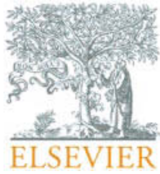

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

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

Document heading

Polymorphisms of the oxidant enzymes glutathione S-transferase and glutathione reductase and their association with resistance of *Plasmodium falciparum* isolates to antimalarial drugs

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ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form 20 July 2010
Accepted 17 August 2010
Available online 20 September 2010

Keywords:
Plasmodium falciparum
Drug resistance
Glutathione reductase (*Pf*GR)
Glutathione S-transferase (*Pf*GST)

ABSTRACT

Objective: To investigate the association between amplification of the two regulatory genes controlling glutathione (GSH) levels, glutathione reductase (*Pf*GR) and glutathione S-transferase (*Pf*GST) genes and sensitivity of *Plasmodium falciparum (P. falciparum)* isolates collected from different malaria endemic areas of Thailand to standard antimalarial drugs. **Methods:** A total of 70 *P. falciparum* isolates were collected from endemic areas of multi-drug resistance (Tak, Chantaburi and Ranong Provinces) during the year 2008-2009. The *in vitro* assessment of antimalarial activity of *P. falciparum* clones (K1- and Dd2 chloroquine resistant and 3D7-chloroquine sensitive) and isolates to chloroquine, quinine, mefloquine and arteusnate was performed based on SYBR Green modified assay. **Results:** 68 (97.14%), 11 (15.71%) and 28 (40%) isolates respectively were classified as chloroquine-, quinine- and mefloquine-resistant isolates. With this limited number of *P. falciparum* isolates included in the analysis, no significant association between amplification of *Pf*GST gene and sensitivity of the parasite to chloroquine, quinine, mefloquine and quinine was found. Based on PCR analysis, Dd2, K1 and 3D7 clones all contained only one copy of the *Pf*GST gene. All isolates (70) also carried only one copy number of *Pf*GST gene. There appears to be an association between amplification of *Pf*GR gene and chloroquine resistance. The 3D7 and Dd2 clones were found to carry only one *Pf*GR gene copy, whereas the K1 clone carried two gene copies. **Conclusions:** Chloroquine resistance is likely to be a consequence of multi-factors and enzymes in the GSH system may be partly involved. Larger number of parasite isolates are required to increase power of the hypothesis testing in order to confirm the involvement of both genes as well as other genes implicated in glutathione metabolism in conferring chloroquine resistance.

1. Introduction

Malaria has always been a major killer of populations throughout the tropics for thousands of years. Despite important advances in our understanding of the disease, it continues to be one of the greatest causes of serious illness and death in the world. Over 75% of 2-3 million deaths occurring in African children, and about 500 million new cases reported annually is a challenge to drug therapy and discovery. Of the four species of human malarial parasite, *Plasmodium falciparum (P. falciparum)* is remarkable for its high fatality particularly among young children and alarming development of resistance to antimalarial drugs. Resistance to antimalarials is spreading throughout the world and is impeding efforts to malaria control[1]. Resistance of *P. falciparum* to the most affordable drugs such as chloroquine and sulfadoxine/pyrimethamine and mefloquine has become widespread throughout Africa and Asia, in particular Southeast Asia[1].

Malaria-infected erythrocytes are under oxidative stress produced by the digestion of host's hemoglobin. The oxidant enzymes in glutathione (GSH) system, i.e, glutathione reductase (*Pf*GR) and glutathione S-transferase (*Pf*GST) are detected in malaria-infected cells[2-5]. Difference in the levels of these enzymes were shown in *P. falciparum* isolates with different sensitivity to chloroquine[4, 6-7]. This may imply the importance of these enzymes in survival

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of malaria parasites, including their possible involvement in conferring antimalarial drug resistance. Any compound inhibiting these molecules would eventually lead to parasite death, and thus be a promising antimalarial drug candidate. The objective of the study was to investigate the association between amplification of *Pf*GR and *Pf*GST genes and sensitivity of *P. falciparum* isolates collected from different malaria endemic areas of Thailand that were resistant to standard antimalarial drugs chloroquine, quinine, mefloquine and artesunate.

