Mutation in pfmdr1 gene in chloroquine-resistant Plasmodium falciparum isolates, Southeast Iran

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Summary
Objectives: The main objective of the present study was to detect point mutations at positions 86, 184, 1034, 1042, and 1246 of the Plasmodium falciparum multidrug resistance gene (pfmdr1) in blood samples collected from malaria patients in Chabahar, a harbor city located in Southeast Iran.

Methods: Twenty-six blood samples from patients infected with P. falciparum, who had a chloroquine (CQ) response failure, were collected pre-treatment. Following treatment with CQ, drug susceptibility was assessed using an in vivo test. Molecular detection of single nucleotide polymorphisms (SNPs) was carried out using the LightCycler hybridization probe assay.

Results: The pfmdr1 N86Y mutation was found in six isolates (23.1%). Mutations at the four other positions were not observed in any isolates.

Conclusion: The present study showed no mutation at codon positions 184, 1034, 1042, and 1246 of pfmdr1 in any of the Iranian P. falciparum isolates; thus these alleles cannot serve as markers for CQ resistance in Iran.

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Introduction
Malaria still remains one of the leading causes of morbidity and mortality in the world. Of the approximately 50 million deaths worldwide each year, about 2 million are attributed to malaria. Drug resistance is one of the major factors contributing to the resurgence of malaria, especially resistance to the most affordable drugs, such as chloroquine (CQ). Prior to the emergence of resistance, CQ was considered a very effective, safe, and inexpensive antimalarial drug. However, resistance to CQ developed in the early 1960s at two foci, one in Southeast Asia and the other in Latin America, and has spread to all areas where malaria is present. It has been estimated that mortality from falciparum malaria increases up to five-fold in areas where resistance to the antimalarial CQ is established. To reduce the mortality rate, understanding the mechanisms of such resistance and the development of new treatments, including new drugs, are urgently required.
Great progress has been made recently in studying the mechanisms of drug action and drug resistance in malaria parasites. These efforts are highlighted by the demonstration of mutations in multiple-drug resistant genes. The emergence and spread of multidrug-resistant *Plasmodium falciparum* has severely limited the therapeutic options for the treatment of malaria.

Mutations in two genes, *P. falciparum* chloroquine resistance transporter (*pfcrt*) and *P. falciparum* multidrug resistance (*pfmdr1*) of *P. falciparum* have been reported to be linked to CQ resistance. It has been proposed that point mutations in the *pfmdr1* gene producing amino acid changes at positions 86, 184, 1034, 1042, and 1246 are associated with CQ resistance. Results obtained from *pfmdr1* single nucleotide polymorphism (SNP) studies have indicated a significant role for the N86Y mutation in contributing to resistance to chloroquine. A point mutation (N86Y) resulting in an amino acid change from asparagine to tyrosine at position 86 in the *pfmdr1* gene has been reported to be associated with in vitro chloroquine resistance in isolates originating from various regions of Asia and Africa.

In Iran *P. falciparum* transmission mainly occurs in the southeast of the country. Chabahar is a harbor city located in Southeast Iran, where more than 85% of annual malaria cases occur. *P. falciparum* chloroquine resistance cases have been reported from Southeast Iran since the early 1980s. In a study carried out by Ursing et al. (2006), polymorphisms in the *pfmdr1* gene were detected by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) and sequencing methods. The main objective of the present study was to detect point mutations at positions 86, 184, 1034, 1042, and 1246 of the *P. falciparum* multidrug resistance 1 gene (*pfmdr1*) in *P. falciparum* isolates collected from malaria patients in Chabahar, in order to evaluate if *pfmdr1* point mutations could be considered as a useful tool in monitoring emergence of CQ and related drugs resistance.

