DECREASED PREVALENCE OF THE PLASMODIUM FALCIPARUM CHLOROQUINE RESISTANCE TRANSPORTER 76T MARKER ASSOCIATED WITH CESSATION OF CHLOROQUINE USE AGAINST P. FALCIPARUM MALARIA IN HAINAN, PEOPLE’S REPUBLIC OF CHINA

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Abstract. The use of chloroquine treatment for Plasmodium falciparum malaria was abandoned in China in 1979 because of widespread drug resistance. Subsequent studies found decreases in the prevalence of chloroquine-resistant strains. To evaluate these decreases and assess the current status of chloroquine sensitivity in Hainan, China, we determined the prevalence of the P. falciparum chloroquine resistance transporter (PICRT) 76T marker in the DNA of blood samples collected from 1978 to 2001. Results showed the presence of PICRT 76T in 101 of 112 samples (90%) from 1978 to 1981, 30 of 43 samples (70%) from 1986, 22 of 34 samples (65%) from 1997 to 1998, and 37 of 68 samples (54%) from 2001. The prevalence of PICRT 76T thus progressively decreased after chloroquine was discontinued as a treatment for P. falciparum malaria (χ² = 5.2, P < 0.022 [1978–1981 versus 1986]; χ² = 7.4, P < 0.006 [1978–1981 versus 1997–1998]; and χ² = 28.8, P < 0.0001 [1978–1981 versus 2001]). Reduced prevalence of the PICRT 76T marker is consistent with greater rates of chloroquine sensitivity from in vitro drug assays of blood samples in 1997 and 2001. Monitoring for continued decreases will provide valuable information for future drug-use policies in China.

INTRODUCTION

In 1979, in view of the widespread resistance of malaria strains to chloroquine treatment in Hainan, People’s Republic of China, the use of chloroquine against Plasmodium falciparum was abandoned and replaced by use of a new bisquinoline antimalarial drug, piperaquine.1 Subsequent in vitro microtest surveys suggested that the prevalence of chloroquine-resistant P. falciparum strains decreased from 98% in 1981 to 61% in 1991, and correspondingly by the four-week in vivo test the rate of chloroquine-resistant P. falciparum malaria decreased from 84% to 40% in 1991.2,3 Another study found in vitro chloroquine resistance rates had decreased from 96% in 1974–1983 to 71% in 1999–2001.4

Recent identification of the role of the P. falciparum chloroquine resistance transporter (PICRT) mutations in chloroquine resistance has provided a molecular marker for P. falciparum strains that can fail chloroquine treatment.5–7 The PICRT protein is altered in naturally chloroquine-resistant P. falciparum parasites by multiple mutations that always include a key charge change from a lysine-to-threonine substitution at PICRT position 76 (K76T). The patterns of the mutations that accompany K76T depend on their geographic origin and probably represent accommodative changes that help preserve the native function of the transporter. To investigate the decrease in chloroquine-resistant P. falciparum in Hainan, we initiated a study of PICRT 76T rates and the decreases in chloroquine resistance indicated by this marker since 1978.

MATERIALS AND METHODS

Samples. Samples in this study included blood smears on glass slides and filter paper from subjects enrolled in the Tianan, Nandao, and Dongfang counties of southern Hainan province, where P. falciparum malaria is endemic. Protocols associated with sample collection were reviewed and approved by the institutional review board of Guangzhou University of Traditional Chinese Medicine (Guangzhou, People’s Republic of China). Information generated in this study was not linked to the original clinical records or in a form associable with specific individuals.

Glass slides collected in 1978, 1979, 1980, 1981, 1986, 1997, and 1998 were wrapped separately with paper and preserved in tightly sealed glass bottles. The slides from 1978, 1979, 1980, and 1981 were preserved from cases of uncomplicated and complicated P. falciparum malaria, including a case of cerebral malaria. Slides from 1986, 1997, and 1998 were from cases of uncomplicated malaria that had been used to provide samples for in vitro sensitivity tests. All samples were taken prior to the initiation of malaria treatment. Parasitemias were reconfirmed by microscopic examination of thick and thin blood smears.

Filter paper blood samples were collected in year 2001 from patients presenting with malaria symptoms and blood smears positive for P. falciparum. For this purpose, drops of peripheral blood were placed on 1.5 × 7.0-cm strips of Whatman (Brentford, United Kingdom) 3MM filter paper so that the blood covered half the length of the strip. The strips were then air-dried and kept in plastic bags until use.

