Molecular Assessment of Artemisinin Resistance Markers, Polymorphisms in the K13 Propeller, and a Multidrug-Resistance Gene in the Eastern and Western Border Areas of Myanmar

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Background. As K13 propeller mutations have been recently reported to serve as molecular markers, assessment of K13 propeller polymorphisms in multidrug-resistant gene in isolates from Myanmar, especially the eastern and western border areas, is crucial if we are to understand the spread of artemisinin resistance.

Methods. A 3-day surveillance study was conducted in the eastern and western border areas in Myanmar, and K13 propeller and Plasmodium falciparum multidrug resistance-associated protein 1 (pfmrp1) mutations were analyzed.

Results. Among the 1761 suspected malaria cases screened, a total of 42 uncomplicated falciparum cases from the eastern border and 49 from the western border were subjected to 3 days of surveillance after artemether-lumefantrine treatment. No parasitic case showing positivity on day 3 was noted from the western border, but 26.2% (11/42) of cases were positive in the eastern border. Although we found no marked difference in the prevalence of the pfmrp1 mutation in the eastern and western borders (36% vs 31%, respectively), K13 mutations were more frequent in the eastern border area (where the 3-day persistent cases were detected; 48% vs 14%). C580Y, M476I, A481V, N458Y, R539T, and R516Y accounted for 68.9% of all K13 mutations significantly associated with day 3 parasitaemia.

Conclusions. The K13 mutations were significantly associated with day 3 parasitaemia, emphasizing the importance of K13 surveillance. The low prevalence of K13 mutations and the absence of day 3 parasitaemic cases indicate that artemisinin resistance may not have spread to the western Myanmar border region. Although analysis of multiple K13 mutations is challenging, it should be done at various sentinel sites in Myanmar.

Keywords. Plasmodium falciparum; K13 propeller; pfmrp1; artemisinin resistance; Myanmar.

Artemisinin-based combination therapy (ACT) is recognized as the most effective pharmacological treatment of Plasmodium falciparum infection [1, 2]. However, an alarming global report of reduced susceptibility of P. falciparum to artemisinin at the Thai–Cambodian border in 2007 [3] and southern Myanmar in 2011 [4], has threatened the hope for malaria elimination [2]. The emergence of the artemisinin resistance in the Greater Mekong Subregion is of great concern because resistance to other antimalarial drugs was first detected in this region [5].

It is accepted that artemisinin resistance involves genetic contributions and possible candidate markers have been reported, which include P. falciparum...
multidrug resistance protein 1 (pfmdr1) [6, 7], P. falciparum chloroquine resistance transporter (pfcrt) [8], sarcoplasmic/endoplasmic reticulum calcium ATPase 6 (ATPase6) [9], and P. falciparum multidrug resistance-associated protein 1 (pfmrp1) [5]. However, their role in surveillance of artemisinin resistance was found to be controversial [9–12]. No associated change for artemisinin resistance was detected in the coding sequences of the pfmdr1, pfcrt, and pfatp6 genes [13]. However, the pfmrp1 gene was found to be associated with both in vitro and in vivo reduced susceptibility to many antimalarial drugs, including artemisinin and lumefantrine [14–16], indicating a potential molecular marker for the artemether-lumefantrine combination.

Recently, a K13 propeller polymorphism has been found to be a useful molecular marker for large-scale surveillance efforts [17]. This K13 propeller polymorphism is associated with day 3 persistence of the parasites in Cambodia [18], and spread of artemisinin-resistant falciparum malaria and of the K13 propeller mutation in Southeast Asia was recently reported [19]. Rapid initial parasite clearance is a hallmark of artemisinin treatment, and parasitemia should be undetectable by 72 hours after artemisinin combination treatment [20].

In Myanmar, ACT has been deployed for treatment of malaria since 2002, and artemether-lumefantrine, artesunate-mefloquine, and dihydroartemisinin-piperaquine combinations have been recommended for uncomplicated falciparum malaria. Despite ACT deployment and insecticide-treated bed net distribution, parasite positivity on day 3 was detected in >10% of isolates from the Myanmar–Thai border during 2009 [2]. Because day 3 positivity serves as an early warning signal to monitor treatment failure by artemisinin derivatives [17], a day 3 surveillance study was implemented to observe whether new foci of artemisinin resistance had arisen [21]. In this study, we evaluated the polymorphism of K13 propeller and pfmrp1 genes in isolates from eastern and western border areas of Myanmar, which is crucial to understand the spread of artemisinin resistance.

