Infection, Genetics and Evolution 20 (2013) 407–412

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Reduced heterozygosity at intragenic and flanking microsatellites of *pfcrt* gene establishes natural selection based molecular evolution of chloroquine-resistant *Plasmodium falciparum* in India

Prashant K. Mallick [a], Ruchi Singh [a,b,*], Om P. Singh [a], Ashok K. Singh [c], Virendra K. Bhasin [c,*], Neena Valecha [a]

[a] National Institute of Malaria Research, Indian Council of Medical Research, Dwarka Sector-8, New Delhi 110077, India
[b] National Institute of Pathology, Indian Council of Medical Research, New Delhi 110029, India
[c] Department of Zoology, University of Delhi, New Delhi 110007, India

ARTICLE INFO

Article history:
Received 30 July 2013
Received in revised form 24 September 2013
Accepted 1 October 2013
Available online 12 October 2013

Keywords:
Chloroquine resistance
pfcrt gene
Microsatellite
Heterozygosity
Plasmodium falciparum
India

ABSTRACT

The positive selection of a nucleotide substitution in exon 2 of *Plasmodium falciparum chloroquine resistance transporter* (*pfcrt*) gene (mutation responsible for chloroquine resistance) causes a reduction in variation of neutral loci close to the gene. This reduction in allelic diversity around flanking regions of *pfcrt* gene was reported in worldwide chloroquine resistant isolates and referred as selective sweep. In *Plasmodium falciparum* isolates of India, the selective sweep in flanking loci of *pfcrt* gene is well established, however, high allelic diversity observed in intragenic microsatellites of *pfcrt* gene implied an ongoing genetic recombination. To understand, if molecular evolution of chloroquine-resistant *P. falciparum* isolates in India follow a selective sweep model, we analyzed genetic diversity at both seven intragenic and seven flanking microsatellites of *pfcrt* (−24 to +106 kb) gene in chloroquine sensitive and resistant parasites originating from high and low transmission areas. We observed low expected heterozygosity at all loci of resistant *pfcrt*-haplotypes (H_e = 0–0.77) compared to the wild-type (H_e = 0.38–0.96). Resistant SVMNT from high transmission areas showed significantly higher mean H_e (P = 0.03, t-test) at both intragenic and *pfcrt*-flanking loci (−24 to +22 kb) in comparison to low transmission areas. Our observation of reduction in variation at both intragenic and flanking loci of mutant *pfcrt* gene confirmed the selective sweep model of natural selection in chloroquine resistant *P. falciparum* isolates in India.

1. Introduction

Genetic cross studies in *Plasmodium falciparum* had identified the *Plasmodium falciparum chloroquine resistance transporter* (*pfcrt*) gene, located on chromosome 7 as a major candidate imparting chloroquine (CQ) resistance (Su et al., 1997; Wellems et al., 1991). Later, molecular studies established a key substitution of amino acid at 76th codon (K76T) of *pfcrt* gene, which turned out to be hallmark of CQ-resistance (Djimde et al., 2001; Fidock et al., 2000; Sidhu et al., 2002). Microsatellite mapping on chromosome 7 of *P. falciparum* revealed a reduced allelic diversity in flanking (±100 kb) regions of various resistant *pfcrt*-haplotypes (amino acids at codon 72–76 in *pfcrt* gene, i.e., SVMNT and CVIET), that suggests a selective sweep or genetic hitchhiking model of molecular evolution in CQ-resistant parasites (Wootton et al., 2002). This reduction in variation at microsatellites flanking mutant *pfcrt* gene have been extensively used to understand the origin and spread of CQ-resistant parasites in different malaria endemic countries (Anderson, 2004; Chen et al., 2005; Mehlotra et al., 2005; Wootton et al., 2002). In addition, high allelic diversity was also reported in microsatellites present at two different introns (intragenic region) of mutant *pfcrt* gene (DaRe et al., 2007; Vinayak et al., 2006). This implied continued genetic recombination inside the *pfcrt* gene, inspite of fixed flanking regions observed in two resistant *pfcrt*-haplotypes (CVIET and SVMNT) of Indian *P. falciparum* isolates (Lumb et al., 2012; Mixson-Hayden et al., 2010; Vinayak et al., 2006). The observed level of variation at intragenic loci could be related to rate of malaria transmission and indeed differed from the globally accepted selective sweep model for CQ-resistant parasite. However, all these previous assessment of the impact of CQ selection on *pfcrt* in Indian isolates either involves the *pfcrt*-flanking microsatellites or the intragenic microsatellites.

