ANALYSIS OF MEfloQUINE RESISTANCE AND AMPLIFICATION OF pfmdr1 IN MULTIDRUG-RESISTANT PLASMODIUM FALCIPARUM ISOLATES FROM THAILAND

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Abstract. Resistance to quinoline-containing compound has been associated with the Plasmodium falciparum multidrug resistance 1 (pfmdr1) gene. We analyzed wild P. falciparum isolates with high levels of chloroquine and mefloquine resistance for their macrorestriction maps of chromosome 5 and sequence of pfmdr1. Two types of chromosome 5 amplification were found. Eleven of 62 resistant isolates displayed Bgl I fragments larger than 100 kb. Twenty-nine isolates possessed multiple copies of the fragments. We failed to detect any amplification of this region on chromosome 5 in 22 mefloquine-resistant isolates, suggesting that other mechanisms can mediate the mefloquine-resistant phenotype. There was no direct association between pfmdr1 mutations and chloroquine sensitivity. Resistant lines could have Asn-86 and Tyr-184 or Phe-184, the predicted sequence of those chloroquine-sensitive isolates. No mutation at Asn-1042 and Asp-1246 was detected among these chloroquine-resistant isolates. Therefore, a few base substitutions in the pfmdr1 gene may not be sufficient to account for all chloroquine-resistant phenotypes.

In Thailand, malaria continues to be a major public health problem due to the emergence of multidrug-resistant organisms. Increasing levels and prevalence of Plasmodium falciparum resistance to chloroquine and mefloquine 

function, have been reported. This has made the treatment and prophylaxis of malaria increasingly complicated. Although new drugs and several drug combinations are being introduced, it is important to elucidate the molecular mechanism of quinoline drug resistance to develop the appropriate strategies for malaria therapy.

A P-glycoprotein homolog (Pgh 1) has been implicated in chloroquine- and mefloquine-resistant phenotypes of P. falciparum. It is encoded by the pfmdr1 multidrug resistance 1 (pfmdr1) gene and is localized on the membrane of the digestive vacuole of this parasite. High levels of Pgh 1 expression have been shown in drug-resistant cell lines to confer a chloroquine-sensitive phenotype and in yeast to complement the ste 6 function, suggesting a role as a drug transporter. Controversy exists regarding the pfmdr1 gene and chloroquine resistance from the work of Wellens and others in that there is no linkage between pfmdr1 and such resistance. In addition, in vitro selection of parasites for increased chloroquine resistance resulted in parasites with higher sensitivity to mefloquine. Suprisingly, chromosome 5 of these parasite clones demonstrated deamplification and the expression of Pgh 1 was also decreased.

In subsequent drug selection studies, the higher levels of mefloquine resistance have been associated with amplification of pfmdr1. Data from several field isolates with mefloquine resistance suggested that the amplification of pfmdr1 and an increase in mRNA expression is associated with natural resistance. Furthermore, cross-resistance to other related drugs such as halofantrine and quinine was also observed.

In this study, we demonstrated that there was no absolute correlation of amplification of the region of the genome surrounding pfmdr1 in P. falciparum isolates with mefloquine resistance from Thailand. Macrorestriction maps showed that there were 2 patterns of amplification. Some parasite isolates demonstrated an increase in number of a 100-kb Bgl I fragment, while the others demonstrated an increase in size of a Bgl I fragment. Our nucleotide sequence data also showed no association of intragenic mutation of pfmdr1 at the predicted site with chloroquine resistance.

MATERIALS AND METHODS

Parasites. Sixty-four fresh clinical isolates of P. falciparum used in this study were isolated from patients admitted to the Bangkok Hospital for Tropical Diseases between April 1994 and December 1995. The study was reviewed and approved by the Ethical Committee of Mahidol University (Bangkok, Thailand). Five milliliters of venous blood were collected after informed consent was obtained from all patients. The isolates were cultivated in vitro in RPMI medium-HEPES, 5.8% NaHCO3 with 10% human serum as described. The malaria parasites were not grown under drug pressure and were in short-term cultures. The FAC8/ItG2 and W2/Indochina clones were used as controls in the chromosome mapping and the drug sensitivity assays.

Drug sensitivity assay. The sensitivity assay was done using a modification of microdilution technique of Desjardins and others. Briefly, the assay was done in 96-well plates with 1% erythrocyte suspension at a parasitemia of 0.5–1%. Several dilutions of the drug being tested were done in duplicate for each assay. The parasites were incubated at 37°C in a candle jar for 18 hr. To assess parasite growth, 3H-hypoxanthine (1 μCi/well; Amersham, Buckinghamshire, United Kingdom) was added. The plates were frozen to terminate the incubation after 24 hr of additional incubation. After thawing, the contents were collected onto glass-fiber paper, washed with distilled water, and dried using a cell harvester (11025; Skatron, Lier, Norway). The incorporation of 3H-hypoxanthine was quantitated using a liquid scintillation counter.

