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Original Article

Variation of the Chloroquine Resistance Transporter (*Crt*) Gene in Chloroquine-Resistant and Chloroquine-Sensitive *Plasmodium berghei*

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Abstracts

Background: The emergence and spread of chloroquine resistant *Plasmodium falciparum* in the world stimulated some investigators to consider different aspects of chloroquine resistance in human and rodent *Plasmodia*. Using animal *Plasmodia*, particularly primate and rodent *Plasmodia* can be useful model for human *Plasmodia* studies. In this study we have tried to consider and compare the sequence of chloroquine resistance transporter (*crt*) gene among chloroquine-resistant and chloroquine–sensitive strains of *Plasmodium berghei*.

Methods: This experimental study was performed at the Malaria Laboratory of School of public health. DNA was extracted from two strains of *P. berghei* which their resistance and sensitivity had been demonstrated in mice with treatment by chloroquine. By using specific primer for *crt* gene some parts of this gene were amplified by PCR, and obtained fragments were then sequenced and compared.

Results: There were considerable differences in *crt* gene between two strains. Sequenced 1212 bp of *crt* gene fragment in the two strains showed 43 differences at nucleotides level and 16 differences in presumed coding amino acids.

Conclusion: *crt* can be addressed as a considerable gene which involves in induction of resistance to chloroquine in *P. berghei*, as *P. falciparum*. The results increased such a promise that considering *crt* gene in chloroquine-sensitive and chloroquine-resistant *P. berghei* can prepare suitable and helpful fields for more understanding the molecular aspects of chloroquine-resistance in *Plasmodia* and reversing the effectiveness of 4-aminoquinolines particularly chloroquine for treatment of drug resistant *Plasmodia*.

Keywords: Chloroquine resistance, *Plasmodium berghei*, *Crt* gene

Introduction

Malaria is one of the world wide parasitic diseases which threaten life of more than hundred million people at the malarious areas each year (1). Although attempts for treatment of malaria are extensively conducted at most of the malarious areas, it still remains uncontrolled to date due to various reasons such as spread of pesticide resistant mosquito vector, lack of suitable vaccine and emergence of drug resistance in human *Plasmodia* particularly in *Plasmodium falciparum* from the early 1960s up to now (2-4). The spread of chloroquine resistant strains of *P. falciparum* stimulated many researchers to investigate the mechanism(s) involved in chloroquine resistance. The first researches suggested that chloroquine-resistant phenotype is characterized by a greatly increased drug efflux from the parasite (5, 6).

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Studies at the molecular level showed that a number of candidate genes such as, *crt* (chloroquine resistance transporter), mdr1 (multidrug resistance 1) and cg2 might be responsible for chloroquine-resistance in *P. falciparum* (7, 8). It was found that some, if not all; resistant lines are associated with amplification of pfmdr1 genes and increased mdr1 expression (9). Moreover, recently many useful investigations were conducted on the role of *crt* gene in chloroquine resistance in *P. falciparum* (6, 7, 10-13). However in this study the situation of *crt* gene in chloroquine-resistant and chloroquine-sensitive *P. berghei*, as a suitable model for human *Plasmodia*, using PCR technique was studied.

Materials and Methods

Parasites and drug:

In this experimental study we produced a chloroquine-resistant *P. berghei* isolate derived from chloroquine-sensitive *P. berghei* (NICD strain infective to mice and rats, developed at the Haffkine Institute, India (Dedicated by Prof. Edrrisian from Tehran University of Medical Sciences), using by 2٪ relapse technique (14). Briefly, male surie mice were infected with 10^7 *P. berghei* parasites via the intra peritoneal route. One hour after injection, the infected mice treated with a single dose of 5 mg/kg chloroquine diphosphate (SIGMA: C6628) in 0.1 ml physiological saline by injection subcutaneous. Daily rate of parasitemia was counted by microscopy in Giemsa stained thin blood smear collected from tail of the mice. When parasitemia reached to 2٪ the infected blood was collected directly from heart and diluted in physiological saline. 0.1 ml of the suspension containing 10^7 parasites was injected intraperitoneal into the next group of mice for the second passage. The infected mice were treated with 10 mg/kg chloroquine. The induction of resistance was repeated as before with 15, 20, 30, 40, 50, and 60 mg/kg chloroquine subsequently.