Extraction of DNA. The DNA from filter paper and glass slides was obtained by recommended protocols and reagents of QIAamp DNA mini kit (Qiagen, Valencia, CA). Supplied ATL buffer (60–90 μL) was applied to the blood smears on the slides, which were then placed on a block heater at 80°C. The buffer was taken up and reapplied by pipette several times to wash the heated smears and then collected in a 1.5-mL microcentrifuge tube. After this process was repeated 2–3 times to obtain a volume of 180 μL, the DNA was purified, and 5 μL of the resulting solution were used for polymerase chain reaction (PCR) amplification. Blank slides and blood spots from blank (non-parasitized) wells of in vitro tests were used as negative controls in the DNA extraction and PCR amplification procedures to confirm lack of contamination.

Nested PCR and mutation-specific amplifications. Flanking primers CRTP1 5’-CCGTAAATAAATTACCCAGCAG-3’ and CRTP2 5’-CGGATGTACAAAACCTATAGT-
cycles at 94 °C amplification stages were at 94 °C/H11032. 

The resulting 150-basepair product were treated with 0.5 units of Exo treatment with SAP/10% rabbit serum. After 50-1.5 minutes. Product from this PCR (1 μL) was then used in two follow-up, nested, allele-sensitive PCR amplifications to detect the codons for PCRT 76K or 76T. These diagnostic PCR amplifications used a common inner primer (CRTP3 5′-TGCTCATGTGTTAAC-3′) coupled with either CRTP4m 5′-GTTCTTTAGCATAAATTG-3′ (detects the 76T codon) or CRTP4w 5′-GTTCTTTAGCATAAATTCT-3′ (detects the 76K codon). The PCR stages for these diagnostic amplifications were at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 47°C for 20 seconds, and 60°C for 1 minute. Purified genomic DNA from P. falciparum clones HB3 (chloroquine sensitive) and Dd2 (chloroquine resistant) were used as positive controls, and water, extracted uninfected blood smears, and uninfected blood spots on filter paper were used as negative controls. The PCR products from the amplification reactions were evaluated by electrophoresis on 2% agarose gels.

Detection of codons for PCRT 76K and 220A and for the P. falciparum P glycoprotein homolog 1 (Pfpgh-1) 86Y by restriction digestion of PCR products. The wild-type codon for PCRT 76K was detected by a primary round of PCR amplification with primers CRTP1 and CRTP2 as described above, followed by a second, nested PCR amplification in a 25-μL volume with two internal primers flanking codon 76: CRTD1 5′-TGCTCATGTGTTAAC-3′ and CRTD2 5′-CAAACATATACTAC-3′. The PCR amplification stages were at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, 48°C for 20 seconds, and 60°C for 1 minute. Eight microliters of the reaction mixture was treated directly with 0.5 μL of the restriction enzyme ApoI for one hour at 37°C. 

Decreasing prevalence of the codon 76T marker following the discontinuance of chloroquine use against P. falciparum malaria in Hainan. Figure 1 summarizes the relative prevalence of the codons for PCRT 76K and 76T in blood smears from 1978–1981, 1986, and 1997 and in filter papers from 2001, as determined by PCR amplification and restriction with ApoI. No or partial ApoI cutting indicating the presence of codon 76T was obtained with 101 (90%) of 112 samples from 1978–1981; results from 5 of these 101 samples
suggested the mixed presence of the 76T and 76K codons. This evidence of the 76T codon decreased to 30 (70%) of 43 samples from 1986, 22 (65%) of 34 samples from 1997–1998, and 36 (53%) of 68 samples from 2001, including 3 samples with codons for both 76K and 76T). Prevalence of the 76T codon indicative of chloroquine resistance thus decreased from more than 90% to less than 60% in the 20 years following discontinuance of the use of chloroquine against *P. falciparum*.\(^\text{3}\)\(^\text{16}\)

The close association of PICRT 76T and 220S noted in previous reports\(^\text{5}\)\(^\text{,6}\)\(^\text{,10}\) was also evident in these surveys. Of the DNA samples successfully amplified from the 2001 filter papers for *Bgl* I restriction testing, 30 of 30 that contained the 76K codon showed cutting indicative of the wild-type codon 220A. Conversely, 29 of 29 successful amplification products from samples containing the 76T codon showed no *Bgl* I cutting and therefore contained the 220S codon.