METHODS

Study Population

In June–December 2013, a day 3 surveillance study was carried out in Kyitedon Township, in Kayin State in the southeastern part of Myanmar close to the Myanmar–Thai border (16°0’N and 98°23’E), and in Paletwa Township, in Chin State in the northwestern part of Myanmar close to the Myanmar–Bangladeshi border (21°18’N and 92°51’E) to explore the spread and magnitude of artemisinin resistance in these areas. Paletwa Township in Chin State and Kyitedon Township in Kayin State were purposely selected because these townships are cross-border areas where many mobile and migrant workers lived and it has the highest prevalence of malaria in these study areas (Figure 1). In this study, a total of 1761 febrile cases were initially screened using a malaria rapid diagnostic test (SDFK80, Standard Diagnostic Inc, Gyeonggi-do, Korea) and confirmed by a 3% Giemsa-stained peripheral blood smear examination using microscopy. Inclusion and exclusion criteria were based on the World Health Organization (WHO) standardized protocol for therapeutic efficacy study [22]. Inclusion criteria included minimum age 2 years, monoinfection with P. falciparum, axillary temperature >37.5°C or history of fever in the last 48 hours, ability to tolerate oral ingestion, provision of written informed consent, and no history of taking any antimalarial within the previous 14 days. Exclusion criteria included severe malaria defined by WHO [22], mixed infection with other Plasmodium species, pregnancy, lactation, malnutrition, other chronic infection, and known hypersensitivity to any artemisinin compound. All of the cases with mixed infection at admission or patients not meeting inclusion criteria were treated in accordance with the national antimalarial treatment guideline and excluded from further analysis (Figure 2). Written informed consent was obtained from all participants. Ethics approval to conduct this study was obtained from the institutional ethical review committee of the Department of Medical Research (Lower Myanmar), Myanmar.

Study Procedures

All of the recruited patients were treated with Artesan, which contains artesunate and lumefantrine (Ajanta House, Mumbai, India; lot number P0852C; expiration date February 2014) under direct observation twice daily for a 3-day course. The dose was calculated according to the body weight of the participants. Microscopic examination of peripheral blood smears was conducted on day 0 and day 3 after treatment. Only the patients with P. falciparum monoinfection were included for further analysis. Dried blood spots on filter papers (Whatman 3MM, Sigma-Aldrich, St Louis, Missouri) were collected on day 0 and day 3. Filter papers were air-dried and stored in individual, clean, sealed plastic bags with a desiccant until analysis.

Sample Size Determination

The proportion of day 3 persistence of parasitemia was assumed at 10%, and 95% confidence intervals with precision 10%; the sample size was calculated by classical statistical method for estimating the population proportion. At least 35 cases in each sites were needed to be included in this study.

Laboratory Procedures

Parasite density was determined by microscopic examination of blood smears and was calculated according to the WHO recommendation [22]. In brief, parasites were counted against the white blood cells (WBCs) in the thick film of Giemsa-stained blood smears. Parasite density was calculated assuming 6000
WBCs/µL of blood. If the parasite count was <10 in the first 200 WBCs, counting continued up to 500 WBCs. The blood smear was checked by 2 independent WHO-certified microscopists to validate the result.

For all study samples, parasite DNA was extracted from filter paper using a QIAamp Blood Mini Kit (Qiagen, Valencia, California) and kept at −20°C until use. The *P. falciparum* infection and day 3 persistence of the parasitemia were confirmed by nested polymerase chain reaction (PCR) amplification of the small subunit ribosomal nucleic acid (18S rRNA) genes as described previously [23].

**Amplification and Sequencing of pfmrp1 and K13 Propeller Genes**

The fragment of *pfmrp1* and K13 propeller gene were amplified by PCR with respective oligonucleotide primers as previously described [18]. For *pfmrp1*, the primers we used were forward: 5’-TGTAACATCTATAGTAATGCAATTGCTGG-3’ and reverse: 5’- TTGTGTGTATTACATTTAATTTTTC-3’.

