrived. The recent large multina-
tional Tracking Resistance to Artemisinin Collaboration (TRAC)
study (10), which conducted detailed parasite clearance assess-
ments and genotyping, clearly associated *Pfk-13* propeller region
mutations with slow parasite clearance in Southeast Asia, but such
mutations were also found in the Democratic Republic of the
Congo, where they were not associated with resistance. Other
genotyping studies suggested a low background frequency of such
mutations in parasite populations across the world (18, 19). How-
ever, a recent study in sub-Saharan Africa identified novel coding
substitutions of unclear phenotypes (20). Clearly, more informa-
tion is needed, but it seems at present that other genetic changes in
*P. falciparum* may be required in order to confer a stable artemis-
in-resistant phenotype. There is an urgent need to assess with
clinical and laboratory phenotyping in addition to genotyping
whether artemisinin resistance is present in this region.

<table>
<thead>
<tr>
<th>Table 1: Point mutations in <em>Plasmodium falciparum</em> k-13 gene from Indian clinical isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pfk-13</em> point mutation description (nonsynonymous)<em>b</em></td>
</tr>
<tr>
<td>G449A</td>
</tr>
<tr>
<td>I543T</td>
</tr>
<tr>
<td>D584V</td>
</tr>
<tr>
<td>No. isolates (n = 380)</td>
</tr>
</tbody>
</table>

*a* Bold type indicates novel point mutation. An empty cell indicates no point mutation.

*b* Mutation in *Pfk-13* that confers ART resistance in Cambodian *P. falciparum* isolates (16).

Mishra et al.
### TABLE 2 Point mutations in *Plasmodium falciparum* k-13 gene from India

<table>
<thead>
<tr>
<th>Study site</th>
<th>Yr of sample collection</th>
<th>Sample size (no.)</th>
<th>k-13 mutation description(^a)</th>
<th>G533A</th>
<th>S549Y</th>
<th>R561H</th>
<th>A578S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gomati, Tripura</td>
<td>2012(^b)</td>
<td>38</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2013(^c)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lunglei, Mizoram</td>
<td>2011(^c)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2012(^d)</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2013(^c)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Karbi Anglong, Assam</td>
<td>2012(^c)</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>West Garohills, Meghalaya</td>
<td>2010(^e)</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Changlang, Arunachal Pradesh</td>
<td>2012(^f)</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jalpaiguri, West Bengal</td>
<td>2011(^c)</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Kolkata, West Bengal</td>
<td>2010(^b)</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sundergarh, Odisha</td>
<td>2012(^c)</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simdega, Jharkhand</td>
<td>2011(^c)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kanark, Chhattisgarh</td>
<td>2011(^c)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betul, Madhya Pradesh</td>
<td>2011(^c)</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surat, Gujarat</td>
<td>2010(^b)</td>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vishakhaptnam, Andhra Pradesh</td>
<td>2010(^b)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pratapgarh, Rajasthan</td>
<td>2010(^b)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gadchiroli, Maharashtra</td>
<td>2010(^b)</td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>384</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\) Bold type indicates novel point mutation. An empty cell indicates no point mutation.

\(^b\) See references 16 and 17.

\(^c\) Our unpublished data.

### TABLE 3 Point mutations in *Plasmodium falciparum* k-13 gene from ACT resistance cases

<table>
<thead>
<tr>
<th>Study site</th>
<th>Clinical outcome(^a)</th>
<th>Sample size (no.)</th>
<th>k-13 mutation (no.)</th>
<th>Prevalence of Pfdhfr/Pfdhps mutant genotype (%)*</th>
<th>Reference or source for Pfdhfr/Pfdhps mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gomati, Tripura</td>
<td>ACPR</td>
<td>25</td>
<td>G-533-A S-549-Y R-561-H A-578-S</td>
<td>100 97.3</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>15(^b)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lunglei, Mizoram</td>
<td>ACPR</td>
<td>21</td>
<td></td>
<td>100 97.0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Changlang, Arunachal Pradesh</td>
<td>ACPR</td>
<td>21</td>
<td></td>
<td>100 97.0</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>6(^c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kolkata, West Bengal</td>
<td>ACPR</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Betul, Madhya Pradesh</td>
<td>ACPR</td>
<td>20</td>
<td></td>
<td>80 3.30</td>
<td>21,22</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surat, Gujarat</td>
<td>ACPR</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>3</td>
<td></td>
<td>100 24.60</td>
<td>Our unpublished data</td>
</tr>
<tr>
<td>Gadchiroli, Maharashtra</td>
<td>ACPR</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>ACPR</td>
<td>146</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>37(^d)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) ACPR, adequate clinical and parasitological response; TF, treatment failure.

\(^b\) Two samples were not PCR amplified.

\(^c\) Three samples were not PCR amplified.

\(^d\) Five samples were omitted from total; shaded areas represent northeastern states.

\(^e\) ND, not done.
In the northeastern states, the higher prevalence of point mutations in the PfΔhps and PfΔhfr genes, both coding for essential enzymes in the folate biosynthesis pathway, is likely to be responsible for ACT treatment failures in this region (17).

All the NS substitutions were observed in northeastern states, with one in Jalpaiguri, which is the gateway to northeastern states (Assam). Treatment responses were good in each of the four patients harboring NS Pfkh mutations, despite the declining efficacy of the partner drug sulfadoxine-pyrimethamine in northeast India.

The history of the introduction of chloroquine resistance to India suggests that the northeastern region is the gateway and therefore a likely physical route for possible introduction of artemisinin-resistant \textit{P. falciparum} strains in the near future. Artemisinin-resistant parasites are prevalent in adjacent Myanmar. Continued monitoring of ART resistance in clinical studies measuring parasite clearance half-lives supported by molecular genotyping is required, particularly in the northeastern states of India. This study had a few limitations. Most of the treatment failure samples from patients were late treatment failures that were due to the partner drug component of ACT rather than to artemisinin. To study had a few limitations. Most of the treatment failure samples from patients who failed the treatment remains inconclusive. The present observations serve as a necessary baseline. Further phenotyping and genotyping studies are needed to determine whether artemisinin resistance has spread to or emerged in northeast India.

ACKNOWLEDGMENTS

The financial support of the World Bank through the National Vector Borne Disease Control Programme (Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India) is gratefully acknowledged.

We thank the patients for their cooperation during the study. We also thank the NIMR field units and NVBDCP regional teams for their hard work.

This paper was cleared by the NIMR’s publication screening committee (030/2014).

We declare no conflicts of interest.

REFERENCES


