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Investigation on the involvement of glutathione (GSH) system in the mechanism of antimalarial action and its possible role in conferring resistance of *Plasmodium falciparum* to antimalarials

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Abstract

The oxidant enzymes of *Plasmodium falciparum* such as glutathione reductase (*Pf*GR) and glutathione *S*-transferase (*Pf*GST) may play important role in survival of malaria parasites including their possible involvement in conferring antimalarial drug resistance. This study was aimed to investigate the mutations of regulatory gene controlling glutathione level in a total of 34 *P. falciparum* isolates collected during 2006-2009 from endemic areas of multidrug resistance *P. falciparum* in Thailand. *In vitro* sensitivity testing of each *P. falciparum* isolate to the four antimalarial drugs, chloroquine (CQ), quinine (QN), mefloquine (MQ) and artesunate (ARS) was performed by using SYBR green modified assay, and 50% inhibitory concentration (IC_{50}) of each drug was determined. Real-time quantitative PCR was performed in DNA extracted from all parasite isolates using IQ SYBR green supermix. Allele-specific oligonucleotides from the gene encoding *Pf*GR and *Pf*GST were selected based on the coding sequence alignment of 3D7 parasites. The amplification of copy number of *Pf*GR and *Pf*GST gene were determined. Median (range) IC_{50} of CQ, QN, MQ and ARS were 86.33 (39.18-189.86), 387.79 (75.92-870.80), 45.87 (9.26-101.56) and 2.26 (0.83-5.21) nM, respectively. All isolates were resistant to CQ. Median (range) IC_{50} of the 24 QN-sensitive (70.6%) and 10 QN-resistant (29.4%) isolates were 262.63 (75.92-491.43) and 688.19 (520-870) nM, respectively. Median (range) IC_{50} of the 16 MQ-sensitive (47.1%) and 18 MQ-resistant (52.9%) isolates were 21.17 (9.26-32.75) and 67.82 (36.82-101.56) nM, respectively. None had more than one copy number of either *Pf*GR or *Pf*GST gene. No associations were observed between sensitivity of parasite isolates to the four antimalarials, and amplification of both *Pf*GR and *Pf*GST genes. Larger number of parasite isolates is required to increase power of the hypothesis testing in order to confirm this finding.

Keywords : *Plasmodium falciparum*, SYBR green assay, real-time PCR, glutathione reductase, glutathione S-transferase, gene amplification

Introduction

Malaria-infected erythrocytes are under oxidative stress produced by the digestion of host's hemoglobin. The oxidant enzymes such as glutathione reductase (*Pf*GR) and glutathione *S*-transferase (*Pf*GST) are detected in malaria-infected cells to reduce oxidative stress in the cell (1). Glutathione reductase (GR) belongs to the pyridine nucleotide-disulfide oxidoreductase family of homodimeric flavoenzymes which include lipoamide dehydrogenase and thioredoxin reductase. Both human GR (hGR) and *P.falciparum* GR (*PfGR*) are essential for the survival of the malarial parasite within the human erythrocyte. In addition, glutathione *S*-transferases (GST) also serves as the intracellular detoxification enzyme; it detoxifies lipid peroxidation products which lead to the inactivation and immobilization of these products (2). Recently, the association between the level of these enzymes in *P.falciparum* isolates and sensitivity of the parasites to chloroquine has been reported (3-4). This may imply the importance of these enzymes in survival of malaria

parasites, as well as their possible involvement in conferring antimalarial drug resistance (5). Compounds which act on these enzyme targets would eventually lead to parasite death, and thus, would be promising antimalarial drug candidates.

The aim of the study was to investigate the association between genetic polymorphisms of glutathione reductase (*Pf*GR) and glutathione *S*-transferase (*Pf*GST) in *P.falciparum* isolates collected from different malaria endemic areas of Thailand in 2009 and susceptibility of the parasites to currently available antimalarial drugs.

Methods

The mutations of regulatory gene controlling glutathione level was investigated in a total of 34 *P.falciparum* isolates collected from endemic areas of multidrug resistance in Thailand (Tak and Ranong Provinces) during the year 2006-2009. Prior to the study, approval of the study protocol was obtained from the Ethics Committee of Ministry of Public Health of Thailand. All *P.falciparum* isolates were adapted to culture *in vitro* according to the method of Trager and Jensen (6). *In vitro* sensitivity testing of each *P.falciparum* isolate to the four antimalarial drugs, chloroquine (CQ), quinine (QN), mefloquine (MQ) and artesunate (ARS) was performed using SYBR green modified assay (7), and 50% inhibitory concentration (IC_{50}) of each drug was determined. Threshold IC_{50} value of CQ, QN and MQ resistance were 30, 500 and 35 nM, respectively. There is no defined threshold IC_{50} value for ARS (8).

DNA sequences of gene controlling GSH level in K1 clone *P.falciparum* (CQ resistant clone) were determined. Comparison of these sequences with 3D7 *P.falciparum* clone from malaria genome project database were performed in order to search for allele-specific sequence(s) involving *Pf*GR and *Pf*GST gene. Allele-specific oligonucleotides from the gene encoding *Pf*GR and *Pf*GST were selected based on the coding sequence alignment of 3D7 parasites. Real-time quantitative PCR was performed in DNA extracted from all parasite isolates using IQ SYBR green supermix and amplification of copy number of *Pf*GR and *Pf*GST gene was performed. The $2^{-\Delta\Delta Ct}$ method of relative quantification was adapted to estimate copy numbers of *Pf*GR and *Pf*GR in *P.falciparum* genes (9). Chi-square test was used to assess significant association between sensitivity of parasite isolates and amplification of *Pf*GR and *Pf*GR genes.