DNA extraction and PCR amplification of crt gene and sequencing:

DNA was extracted from chloroquine-resistant and chloroquine-sensitive *P. berghei* infected blood by Flexi Gene DNA kit according to the manufactures instructions (QIAGEN, Germany). A PCR was used to amplify some region of the *crt* gene in two strains. Two set of primers
AS_3 5' GTTCTCAACAATTGTATTTTCTCC 3' and S_3 5' TTTGCAAAAAGAACCCTGAA 3' were designed according to mRNA fragment of *crt* gene of *P. berghei* (15). Amplification reaction was performed in volume of 50 µL. Two µL of isolated DNA were added to a PCR Master Mix, containing 4.0 mM $MgCl_2$, 200µM dNTPs, 20 pmol of each primer and 2U of Taq polymerase (Roch, Germany) in the PCR buffer. Reaction were over laid with 50 µL of mineral oil and amplified in a Thermocycler (Techne USA) as follows: initial denaturation at 95 ^{0C} for 5 min followed 35 cycles of 95 ^{0C} for 1 min, 60 ^{0}C for 1.5 min, and 72 ^{0}C for 2 min and additional extension at 72 ^{o}C for 8 min. The PCR products were run with a 100 bp ladder on a 1.2٪ agarose gel containing ethidium bromide for 1 hour at 70V. The gel was observed on a UV transilluminator and then, digital photograph was prepared. The product of two strains of *P. berghei* was sequenced and repeated at least two at MWG biotechnology center in Germany.

Results

Using 2٪ relapse technique, we produced an experimental chloroquine-resistant *P. berghei*. Chloroquine sensitivity of two strains was tested in mice treated with a single dose of 50 mg/kg. By using PCR we could amplify PCR fragments in both sensitive and resistant strains of *P. berghei* (Fig. 1). Each PCR fragments was sequenced and the homology study of these two PCR fragments revealed significant identification with other *crt* genes in *Plasmodium* species. Blast searches were performed at

www.ncbi.nlm.gov/nocleotide Blast. The both sequenced *crt* genes were submitted to Gene Bank databases and released for public access under, accession numbers EU414204.1 for sensitive strain (TUMS/PB/NR), and EU414205.1 for resistant strain (TUMS/PB/R). The sequence of *crt* gene was aligned with mRNA fragment of *crt* gene in *P. berghei* (accession number XP-676387.1), to predicted a possible exon/intron structure for pb*crt*.

Table 1 shows the lengths of the predicted exon/intron pb*crt*. Out of 13 exons and 12 introns available in *crt* gene structure, we could sequenced only 6exons and 5 introns in this study. Analysis of these two genes which from now is named as TUMS PB/R for resistant strain and TUMS PB/NR for sensitive, genes showed 96٪ homology between them. Alignment of these two sequenced *crt* gene, indicated that the strains were different in 43 nucleotides which 25diversities are in 6 exons and 19 diversities in 5 introns. The amount of 546 nucleotides within 6 exons which has be described here, encodes 182 amino acids from the total of 424 amino acids of *crt* gene. In our study, 182 sequenced amino acids showed 91٪ similarity between the two strains. An alignment of the sequenced amino acid in two strains shown in Fig. 2, indicated this two strains were different in 16 amino acids.

Table 1: Exon and intron positions in *crt* gene characterized *Plasmodium berghei*

5'-UT	**Exon length (bp)**	***Intron length (pb)***
1	141	*108*
2	95	*119*
3	130	*122*
4	72	*174*
5	76	*143*
6	32	
Total coding region	546	
Total introns		*666*

