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Sequence Analysis and Genotypes of Glutamate Rich Protein of *Plasmodium falciparum* Isolates from Different Malaria Endemic Areas in China

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Objective To sequence the gene encoding glutamate rich protein (GLURP) and identify the genotypes of geographically different *Plasmodium falciparum* (*P. f*) isolates from China. **Methods** The gene of R2 repeat region of GLURP was amplified by nested polymerase chain reaction and cloned into T-vector. The nucleotide sequence of GLURP gene was determined by automatic sequencer (Dideoxy termination method) and analyzed by DNA Star software. **Results** At least 7 different GLURP genotypes ranging from 600 bp to 1 500 bp were found in Yunnan and Hainan provinces. R2 region of GLURP gene consisted of several repeat units. Each repeat unit was composed of 19-20 residues which were shown to be highly conserved. GLURP gene was also size polymorphic due to differences in the number of repeat units, whereas the repeat sequence was conserved. Sequence analysis showed that DNA sequences and deduced amino acid sequences were highly homologous among the geographically dispersed isolates or various isolates from the same geographical region. No obvious differences were found in the GLURP gene sequences among geographically different isolates. **Conclusion** GLURP gene is highly structure conserved and size polymorphic, and so is useful in searching for malaria vaccine candidate antigen and developing a genotyping method for malaria research.

Key words: GLURP; *Plasmodium falciparum*; Malaria

INTRODUCTION

Glutamate rich protein (GLURP) is an exoantigen of *Plasmodium falciparum*. It is widely presented in the parasitophorous vacuole of the liver stage schizont, the erythrocytic schizont and on the surface of the released merozoite. Because GLURP is highly immunogenic and can be recognized by immune sera, it might be a promising vaccine candidate antigen of malaria vaccine.

This protein has been identified and gene for GLURP cloned[1]. It has been reported that vaccinia virus-expressed GLURP product or recombinant GLURP produced in *E. coli* can induce a humoral immune response against GLURP derived from blood-stage parasites[2,3]. Synthetic *P. falciparum* GLURP peptides have been used in evaluating

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antibody reactivities from different malaria endemic areas[4,5]. Result from Clinical research has shown that there is a strong correlation between protection against malaria attacks and levels of immunoglobulin against GLURP. Thus GLURP may play a role in the induction of protective immunity against *P. falciparum* malaria[6].

Moreover, knowledge of the nature and extent of genetic diversity within *P. falciparum* is essential in understanding the mechanisms underlying the pathology of malaria, the acquisition of immunity, and the development of a malaria vaccine. GLURP gene has been shown to be a good genetic marker in distinguishing different *P. falsiparum* genotypes[7].

Although some research work on GLURP has been done abroad, no report on this protein has ever been published in China so far. In the present report, we have cloned and sequenced the R2 region genes of GLURP of geographically different *P. falsiparum* from Yunnan and Hainan provinces of China. In addition, the genetic composition of *P. falsiparum* population in China by using GLURP gene as a genetic marker has been described for first time. The purpose of our research is to provide a theoretical basis for developing malaria vaccine and to establish a new malaria genotyping method in China.

MATERIAL AND METHODS

Sample Collection

Blood samples were collected from local resident patients living in Mengla County, Yunnan Province and Dongfang County, Hainan Province by finger-prick or venepuncture[8] respectively. Thin and thick blood smears were made before aliquoting the blood. The samples were stored on ice, transported to our laboratory and were stored at -40℃ for preparation of the DNA template. Those samples which were confirmed to be positive for *P. falciparum* by both microscopy and PCR assay were used in our experiments. Blood samples collected from healthy blood donors in non-endemic region were used as negative control.

DNA Template Preparation

DNA was purified as previously described[9]. Briefly, 200 μl aliquot blood was thawed on ice before being mixed with cold phosphate-buffered saline. The parasites were recovered by the centrifugation (5 min, 6 000 × *g*)following saponin lysis of the erythrocyte (final concentration 0.05%). After incubation in lysis buffer (10 mmol/L Tris-HCl, pH 8.0, 20 mmol/L EDTA, pH 8.0, 0.5% SDS and 0.5 mg/ml pronase E) 37℃ 4 hours, DNA was purified by phenol extraction and ethanol precipitation. The DNA was air dried and resuspended in TE buffer (10 mmol/L Tris-HCl, pH 8.0, 0.1 mmol/L EDTA, pH 8.0).