Results

Median (range) IC_{50} values of CQ, QN, MQ and ARS were 86.33 (39.18-189.86), 387.79 (75.92-870.80), 45.87 (9.26-101.56) and 2.26 (0.83-5.21) nM, respectively. All isolates (100%) were resistant to CQ. Twenty-four isolates [70.6%: IC_{50} 262.63 (75.92-491.43) nM] and 10 isolates [29.4%: IC_{50} 688.19 (520-870) nM] were defined as QN-sensitive and QN-resistant, respectively. For sensitivity to MQ, 16 isolates [47.1%: IC_{50} 21.17 (9.26-32.75) nM] and 18 isolates [52.9%: IC_{50} 67.82 (36.82-101.56) nM] were categorized as MQ-resistant and MQ-sensitive, respectively (Table 1). All isolates carried only one copy number of either *Pf*GR or *Pf*GST gene.

Discussion

Results from *in vitro* sensitivity test suggest virtually 100% of CQ-resistant isolates. It is noted however for the improved sensitivity of CQ in these isolates compared with that was previously reported in isolates collected from the same area during 1994-1999. [median (range) IC_{50} 157.05 (105.75-198.73 nM)] (10-11). Sensitivity of parasite isolates to QN was more or less stable since 1994 [median IC_{50} 403.87 (312.88-493.54) nM] (10-11). Interestingly, sensitivity to MQ was markedly improved, with the IC_{50} reduced by about 50% since 1994 [median (range) IC_{50} 95.48 (75.90-122.42) nM] (10-11). For ARS, median (range) IC_{50} values were comparable with that reported during 1991-1992, 1994, and 1999 [2.58 (1.80-3.71), 3.39 (2.67-4.29), and 2.19 (1.67-2.87) nM, respectively] (10-11). No

isolate carried more than one copy number of *Pf*GR and *Pf*GST genes. No association was observed between sensitivity of parasite isolates to the four antimalarials, and amplification of both *Pf*GR and *Pf*GST genes. However, larger number of parasite isolates is required to increase power of the hypothesis testing in order to confirm this finding.

Table 1 Number and percentage of sensitive and resistant groups classified based on results from *in vitro* sensitivity test

Drug	Drug susceptibility test			
	Sensitive group		Resistant group	
	Number (%)	Median (Range) (nM)	Number (%)	Median (Range) (nM)
CQ	0 (0%)	-	34 (100%)	86.33 (39.18-189.86)
QN	24 (70.6%)	262.63 (75.92-491.43)	10 (29.4%)	688.19 (520-870)
MQ	16 (47.1%)	21.17 (9.26-32.75)	18 (52.9%)	67.82 (36.82-101.56)

Conclusion

In this study with limited number of *P.falciparum* isolates under investigation, lack of association between amplification of either *Pf*GR or *Pf*GST gene was observed. CQ resistance was virtually 100%, whereas MQ and QN resistance were 52.9 and 29.4%, respectively.

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References

1. Luersen K. *Plasmodium falciparum*-infected red blood cells depend on a functional glutathione de novo synthesis attributable to an enhanced loss of glutathione. Biochem J 2000; 346: 545-52.
2. Kavishe RA, Koenderink JB, Mccall MBB, Peters WHM, Mulder B, et al. Short report: Severe *Plasmodium falciparum* malaria in Cameroon: Associated with the glutathione S-transferase M1 null genotype. Am J Trop Med Hyg 2006; 75(5): 827–9.
3. Rojpibulstit P, Kangsadalampai S, Ratanavalachai T, Denduangboripant J and Chavalitshewinkoon-Petmitr P. Glutathione –*S*- transferase from chloroquine resistant and –sensitive strains of *Plasmodium falciparum*: What are their differences? Southeast Asain J Trop Med Public Health 2004; 35(2): 292-9.
4. Meierjohann S, Walter RD and Muller S. Regulation of intracellular glutathione levels in erythrocytes infected with chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. Biochem J 2002; 368: 761-8.
5. Bloland PB. Drug resistance in malaria. World Hlth Organ 2001; 5-15.
6. Trager W and Jensen JB. Human malaria parasites in continuous culture. Science 1976; 193: 674-5.
7. Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, and Waters NC. Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. Antimicrob Agents Chemother 2007; 51 (6): 1926–33.

8. Ceruttij JC, Marques C, Alencar FE, Durlacher RR, Alween A, Segurado AA, Pang LW, Zalis MG. Antimalarial drug susceptibility testing of *Plasmodium falciparum* in Brazil using a radioisotope method. Mem Inst Oswaldo Cruz 1999; 94: 803–9.
9. Ferreira ID, Rosário VE and Cravo PVL. Real-time quantitative PCR with SYBR green I detection for estimating copy numbers of nine drug resistance candidate genes in *Plasmodium falciparum*. Mal J 2006; 5: 401-8.
10. Wongsrichanalai C, Wimonwattrawatee T, Sookto P, Laoboonchai A, Heppner DG, Kyle DE, Wernsdorfer WH. *In vitro* sensitivity of *Plasmodium falciparum* to artesunate in Thailand. Bulletin of the World Hlth Organ, 1999; 77: 392-8.
11. Noedl H, Faiz MA, Yunus EB, Rahman MR, Hossain MA, Samad R, Miller RS, Pang LW, Wongsrichanalai C. Drug-resistant malaria in Bangladesh: An *in vitro* assessment. Am J Trop Med Hyg 2003; 68: 140–2.