The 50% inhibitory concentration (IC50) was defined as the concentration of an anti-malarial drug producing 50% inhibition of 3H-hypoxanthine uptake. This was estimated by non-linear regression analysis. Chloroquine sulfate, quinine hydrochloride, and amodiaquine were obtained from Sigma.
TABLE 1

Mean and range of in vitro drug sensitivity test (IC50) of Thai isolates of *Plasmodium falciparum*.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLQ</td>
<td>45.2</td>
<td>26–74</td>
</tr>
<tr>
<td>MFQ</td>
<td>11.8</td>
<td>9–24</td>
</tr>
<tr>
<td>HAL</td>
<td>2.6</td>
<td>1.6–4.2</td>
</tr>
<tr>
<td>QUIN</td>
<td>144</td>
<td>47–294</td>
</tr>
<tr>
<td>AMO</td>
<td>15.4</td>
<td>10.2–20</td>
</tr>
</tbody>
</table>

* A total of 64 isolates were obtained from patients admitted to the Bangkok Hospital for Tropical Diseases during malaria season of 1994 to 1995. IC50 = 50% inhibitory concentration; CLQ = chloroquine; MFQ = mefloquine; HAL = halofantrine; QUIN = quinine; AMO = amodiaquine.

**RESULTS**

In this study, we determined drug sensitivity of 64 *P. falciparum* isolates from Thailand to aminoquinoline drugs as described in the previous section. There were 2 chloroquine-sensitive lines obtained from a Laos patient and a patient who lived in a province near Thailand-Laos border admitted to the Bangkok Hospital for Tropical Diseases. These 2 isolates were used as chloroquine-sensitive controls. However, after the levels of mefloquine resistance were determined, these 2 isolates demonstrated low levels of resistance (9 and 11 ng/ml). The remaining 62 isolates were resistant to both drugs at different levels. In resistant lines, chloroquine resistance ranged from 26 to 74 ng/ml while the IC50 of mefloquine ranged from 9 to 24 ng/ml (Table 1). We also observed that *P. falciparum* isolates with high levels of chloroquine resistance rarely demonstrated high levels of mefloquine resistance.

To investigate whether there was any amplification of the region containing *pfmdr1* on chromosome 5 among mefloquine-resistant isolates, PFGE was performed on whole and digested chromosomes. An autoradiogram of *Bgl* I-digested chromosomes probed with *pfmdr1* demonstrated that several isolates have *pfmdr1* on 100-kb *Bgl* I fragments (Figure 1). However, approximately 17.7% (11 of 62) of the isolates analyzed displayed larger *Bgl* I fragments (Figure 1). The FAC8 control (Figure 1, lane A) and other Thai isolates (Figure 1, lanes B–F) had *pfmdr1* on 100-kb *Bgl* I fragments, whereas *Bgl* I fragments of SL 111, SL 114, and SL 118 had increased in size to 160, 180, and 180 kb, respectively. Twenty-nine of those isolates with *pfmdr1* on chromosome 5 had increased in size to 160, 180, and 180 kb, respectively.

**Pulsed-field gel electrophoresis (PFGE) and chromosome mapping.** Pulsed-field gel electrophoresis was performed in a CHEF DRIII apparatus (Bio-Rad, Hercules, CA). Running conditions were as described by Cowman and others and sizes were determined by comparison with *Saccharomyces cerevisiae* and bacteriophage concatamers (Promega, Madison, WI). The DNA probes were labeled with α-32P-dATP and hybridized as described.8

Quantification of *pfmdr1* copy number. Genomic DNA of Percoll gradient-purified trophozoites was digested with Eco RI and *Bam* HI and subjected to electrophoresis on a 1% agarose gel. The DNA fragments were transferred to a nylon membrane and hybridized with a radiolabeled *pfmdr1* probe.5 The copy number of *pfmdr1* was compared with that of the circumsporozoite gene, which occurs in a single copy in the genome.14

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Quantification of the copy number of *pfmdr1* was also performed to verify the results obtained by PFGE under the conditions used. Those isolates with larger *Bgl* I fragment or with multiple copies of the 100-kb *Bgl* I fragment were all mefloquine resistant. However, amplification of this region was not found in 2 mefloquine-resistant isolates (moderate to high levels of resistance) because they contained a
single copy of the 100-kb Bgl I fragment. No amplification was also found in the 2 control isolates that were chloroquine-sensitive and showed low-level resistance to mefloquine. The SL 123 and SL 127 (Figure 2, lanes D and E) isolates represent those with no amplification of chromosome 5 in this particular region.

We also attempted to determine the association of point mutation at 5 discrete positions in pfmdr1 by analyzing the sequence of 35 isolates. Nucleotide differences only at amino acid positions 86 and 184 were observed. These data are shown in Table 2.