For K13 putative gene, forward: 5’-CGGAGTGAACAAA TCTGGGA-3’ and reverse: 5’-GGGAATCTGGTGGTTAACA GC-3’ for nested-1, and forward: 5’-GCGAAGCTGCCATT CATTG-3’ and reverse: 5’-GGCTTGTGGAAGAGAGCA A3’ for nested-2, were used. Amplification was performed using an Accupower premix (Bioneer, Daejon, Korea) in a final volume of 20 µL, which included 250 nM primers, 0.25 mM of each deoxyribonucleoside triphosphate, 10 mM Tris-hydrochloric acid (pH 9.0), 30 mM magnesium chloride, 1.0 units of Taq polymerase, 250 nM of each primer, and 2 µL of genomic DNA template. Reaction conditions for *pfmrp1* amplification consisted of an initial denaturing at 94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute, and a final extension step of 7 minutes at 72°C. For nested-1 of the K13 propeller gene amplification, initial denaturation at 95°C for 5 minutes was followed by 30 cycles of 95°C for 30 seconds, 59°C for 30 seconds, 59°C for 2 minutes, 72°C for 2 minutes, and a final extension of 72°C for 10 minutes. Using 1 µL of the

Figure 1. Map of the areas surveyed in Kayin State (eastern border area) and Chin State (western border area), Myanmar.
nested-1 product as a template, the same conditions were applied for the nested-2 PCR except for an annealing temperature of 60°C and 35 cycles. After 1% agarose gel electrophoresis, PCR products were visualized by staining with diluted ethidium bromide solution. PCR products were purified using a MEGA quick-spin Total Fragment DNA Puriﬁcation Kit (iNtRON Biotechnology, Sangdaewon, Korea) and sequenced both directions with respective forward and reverse primers by a commercial sequencing company (Genotech, Daejeon, Korea).

**Sequence Analysis and Statistics**

All of the nucleotide and amino acid sequences were compared with the reference sequence of pfmrp1 (Gene ID: PF3D7_0112200) and K13 propeller protein (Gene ID: PF3D7_1343700) from www.plasmodb.org and aligned by using software in the Lasergene Genomic Suite (MegAlign, version 7.1, DNASTar, Madison, Wisconsin). All statistical analyses were performed using SPSS software (version 22.0, IBM SPSS Statistics, Armonk, New York). Frequencies of mutations and haplotypes among the groups were compared using $\chi^2$ and Fisher exact tests with a 2-sided confidence interval at the 95% confidence level.

**RESULTS**

**Demographic Characteristics of the Participants**

In this study, we screened a total of 940 patients from 56 villages in Kayin State (eastern border) and 821 patients from 30 villages in Chin State (western border). Among them, 42 patients (46.2%) in Kayin State (eastern border) and 49 (53.8%) in Chin State (western border) were successfully monitored up to day 3, and the sequences for both pfmrp1 and K13 propeller putative genes were amplified. Among them, 11 cases from Kayin State (eastern border) were parasite positive on day 3, whereas there were no positive cases on day 3 in cases from Chin State (western border) (Table 1). There was no significantly different day 0 parasite counts between the 2 study sites ($P = .198$). Similarly, there was no significantly different between the day 0 parasite count vs K13 propeller mutation ($P = .965$) or pfmrp1 polymorphism ($P = .304$).

