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Original Article

Detection of K76T Mutation in pfcrt Gene as an Applicable Genetic Marker for Prediction of Chloroquine Resistant falciparum Malaria in Isolates from an Endemic District of Iran

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Abstract

Background: This study investigated the association between pfcrt, T76 allele and chloroquine resistance in patients with falciparum malaria. Molecular assays for point mutations on drugs resistance-related genes are applied tools for monitoring emerging resistance and surveillance malaria control strategies in endemic areas. The mutant genotype at codon 76 of *Plasmodium falciparum* chloroquine resistance transporter gene (*pfcrt*) has been proposed as a molecular marker for the faster detection of chloroquine resistance in field.

Methods: In 64 samples from patients with uncomplicated falciparum malaria from Sarbaz district in southeast of Iran, the clinical response to chloroquine and the prevalence of K76T mutations in *pfcrt* gene were investigated by *in vivo* and nested-PCR followed restriction enzyme digestion methods.

Results: The occurrence of the K76T mutation was very high (60 of 64, i.e. 93.75%) among these filed isolates. Only 4 of 64 isolates harbored wild type K76 codon and no case was a mixed of K76 and 76T codons. All of the 22 (100%) chloroquine-resistant and 16.7% of sensitive isolates were found to harbor the 76T mutation and none was found to contain the wild type (K76) allele.

Conclusions: The frequency of chloroquine resistance associated point mutation K76T, in *pfcrt* gene in this region suggest that detection of this mutation can be applied for predicting chloroquine resistance in epidemiologic settings with sufficiently high sensitivity to make it an attractive alternative to time and labor-consuming *in vivo* trials.

Keywords: Plasmodium falciparum, *chloroquine resistance*, *pfcrt*, *K76T*, *Iran*

Introduction

Chloroquine (CQ) and other quinoline-based drugs have been used for the prophylaxis and treatment of malaria for more than 50 years in all of the malarious countries because of its cost effectiveness, few side effects, and easy availability. The tremendous success of chloroquine and its heavy use through the decades eventually led to chloroquine resistance in *Plasmodium falciparum* which is responsible for fatal malaria in humans. Foci of resistant *P. falciparum* were detected in Colombia and at the Cambodia-Thailand border during the late 1950s and then resistant strains from these foci spread through the world (1).

Genetic polymorphism associated with the chloroquine resistance (CQR) phenotype in *P. falciparum* has been identified in the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene,

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located on chromosome 7 (2-4). The amino acid substitution at *pfcrt* codon 76, K (lysine)→T (threonine) has been shown to have the strongest association with the CQR phenotype (3, 5-12). The *pfcrt* gene encodes an integral membrane protein, which is localized to the parasite digestive vacuole where haem molecules released during haemoglobin digestion are detoxified by the formation of haemozoin, also known as malaria pigment; CQ is suggested to interfere with this process (13, 15). *Plasmodium falciparum* CQR is suggested to involve mechanisms whereby pH sensitive physiologic processes inhibit formation of toxic CQ: haematin complexes in favor of haemozoin, or CQ efflux reduces drug concentration to the levels that are no longer parasiticidal (14-16). In addition to *pfcrt*, *P. falciparum* multidrug resistance (*pfmdr1*, chromosome 5 and nine other putative transporter genes have been implicated in CQR (7, 17). Polymorphisms in *pfmdr1* gene play a modulatory role in chloroquine resistance (18).

In Iran, resistance of *P. falciparum* to chloroquine was first observed in the district of Iran-Shahr, in Sistan & Baluchestan Province in 1983 (19) and later in Bandar_Abbas district, in Hormozgan Province (20, 21). In 2005 National Malaria Control Programme reported 18966 malaria cases that more than 75% were autochthonous. Eleven percent of total reported malaria cases were caused by *P.falciparum*. According to the current reports **(**22**)**, 90% of cases are from three provinces in the southeast part of the country: Sistan & Baluchestan, Hormozgan and Kerman. In these three provinces, the major peak of malaria transmission occurred between September and November and a large proportion of the malaria cases diagnosed, and an increased risk of local transmission has been observed. These areas incorporated less than 5% of Iran's total population, but contain more than 85% of the total incidence of malaria cases in the whole country with *P. vivax* and *P. falciparum* are both present. The Sistan & Baluchestan province is the most important area with more than 60% of all cases (23-24).