Sensitive isolates displayed the same amino acids as the previously predicted chloroquine-sensitive phenotypes. Sixteen chloroquine-resistant lines had Asn-86 and either Tyr-84 (7 isolates) or Phe-184 (12 isolates). Thus, by the criteria used, these isolates would have been chloroquine sensitive. Forty-four of 33 chloroquine-resistant isolates had Tyr-86 and Phe-184, a characteristic of the previously reported resistant isolates.

### Discussion

It was previously demonstrated that *P. falciparum* clones that were subjected to mefloquine in vitro had amplified a pfmdr1 gene and expressed high levels of Pgh1. To evaluate this linkage in natural isolates, we performed chromosome analysis of field isolates from Thailand. Two types of chromosome 5 amplification were found. One was an increase in size of a Bgl I fragment similar to those of K1mef and K1mef5, the mefloquine-selected lines. The other was the same as those of the W2 series in that there were more than 1 amplicon defined by the Bgl I sites. More isolates displayed the latter pattern (29 of 62 isolates). These parasites were all mefloquine resistant and displayed high levels of pfmdr1 expression. In particular, isolates SL 114 and SL 118 showed increases in both size and number of amplicons. These results confirm the association of mefloquine resistance and amplification of chromosome 5 previously reported by Wilson and others. However, there were certain isolates (22 of 62) that did not demonstrate any amplification yet demonstrated mefloquine resistance. Based on our results, there must be other mechanisms that mediate mefloquine resistance. One might be an interplay between mechanisms of mefloquine and chloroquine resistance as suggested by Lim and others. In that study, selection for increased mefloquine resistance of *P. falciparum* FAC8 did not alter the copy number or the level of expression of pfmdr1. In addition, the level of chloroquine resistance of these selected parasites showed a small increase during selection. Thus, no inverse relationship involving pfmdr1 between mefloquine and chloroquine was shown in mefloquine-selected FAC8 lines as previously reported.

Several investigators have also suggested that resistance to mefloquine, halofantrine, and quinine are linked. We found that the IC50 of field isolates for halofantrine usually increased as the parasites become more resistant to mefloquine. No similar observation has been noted in the case of quinine resistance. It is also important to note that from our experience, the IC50 for quinine of Thai isolates did not correlate well with the efficacy of treatment. In this case, it is difficult to make any sensible conclusion on cross-resistance of mefloquine and quinine.

There was no direct association between pfmdr1 mutations and chloroquine sensitivity. Resistant lines could have Asn-86 and Tyr-184 or Phe-184. In addition, several isolates had Tyr-86 and Phe-184, a variation previously noted by Foote and others as a chloroquine-resistant isolate. The discordance between the prediction based on the allelic form of pfmdr1 and chloroquine resistance has been shown elsewhere. No mutation at Asn-1042 and Asp-1246 was detected among the Thai chloroquine-resistant isolates of *P. falciparum*, although earlier studies have reported this. Similarly, Cox-Singh and others found no mutations of this type in isolates from Malaysia. Thus, there is no simple association between mutation at positions 1042 and 1246 and chloroquine resistance.

In conclusion, this study of Thai isolates has shown that there are 2 types of chromosome 5 amplification at Bgl I sites in natural isolates with mefloquine and halofantrine resistance. Second, amplification and overexpression of pfmdr1 are not always necessary for mefloquine resistance, since some resistant isolates displayed no amplification. 

### Table 2

Sequence analysis of pfmdr1 from Thai isolates of *Plasmodium falciparum*

<table>
<thead>
<tr>
<th>Isolate/Amino acid residue</th>
<th>86</th>
<th>184</th>
<th>1034</th>
<th>1042</th>
<th>1246</th>
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</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>AAT</td>
<td>TAT/TTT</td>
<td>AGT</td>
<td>AAT</td>
<td>GAT</td>
</tr>
<tr>
<td>Resistant/Asia</td>
<td>TAT</td>
<td>TAT/TTT</td>
<td>AGT</td>
<td>AAT</td>
<td>GAT</td>
</tr>
<tr>
<td>CLQ/S (2)‡</td>
<td>AAT</td>
<td>TTT</td>
<td>AGT</td>
<td>AAT</td>
<td>GAT</td>
</tr>
<tr>
<td>CLQ/R (7)†</td>
<td>AAT</td>
<td>TAT</td>
<td>AGT</td>
<td>AAT</td>
<td>GAT</td>
</tr>
<tr>
<td>CLQ/R (12)‡</td>
<td>AAT</td>
<td>TTT</td>
<td>AGT</td>
<td>AAT</td>
<td>GAT</td>
</tr>
<tr>
<td>CLQ/R (14)†</td>
<td>TAT</td>
<td>TTT</td>
<td>AGT</td>
<td>AAT</td>
<td>GAT</td>
</tr>
</tbody>
</table>

* CLQ = chloroquine; S = sensitive; R = resistant.
† Number of isolates.
nally, Thai isolates do not corroborate the association between the alleles of pfmdr1 and chloroquine resistance.

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