**Materials and methods**

**Blood samples**

Twenty-six blood samples were taken from patients infected with *P. falciparum*, who had a CQ response failure, living in Chabahar, Southeast Iran from March to December 2004. Patients were selected from those referred to the City Public Health Center for malaria treatment. Sampling (5 ml blood) was done only from patients who gave consent to participate in the study. The mean age of patients was 35 years (range 12—60 years). The infection was assessed by microscopic examination of thick and thin smears before starting treatment. All the patients were treated with CQ (25 mg/kg over 3 days), the first-line treatment according to the Iranian Ministry of Health guidelines at the time of the study. CQ susceptibility was assessed using an in vivo test. A period of 28 days of in vivo testing was conducted according to the World Health Organization (WHO) protocol to assess the efficacy of standard supervised CQ therapy.

Discrimination between recrudescence and new infection was done based on the patient’s history during the 28-day follow-up period. The study received ethical clearance from the institutional ethical review board (Ethical Approval Reference Committee, Medical Sciences Faculty).

All the blood samples for PCR were taken before treatment. The samples were transferred to the Parasitology Laboratory of Tarbiat Modares University and stored at −20 °C until use for the molecular study.

**Plasmodium falciparum reference strains**

Three *P. falciparum* strains adapted to culture medium were selected as reference strains. 3D7 was the CQ-sensitive strain while W2 and 7G8 were selected as CQ-resistant strains. These strains were generously provided by F. de Monbrison from Claude Bernard University, Lyon, France. The dispatched reference isolates were spotted on Whatmann filter paper and air-dried. The DNA was isolated from the dried spots and blood samples using QIAamp DNA mini kit (Qiagen, Germany).

**Detection of pfmdr1 mutation**

Detection of mutations was carried out with LightCycler using hybridization probes according to the method described by de Monbrison et al. (2003). For *pfmdr1* mutations, hybridization probes consisted of two different oligonucleotides that bind to an internal sequence amplified by forward and reverse primers. The sensor probe, labeled at the 3’ end with fluorescein, is designed to the mutation sites. The anchor probe, labeled at the 5’ end with LightCycler Red 640 and phosphorylated at the 3’ end to prevent extension by Taq polymerase, is designed to the conserved sequences adjacent to the mutation sites. Both probes, localized on the same DNA strand, could hybridize in a head-to-tail arrangement, bringing the two fluorescent dyes into close proximity.

Primers and probes were designed according to the de Monbrison study, checked with the GenBank facility for accuracy, and then synthesized by TIB MOL BIOL (Berlin, Germany) to detect mutations of *pfmdr1* (GenBank Accession No. X56851).

**PCR amplification**

PCR master mix was prepared using the Roche LC kit (Roche Molecular Biochemicals, Germany). For *pfmdr1* codons 86 and 184, the PCR program was as follows: 9 min at 95 °C, 35 cycles of 95 °C for 10 s, 54 °C for 10 s, and 65 °C for 40 s; followed by a melting curve analysis from 35 °C to 75 °C at 0.2 °C/s. For *pfmdr1* codons 1034—1042 and 1246, the conditions for cycling were: 9 min at 95 °C, 40 cycles of 95 °C for 10 s, 54 °C for 10 s, and 65 °C for 55 s; followed by a melting curve analysis from 35 °C to 80 °C at 0.2 °C/s.

Reference isolates were included in each run as reference controls. For recovery of DNA fragments from agarose gel, a PCR purification kit (Fermentas, Lithuania) was used. Sequencing of both strands was done using the primers by MWG Company (Basel, Switzerland). Purified PCR products were cloned into a 2886 bp PT Z57R/T vector (Fermentas, Lithuania). The ligated product was transformed into *Escherichia coli* TG1 competent cells, and the plasmids from positive colonies were selected for sequencing.
Results

In the present study, a point mutation in the *pfmdr1* gene of *P. falciparum* isolates was successfully detected. As shown in Table 1, our isolates showed mutant nucleotide only at position 86. The 3D7 reference strain showed the wild *pfmdr1* genotype at all codon positions: 86, 184, 1034, 1042, and 1246. The W2 reference strain showed mutant nucleotide at position 86, while the 7G8 reference strain showed mutant genotype at codon positions 184, 1034, 1042, and 1246 as well as wild genotype at codon position 86.

The frequency of the *pfmdr1* mutation among the 26 human blood samples is shown in Table 2. The *pfmdr1* N86Y mutation was found in six isolates (23.1%). In the four other positions (184, 1034, 1042, and 1246) no mutations were observed.

