MAJOR ARTICLE

# A Single Mutation in K13 Predominates in Southern China and Is Associated With Delayed Clearance of *Plasmodium falciparum* Following Artemisinin Treatment

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***Background.*** Artemisinin resistance in *Plasmodium falciparum* has emerged in Southeast Asia and poses a threat to malaria control and elimination. Mutations in a *P. falciparum* gene encoding a kelch protein on chromosome 13 have been associated with delayed parasite clearance following artemisinin treatment elsewhere in the region, but not yet in China.

***Methods.*** Therapeutic efficacy studies of artesunate and dihydroartemisinin-piperaquine were conducted from 2009 to 2012 in the Yunnan Province of China near the border with Myanmar. K13 mutations were genotyped by capillary sequencing of DNA extracted from dried blood spots collected in these clinical trials and in routine surveillance. Associations between K13 mutations and delayed parasite clearance were tested using regression models.

***Results.*** Parasite clearance half-lives were prolonged after artemisinin treatment, with 44% of infections having half-lives >5 hours (n = 109). Fourteen mutations in K13 were observed, with an overall prevalence of 47.7% (n = 329). A single mutation, F446I, predominated, with a prevalence of 36.5%. Infections with F446I were significantly associated with parasitemia on day 3 following artemisinin treatment and with longer clearance half-lives.

***Conclusions.*** *Plasmodium falciparum* infections in southern China displayed markedly delayed clearance following artemisinin treatment. F446I was the predominant K13 mutation and was associated with delayed parasite clearance.

***Keywords.*** artemisinin resistance; China; kelch 13; malaria; *Plasmodium falciparum*.

Artemisinin-based combination therapies (ACTs) are the first-line treatment in almost all malaria-endemic countries and are critical for control and elimination of *Plasmodium falciparum* malaria globally. Artemisinin resistance was reported first in western Cambodia [1, 2] and has been reported in other areas of Southeast Asia [3–8]. Genome-wide association studies conducted in clinical isolates from Southeast Asia identified a region of *P. falciparum* chromosome 13 that was associated with delayed parasite clearance [9, 10]. Mutations in the propeller region of a kelch protein (K13) within the associated region were identified in laboratory-selected artemisinin-resistant isolates [11] and confirmed through in vitro and ex vivo association studies [12]. To date, at least 54 mutations have been identified within K13, including 41 mutations in the propeller domain, the domain in which most resistance mutations have been located [6, 11–15].

Received 11 December 2014; accepted 16 April 2015; electronically published 24 April 2015.

Presented in part: Annual meeting of the American Society of Tropical Medicine and Hygiene, Washington D.C., 13–17 November 2013; Annual meeting of the American Society of Tropical Medicine and Hygiene, New Orleans, Louisiana, 2–6 November 2014.

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**The Journal of Infectious Diseases® 2015;212:1629–35**



DOI: 10.1093/infdis/jiv249

*Plasmodium falciparum* malaria is currently only found in 1 province in China, the Yunnan Province, which borders Myanmar, Vietnam, and Laos. The national malaria treatment policy of China was updated in 2009, and the first-line drugs used to treat falciparum malaria since then have been ACTs, which include dihydroartemisinin-piperaquine, artesunate-amodiaquine, artemisinin-naphthoquine phosphate, and artemisinin-piperaquine [3].

This study was designed to evaluate clinical outcomes after 7-day artesunate monotherapy or standard 3-day treatment with dihydroartemisinin-piperaquine for uncomplicated *P. falciparum* malaria at 3 sites in the Yunnan Province along the China-Myanmar border. Sequencing of the propeller domains of the K13 gene was performed to identify mutations and test their association with delayed parasite clearance.

## METHODS

### Therapeutic Efficacy Studies and Routine Surveillance

#### *Study Sites and Design*

The clinical studies were 1-arm prospective evaluations of clinical and parasitological responses to directly observed treatment for uncomplicated malaria, using a standard World Health Organization (WHO) therapeutic efficacy protocol [16]. The studies were conducted in Yingjiang, Tengchong, and Menglian counties (Figure 1) between 2009 and 2012. In addition to these therapeutic efficacy studies, samples were obtained from routine malaria surveillance in Yingjiang and Tengchong counties from 2010 to 2013, as well as in Hainan Province (Figure 1) in 2007.

