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Mutation analysis in *pfmdr1* and *pfmrp1* as potential candidate genes for artemisinin resistance in *Plasmodium falciparum* clinical isolates 4 years after implementation of artemisinin combination therapy in Iran

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ABSTRACT

The emergence and spread of *Plasmodium falciparum* resistant to the commonly used anti-malarial drugs is a major challenge in the control and elimination of malaria. The present study provides information on genetic analysis in multidrug resistance 1 (*pfmdr1*) (N86Y/Y184F/S1034C/N1042D/F1226Y/D1246Y) and multidrug resistance protein 1 (*pfmrp1*) (H191Y/S437A/I876V/F1390I/K1466R) genes that are probably associated with artemisinin as well as chloroquine resistance transporter (*pfcrt*) 76T in *P. falciparum* clinical isolates ($N = 200$) exposed to artemisinin-based combination therapy (ACT) 4 years after its adoption in Iran. Also, the copy number of *pfmdr1* gene was screened for its association with *pfmdr1* mutations to incriminate artemisinin resistance. By using nested PCR-RFLP and sequencing analysis, none of the samples had any mutation at codons 1034, 1042, 1226 and 1246 of *pfmdr1*, while 86Y and 184F mutations were detected in 46% and 2% of the examined samples, respectively. Also, no significant difference was identified among analyzed samples collected before (baseline, 2002–2005) and after adoption of ACT (2007–2010) ($P > 0.05$). As with *pfmrp1* gene, the mutations at positions 191Y (76.5%), 437A (69.5%), 876V (64.5%) and 1390I (17%) were detected and no samples displayed mutation at codon 1466R. In total, 42.5% of the examined isolates carried both *pfmdr1* 86Y and *pfcrt* 76T and none of the parasites simultaneously harbored *pfcrt* 76T, *pfmdr1* 86Y, 184F and *pfmrp1* 191Y, 437A, 876V, 1390I mutations. In addition, the copy number of *pfmdr1* gene ($N = 1$) was similar as a sensitive isolate, 3D7, to artemisinin. In summary, none of the potential mutations associated with artemisinin and its derivatives resistance was significantly changed 4 years after adoption of ACT in Iran.

1. Introduction

Elimination of malaria has become one of the most important goals of World Health Organization (WHO) (Guerra et al., 2008); however, the emergence and spread of *Plasmodium falciparum* and *Plasmodium vivax* resistant to the commonly used anti-malarial drugs is a major challenge in the control and elimination of malaria (Olliaro, 2005; The malERA Consultative Group on Drugs, 2011). Because of the resistance of these parasites to different anti-malarial drugs such as chloroquine (CQ) and sulfadoxine–pyrimethamine (SP), combination therapies rather than monotherapies have been suggested to be highly powerful for efficient management of malaria (Halim et al., 2006). Therefore, artemisinin-based combination therapies (ACTs) have proven to be extremely effective for management of uncomplicated malaria as global efforts to control and eliminate malaria worldwide (Nosten and White, 2007; White, 2008; WHO, 2008, 2011).

Several reports of the slow clearance rate of *P. falciparum* parasites to artemisinin have been recently found in known foci in Cambodia and Thailand, and new suspected foci in Myanmar and Vietnam (Mugittu et al., 2006; Dondorp et al., 2009; WHO, 2011) that is disastrous for global control and elimination programs (Laufer et al., 2007; Dondorp et al., 2009). Therefore, due to the essential role of ACT in malaria control and elimination, regular monitoring of both artemisinin and its partner drugs provides essential data to the health authorities responsible for ensuring access to effective anti-malarial drugs. In the light of this fact, understanding the mechanisms of artemisinin drug resistance and providing information about molecular markers, which contribute to the artemisinin resistance and its derivatives before appearance of treatment failures, could be essential to accelerate elimination program nationwide (Afonso et al., 2006; Dahlström et al., 2009; Imwong et al., 2010).