Primer Synthesis

The sequence of the oligonucleotide primers used in this study was designed on the basis of the published sequence of the GLURP gene of *P. falciparum*[11]. Two pairs of primers were employed for amplification of the polymorphic repeat region R2 of the GLURP gene. The sequences of the oligonucleotide primers were as follows:

G-OF:5'-TGAATTTGAAGATGTTCACACTGAAC-3'; G-OR:5'-GTGGAATTGCTTT TTCTTCAACACTAA-3'; G-NF:5'-TGTTCACACTGAACAATTAGATTTAGATCA-3'. The sequence of G-NR was the same as that of G-OR. Synthesis of the primers was performed by SBS company.

PCR Amplification and Product Analysis

In order to increase the sensitivity of genotyping, nested PCR amplification protocol was employed[8]. All PCR reactions were carried out in a total volume of 20 μl. Nested PCR amplification was performed by two amplification reactions. PCR amplification was carried out in 1 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.3, 0.1 mg/ml gelatin, 250 μl of each dNTP and 0.4 unit of Tag polymerase (gene company). GLURP gene includes 2 repeat regions, R1 and R2. In a first reaction, the region that spans the polymorphic region of GLURP-a region covers R1 and R2 sections was amplified by the primer pair G-OF and G-OR. Using as a template the product generated in the first reaction, the second nested reaction was then performed using the primer pair G-NF and G-OR in order to amplify R2 block of GLURP. Primers were used at an individual final concentration of 250 nmol/L. Amplifications were performed on a PTC-100 thermocycler (M. J Research Inc. USA). An initial denaturation period of 5 min at 95℃ preceded the amplification cycles: annealing for 2 min at 58℃, extension for 2 min at 72℃, denaturation for 1 min at 94℃. Twenty-five cycles were performed for the first reaction and 30 cycles for the nested reaction. The last extension was carried out for 5 min. After the addition of 5 μl of loading buffer to the amplified product, 15 μl were analysed by 1% agarose gel electrophoresis in TBE buffer. DNA was visualized and analyzed by gel documentation system (UVP GDS 8 000, U. K.) after staining with ethidium bromide.

Cloning and Sequencing

DNA fragments of 600 bp and 800 bp amplified from Yunnan and Hainan samples were purified (QIA quick PCR purification kit, Qiagen) and cloned into T-vector (PGEM-T vector system, Promaga). Plasmids with insert were selected by LacZ fusion protein and target DNA fragments were identified by PCR. The nucleotide sequences were determined with dideoxy chain termination method, using automatic sequencer.

DNA Sequence Analysis

The gene sequence was analyzed by DNA star software and GenBank database.

RESULTS

Genotyping of P. falciparum

The R2 polymorphic region of GLURP allele was amplified by the nested PCR for genotyping of *P. falciparum*. Conspicuous polymorphism of GLURP alleles in natural population was found. 55 GLURP alleles were detected in 55 different *P. falciparum* isolates from Yunnan and Hainan provinces. At least 7 different GLURP genotypes ranging from 600 bp to 1 500 bp were found among the above-mentioned alleles according to different DNA sizes. Of them, the most frequently found allele was a variant of 600 bp, more frequent was that of 800 bp, and the least allele was that of 1 500 bp (Table 1).

Characterization of P. falciparum GLURP -R2 Gene

To identify the inserts of the recombinant plasmids, positive plasmids from 4 isolates from Yunnan and Hainan were amplified by PCR using GIF and GOR primers. The PCR products showed that the amplified fragments corresponded to predicted insert size (Fig. 1).