#### *Recruitment of Patients*

For therapeutic efficacy studies, patients aged 6 months or older with fever (axillary temperature ≥37.5°C) or history of fever in the previous 48 hours were screened for inclusion. Enrolled patients had monoinfection with *P. falciparum*, parasitemia levels between 500 and 100 000 asexual parasites/μL, no history of antimalarial use in the last 14 days, and no signs of severe malaria or danger signs. All routine surveillance samples were obtained through passive case detection at clinics. Patients self-referring for evaluation of fever were evaluated for malaria infection by microscopy and/or rapid diagnostic tests. Those testing positive for malaria were referred to appropriate local health providers and asked to contribute dried blood spot samples.

#### *Treatment of Patients and Follow-up*

After written informed consent was provided, a detailed medical history, clinical examination, and both thick and thin blood films were performed for participants in therapeutic efficacy studies. In the 2009 study in Yingjiang, artesunate was administered at a total dose of 16 mg/kg over 7 days (1st day: 4 mg/kg; 2nd–7th days: 2 mg/kg/day). In each of the other studies, dihydroartemisinin-piperaquine was administered at a total adult dose of 2.5 mg/kg dihydroartemisinin and 20 mg/kg piperaquine for 3 days. One tablet of dihydroartemisinin-piperaquine contained 40 mg base dihydroartemisinin and 320 mg piperaquine phosphate. All the antimalarial drugs were provided by the WHO.

Clinical and laboratory tests, including axillary temperature measurement and thick/thin blood smear preparation and examination, were performed at least daily (every 12 hours for the first 48 hours) while the study participants stayed in the hospital for antimalarial drug treatment (7 days for those treated with artesunate and 3 days for those treated with dihydroartemisinin-piperaquine). With the first day of treatment being day 0, postdischarge parasite examinations were also performed on days 14, 21, and 28 for artesunate-treated patients, and on days 7, 14, 21, 28, 35, and 42 for dihydroartemisinin-piperaquine-treated patients. Giemsa-stained blood smears were prepared for parasite density determination and speciation. Parasites were counted against white blood cells (WBCs) [17] (Supplementary Methods). Smears were read by 2 experienced microscopists, and discrepant results were adjudicated by expert microscopists certified as Level 1 by the WHO. Smears were considered negative when no asexual parasites were found after counting 1000 WBCs. Polymerase chain reaction (PCR) analysis was conducted to differentiate recrudescence (same parasite strain) from newly acquired infection with parasite genes *msp1*, *msp2*, and *glurp* [18].

#### *Outcome Measures*

Treatment efficacy was evaluated based on clinical and parasitological outcomes and study end points in accordance with WHO guidelines for therapeutic efficacy monitoring. Study end points included treatment failure, completion of follow-up without treatment failure, loss to follow-up, and withdrawal from the study. Outcomes were classified as early treatment failure, late clinical failure (LCF), late parasitological failure, and adequate clinical and parasitological response.

### Ethical Considerations

The study was reviewed and approved by the ethical review committees of the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (China CDC), and of the WHO Western Pacific Regional Office. Informed written consent was obtained from patients or guardians of minor patients. Therapeutic efficacy studies were also registered as clinical trials at https://www.anzctr.org.au under the numbers ACTRN12610001008011 and ACTRN12610001028099.

### Genotyping of K13

#### *Sample Collection and DNA Extraction*

Dried blood spots on filter paper (Whatman 903, GE Healthcare) were collected from all participants in the therapeutic efficacy study before they received antimalarial drug treatment, as well as from individuals with *P. falciparum* confirmed by

microscopy or rapid diagnostic test identified through routine surveillance. The routine surveillance samples were from Hainan Province in 2007 and therapeutic efficacy study sites from 2009 to 2013. DNA was extracted from dried blood spots following manufacturer's instructions (QIAamp 96 DNA Blood Kit, Valencia, California).