Melting temperatures are reported as means ± standard deviation from four independent assays. M*: mutant in that position.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Codon positions</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pfmdr1</em></td>
<td>Wild (N86)</td>
<td>20 (76.9)</td>
</tr>
<tr>
<td></td>
<td>Mutant (Y86)</td>
<td>6 (23.1)</td>
</tr>
<tr>
<td></td>
<td>Wild 184, 1034,</td>
<td>26 (100)</td>
</tr>
<tr>
<td></td>
<td>1042, 1246</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutant 184, 1034,</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>1042, 1246</td>
<td></td>
</tr>
</tbody>
</table>

Melting curve analysis of isolates and reference strains for detection of the N86Y *pfmdr1* mutation is shown in Figure 1. According to the results, the isolates show A to T nucleotide exchange. These types of alleles showed a perfect match with sensor probe. Their Tm curves are similar to W2 and 7G8 strains.

GHA86-R1 15……380 of trace file. (GenBank accession no. **X56851**)

ATTTTTATTAATGACAAATAATAACCCCTAAAAGGAAGTGGCAATATGTAATAAATGTTTATAAATTTCTGATCCAAATCTCCTGAACTCACCCTGTCTTCAAATATAAAAATCTAATATCAGATTTACCATGAGATATATTATCATGAAAAATGTGCACTCGTAAAAAAACACTCTCTTAAATTTCAAGCTTTTTAAATTTTTTATTTTTGATGTAAATATTACACATATATAACTTTGATACATTGATAATATAATTGTACTAACAACCTTAGATATCTAAATGATATATAGGATATATATCATACCATCTAATATACATGTGTTTTTATATTACACAAACACAGATATAAAAAAGGTAATGTG

**Figure 1** DNA sequencing of *pfmdr1* gene with polymorphic codon at position 86.

**Table 1** Melting temperatures of different alleles of *pfmdr1* codons (N86Y, Y184F, S1034C, N1042D, D1246Y) in three reference strains and isolates

<table>
<thead>
<tr>
<th><em>pfmdr1</em> gene codons</th>
<th>Melting temperatures (°C) of alleles</th>
<th>3D7</th>
<th>W2</th>
<th>7G8</th>
<th>Wild-type</th>
<th>Mutant-type</th>
</tr>
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<tbody>
<tr>
<td>Codon 86</td>
<td>50.56 ± 0.4</td>
<td>54.7 ± 0.6 (M*)</td>
<td>50.65 ± 0.5</td>
<td>50.36 ± 0.11</td>
<td>54.15 ± 0.11 (M*)</td>
<td></td>
</tr>
<tr>
<td>Codon 184</td>
<td>53.57 ± 0.5</td>
<td>53.55 ± 0.58</td>
<td>57.90 ± 0.5 (M*)</td>
<td>52.48 ± 0.31</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Codon 1034–1042</td>
<td>57.13 ± 0.65</td>
<td>57.14 ± 0.75</td>
<td>64.08 ± 0.7 (M*)</td>
<td>56.79 ± 0.15</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Codon 1246</td>
<td>52.54 ± 0.22</td>
<td>52.57 ± 0.26</td>
<td>57.85 ± 0.5 (M*)</td>
<td>52.42 ± 0.06</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Melting temperatures are reported as means ± standard deviation from four independent assays. M*: mutant in that position.

**Table 2** Frequency of codon positions of Pfmdr1 mutation among 26 human blood isolates from Chabahar, Southeast Iran

<table>
<thead>
<tr>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Figure 2** Melting curve analysis of patient isolates and reference strains for detection of N86Y mutation in *pfmdr1* gene. Right curve from top to bottom (mutant): KhT, Gha, SaS, W2 (Ref. strain), ZaB, RbB, 44. Left curve from top to bottom (wild type): 7G8 (Ref. strain), RbB, HaD, SaR, ZaA, AbB, GhSA, LaF, 3D7 (Ref. strain).
pfmdr1 gene mutation in P. falciparum Iran

Discussion

In the past, malaria was highly endemic in most parts of Iran. In 1924, it was estimated that out of a population of 13 million, 4–5 million people had clinical malaria. An organized malaria control program was started before the Second World War, and in 1957 a strategy of malaria eradication was endorsed by the Government.10 Now, the Iranian Government belongs to the WHO Eastern Mediterranean Region (EMR) and sustains a strong malaria control program.