2. Materials and methods

2.1. Parasite cultivation

A total of 70 *P. falciparum* isolates were collected from endemic areas of multidrug resistance (Tak, Chantaburi and Ranong Provinces) during the year 2008-2009. Prior to the study, approval of the study protocol was obtained from the Ethics Committee of the Ministry of Public Health, Thailand. Written informed consent for study participation was obtained from all patients prior to blood sample collection. K1 and Dd2 (chloroquine-resistant reference clones) and 3D7 (chloroquine-sensitive reference clone) *P. falciparum* clones including all isolates were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% human B serum and 25 mM HEPES. Cultures were maintained at 37 ℃ under an atmosphere of 5% O_2, 5% CO_2, and 90% N_2[8]. The level of parasitemia in the cultures was maintained between 2 and 10%, with 5% hematocrit.

2.2. Preparation of drug solutions

Standard powder of tested antimalarial drugs, i.e., chloroquine, quinine, mefloquine and artesunate were prepared in 50% ethanol at the concentration of 10 nM. On the day of experiment, stock solutions were diluted with serum-free medium in order to obtain required concentrations (1 000, 4 000, 400 and 100 nM for chloroquine, quinine, mefloquine and artesunate, respectively).

2.3. In vitro antimalarial sensitivity test

The *in vitro* assessment of antimalarial activity of *P. falciparum* clones and isolates to chloroquine, quinine, mefloquine and artesunate was performed based on SYBR Green fluorescence-based assay[9]. Experiment was done in triplicate for each isolate/clone. Parasites used in all experiments were synchronized to the ring stage according to the method of Lambros and Vanderburg using 5% (w/v) sorbital[10]. For investigating antimalarial activity, an aliquot of parasite inoculum (10 μL) with 1% parasitemia and 20% hematocrit was added into each well of microtiter plate. The test wells consisted of varying concentrations of each drug. All experiments were performed in triplicate. The microtiter plates were placed in a candle jar with atmosphere consisting of 3% O_2, 90% NO_2 and 7% CO_2. Following a 48 hr incubation period, the plates were removed from the incubation box and 100 μL of the fluorescent hemolysis reagent was added to each well. The plates were then incubated at 37 ℃ for an additional 1 h for optimal development of fluorescent dye. Fluorescent intensity was then read by Varioskan Flash Machine (Thermo Electron Incorporation, USA) at the excitation and emission wave lengths of 485 and 530 nm.

The percent growth was compared between numbers of schizont in each drug-containing well and the control well. Concentration-response analysis was performed using Grafit™ (Erithacus Software Ltd., U.K) in order to obtain fifty percent inhibitory concentration (IC_{50}) values.

Threshold IC_{50} values for chloroquine, quinine, and mefloquine were 25 500 and 24 nM, respectively[11-12]. For artesunate, there is currently no defined threshold IC50 value.

2.4. Analysis of the amplification of PfGST and PfGR genes of P. falciparum isolates

Analysis of the copy numbers of the two regulatory gene controlling glutathione levels, *Pf*GST and *Pf*GR was performed and used as template to design primers for investigating copy numbers of these genes in all *P. falciparum* isolates and the reference clones. DNA of *P. falciparum* isolates was extracted from *in vitro* culture by chelex-resin modified technique[13].

Allele-specific oligonucleotides from the genes encoding *Pf*GR and *Pf*GST were selected according to the report of Ferreira and colleagues[14], using *Pf* β-actin as house keeping gene control. The forward and reverse primers for *Pf*GST, *Pf*GR and β-actin genes were as follows:

*Pf*GR: 5′-GCA GTG GCC TTA AAA ATG AAT G-3′ and 5′-GCT GTA GGA TGT ATA GGT ATG G-3′. The target fragment is 68 bp.