Until recently, our knowledge of the epidemiology of drug- resistant malaria was based on the collection of *in-vivo* data from symptomatic patients to whom different antimalarial drugs were administered and, to a lesser extent, on *in-vitro* drug sensitivity assays. Regarding the limitations of these methods for studying drug-resistant malaria, we determined the regional prevalence of the mutant allele of *pfcr*t gene associated with resistance to chloroquine in the *P. falciparum* population for estimation levels of resistance to chloroquine (i.e., the genotype-resistance index,GRI) in an endemic area of Iran.

Materials and Methods

Study site and subjects

This study was conducted at Pishin and Rask health centers located in the Sarbaz district in Sistan & Baluchestan Province, a borderland of Iran with Pakistan and Afghanistan. Malaria transmission occurs during the whole year with two peaks (May-Jun**)** and (Oct-Nov) (24). The tribulation encountered here are resistance of *P. falciparum* to drugs and that of vectors to insecticides (25-28). Patients were enrolled for the study if they met the following criteria: native resident, current fever or history of fever within the past 24hr chills, headache, mono-infection with *P. falciparum*, Parasitemia ranged 1000-100,000 parasite/ μL of blood. Patients with pregnancy sever or complicated malaria and chloroquine treated infection were excluded from the study (29). Parasite count was made in the thick and thin blood smears stained with Giemsa. Asexual parasites were counted against at least 200 leukocytes and then converted to the number of parasites per micro liter of blood. A total of 71 clinical isolates were collected from patients. Of them, 28 patients on day 0 were treated under supervision, with CQ(Pars Daru,Tehran),given at dose of 10mg/kg on each of days 0 and 1 and5mg/kg on day2.They were given clinical examination, with the recording of axillary's temperatures, on days 0, 1, 2, 3, 7,

14, 21 and 28, and parasitological examination, for *P.falciparum* parasitemias,on days 0, 2, 3, 7, 14, 21, and 28.For each patient, the therapeutic response to CQ by day 28 was classified as an early treatment failure(ETF) ,a late clinical failure(LCF) a late parasitological failure(LTF),or an adequate clinical and parasitological response(ACPR).Before treatment, from all 71 patients 2ml venous blood were collected in tubes containing anti coagulant solution and stored at -20 ºC until use. This study was approved by the Ethical Review Committee of Research in Tehran University of Medical Sciences, Iran.