* Corresponding authors. Address: National Institute of Pathology (ICMR), Safdarjung Hospital Campus, New Delhi 110029, India. Tel.: +91 11 26198402x401; fax: +91 11 26198401 (R. Singh).
E-mail addresses: ruchisp@gmail.com, ruchis@icmr.org.in (R. Singh), virendrabhasin@gmail.com (V.K. Bhasin).

1567-1348/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.meegid.2013.10.001

A study involving simultaneous evaluation of both intragenic and flanking microsatellites of *pfcrt* gene is warranted to comprehend the ambiguity about the natural selection on Indian CQ-resistant parasite. Here, we investigated the polymorphism at intragenic microsatellites in seven different introns and seven microsatellites flanking *pfcrt* gene to infer the role of natural selection in the evolution of CQ-resistant *P. falciparum* isolates in India.

2. Materials and methods

2.1. Selection of isolates

The samples were collected between the year 2002–2006 during a project on therapeutic efficacy of chloroquine and the study was approved by the ethics committee of the National Institute of Malaria Research, New Delhi (Valecha et al., 2009). A set of 86 single-clone (single genotype) *P. falciparum* isolates from different malaria endemic regions of India were retrospectively selected from our previous studies (Mallick et al., 2012, 2013). The screening of single-clone isolates was based on *msp-1* and *msp-2* allelic family genotyping (Snounou et al., 1999), and further genotyped at three established neutral microsatellites at chromosomes 5 (TAA81), 11 (ARA2) and 13 (TAA60) (Anderson et al., 1999). The details of distribution of *pfcrt*-haplotypes in these single-clone isolates are illustrated in Table 1.

2.2. PCR amplification of microsatellite loci

Eight suitable microsatellite repeats (dinucleotide repeat units $\geqslant 5$) were identified inside eight different introns (namely 1, 2, 3, 4, 5, 6, 9 and 12) of *pfcrt* using tandem repeat finder version 4.00 (Benson, 1999). All the intragenic microsatellites except intron 3 were successfully amplified by PCR and sequenced to determine the number of dinucleotide repeats in Indian isolates. We also sequenced eight isolates representing Southeast Asia: INDO (MRA819G), V1/S (MRA820G), Dd2 (MRA150G); South America: HB3 (MRA155G), 7G8 (MRA152G); Africa: KMWII (MRA821G), D6 (MRA398) and 3D7 (MRA102G). The sequences of designed primers for PCR and amplifying conditions are listed in Table S1. The various nucleotide sequence observed are available in GenBANKTM under accession numbers KC593454–KC593511 (for Indian isolates) and KC859322–KC859376 (for worldwide isolates). In addition to intragenic microsatellites, six close microsatellites (B5M97 at −24 kb, B5M77 at −20 kb, 2E10 at −1 kb, 9B12 at +1 kb, PE12a at +6 kb, 2H4 at +22 kb) flanking the *pfcrt* gene and one farthest locus PE14f at +106 kb, all supposed to be under selective sweep were analyzed as described earlier (Wootton et al., 2002).

Table 1
Distribution of various *pfcrt*-haplotypes among single-clone Indian *P. falciparum* isolates.