Although, there has been a decreasing trend in the malaria incidence in recent years, the southeastern corner of Iran, which consists of Sistan and Baluchistan Province, Hormozgan Province, and the tropical part of Kerman Province with a combined population of approximately 3 million, is considered to be a ‘refractory malaria region’. The annual parasite incidence (API) was reported to be 8.74 per 1000 population in 1997. In this part of the country, malaria belongs to the oriental type; hence it is difficult to control. Inherent problems are the drug resistance of P. falciparum and the vector resistance to insecticides, with the additional complication of the importation of malaria, mostly P. falciparum, from Afghanistan and, to a lesser extent, Pakistan.9

According to an Iranian Ministry of Health report, less than 15,000 malaria cases were detected in the malaria region in 2006; of these, 30% were not in Iranian people. In our study 19%, 80%, and 1% of malaria infected cases detected were due to P. falciparum, Plasmodium vivax, and mixed infection, respectively. Since the early 1980s, the major problem in the region has been considered to be CQ resistance.4–6

Mutations in the P. falciparum multidrug resistance gene (pfmdr1) of P. falciparum have been reported to be linked to chloroquine resistance. It has been indicated that P. falciparum can show mutation in its genome. For its survival it can delete certain genes (or a portion) that are not required for its growth and it has the capability to regulate its genes under various stages of its life cycle and under unfavorable environmental conditions. Similarly, under drug pressure it allows mutations to settle in the target genes.4 Epidemiological studies have shown that the frequency of chloroquine-resistant mutants varies among isolated parasite populations in most malarial endemic countries.

pfmdr1 point mutations depend on the parasite genetic background and can significantly affect parasite susceptibility to a wide range of antimalarials in a strain-specific manner. Some field studies support the linkage of this mutation with chloroquine resistance while others do not. Results obtained from some pfmdr1 SNP studies indicate a significant role for the N86Y mutation in contributing to resistance to chloroquine.10 P. falciparum isolates from Malaysia, Indonesia, Guinea-Bissau, Nigeria, and Sub-Saharan Africa have shown the N86Y mutation among the chloroquine-resistant. A high prevalence of mutations in pfmdr1 was observed in Cambodian isolates.11 In Thailand, pfmdr1 Y86 and 1034C mutants have been reported at a significant level.12 But studies from Uganda, Laos, Cameroon, South Africa, Brazil, and the Peruvian Amazon have reported that this mutation is not predictive of treatment outcome.13–19 Some studies have shown that the pfmdr1 Y86 mutation causes an increase in sensitivity to mefloquine and artemisinin.18–20 and this could be considered in drug treatment strategies in endemic areas.

In the present study, among 26 CQ-resistant isolates, six isolates showed N86Y mutations in the pfmdr1 gene. In a study carried out by Ursing et al. (2006) in 100 isolates from the same area, polymorphism at codon position 86 of the pfmdr1 gene was reported, while the 184F allele was observed in 92% of the analyzed infections and no variation was found in the SNPs of pfmdr1 at positions 1034, 1042, and 1246. They used PCR-RFLP and sequencing methods for their investigation.6

In conclusion, the present study showed no mutation in pfmdr1 at codon positions 184, 1034, 1042, and 1246 in any of the Iranian P. falciparum isolates, and thus these alleles cannot serve as markers for CQ resistance in Iran. In contrast, N86Y mutations may be considered as resistance markers for studying CQ resistance in endemic areas. Based on the present results, it is proposed that pfmdr1 alone is not sufficient to provide chloroquine resistance and that other gene mutations, such as in pfcrt are necessary for the determination of drug resistance.

Acknowledgment

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Conflict of interest: The authors have no conflicts of interest concerning the work reported in this paper.

References