DNA extraction and molecular analysis

Parasite genomic DNA was extracted directly from 100-200µL of infected blood using the QIAamp DNA blood mini kit (Qiagen, Valencia, CA) according to the manufacture's protocol. A polymerase chain reaction (PCR) and restriction digestion protocol for the detection of pfcrt gene was modified from the methods previously described (7). To amplify the pfcrt gene, PCR was carried out using forward primer, cr1: (CAT TGT CTT CCA CAT ATA TGA CAT AAA) and reverse, cr4: (GAT CTC TAT ACC ATT ATT CCT) in initial amplification. The final concentration of the PCR was 1x PCR buffer, 2mM Mgcl2, 2.5mM dNTP, 10 picoM of each primer (F/R), and 0.05 units/µL of Taq polymerase. Four micro liters of DNA template was added to a reaction volume of 16 µL and conditions were one cycle at 94 º C for 2 minutes an amplification of 30 cycles(94 ºC for 30 seconds, 52 ºC for one minute and 70 ºC for 4:30 minutes. The nested amplifications were carried out in a 20-µL reaction volume containing 1x PCR buffer, 2 mM MgCl2, 2.5 mM dNTPs, 10 picoM of each primer (F/R), and 0.05 units/µL of Taq polymerase. The product of the first amplification after dilution was used as the template for the second PCR. For nested PCR, forward primer was cr2:(TTT CCC TTG TCG ACC TTA ACA GAT GGC) and reverse was cr6:(CGG ATG TTA CAA AAC TAT AGT TAC C) and conditions were one cycle at 94 ºC for 2:30 minutes an amplification of 35 cycles (94 ºC for 35 seconds, 52ºC for one minutes and 60ºc for 2 minutes). Then nested PCR products including codon 76 in the pfcrt gene was digested with the restriction enzyme Apo1 (New England Biolabs, Inc., Beverly, MA).This enzyme digests K76 but not 76T Thus, the cleavage of the amplicon into 2 fragments (158 and56bp) indicates the presence of the wild-type codon K76. PCRs and digestion products were resolved on 2.5% agarose gel containing ethidium bromide and visualized under UV light. FCRC3 strain DNA as a positive and water as negative controls were included with each set of PCR reaction. The PCR reagents were obtained from the TaKaRa Shuzo Co. (Kyoto, Japan).

Chi-square and Anova one way tests were applied to calculate significance of the results.

Results

A total of 71 clinical isolates were collected from the slide positive *P. falciparum* malaria patients. During the study period, data from 7 patients' samples finally excluded from analysis due to incomplete clinical histories and an inability to amplify gene product. The mean age of the study population was 28 years [4-60yr], and 43(67.2%) of patients were male. All 64 samples successfully amplified and produced the expected 214 bp amplicon (Fig. 1). A subset of 28 from 64 patients participated in the *in vivo* chloroquine assessment studies (Table 1). Results show that 6 (21.4%) patients were responding to chloroquine. Among non-responders, 5(17.9%) were of early treatment failures. We have analyzed the K67T mutations in the *pfcrt* gene of all the 64 clinical isolates of *P. falciparum* with known (28 cases) and unknown (36 cases) *in vivo* chloroquine susceptibility profile. Results are shown in Table 2. The occurrence of the K76T mutation was very high (60 of 64, i.e. 93.75%) among these isolates. Only (4 of 64) isolates harbored wild type K76 codon and

no case was a mixed of K76 and 76T codons. All of the 22 (100%) chloroquine-resistant isolates, irrespective of early or late treatment failure, were found to harbor the 76T mutation and none was found to contain the wild type (K76) allele and only one isolate from the chloroquine responder group (16.7%) harbored this 76T mutation (Table 2). According to the genotype resistance index (GRI) and genotype failure index (GFI) models (30), we found 1.7 and 1.05 for GRI and GFI, respectively. We also investigated the impact of age, sex, baseline temperature, pre-treatment parasite density and study site on the prevalence of K76T mutations, but no association significantly was found between these variables and K76T mutations.

Table1: *In vivo* chloroquine response among *P.falciparum* malaria patients in Sarbaz district of Iran.

Characteristics	**Results**
Number of patients	28
Parasitologic response;	
Sensitive (%)	21/4
Resistant (%)	78/6
Early treatment failure (%)	17/9
Late treatment failure (%)	57/1

Table 2: The K76T mutations in *Pfcrt* gene among clinical isolates of *P.falciparum* from Sarbaz District of Iran.

***In vivo* choloroquine susceptibility**	**No. of samples**	***pfcrt* Alleles**		
		Wild (K76)	**Mutant (T76)**	**Mixed**
(A) Sensitive	6	1 (16/7%)	5 (83/3%)	None
(B) Resistance	22	None	22 (100%)	None
(C) Not known*	36	3 (8/3%)	33 (91/7%)	None
Total	64	4 (6/25%)	60 (93/75%)	None

Isolates in categories A and B were collected in 2003
* Chloroquine susceptibility was not determined. These isolates were collected in 2004