**Sequence analysis of DNA of mutant pfcr alleles.** Mutant *pfcr* sequences spanning codon 76 were amplified from 32 selected DNA samples (selected at random from all years except 1979) and subjected to direct DNA sequencing. All were found to contain the four codons predicting the amino acid sequence 72-CVIET-76 characteristic of chloroquine-resistant parasites from southeast Asia.\(^\text{6}\)\(^\text{,10}\)

We also checked the *pfcr* gene in six randomly selected 1986 samples for sequence differences in other regions known to harbor mutations in chloroquine-resistant parasites.\(^\text{5}\)\(^\text{,10}\) In all cases, the codon data identified mutations representative of the southeast Asian origin of chloroquine resistance: M74I, N75E, K76T, A220S, Q271E, N326S, I356T, and R371L. The sequences contained none of the mutations selected in other geographic foci of chloroquine resistance (parasites from the foci in South America, The Philippines, and Papua New Guinea have such mutations as C72S, H97Q, A144T, L160Y, N326D, I356L, and R371T).\(^\text{5}\)\(^\text{,10}\)\(^\text{,11}\) The sequences also showed no evidence for the S163R mutation recently reported to return chloroquine-resistant parasites to the chloroquine-sensitive phenotype.\(^\text{12}\) We did not check more recent DNA samples for the S163R mutation because all 1997 and 2001 samples containing the 76T marker were chloroquine-resistant by *in vitro* microtests (next section and Table 1).

**Association of the PICRT 76T marker with *in vitro* chloroquine resistance in Hainan.** Table 1 shows the molecular marker results from 80 *P. falciparum* samples tested for *in vitro* drug response in 1997 and 2001; 22 of these samples were determined to be chloroquine-sensitive and 58 chloroquine-resistant by World Health Organization microtest criteria. Amplification products from all the chloroquine-sensitive samples carried the codon for PICRT 76K, but not 76T. Of the 58 samples reported chloroquine-resistant by *in vitro* testing, 50 were found to carry the 76T codon, 5 were found to contain mixed 76T/76K codons, and 3 were discordantly found to contain the 76K codon. It was not possible to recover the frozen parasites and confirm the discordant drug test results from the three samples containing the 76K codon.

**Lack of an association between Pfpgh-1 86Y polymorphism and chloroquine resistance in Hainan.** The 86Y polymorphism of Pfpgh-1 (encoded by the *pfmdr1* gene) in southeast Asia has been associated with chloroquine resistance in some studies,\(^\text{13}\) but not in others.\(^\text{14}\)\(^\text{–}\)\(^\text{17}\) To test for possible association of this polymorphism with decreasing chloroquine resistance in Hainan, we checked *pfmdr1* segments amplified from the DNA samples for *Afl* III restriction. Of 104 samples tested, only 2 of 20 samples from 1986 showed cutting indicative of the 86Y polymorphism (Figure 2). To ensure that the 86Y polymorphism was not being missed because of inadequate restriction conditions or other reasons, we randomly selected and sequenced 16 of the amplification products from the 1986 and 2001 DNAs. None of these samples showed evidence of the codon for Pfpgh-1 86Y.

**DISCUSSION**

Decreasing prevalence of the PICRT 76T marker in Hainan is consistent with the decrease in chloroquine-resistant *P. falciparum* strains reported since 1981. Although the rates of resistance in Hainan are expected to vary by region, our finding of a decrease in PICRT 76T prevalence from 90% in the late 1970s to 65% in the late 1990s and to 54% in 2001 is consistent with microtest assays showing 98% resistant strains.

**Table 1**

*Plasmodium falciparum* chloroquine resistance transporter (PICRT) markers and reported *in vitro* microtest outcomes from *P. falciparum* samples on Hainan Island in 1997 and 2001

<table>
<thead>
<tr>
<th></th>
<th>Reported as <em>in vitro</em> microtest outcome</th>
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<tr>
<td></td>
<td>2001</td>
</tr>
<tr>
<td>PICRT 76K</td>
<td>16</td>
</tr>
<tr>
<td>Mixed 76K/76T</td>
<td>0</td>
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<tr>
<td>PICRT 76T</td>
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in 1981 and 61% and 71% resistant strains in recent years.\textsuperscript{2\textendash}4 In contrast, Pfpg-1 86Y, a polymorphism in southeast Asia that has been associated with chloroquine resistance in some studies,\textsuperscript{13} but not others,\textsuperscript{14\textendash}17 was absent from nearly all Hainan \textit{P. falciparum} samples and showed no correlation with the changes in prevalence of chloroquine resistance.