	Area (year)	*n*	*Pfcrt*-haplotype		
			CVMNK	CVIET	SVMNT
HTA*	Odisha (2003)	17	3	9	5
	Jharkhand (2006)	14	3	9	2
	Assam (2002)	7	–	5	2
	West Bengal (2003)	7	–	2	5
	Maharashtra (2002)	4	1	–	3
	Chhattisgarh (2006)	6	5	–	1
	Car Nicobar (2002)	4	1	1	2
LTA*	Goa (2004)	6	–	–	6
	Gujarat (2005)	5	–	–	5
	Rajasthan (2003)	10	1	–	9
	Tamil Nadu (2004)	6	–	–	6

n is the no of single-clone isolates.
The year of collection of isolates is provided in the closed brackets.
* HTA, high transmission areas; LTA, low transmission areas.

2.3. Statistical analysis

Genetic diversity was evaluated for all the microsatellite loci in terms of expected heterozygosity (H_e) calculated as $[n/(n-1)][1-\Sigma p_i^2]$, where n is the number of isolates and p_i is the frequency of the ith allele. The sampling variance of H_e was calculated as $[2(n-1)/n^3]\,(2(n-2)\,[\sum p_i^3 - (\sum p_i^2)^2])$ (Nash et al., 2005). The level of genetic variation at each locus was compared among various *pfcrt*-haplotypes. In previous studies, SVMNT haplotype was found predominantly across India (Keen et al., 2007; Vathsala et al., 2004) and ubiquitously in low transmission areas (Mallick et al., 2012). Thus, genetic variation in SVMNT haplotype was also compared among previously classified high and low transmission areas (Mallick et al., 2012). The statistical significance of differences in mean H_e between various *pfcrt*-haplotypes and differences in allele frequency were calculated using *t*-test and Fisher's exact test, respectively (GraphPad Prism 5.0).

2.4. Linkage disequilibrium

The 6-locus microsatellite haplotypes of intragenic region and 5-locus microsatellite haplotypes of flanking (−24 to +6 kb) region was obtained from Arlequin software (Excoffier et al., 2005). LIAN version 3.5 (Haubold and Hudson, 2000) was used to analyze the evidence of linkage disequilibrium (LD) at 6-locus intragenic haplotypes as well as 5-locus *pfcrt*-flanking microsatellite haplotypes obtained above for various *pfcrt*-haplotypes (CVMNK, CVIET and SVMNT). The strength of LD was calculated as standardized index of association (I_A^S) for 6-locus intragenic haplotypes and 5-locus *pfcrt*-flanking microsatellite by LIAN. The null hypothesis of complete linkage equilibrium ($I_A^S = 0$) was tested by Monte-Carlo simulations using 10,000 random permutations of the multi-locus haplotypes.

2.5. Genetic relationship

The genetic relationship was assessed between the various *pfcrt*-haplotypes observed in Indian and worldwide isolates analyzed in this study. A median joining network was constructed using 6-locus haplotypes (obtained from Arlequin) observed at intragenic region of *pfcrt* gene in NETWORK version 4.5.1.0 (http://www.fluxus-engineering.com/sharenet_rn.htm). All the 6-locus intragenic microsatellite haplotypes used in NETWORK program is tabulated in Table S2.

3. Results and discussion

3.1. Allelic diversity at intragenic microsatellite loci

The number of alleles observed at each intron ranged from the single allele at intron 5 to 12 alleles at intron 2. The monomorphic nature of intron 5 may be due to short repeat array (5AT), as microsatellite polymorphism varies with the length of array with high variations observed in loci with long repeat array compared to short repeat arrays (Schlotterer, 2000). Two predominant alleles observed at each of the introns were associated with two resistant haplotypes (SVMNT/CVIET) ($P < 0.0001$, Fisher's exact test) (Table 2). The observed association of SVMNT and predominant allele 21AT repeats and association of CVIET with 15AT repeats at intron 2 had been reported earlier (Vinayak et al., 2006). Similarly, 12TA and 15TA repeats at intron 4 were noted to be predominantly associated with SVMNT and CVIET, respectively. This