The association of molecular markers and drug resistance in the field isolates has been controversial for some anti-malarial drugs. CQ resistance is associated with single nucleotide polymorphisms (SNPs) in *P. falciparum*-CQ resistance transporter (*pfcrt*) and

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-multidrug resistance 1 (*pfmdr1*) genes (Cooper et al., 2005; Duraisingh and Cowman, 2005; Picot et al., 2009) and SP resistance is associated with quintuple mutations in *pfdhfr* and *pfdhps* genes (Gregson and Plowe, 2005). Although the molecular basis of artemisinin resistance is not fully elucidated, several current molecular studies of *P. falciparum* isolates have proposed that mutations in several candidate genes could be associated with artemisinin resistance. Up to now, a few genes have been suggested to be associated with artemisinin resistance and these genes are *P. falciparum ATPase6* (*pfatpase6*) (Eckstein-Ludwig et al., 2003; Jambou et al., 2005; Uhlemann et al., 2005; Price et al., 2004), *pfmdr1* (Cowman and Karcz, 1993; Duraisingh et al., 2000; Sidhu et al., 2006; Lim et al., 2009) and *P. falciparum* multidrug resistance protein 1 (*pfmrp1*) (Klokouzas et al., 2004; Mu et al., 2003; Dahlström et al., 2009; Raj et al., 2009; Veiga et al., 2011).

pfmdr1 gene, located on chromosome 7, encodes an ATP-binding cassette (ABC) transporter resident on the digestive vacuole and has received the most attention. Several point mutations (at codons N86Y, Y184F, S1034C, N1042D and D1246Y) in *pfmdr1* from wild-type field isolates are associated with changed sensitivity to different anti-malarials drugs, such as CQ, mefloquine (MQ), quinine (QN), halofantrine (HF), and artemisinins (Foote et al., 1990; Duraisingh et al., 2000; Reed et al., 2000; Sidhu et al., 2005; Pickard et al., 2003). In a recent study of field isolates from the Thai–Myanmar border, novel polymorphism in this gene (*pfmdr1* F1226Y) was associated with reduced *in vitro* artemisinin susceptibility (Veiga et al., 2011). More importantly, *pfmdr1* amplification has been suggested to be a key determinant for both *in vivo* and *in vitro* resistance to MQ and HF (Wilson et al., 1993; Cowman et al., 1994; Price et al., 1999, 2004) and with increased risk of therapy failures to artesunate-MQ in Cambodia (Alker et al., 2007; Shah et al., 2008; Wongsrichanalai and Meshnick, 2008; Price et al., 2006; Lim et al., 2009). Moreover, *in vivo* selection of the *pfmdr1* 86N allele after artemether–lumefantrine (LUM) treatment has been reported in African isolates (Sisowath et al., 2005). Therefore, *pfmdr1* gene may play a role in reducing parasite susceptibility to ACT.

The *pfmrp1* gene is located on chromosome 1 and encodes PfMRP1, a member of the ABC transporter superfamily (Bouige et al., 2002). Little is known about the possible involvement of PfMRP1 in drug resistance. An *in vitro* study by Mu et al. (2003) suggested an association of the *pfmrp1* SNPs H191Y and S437A with CQ and QN drugs; however, this finding has not been confirmed by others yet. Besides, Raj et al. (2009) have recently showed that disruption of *pfmrp1* in CQ-resistant *P. falciparum* isolates made this parasite more sensitive to CQ, QN, piperaquine, primaquine and artemisinin. This issue may suggest the role of PfMRP1 in the sensitivity of the parasite to antimalarial drugs possibly by pumping drugs outside the parasite. Recent studies have suggested that mutations at codons I876V and F1390I in *PfMRP1* might be associated with *in vivo* and *in vitro* susceptibility to ACT response (Dahlström et al., 2009; Veiga et al., 2011).

In Iran due to CQ resistance, the national drug policy for malaria control recommended CQ–SP as the first-line treatment for uncomplicated *P. falciparum* cases in 2005. Subsequently, in 2007, CQ treatment regime for uncomplicated *P. falciparum* malaria was terminated because of inadequate efficacy of treatment with CQ–SP; therefore, SP–artesunate was introduced as the first-line anti-malarial treatment. As a result, with increase in the use of SP–artesunate in the country, *P. falciparum* isolates are increasingly being exposed to drug pressure of SP–artesunate and subsequently, it may cause the emergence of parasite resistance to both drugs due to the selection (Meshnick, 2002).