**pfmrp1 Gene Analysis**

Eleven pfmrp1 single-nucleotide polymorphisms (SNPs) were detected in 32 isolates of which 18 (56.3%) were from Kayin State (eastern border) and 14 (43.7%) were from Chin State (western border). Among the SNPs, 4 of the 11 were synonymous mutations, and F1390I (10 isolates), E1484G (9 isolates), and I1444T (6 isolates) were major nonsynonymous SNPs (GenBank accession numbers KM108341–KM108347). Among the F1390I isolates, only 1 was parasite positive on day 3 and this...
SNP was found not to be significantly associated with the day 3 parasite-positive state \((P = .659)\). Of the F1390I mutations, 6 of 11 isolates were from Kayin State (eastern border) and 4 of the 11 were from Chin State (western border), so this mutation was widely distributed in different regions (Figure 3). Another SNP in pfmrp1, E1484G, was detected in 9 isolates from Kayin State (eastern border), of which 3 isolates were from patients with day 3 persistence of parasitemia, but the association was not significant \((P = .675)\). We also detected 6 isolates of I1444T, 2 isolates each of R1449T and I1464L, and 1 isolate each of L1374S and V1376L from Chin State (western border) (Table 2). However, no mutation in pfmrp1 was found to be associated with day 3 parasite positivity \((P = .950)\) (Table 3).

**K13 Propeller Gene Analysis**

In this study, synonymous and 16 nonsynonymous mutations of the K13 propeller domain were detected in 29 isolates, of which 22 (75.9%) were from Kayin State (eastern border) and 7 (24.1%) were from Chin State (western border) (Figure 3) (GenBank accession numbers KM192267–KM192281). Among them, 6 SNPs—namely, C580Y, M476I, A481V, N458Y, R539T, and R516Y—account for 68.9% of all K13 SNPs. The K13 propeller mutation was significantly associated with the day 3 persistence of parasitemia in vivo in patients from Kayin State (eastern border) \((\text{odds ratio} = 6.23; \ P = .035)\). Six isolates of C580Y, and 5 of M476I were found in this study, and interestingly, all of the most prevalent SNPs (ie, C580Y, M476I, A481V, N458Y, and R539T) were detected only in isolates from Kayin State (eastern border) where day 3 persistence of parasitemia was high. However, no single SNP was found to be associated with the day 3 persistence of parasitemia in this study.

**DISCUSSION**

The emergence and spread of artemisinin resistance is the most important obstacle for malaria control and prevention [5]. Despite efforts to contain the artemisinin-resistant parasites in the regions where resistance has been documented [24], it is difficult to measure the magnitude and spread of the artemisinin-resistant parasites in certain regions. The methods available for assessing efficacy and resistance to antimalarials are the study of therapeutic efficacy in vivo human studies, tests of in vitro susceptibility [25], and molecular markers [26]. Although WHO currently recognized the vivo human study, at least 28 days of follow-up is the most reliable and practical methods [2, 22], and there are limitations to conducting huge number of cases, especially in the areas where many migrant and mobile populations stayed. Alternatively, day 3 surveillance studies have been conducted effectively even by community health workers because of the short follow-up [27]. However, day 3 surveillance studies cannot replace the standard 28- to 42-day follow-up studies [5]. Molecular marker analysis can be applied to different geographical areas handling the huge number of samples, but it needs to be confirmed by vivo human studies. This study filled this gap by a new approach, 3-day surveillance in falciparum malaria patients and further molecular analysis.

Among the molecular markers of drug resistance, pfmrp1 is a transporter gene belonging to the ABC superfamily [28] and is assumed to be a candidate marker for potential artemisinin resistance [15]. This gene may have a role in the response to a variety of antimalarials because its knockout results in increased susceptibility to a variety of antimalarials [29]. One of the SNPs found in pfmrp1, F1390I, is significantly associated with the half-maximal inhibitory concentration for artemisinin, mefloquine, and chloroquine [15]. However, this mutation has been widely reported in different regions including west Asia and Africa, where there are no known cases of artemisinin resistance [16]. In this study, pfmrp1 mutations were detected not only in isolates from Kayin State (eastern border) where high prevalence of day 3 persistence of parasitemia exists, but also in Chin State (western border) where there is...
no day 3 persistence after treatment. Therefore, it may not be a suitable candidate for molecular surveillance of artemisinin-resistant falciparum malaria.