### *Nested PCR and Sequencing*

A nested PCR amplification method was used following previously reported protocols with some minor modifications [11]. PCR products were purified using filter plates (Edge Biosystems, Gaithersburg, Maryland) and directly sequenced and analyzed on an ABI 3730XL automatic sequencer as recommended by the manufacturer. (Primers for nested PCR and sequencing PCR and cycling conditions are listed in Supplementary Table 1).

### Statistics and Analyses

Clinical and demographic data were double-entered into a Microsoft Excel database. Parasite clearance half-lives were estimated from serial parasite counts using the publicly available WorldWide Antimalarial Resistance Network (WWARN) Parasite Clearance Estimator [19] and confirmed with a different clearance half-life calculator made available by the WHO Global Malaria Programme's Drug Resistance and Containment Unit.

The output sequence data were assembled, edited, and aligned using Sequencher (version 5.1) software. All mutations were assessed by comparing each sequence to the 3D7 reference (PF13_0238). Logistic regression was used to estimate the association between parasite clearance half-life >5 hours or day-3 parasitemia and the presence of any K13 mutation or the F446I mutation in infections for which parasite clearance half-lives measured. Odds ratios (ORs) and 95% confidence intervals were estimated using SAS (version 9.2) software. Sequences were deposited in GenBank under accession numbers KM591247–KM591575.

## RESULTS

### Characteristics of the Patients

A total of 218 participants from Yingjiang, Tengchong, and Menglian counties were enrolled in the therapeutic efficacy studies from 2009 to 2012. The average ages of participants from different sites ranged from 22.7 to 32.7 years. Most participants were young adults: less than 2% were aged 5 years or less, and 20.1% were aged 5–15 years. Men accounted for 66.1% of participants. Most (89.5%) participants had axillary temperatures ≥37.5°C before treatment, and the geometric mean parasite density of infections from each site ranged from 11 257/μL to 69 535/μL (Table 1).

### Outcome of Therapeutic Efficacy Study

All participants received directly observed antimalarial treatment with artesunate or dihydroartemisinin-piperaquine. The PCR-corrected efficacy of artesunate and dihydroartemisinin-piperaquine were 97.7%–100%. In Yingjiang in 2009, there was 1 LCF and 1 late parasitological failure, with parasitemia on follow-up days 20 and 27, respectively, following artesunate treatment. The third participant experienced an early treatment failure with parasitemia and axillary temperature ≥37.5°C on day 3 following treatment with dihydroartemisinin-piperaquine. The 2 participants with recurrent parasitemia were confirmed to have new infections by PCR [18] (Table 2). Twelve of 65 participants (18%) were still positive for parasitemia on day 3 following a 7-day course of artesunate, and 5 of 80 participants (6%) were still positive for parasitemia on day 3 after dihydroartemisinin-piperaquine treatment.

### Parasite Clearance Half-life

Parasite clearance half-lives were calculated based on the parasitemia at 0, 12, 24, 36, 48, and 72 hours [19]. Infections displayed short clearance half-lives after dihydroartemisinin-piperaquine treatment in different sites and different years, with a median parasite half-life of less than 5 hours. Parasites from Yingjiang County displayed prolonged parasite clearance half-lives 5.08 hours (interquartile range: 3.11–6.43) following 7 days of artesunate monotherapy (Figure 2*A*). There were no significant differences in the parasite clearance half-life between sites and times (Student *t* test; $P > .05$). The proportions of participants with different parasites clearance half-lives are shown in Figure 2*B*. Clearance half-lives were similar using both the WWARN and WHO calculators ($P > .05$, data not shown).