*Pf*GST: 5′-GAT GCA AGG GGT AAA GCT G-3′ and 5′-GGG TAC TTG CTC AAA AGG AG-3′. The target fragment is 150 bp.

Pf β-actin: 5′-GGA CAC ATA TTG TGC CTG C-3′ and 5′-CTC CAC TAT CTA ACA CAA TAC C-3′. The target fragment is 90 bp.

Real-time quantitative PCR was performed in DNA extracted from all parasite isolates using Platinum SYBR™ Green qPCR Supermix-UDG (Invitrogen, USA) and amplification of the copy numbers of *Pf*GR, *Pf*GST and β-actin genes was performed in the iCycler IQ (Bio-Rad, USA) using the default thermocycler program for all genes: 10 min of pre-incubation at 95 ℃, followed by 40 cycles for 15 sec at 95 ℃, and 1 min at 58 ℃. Individual real-time PCR reaction was carried out in 25 μL volume of the mixture of Platinum SYBR™ Green qPCR Supermix-UDG containing 1x buffer (10x), 3.5 mM $MgCl_2$, 200 μM dNTPs, 0.025 U/μL Platinum™ Taq DNA polymearse enzyme and 1:66000 SYBR Green™, 1 μM of sense and antisense primers, and DNA template (3 μL) in a 96-well plate (Genuine Axygen Quality, USA). At the end of each reaction, cycle threshold (Ct) was manually set up at the level that reflected the best kinetic PCR parameters and melting curves were acquired and analyzed. The $2^{-\Delta\Delta Ct}$ method of relative quantification was adapted to estimate copy numbers of *Pf*GR and *Pf*GST in *P. falciparum* genes[14]. Genomic DNA extracted from *P. falciparum* 3D7, known to harbor a single copy of *Pf*GR and *Pf*GST, was used as calibrator, while *Pf*-β-actin served as the house-keeping gene in all real-time PCR experiments. The ΔΔCt calculation for the relative quantification of target was used as follow: ΔΔCt = (Ct, target gene − Ct, *Pf*-β-actin) x − (Ct, target gene-Ct, *Pf*-β-actin)y, where x = unknown sample and y = *P. falciparum* 3D7). Result for each sample was expressed as N-fold changes in x target gene copies, normalized to *Pf*-β-actin relative to the copy number of the target gene in *P. falciparum* 3D7, according

to the following equation: amount of target = $2^{-\Delta\Delta Ct}$[14–15]. In each experiment, each individual sample was run in duplicate wells and the Ct of each well was recorded at the end of the reaction. Result for each sample was expressed as the N–fold copy number of a given gene relative to *P. falciparum* 3D7. Assay was repeated if amplification curve did not reflect exponential kinetic parameters or if the N–fold copy number of a given gene was lower than 0.7 or higher than 1.3. In case where N–fold was comprehended between those values (0.7 <N–fold< **1.3**), it was accepted that the test sample harbored a single copy of the target gene (N–fold = 1).

2.5. Statistical analysis

Non–parametric statistics were applied for analysis of data which were not normally distributed. Quantitative data were presented as median (range) values, whereas qualitative data were presented as number (%) values. The analysis of the association between genetic polymorphisms (gene amplification) of *Pf*GR and *Pf*GST and sensitivity of *P. falciparum* isolates to the tested antimalarials was performed using *Chi*–square test at a statistical significance level of α =0.5.