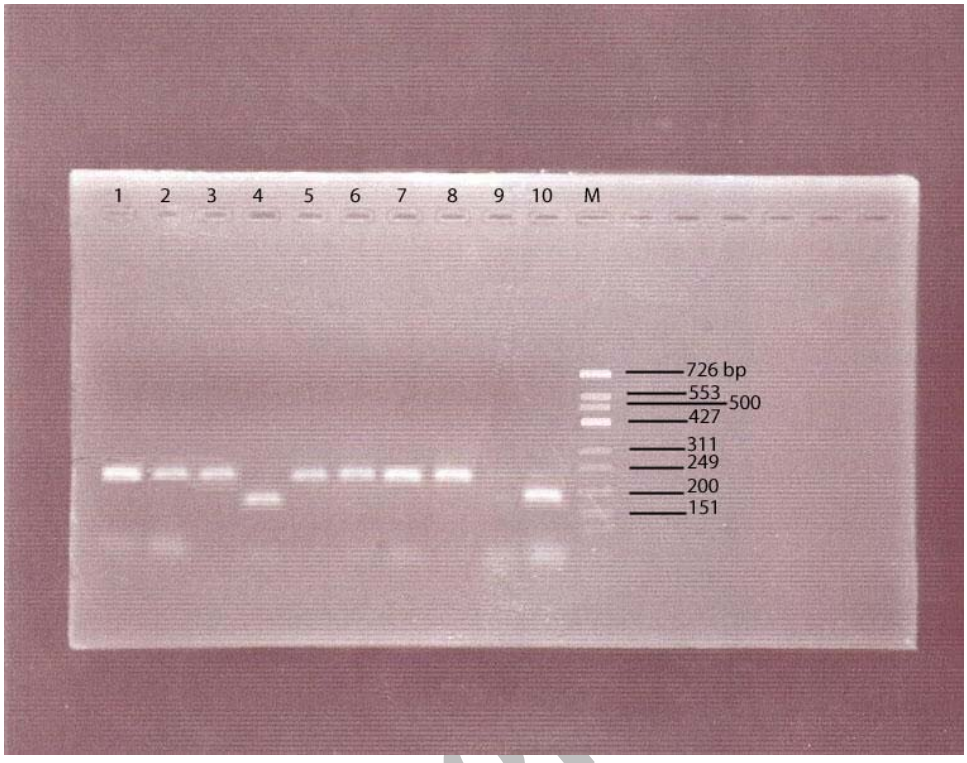

Fig.1: Electrophoretic results of representative *pfcrt* amplicons (214bp) from chloroquine study specimens after digestion whit Apo1. Lanes1-8 are study specimens; lane 4 is wild-type (158 and 56 bp). Lane 9 is negative control; lane 10 is positive control strain FCR3; lane M is marker (TAKARA BIO INC., Japan)