The decrease in chloroquine-resistant \textit{P. falciparum} in Hainan has evidently been less rapid than that reported recently from Malawi, where a switch from chloroquine was followed by a decrease in the PFCRT 76T marker from 85% in 1993 to 13% in 2000.\textsuperscript{18} The explanation for this less rapid decrease may involve several factors. First, after the 1979 discontinuation of chloroquine treatment against \textit{P. falciparum} malaria, use of the drug was still recommended for \textit{P. vivax} malaria in Hainan. Not all chloroquine pressure against \textit{P. falciparum} would therefore have been removed since chloroquine was probably used against undifferentiated cases of malaria and perhaps some mixed \textit{P. falciparum}-\textit{P. vivax} infections, which occur in regions where both species are transmitted.\textsuperscript{19,20} Second, local authorities of health sectors in some areas of Hainan revived the use of chloroquine against \textit{P. falciparum} for periods of time in the 1990s (Wang X, unpublished data). Third, widespread use in China of piperaquine, a bisquinoline containing structural components of chloroquine, may provide some positive selection pressure for PFCRT mutations, even though cross-resistance between these two antimalarial drugs seems to be low in \textit{P. falciparum}.\textsuperscript{21,22}

Data from 3 of 80 samples in our study showed discordances between their PFCRT type and their chloroquine response as determined by the World Health Organization \textit{in vitro} microtest method. Independently repeated PCR experiments confirmed the PFCRT types of the three samples, but we were unable to obtain viable parasites from cryopreserved samples to retest their chloroquine responses. Discordances commonly result from inaccuracies of microtest methods on parasites from patient blood samples that have not been adapted to continuous \textit{in vitro} culture.\textsuperscript{7,23} Thus, while tests can generally give good indications in epidemiologic surveys, the accuracies of individual assignments (in terms of inhibitory concentration) can be thrown off by parasite sample variability, inadequate or incorrect \textit{in vitro} test conditions, inaccuracy in microscopy, and humoral factors that can carry over from the sample and act with the drug to affect parasite maturation.\textsuperscript{24} It is unlikely that the three discordances in our study can be explained by an alternative mechanism of chloroquine resistance not involving the K76T mutation in PFCRT. No such mechanism has been identified for \textit{P. falciparum} parasites from any malarious region, and a single mechanism of chloroquine resistance in Hainan is consistent with the sweep of mutant parasites from a single focus in southeast Asia that eventually expanded across Asia, the Indian subcontinent, and into Africa.\textsuperscript{10}

Clinical studies have consistently found the prevalence of the PFCRT 76T marker to be greater than the rate of chloroquine failure in study groups, indicating the presence of additional host and parasite factors that contribute to the clearance of chloroquine-resistant parasites.\textsuperscript{7} In 1991, studies from Hainan reported a rate of \textit{in vitro} chloroquine resistance about 1.5 times higher than the rate of \textit{in vivo} treatment failures.\textsuperscript{2,3} At these comparative rates, a 50\textendash}60% prevalence of PFCRT 76T in Hainan today suggests that roughly one-third to half of \textit{P. falciparum} malaria cases would be expected to fail chloroquine treatment.

The recovery of chloroquine-sensitive parasite populations in Hainan after removal of drug pressure suggests a slight advantage of the native PFCRT molecule over its mutant forms and points to the possible value of drug rotation strategies in antimalarial policies. However, our view is that any reintroduction of chloroquine as monotherapy remains out of the question, while the possibility of its use in a drug combination will require much lower rates of resistance as well as a demonstrable advantage over alternatives. Combination treatments that include an artemisinin derivative with other antimalarials such as piperaquine,\textsuperscript{25,26} presently provide stronger options the treatment of drug-resistant malaria in southeast Asia.

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**Figure 2.** Prevalence of the \textit{Plasmodium falciparum} P glycoprotein homolog 1 (Pfpg-1) 86N and 86Y markers in samples from malaria cases in Hainan, People’s Republic of China, 1978–2001.