Recently, mutations in a putative kelch protein (K13) situated within chromosome 13 was found to be strongly associated with delayed clearance of falciparum malaria parasites [18]. The K13 propeller gene is an exon that codes for the kelch propeller protein [30]. K13 propeller mutations were found to be highly and constantly associated with day 3 parasitemia, indicating that it is an useful molecular tool for surveillance of artemisinin resistance [18]. However, all of the K13 propeller mutant isolates were not day 3 persistent cases and 2 of the 11 day 3 parasite-positive cases were wild type in this study. Therefore, the K13 propeller mutation should be interpreted with caution, although significant association was observed between the K13 propeller mutation and day 3 persistence of parasitemia. In this study, among the K13 SNPs, C580Y was the most prevalent and readily detected mutations in areas of Cambodia where artemisinin resistance was found. A previous study found that the parasite clearance half-life in patients with the wild-type K13 propeller was shorter than those with SNPs such as C580Y, R539T, or Y493H [18]. In this study, although Y493H was not detected, M476I appeared as the second most common SNP in isolates from eastern Myanmar border.

### Table 2. Single-Nucleotide Polymorphisms in K13 Propeller and pfmrp1

<table>
<thead>
<tr>
<th>Target Genes</th>
<th>Codon Position</th>
<th>Reference Sequence</th>
<th>Mutant Type Sequence</th>
<th>No. of Isolates/Total No. (%)</th>
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<td></td>
<td>Amino Acid</td>
<td>Nucleotide</td>
<td>Amino Acid</td>
<td>Nucleotide</td>
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<tr>
<td><strong>K13</strong></td>
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<td></td>
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<tr>
<td>580</td>
<td>C</td>
<td>TGT</td>
<td>Y</td>
<td>TAT</td>
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<tr>
<td>476</td>
<td>M</td>
<td>ATG</td>
<td>I</td>
<td>ATA</td>
</tr>
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<td>GCT</td>
<td>V</td>
<td>GTT</td>
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<td>I</td>
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<td>441</td>
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<td><strong>pfmrp1</strong></td>
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<td>V</td>
<td>GTT</td>
<td>L</td>
<td>CTA</td>
</tr>
</tbody>
</table>

The changes of the nucleotide from reference sequence to mutant one were shown as bold face letters.

**a** Three of 19 single-nucleotide polymorphisms (SNPs) were synonymous mutation.

**b** Four of 11 SNPs were synonymous mutation.

### Table 3. Association of Mutation on K13 Propeller and pfmrp1 With Day 3 Parasite Positivity After Treatment With Artemisinin-Based Combination Therapy

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Day 3 Positive</th>
<th>Day 3 Negative</th>
<th>Odds Ratio (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K13</td>
<td>81.8 (9/11)</td>
<td>41.9 (13/31)</td>
<td>6.23 (1.15–33.77)</td>
<td>.034</td>
</tr>
<tr>
<td>pfmrp1</td>
<td>27.3 (3/11)</td>
<td>19.4 (6/31)</td>
<td>1.04 (1.24–4.35)</td>
<td>.950</td>
</tr>
</tbody>
</table>

Data are presented as % (No. of mutant cases/total No. of samples) unless otherwise indicated.

Abbreviation: CI, confidence interval.
areas. Interestingly, this M476I mutation was not detected in clinical isolates from Cambodia, where it was found only in vitro; the drug-pressure strain, F32-ART5, and this mutation are situated between the first and second blades of the propeller domain [18]. Further phylogenetic or haplotype network tree analysis is needed to trace the origin of the K13 propeller mutations and to determine whether the artemisinin resistance was spread from Cambodia or whether it emerged independently.

In summary, pfmrp1 mutation was not associated with the delayed parasite clearance in this day 3 surveillance study, whereas K13 propeller mutations were found to be significantly associated with the day 3 persistence of parasitemia after treatment with artemether-lumefantrine. Although multiple mutations in the putative K13 propeller domain cause challenges for artemisinin resistance surveillance, C580Y and M476I were the most prevalent in eastern border areas. The high prevalence of the day 3 parasite positivity and of K13 propeller mutations in isolates from Kayin State near the Myanmar–Thai border emphasizes the importance of containment measures in this region. The low prevalence of K13 propeller mutations and lack of in vivo cases of day 3 parasite positivity in the Chin State (western border) suggest that artemisinin-resistant parasites may not have spread to the western border of Myanmar. To confirm the spread of artemisinin resistance, molecular surveillance for the K13 propeller putative polymorphism in different sentinel sites in Myanmar is recommended.

Notes

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

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References