**Table 1. Characteristics of Patients With *Plasmodium falciparum* in Therapeutic Efficacy Studies**

| Site-year (N) | Age (years) | | | | % Male | Axillary Temperature ≥37.5°C | Geometric Mean Parasite Density (/μL) |
|---|---|---|---|---|---|---|---|
| | <5 | 5–15 | >15 | Mean | | | |
| Yingjiang-2009 (65) | 2 | 19 | 44 | 25 | 73.8 | 48/65 (73.8%) | 69 535 |
| Yingjiang-2010 (30) | 1 | 2 | 27 | 27.1 | 76.7 | 28/30 (93.3%) | 63 140 |
| Yingjiang-2012 (50) | 1 | 10 | 39 | 22.7 | 68.0 | 50/50 (100.0%) | 23 311 |
| Tengchong-2012 (22) | 0 | 0 | 22 | 32.7 | 81.8 | 20/22 (90.9%) | 20 984 |
| Menglian-2009 (51) | 0 | 14 | 37 | 25.3 | 41.2 | 49/51 (96.1%) | 11 257 |

**Table 2. Outcome of Therapeutic Efficacy Studies in Yingjiang, Tengchong, and Menglian Counties From 2009 to 2012**

| Year | Site | Antimalarial Drug Treatment | Day-3 Positive | ACPR | LCF | LPF | ETF | WTH/LFU |
|---|---|---|---|---|---|---|---|---|
| 2009 | Yingjiang | Artesunate | 12/65 (23.5%) | 49/49 (100.0%) | 0/49 (2.0%)[a] | 0/49 (2.0%)[a] | 0/49 (0.0%) | 16/65 (24.6%) |
| 2010 | Yingjiang | DHA-PIP | 3/30 (5.9%) | 24/24 (100.0%) | 0/24 (0.0%) | 0/24 (0.0%) | 0/24 (0.0%) | 6/30 (20.0%) |
| 2012 | Yingjiang | DHA-PIP | 2/50 (3.9%) | 42/43 (97.3%) | 0/43 (0.0%) | 0/43 (0.0%) | 1/43 (2.3%) | 7/50 (14.0%) |
| 2012 | Tengchong | DHA-PIP | 1/22 (2.0%) | 20/20 (100.0%) | 0/20 (0.0%) | 0/20 (0.0%) | 0/20 (0.0%) | 2/22 (9.1%) |
| 2009 | Menglian | DHA-PIP | 0/51 (0.0%) | 51/51 (100.0%) | 0/51(0.0%) | 0/51 (0.0%) | 0/51 (0.0%) | 0/51 (0.0%) |

Abbreviations: ACPR, adequate clinical and parasitological response; DHA-PIP, dihydroartemisinin-piperaquine; ETF, early treatment failure; LCF, late clinical failure; LFU, lost to follow-up; WTH, withdrawn.

[a] Two participants showed LCF and 1 late parasitological failure, with parasitemia on follow-up days 20 and 27, respectively, following artesunate treatment. Genotype analysis was conducted to differentiate recrudescence (same parasite strain) from newly acquired infection with parasite genes *msp1*, *msp2*, and *glurp*.

### Prevalence of K13 Mutations

The gene encoding the K13 protein was successfully sequenced in 329 samples, 124 from therapeutic efficacy studies from 2009 to 2012 and 205 from routine surveillance. Fourteen mutations in the K13 propeller domain were observed in 157 samples, and the prevalence of K13 mutations was 47.7% (157/329) (Figure 3). F446I accounted for 73.2% (52/71) of all K13 mutations (Figure 3*B*). The F483S, L492S, F495L, and E556D K13 mutations of K13 have not been reported in previous studies. Among infections with wild-type K13 alleles, 5.7% had a parasite clearance half-life longer than 5 hours, while 62.0% (44/71) of infections with mutant K13 had a parasite clearance half-life longer than 5 hours. The predominant mutation was F446I, which was seen in 44.0% of all samples. C580Y, R539T, and Y493H have


**Figure 1.** Locations for sample collection. Therapeutic efficacy study sites in Yunnan Province (green) include Yingjiang, Tengchong, and Menglian counties, shown in red. Hainan Province and Yunnan Province are shown in blue and green, respectively.