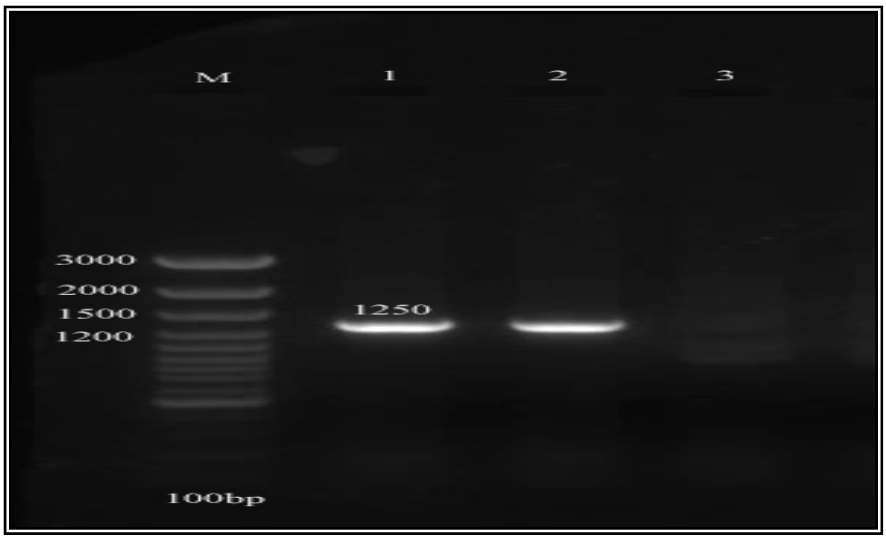

Fig. 1: Electrophoresis results of *crt* gene from *Plasmodium berghei*.
Lane 1 and 2, TUMS PB/NR and TUMS PB/R respectively. Lane 3, Negative control (mouse blood without *Plasmodium berghei* infection)

PbR*CRT*	1	VFQLLYFIYRKTSSSSVYKNESQKNFGWQFFLISLLDASTVIISMIGLTRTTGNIQSFIM	60
PbS*CRT*	1	VFQLLYFIYRKTSSSSVYKNESQKNFGWQFFLISLLDASTVIIRMIGLTRTTGKILSFIM	60
PbR*CRT*	61	QLIIPVNMYFCFMFLGYRYHLFNYLGAFIILITIAVVETFLSFETQGENSIIFNLIMISA	120
PbS*CRT*	61	QLSIPVNMYFCFMSLGYRYHLFNYLGAFIILITIAVVETFLSFETQGENSIIFNLIMISA	120
PbRCT	121	LIPLSVSNMTREVVFKKHKINILRLNAMVILFQFFTSLLVLPVYNIPFLKEIYMPFTEMS	180
PbS*CRT*	121	LIPLNFSNLTREGFFKKHKINFPKFNAMVILFQFFTSLLVLPVYNIPFLKEIYMPFSEMS	180
PbR*CRT*	181	TN 182	
PbS*CRT*	181	TD 182	

Fig. 2: Aligment of predicted amino acid sequence encoded by TUMS PB/NR and TUMS PB/R

Discussion

The main objective of this study was to characterize the mechanism(s) of chloroquine resistance in *P. berghei* at the molecular level. Many researches have recently confirmed that point mutations on *crt* gene are strongly associated with chloroquine resistance in *P. falciparum* (16-19). A Study on the role of *crt* gene in chloroquine-resistant *P. vivax*, showed that chloroquine resistance mechanism in *P. vivax* was different from *P. falciparum* (15). In another study no linkage was observed between chloroquine resistance in *P. chabaudi*, a rodent *Plasmodium*, and *crt* gene (20).

These studies may reflect basic differences in the genetic determinants and molecular mechanisms of chloroquine resistance in *Plasmodia* species. Therefore it seems probably that a multigenic origin is involved in such phenomenon. Our finding in this study, indicated that pb*crt* is orthologues of pf*crt*. Sequence of *crt* gene in chloroquine-resistant and chloroquine-sensitive *P. berghei* strains showed that some mutations have been occurred in pb*crt* Of TUMS/PB/R strain. However, in this study, we have investigated the region consists of 6 exons and 5 introns of *crt* gene that showed 43 different nucleotides Changes in nucleotides resulted in 16 differences at amino acid level in the strains. Since in this work structure of protein

was not analyzed, the determined mutation can partly related to modified structure of protein. It is possible to speculate that *crt* can be a candidate gene for chloroquine resistance in *P. berghei.*Indeed more investigation is needed to understand different aspects of *crt* gene involved in chloroquine resistance in *P. berghei.* Such studies prepare a new approach for reversing resistance and restoring the effectiveness of chloroquine for chemotherapy of chloroquine resistant *Plasmodia*.

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