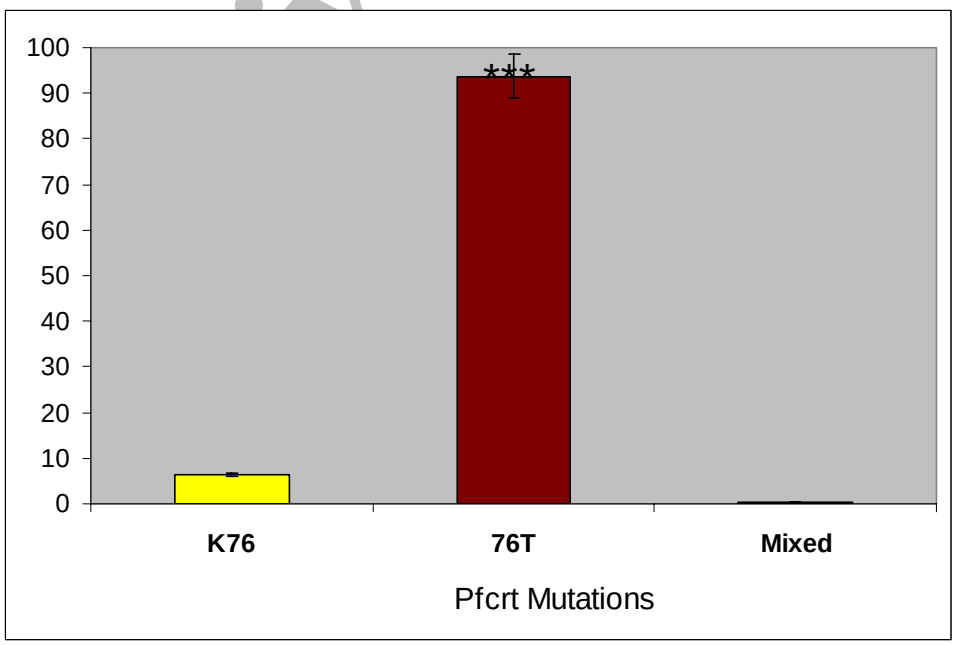

Fig.2: Prevalence of molecular markers of chloroquine–resistant Plasmodium falciparum in the Sarbaz district, Iran (mean±SEM, ****P*<0.001)

Discussion

Standard *in vitro* and *in vivo* studies of resistance to anti-malarial drugs are time consuming and often difficult especially in poor and displaced population (29).

The PFCRT is a novel predicted integral transmembrane protein and the *pfcrt* K76T is a molecular marker for resistance to chloroquine both *in vitro* and *in vivo* (3).

Several field studies in different geographical site confirmed absolute specificity of this marker to clinical chloroquine resistance (2). Ratios of the prevalence of this marker to the prevalence of *in vivo* resistance to chloroquine have been described as a practical means of using marker to estimate levels of resistance to chloroquine (i.e., the genotype-resistance index, GRI (30).

Result of the present study show that the K76T mutation in pfcrt gene is highly predominant in this part of Iran (Table 2). All the patients with a treatment–failure response harbored *P. falciparum* with the *pfcrt* 76T allele. The presence of this allele was predictive of *in vivo* failure in patients from these areas, where the level of transmission, and therefore, presumably, the level of acquired immunity was relatively low (31). This observation, and the similar ones of Babiker *et al.* (11) and Chen *et al*. (12), confirms the potential usefulness of *pfcrt* 76T as predictive marker for CQ-treatment in semi-immune populations (33). In our study, 93.75% of the pretreatment samples showed the mutated variant 76T in *pfcrt* gene and association between pfcrt K76T frequency and sex, age, fever, parasite density and study site were not significant. It could be attributed to exposing the parasite to sub-inhibitory drug concentration and/or heavy drug pressure on the parasites that circulate in this part of Iran, during several years. The stability of *pfcrt* T76 marker across this variation in sampling framework will not result in bias.

According to the study of Ursing *et al*. in Iran (25), the probable common origin of the *pfcrt* indicates the possibility of a drug-resistant gene spreading through the whole region. That is of particular concern due to the recent reports of high prevalence of *in vivo* resistance to amodiaquine in Afghanistan (25). Two indexes GRI and GFI in our study were close to 1 and 2, respectively, mean that in this high level of chloroquine resistance site (28), prevalence of T76 marker would be predicted around 100% that consistent to other molecular studies in Iran (25, 31), and agreement with findings from other areas where clinical chloroquine failure has reached high frequency (10).We believe that in the areas that have been covered by our study, rates of *in vivo* and the prevalence of molecular markers may be more closely matched than in areas of higher transmission (32). On the other hand we may miss some mixed infections because the restriction digestion method is generally less sensitive than direct sequencing method.

In conclusion our result showed that PCR-based technique provides a simple, rapid method of detecting polymorphisms in *pfcr*t gene that affects resistance to chloroquine. Moreover, the results support the hypothesis that molecular basis of chloroquine resistance involve mutation in *pfcr*t gene and that detection of mutated alleleT76 could predict potential chloroquine treatment failures.

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