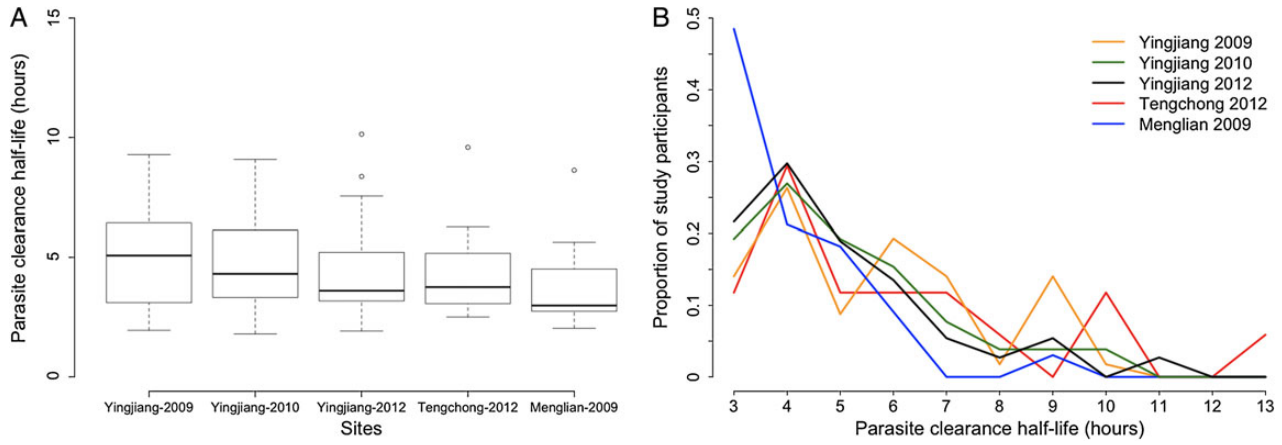


**Figure 2.** Distribution of parasite clearance half-lives by study site (*A*) and proportion of study participants with different parasite clearance half-lives (*B*). Box plots depict interquartile range, with data falling outside this range appearing as open circles.

been previously associated with delayed parasite clearance in Southeast Asia, but C580Y was observed in only 2 samples in southern China. No mutations were found in samples from Hainan Province (n = 14) or Menglian County (n = 9) (Figure 3*B*). We observed only 1 K13 mutation in each infection, consistent with other reports [11]. Details of K13 genotyping by site, study type, and year are provided in Supplementary Table 2.

### Association Between K13 Mutations and Delayed Parasite Clearance

Parasite clearance half-lives were prolonged after artemisinin treatment, with 44% of infections having half-lives >5 hours (n = 109). The presence of any K13 propeller mutation was significantly associated with parasitemia on day 3 after starting artemisinin treatment, as well as with parasite clearance half-life >5 hours in multivariable logistic regression models adjusting for, age, log-transformed parasitemia at diagnosis, and treatment (OR: 104, $P = .0022$ for day 3 parasitemia; OR = 26, $P < .0001$ for parasite clearance half-life). The presence of the F446I mutation was also significantly associated with day-3 parasitemia and clearance half-life >5 hours (OR: 9.1, $P = .0045$; OR: 9.0, $P < .0001$, respectively) (Figure 3*A*).

## DISCUSSION

ACTs are currently relied upon for effective malaria treatment in most areas of the world, and their continuing efficacy is crucial to the success of control and elimination programs [20]. Artemisinin resistance has been identified along the Thailand-Myanmar, Thailand-Cambodia, Vietnam-Cambodia, and Vietnam-Laos borders [21, 22]. Artemisinin resistance was manifested as delayed parasite clearance and correlated with ring-stage survival of artemisinin treatment in vitro [1, 23].