TABLE 1

Genotyping of GLURP Allele From *P. falciparum* Isolates of Yunnan and Hainan Provinces

Genotype	Yunnan Province	Hainan Province
I (1 500 bp)	Nil	1
II (1 000 bp)	2	Nil
III (900 bp)	5	5
IV (800 bp)	7	6
V (750 bp)	1	4
VI (700 bp)	1	2
VII (650 bp)	12	9

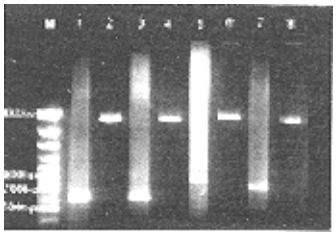

FIG. 1. PCR identification of recombinant plasmids with GLURP gene.
M is 100bp DNA marker (from big size to small size: 3 000bp, 2 000bp, 1 500bp, 1 200bp, 1 031bp, 900bp, 800bp, 700bp, 600bp, 500bp, 400bp).
Lanes 1, 3, 5, 7 were PCR products of Yunnan II, Hainan II, Yunnan I and Hainan I, respectively.
Lanes 2, 4, 6, 8 were cloning plasmids of Yunnan II, Hainan II, Yunnan I and Hainan I, respectively.

Sequence analysis showed that R2 region of GLURP gene consisted of several repeat units, each of them was composed of 19-20 residues which were shown to be highly conserved. DKNEKGQHEIVEVEEILPE or DKNEKGQHEIVEVEEILPED is presented as basic and often a repeat unit. In some units, GQ in the sixth and seventh position is replaced by VE and VQ. Sequence result showed that there was considerable length variation of R2 from isolate to isolate. For instance, sequence data obtained from R2 region of GLURP from Yunnan I, Yunnan II, Hainan I and Hainan II indicated that the size variation was due to differences in the number of repeat units whereas the repeat sequence was highly conserved. The number of repeat units in the R2 region of different isolates was different: that of Hainan I is 8, that of Yunnan I is 7. Both Hainan II and Yunnan II consist of 5 repeat units respectively. The flanking sequences of the R2 region in the above-mentioned 4 isolates was identical (Fig. 2).

Homology Comparison of GLURP-R2 Region From Geographically Different Isolates

The data in Table 2 indicated that DNA sequences and deduced amino acid sequences were highly homologous among various isolates from the same geographical region or from the geographically dispersed isolates. The result suggested that no obvious differences

were found in the GLURP gene sequences between Yunnan and Hainan provinces, and the GLURP gene of geographically different *P. falciparum* isolates was highly conserved.

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HainanI    N. I. I IKQLGPEIVEVEEEIPSELHENEVAHPEIVEIEEVFPEPNQNNEFQEINED   55
YunnanI    QLDLDHKTVDPEIVEVEEEIPSELHENEVAHPEIVEIEEVFPEPNQNNEFQEINED   55
HainanII   QLDLDHKPVDPRNSRS. RNSFRTX. K. SGSSRNC  N. GSFS. PNQNNEFQEINED   55
YunnanII   QLDLDHKTVDPEIVEVEEEIPSELHENEVAHPEIVEIEEVFPEPNQNNEFQEINED   55

HainanI    DKSAHIQHEIVEVEEEILPED DKNEKVEHEIVEVEEEILPE DKNEKGQHEIVEVEEEILPE   113
YunnanI    DKSAHIQHEIVEVEEEILPED DKNEKVEHEIVEVEEEILPE DKNEKGQHEIVEVEEEILPE   113
HainanII   DKSAHIQHEIVEVEEEILPED DKNEKVEHEIVEVEEEILPE DKNEKVQHEIVEVEEEILPE E   114
YunnanII   DKSAHIQHEIVEVEEEILPED DKNEKVEHEIVEVEEEILPE DKNEKVQHEIVEVEEEILPE   113

HainanI    DKNEKVQHEIVEVEEEILPE  DKNEKGQHEIVEVEEEILPE DKNEKVQHEIVEVEEEILPE   170
YunnanI    DKNEKVQHEIVEVEEEILPE  DKNEKVQHEIVEVEEEILPE  DKNEKGQHEIVEVEEEILPE   170
HainanII   DKNEKGQHEIVEVEEEILPED DKNEKGQHEIVEVEEEILPED DKNEKGQHEIVEVEEEILPE   173
YunnanII   DKNEKVQHEIVEVEEEILPED DKNEKVQHEIVEVEEEILPED DKNEKGQHEIVEVEEEILPE   172

HainanI    DKNEKGQHEIVEVEEEILPE  DKNEKAQHEIVEVEEEILPE E DKNEKGQHEIVEVEEEILPE   228
YunnanI    DKNEKVQHEIVEVEEEILPE E DKNEKGQHEIVEVEEEILPE ----------------------   209
HainanII   ---------------------- ---------------------- ----------------------   173
YunnanII   ---------------------- ---------------------- ----------------------   172