3. Results

3.1. Assessment of antimalarial drug sensitivity

Median (range) IC_{50} values of the 70 *P. falciparum* isolates for chloroquine, quinine, mefloquine and artesunate were 67.68 (9.4–189.86), 239.12 (35.10–870.80), 29.33 (3.4–105.44) and 1.91 (0.77–5.77) nM, respectively (Table 1). Median IC_{50} values of chloroquine, quinine, mefloquine and artesunate for the K1 clone were 128.68, 376.27, 10.43 and 1.91 nM, respectively. The corresponding median IC_{50} values for the 3D7 clone were 9.40, 58.57, 20.82 and 2.14 nM, respectively. Representative plots of concentration response curves for chloroquine, quinine, mefloquine and artesunate are shown in Figure 1. IC_{50} values for chloroquine, quinine and mefloquine were categorized based on the sensitivity level to sensitive and resistant groups. Sixty–eight isolates (97.14%) were classified as chloroquine–resistant isolates with median (range) IC_{50} value of 68.14 (26.33–189.86) nM; only 2 isolates (2.86%) were classified as chloroquine–sensitive isolates with median (range) IC_{50} value of 16.77 (9.40–24.14) nM. For quinine, 59 isolates (84.29%) were classified as quinine–sensitive isolates with median (range) IC_{50} value of 220.55 (35.10–494.60) nM; 11 isolates (15.71%) were classified as quinine–resistant isolates with median (range) IC_{50} value of 667.08 (501.49–870.80) nM. For mefloquine, 42 isolates (60.0%) were classified as mefloquine–sensitive isolates with median IC_{50} (range) value of 46.54 (24.33–105.44) nM; 28 isolates (40.0%) were classified as mefloquine–resistant isolates with median (range) IC_{50} value of 13.92 (3.40–22.63) nM.

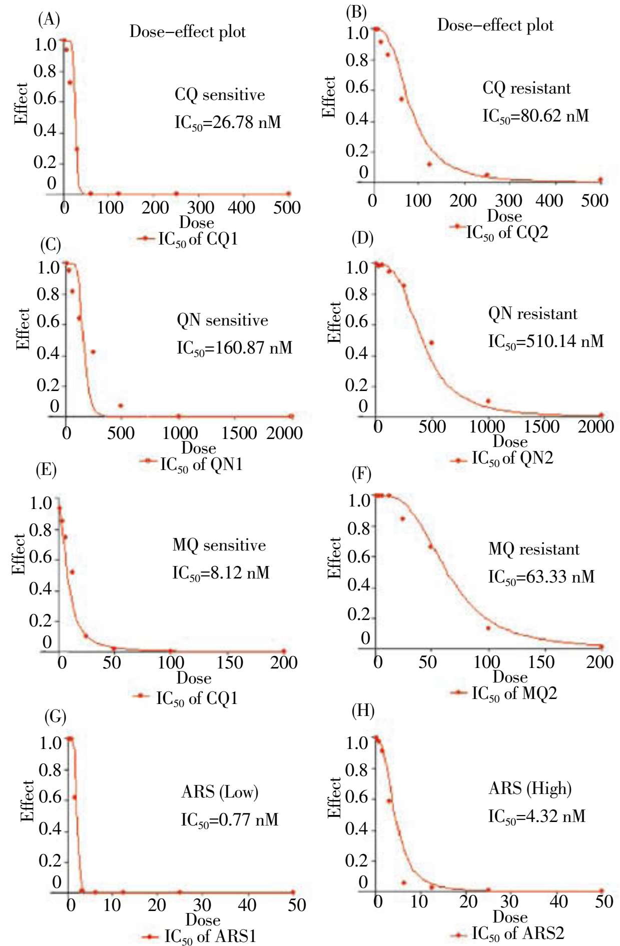

Figure 1. Representative plots of concentration response curves and IC_{50} values for chloroquine (CQ: A, B), quinine (QN: C, D), mefloquine (MQ: E, F) and artesunate (ARS: G, H) in sensitive and resistant isolates.

3.2. Association between amplification of PfGST and PfGR and sensitivity of P. falciparum isolates to antimalarial drugs

Based on PCR analysis, Dd2, K1 and 3D7 clones all contained only one copy of the PfGST gene. All isolates (n=70) also carried only one copy number of PfGST gene. For PfGR however, the 3D7 and Dd2 clones were found to carry only one gene copy, whereas the K1 clone carried two gene copies. Two isolates collected from Tak (Mae–Sot) and Ranong Provinces carried two *Pf*GR gene copies. The median IC_{50} values (from three experiments) of chloroquine, quinine, mefloquine and artesunate for these two isolates were 68.94 vs 109.38, 98.95 vs 258.12, 29.98 vs 17.31, and 0.93 vs 2.19 nM, respectively.