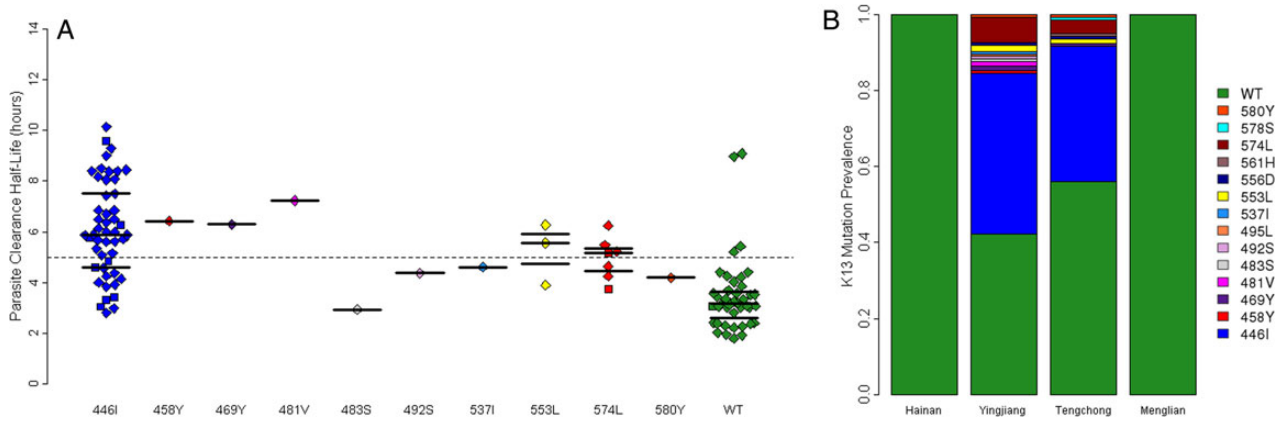


**Figure 3.** Association of K13 mutations with parasite clearance half-lives (*A*) and prevalence of infections with all K13 propeller mutations (*B*). Parasite clearance half-lives with K13 mutations (colors) or wild-type (WT) alleles (green).

Parasites in China have been reported to show decreased sensitivity to artemisinin and delayed parasite clearance time [3, 24]. Our study has shown that parasite clearance time after artesunate and dihydroartemisinin-piperaquine treatment is prolonged, and some infections have parasite clearance half-lives longer than 5 hours. Parasites with K13 mutations are more likely to exhibit parasite clearance half-lives longer than 5 hours compared to those with wild type alleles (Figure 2*A* and Figure 3). One early treatment failure occurred on day 3 following treatment with dihydroartemisinin-piperaquine. Unfortunately, K13 was not successfully sequenced for this treatment failure, which may or may not have represented a case of resistance, because parasite clearance is influenced by many factors, including the pretreatment parasite density, accurate timing of samples, adequate drug levels, and reliable counting [25–27].

This study provides evidence of reduced susceptibility of *P. falciparum* to artemisinin in southern China. All 3 clinical trial sites are located in southern Yunnan Province, bordering Shan and Kachin states in northern Myanmar. *Plasmodium falciparum* infections in Kawthaung, southern Myanmar, displayed markedly delayed clearance following artemisinin treatment, with parasite clearance half-lives being longer than 5 hours, suggesting emergence of artemisinin resistance in southern Myanmar [8]. Delayed parasite clearance suggesting artemisinin resistance was also found in southern Vietnam [13]. Large movements of mobile and migrant populations take place along these border areas frequently [28]. Border malaria will be one of the obstacles in artemisinin resistance contamination and malaria elimination, especially in the Greater Mekong Subregion [29].

The K13-propeller was recently identified as a key determinant of delayed parasitological clearance after artemisinin treatment, and multiple single-nucleotide polymorphisms were associated with delayed clearance in western Cambodia [11]. The validation and molecular surveillance of the K13-propeller sequence in parasite surveys in Southeast Asia and sub–Saharan Africa is crucial to the artemisinin resistance containment efforts being implemented [30]. Numerous K13 propeller-coding polymorphisms were found in sub–Saharan Africa; however, the relationship between these mutations and artemisinin resistance in Africa has yet to be determined [14, 15, 30, 31]. Our study showed 14 different mutations in the K13 propeller domain in southern China with a prevalence of 47.7%. Four mutations of the K13 propeller domain, F483S, L492S, F495L, and E556D, were first reported in this study. The predominant mutation was F446I, which was associated with prolonged parasite clearance half-life and parasitemia on day 3 after treatment. F446I was also observed in a recent study, which found 3 parasites with the F446I mutation, 2 of which had a parasite clearance half-life of <5 hours, suggesting that this mutation might not be associated with artemisinin resistance [6]. With its much larger sample size of infections with this mutation, our study clearly implicates F446I as a resistance marker. Another study that did not assess treatment outcomes found F446I to be common in northeastern Myanmar [32].