Therefore, in the present investigation, molecular analysis of the *pfmdr1* and *pfmrp1* genes as potential markers for ACT tolerance/resistance has been carried out in *P. falciparum* isolates 4 years after introduction of SP–artesunate in the first-line therapy in Iran. We also investigated the copy number of *pfmdr1* gene to screen its association with SNPs to define whether these two candidates may incriminate artesunate resistance. In addition, Laboratory-induced artemisinin resistance in the *Plasmodium chabaudi* model has been demonstrated in a CQ-resistant strain. Hence, it has been suggested that CQ resistance might be a prerequisite for the subsequent development of artemisinin resistance. Therefore, to provide more data on this hypothesis, we simultaneously analyzed *pfcrt* K76T mutation, that plays a central role in the *P. falciparum* resistance to CQ. Finally, as finding surveillance molecular tools for monitoring ACT resistant are research priorities, in order to determine the role of these molecular markers in ACT resistance, the present study was undertaken to compare the similar baseline data obtained from genotyping analysis of *pfmdr1* and *pfmrp1* in *P. falciparum* collected-samples during 2000–2005, before implementation of ACT in the study areas (Ursing et al., 2006; Zakeri et al., 2008).

2. Materials and methods

2.1. Study subjects and parasite detection

Two hundred *P. falciparum* isolates were collected from patients 2–70 years of age, from Chabahar district, in Sistan and Baluchistan province, Iran during 2007 ($N = 51$), 2008 ($N = 37$), 2009 ($N = 34$) and 2010 ($N = 78$). All blood samples were microscopically confirmed for *P. falciparum* infection. One ml blood was collected in tubes containing EDTA before national standard treatment with SP–artesunate, stored at 4 °C, and transported to the main laboratory in Pasteur Institute of Iran. The protocol was approved by the Ethical Review Committee of Research of Pasteur Institute of Iran and a written informed consent was obtained from all patients (adults or parents/legal guardians of children). Parasite DNA was extracted from 300 μl blood samples of either continuous culture of *P. falciparum* (3D7 strain) or field isolates using the commercially available DNA purification kit (Promega, Madison, WI, USA), and kept at −20 °C until use. DNA of *P. falciparum* was detected by nested-PCR amplification of the small subunit ribosomal ribonucleic acid (18ssrRNA) genes as described previously (Snounou et al., 1993).

2.2. pfcrt, pfmdr1 and pfmrp1 genotyping by PCR-RFLP and sequencing

Nested PCRs were performed for *pfcrt* and *pfmdr1* (except for detection of F1226Y) genes on all examined samples based on those published previously (Duraisingh et al., 2000; Zakeri et al., 2008). For *pfcrt*, 10 μl of the PCR products were digested with one unit of *ApoI* (Fermentas, Vilnius, Lithuania) at 37 °C for 4 h. The resulting fragments were 98 bp and 47 bp for *pfcrt* 76 K. For *pfmdr1*, *ApoI* and *NsiI* (Fermentas, Vilnius, Lithuania; New England Biolab, Beverly, MA, USA, respectively) were used to identify polymorphisms at codon 86 according to the manufacturer's instruction. Four enzymes, *TasI*, *DdeI*, *AseI*, and *EcoRV* were used to determine the polymorphisms at codons 184, 1034, 1042, 1246, respectively. Digestions were carried out in 20 μl reactions containing 10 μl PCR products according to the manufacturer's instructions (New England Biolab, Beverly, MA, USA; and/or Fermentas, Vilnius, Lithuania; Invitrogen, Carlsbad, CA, USA). If there was any doubt about the complete digestion, reactions were repeated overnight. Digested products were subjected to electrophoresis on 2–2.5% agarose gels or 2–3% Metaphor agarose gels, and visualized by ultraviolet (UV) transillumination. For detection of *pfmdr1* F1226Y SNP, the following forward and reverse primers