Table 1

Number (%) and median (range) IC_{50} values of chloroquine, quinine and mefloquine in *P. falciparum* isolates classified in sensitive and resistant groups.

Drug	Drug sensitivity test (n=70)			
	Sensitive group		Resistant group	
	Number (%)	Median (range) IC_{50} (nM)	Number (%)	Median (range) IC_{50} (nM)
Chloroquine	2 (2.86)	16.77(9.40–24.14)	68 (97.14)	68.14(26.33–189.86)
Quinine	59 (84.29)	220.55(35.10–494.60)	11 (15.71)	667.08(501.49–870.80)
Mefloquine	28 (40.00)	13.92(3.40–22.63)	42 (60.00)	46.54(24.33–105.44)

4. Discussion

In vitro sensitivity test for a total of 70 isolates collected from malaria endemic areas of multi-drug resistance (Tak and Ranong Provinces) during the year 2008-2009 was performed based on SYBR Green fluorescence-based assay[16]. This fluorescence-based assay is sensitive, accurate, reliable, and less expensive than radio-isotopic[17] and colorimetric-based histidine-rich protein 2 capture ELISA[18] methods and involves only one-step in one plate to obtain results. Due to the fact that mature erythrocytes lack RNA and DNA, binding of the dye is specific for malarial DNA in any erythrocytic stage of *P. falciparum* development[16].

Median (range) IC_{50} values of chloroquine, quinine, mefloquine and artesunate in a total of 70 *P. falciparum* isolates were 67.65 (9.4-189.86), 237.04 (35.10-870.80), 29.48 (3.4-105.44) and 1.90 (0.77-5.77) nM, respectively. Threshold IC_{50} values of 25, 500, and 24 nM were used to classify resistance to chloroquine, quinine and mefloquine, respectively. The sensitivity of *P. falciparum* isolates in Mae-Sot area to chloroquine and quinine appears to be gradually increased when compared with data published during the year 1991-1992[geometric mean (range) IC_{50}: 193.23 (148.24-251.85) nM], 1994[geometric mean (range) IC_{50}: 157.05 (124.11-198.73) nM], and 2003[geometric mean (range) IC_{50}: 120.50 (105.75-137.32) nM][19-20]. Nevertheless, chloroquine resistance has still remained in this area since 67 isolates (97.1%) were indentified as chloroquine-resistant isolates, whereas only 2 isolates (2.9%) were identified as chloroquine-sensitive isolates. The geometric mean (range) IC_{50} values of quinine during the years 1991-1992, 1994 and 2003 were 576.22 (460.51-720.99), 403.87 (330.650-493.54) and 382.34 (312.88-467.22) nM, respectively[19-20]. About 15.94% (11 isolates) were defined as quinine-resistant. For mefloquine however, sensitivity level seems to be improved, but the prevalence of mefloquine-resistant isolates was relatively high (60.8%). Mefloquine sensitivity was declined in 1994[geometric mean (range) IC_{50}: 95.48 (75.90-120.12) nM] and 2003[geometric mean (range) IC_{50}: 98.79 (79.72-122.42) nM] from that in 1991-1992[geometric mean (range) IC_{50}: 67.71 (46.77-96.48) nM][19-20], but was markedly improved during the year 2008-2009. Sensitivity to artesunate was more or less stable with IC_{50} values varying in the narrow range of 0.77-5.77 nM during the year 1991-2009. Although the *in vitro* results obtained from various studies using different methods may not be directly compared, results reported using both the radio-isotopic and colorimetric-based histidine-rich protein 2 capture ELISA methods[19-20] have been evaluated to provide comparable results with the fluorescence-based method used in the present study[21-22].