C580Y, which is the most common mutation in other Cambodian sites, was only observed in 2 samples in our study. Two other resistance-associated mutations common in Cambodia, R539T and Y493H, were not observed. Recently, we have shown that K13 mutations have emerged independently in multiple locations in Southeast Asia, including in Myanmar [12]. The independent emergence of K13 mutations in multiple geographic locations suggests the efforts to eliminate artemisinin-resistant malaria in 1 region may have a limited impact on the emergence of resistance in neighboring countries.

Our study had some limitations. Parasite clearance half-life is typically calculated based on the measuring parasitemia every 6 hours. Our study used the parasitemia every 12 hours, which may have inflated parasite clearance times. In addition, most of our results are based on treatment with dihydroartemisinin-piperaquine, and the use of ACTs as opposed to artesunate monotherapy could underestimate artemisinin resistance.

In conclusion, *P. falciparum* infections in southern China displayed delayed parasite clearance that was associated with a predominant K13 mutation different from those that predominate elsewhere in the region. Our study provides new evidence that artemisinin resistance has emerged and is firmly established in southern China, and supports the idea that different artemisinin resistance mutations arise and predominate, even within the same geographical region. The scaling up of mapping K13 in Southeast Asia in conjunction with therapeutic efficacy studies will guide efforts to eliminate artemisinin resistant malaria in this region. This surveillance should account for the possibility of region-specific, resistance-associated K13 mutations.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

## Notes

***Acknowledgments.*** We thank all the participants involved in the clinical study for their contribution work for the study, and local staff from Yingjiang Center for Disease Control and Prevention (CDC), Tengchong CDC, and Menglian CDC. We thank Amy Mu, Biraj Shrestha, and Gillian Mbambo for technical support, and Nicole Eddington Johnson for administrative support.

***Financial support.*** This work was supported by the World Health Organization (WHO) Mekong Malaria Programme (WP/08/MVP/000512 and WP/10/MVP/005837), the National S&T Major Program (grant 2012ZX10004220), and the Overseas Public Health Training Programme of the Shanghai Health Bureau (grant GWHW201210). The genotyping for this study was funded by the Howard Hughes Medical Institute, the WHO Global Malaria Programme, and a grant from the Bill and Melinda Gates Foundation to the WorldWide Antimalarial Resistance Network. S. T.-H.

and C. G. J. are supported by a grant (R01AI101713) from the National Institute of Allergy and Infectious Diseases at the National Institutes of Health.