GSH appears to be relevant in heme detoxification. Chloroquine and amodiaquine inhibit GSH-mediated heme degradation. Involvement of the two GSH associated enzymes, i.e., *Pf*GST and *Pf*GR has been implicated in *P. falciparum* drug resistance. *Pf*GST is responsible for fighting reactive oxygen species and reduction of oxidative stress. Although the role of PfGST in drug detoxification process in *P. falciparum* is still unclear, several publications strongly support the hypothesis that the chloroquine-resistant mechanism in *P. falciparum* may involve an increase in *Pf*GST enzyme activity[24-26]. Kavishe and colleagues suggested that *Pf*GST polymorphisms could change the enzyme activity, leading to reduced detoxification of the host cell or increased availability of host GSH that might be used by the parasite[27]. There has been limited data on the amplification of *Pf*GST gene in *P. falciparum* isolates in different malaria endemic areas. Based on results obtained from the present study, no association between sensitivity of *P. falciparum* isolates to the four antimalarials and amplification of *Pf*GST gene was observed. No amplification of *Pf*GST gene was observed in any isolate; all carried only one gene copy.

*Pf*GR plays a key role in cellular redox homeostasis catalyzing the reduction of GSSG to GSH and creates an intracellular GSH/GSSG ratio[28]. *Pf*GR is an enzyme that is believed to have a significant role in malarial infection. Since the malarial parasite *P. falciparum* is known to be sensitive to oxidative stress, and thus the antioxidant enzyme *Pf*GR has become an attractive drug target for antimalarial drug development[29]. Nowadays, functional *P. falciparum* glutathione was correlated to human glutathione[30]. Comparing to human glutathione, the *Pf*GR has similar molecular functions as glutathione disulfide reductase activity, oxidoreductase activity, disulfide oxidoreductase activity and metal ion binding. In the present study, 3D7 and Dd2 *P. falciparum* clones (chloroquine-sensitive clones) were found to carry only one *Pf*GR gene copy, while K1 (chloroquine-resistant clone) contained two gene copies. Only two isolates (collected from Tak and Ranong Provinces) were found to carry two copies of PfGR gene. The *in vitro* sensitivity results revealed that apart from K1 chloroquine-resistant clone, both isolates (out of 68 chloroquine-resistant isolates) which carried two copies of *Pf*GR were classified as chloroquine-resistant. The present findings with *Pf*GST support the finding of Ferreira and colleagues who demonstrated that both chloroquine-sensitive (3D7) and chloroquine-resistant (Dd2) *P. falciparum* clones contain only one copy of this gene. However, result of the association with *Pf*GR gene was not in agreement since no isolate (a total of 8 isolates collected from the Democratic Republic of Sao Tomé and Principe (DRSTP, the islands in Western Central Africa) with double gene copies was found in that study[14]. Difference of endemic areas of sample collection and number of isolates should be the reason of this disagreement.

In conclusion, with this limited number of *P. falciparum* isolates included in the analysis, no significant association between amplification of *Pf*GST gene and sensitivity of the parasite to chloroquine, quinine, mefloquine and quinine was found. There appears to be the association between amplification of *Pf*GR gene and chloroquine resistance. Chloroquine resistance is likely to be a consequence of multi-factors and enzymes in the GSH system may be partly involved. Larger number of parasite isolates are required to increase power of the hypothesis testing in order to confirm the involvement of both genes in conferring chloroquine resistance. Analysis of other elements implicated in glutathione metabolism, i.e., γ-glutamyl-cysteinyl synthase, glutathione synthase, and glutathione transporter (MRP) should also be included in conjunction with the levels of reduced and oxidized glutathione.

Role of funding source

The study was supported by Thammasat University and The Commission on Higher Education, Ministry of Education of Thailand.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The study was supported by Thammasat University and The Commission on Higher Education, Ministry of Education of Thailand.

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