***Potential conflicts of interest.*** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

## References

1. Dondorp AM, Nosten F, Yi P, et al. Artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med **2009**; 361:455–67.
2. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM. Evidence of artemisinin-resistant malaria in western Cambodia. N Engl J Med **2008**; 359:2619–20.
3. Huang F, Tang L, Yang H, Zhou S, Sun X, Liu H. Therapeutic efficacy of artesunate in the treatment of uncomplicated *Plasmodium falciparum* malaria and anti-malarial, drug-resistance marker polymorphisms in populations near the China-Myanmar border. Malar J **2012**; 11:278.
4. Phyo AP, Nkhoma S, Stepniewska K, et al. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. Lancet **2012**; 379:1960–6.
5. Amaratunga C, Sreng S, Suon S, et al. Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: a parasite clearance rate study. Lancet Infect Dis **2012**; 12:851–8.
6. Ashley EA, Dhorda M, Fairhurst RM, et al. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med **2014**; 371:411–23.
7. Yeung S, Socheat D, Moorthy VS, Mills AJ. Artemisinin resistance on the Thai-Cambodian border. Lancet **2009**; 374:1418–9.
8. Kyaw MP, Nyunt MH, Chit K, et al. Reduced susceptibility of *Plasmodium falciparum* to artesunate in southern Myanmar. PLOS One **2013**; 8:e57689.
9. Takala-Harrison S, Clark TG, Jacob CG, et al. Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in Southeast Asia. Proc Natl Acad Sci USA **2013**; 110:240–5.
10. Cheeseman IH, Miller BA, Nair S, et al. A major genome region underlying artemisinin resistance in malaria. Science **2012**; 336:79–82.
11. Ariey F, Witkowski B, Amaratunga C, et al. A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. Nature **2014**; 505:50–5.
12. Takala-Harrison S, Jacob CG, Arze C, et al. Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. J Infect Dis **2015**; 211:670–9.
13. Thriemer K, Hong NV, Rosanas-Urgell A, et al. Delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in Central Vietnam. Antimicrob Agents Chemother **2014**; 58:7049–55.
14. Taylor SM, Parobek CM, DeConti DK, et al. Absence of putative *Plasmodium falciparum* artemisinin resistance mutations in sub–Saharan Africa: a molecular epidemiologic study. J Infect Dis **2015**; 211:680–8.
15. Ghansah A, Amenga-Etego L, Amambua-Ngwa A, et al. Monitoring parasite diversity for malaria elimination in sub–Saharan Africa. Science **2014**; 345:1297–8.
16. World Health Organization (WHO). Methods for the surveillance of antimalarial drug efficacy. Geneva, **2009**.
17. World Health Organization (WHO). Malaria microscopy quality assurance manual—Version 1. Geneva, **2009**.
18. Snounou G, Beck HP. The use of PCR genotyping in the assessment of recrudescence or reinfection after antimalarial drug treatment. Parasitol Today **1998**; 14:462–7.
19. Flegg JA, Guerin PJ, White NJ, Stepniewska K. Standardizing the measurement of parasite clearance in falciparum malaria: the parasite clearance estimator. Malar J **2011**; 10:339.
20. Ferreira PE, Culleton R, Gil JP, Meshnick SR. Artemisinin resistance in *Plasmodium falciparum*: what is it really? Trends Parasitol **2013**; 29:318–20.
21. Cara Smith Gueye GNJHA. Emergency response to artemisinin in the greater Mekong subregion. **2014**.
22. Wongsrichanalai C, Sibley CH. Fighting drug-resistant *Plasmodium falciparum*: the challenge of artemisinin resistance. Clin Microbiol Infect **2013**; 19:908–16.
23. Witkowski B, Amaratunga C, Khim N, et al. Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. Lancet Infect Dis **2013**; 13:1043–9.
24. Huang F, Tang L, Yang H, et al. Molecular epidemiology of drug resistance markers of *Plasmodium falciparum* in Yunnan Province, China. Malar J **2012**; 11:243.
25. White NJ. Malaria: a molecular marker of artemisinin resistance. Lancet **2014**; 383:1439–40.
26. Satimai W, Sudathip P, Vijaykadga S, et al. Artemisinin resistance containment project in Thailand. II: responses to mefloquine-artesunate combination therapy among falciparum malaria patients in provinces bordering Cambodia. Malar J **2012**; 11:300.
27. Pradines B, Bertaux L, Pomares C, Delaunay P, Marty P. Reduced in vitro susceptibility to artemisinin derivatives associated with multiresistance in a traveller returning from South-East Asia. Malar J **2011**; 10:268.
28. Wang RB, Zhang J, Zhang QF. Malaria baseline survey in four special regions of northern Myanmar near China: a cross-sectional study. Malar J **2014**; 13:302.
29. Smith Gueye C, Newby G, Hwang J, et al. The challenge of artemisinin resistance can only be met by eliminating *Plasmodium falciparum* malaria across the Greater Mekong subregion. Malar J **2014**; 13:286.
30. Roper C, Alifrangis M, Ariey F, et al. Molecular surveillance for artemisinin resistance in Africa. Lancet Infect Dis **2014**; 14:668–70.
31. Conrad MD, Bigira V, Kapisi J, et al. Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in *Plasmodium falciparum* isolated from Ugandan children. PLOS One **2014**; 9:e105690.
32. Tun KM, Imwong M, Lwin KM, et al. Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: a cross-sectional survey of the K13 molecular marker. Lancet Infect Dis **2015**; 15:415–21.