HainanI    IVEIEEVPSQTNNNENIETIKPEEKKNEFSVEEKAIP   265
YunnanII   IVEIEEVPSQTNNNENIETIKPEDKQE. I. C. RKSNS   246
HainanII   IVEIEEVPSQTNNNENIETIKPEEKKNEFSVEEKAIP   210
YunnanII   IVEIEEVPSQTNNNENIETIKPEEKKNEFSVEEKAIP   209
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FIG. 2. Comparison of deduced amino acid sequences of GLURP gene R2 region from geographically different *P. falciparum* isolates.
The sequences of the repeat units of 19-20 amino acid residues are underlined.
"-": represents deletion of amino acid.

TABLE 2

Homology Comparison of DNA Sequences and Deduced Amino Acids of GLURP Genes From Geographically Different *P. falciparum* Isolates in China

Isolates		Nucleotide Homology	Amino Acid Homology
Yunnan I (740 bp)	Yunnan II (628bp)	97.8%	91.4%
Hainan I (797 bp)	Hainan II (631 bp)	95.6%	76.7%
Yunnan I (740 bp)	Hainan I (797 bp)	96.8%	89.4%
Yunnan II (628bp)	Hainan II (631 bp)	96.7%	82.8%

DISCUSSION

After several decades of research on malaria vaccine, some effective vaccine antigens were identified, such as Merozoite Surface Protein 1[10], Merozoite Surface Protein 2[11] and Circumsporozoit Protein[12]. However, some obstacles remain to be cleared away

because of antigen variation and antigen complexity. Therefore, it is crucial to identify more effective antigens in developing malaria vaccine. Glutamate rich protein, being an antigen associated with mature schizont-infected erythrocyte, has recently been identified as a target antigen for antibodies involved in antibody-dependent cellular inhibition (ADCI)[13], which is believed to be involved in acquired protective immunity[14]. Overseas research showed that the cloned GLURP gene encodes a protein 1 271 amino acid residues long, with a calculated molecular weight of 145 kDa. Sequence of GLURP has two tandem repeats designated as R1 and R2. Human immune response against GLURP is primarily directed against R2 region which is highly immunogenic[15]. Results from immuno-epidemiological studies have recently shown that high levels of anti-GLURP antibodies correlate with a low grade of parasitemia[15,16] and absence of diseases[15]. Our research showed for the first time that the R2 region of *P. falsiparum* GLURP is highly conserved in isolates from different geographical areas in China. Compared with published data, the DNA sequences and deduced amino acid sequence of Chinese isolates are highly homologous with that of overseas isolates[1,17]. The conservation of sequence characteristic of R2 region possesses an advantage in vaccine design, which will be of benefit in the wildly use of malaria vaccine in different endemic areas in China. Therefore, if the GLURP R2 region elicits a biologically active antibody response, it might evoke an anti-parasitic response against a broad range of isolates on a global scale. Recent research results have also shown that levels of IgG1 and IgG3 antibodies against N-terminal region (R0) and R2 region of GLURP, respectively, are significantly correlated to clinical protection from *P. falciparum* in Ghanaian children[16]. Cytophilic antibodies against the R0 and the R2 regions of GLURP contribute to the development of clinical immunity in West African children[18]. The potential importance of anti-GLURP antibodies for immunity to disease is further indicated by the observation that human IgG antibodies against GLURP promote a strong antibody-dependent monocyte-mediated inhibition (ADCI) of parasite growth *in vitro*[13]. Thus, the mechanism of GLURP R2 region in the induction of protective immune responses is worth further studying.

Furthermore, assessment of gene polymorphism in *P. falciparum* vaccine candidate antigens is a relevant consideration in the development of a malaria vaccine. Our research has also found that R2 sequence of GLURP is size polymorphic and there is a considerable length variation of R2 among Chinese isolates. The size variation is due to differences in the number of repeat units whereas the repeat sequence is highly conserved. Based on polymorphic feature GLURP R2 gene can be considered as an useful genetic marker for genotyping field population of *P. falciparum*[19]. Genotyping is a powerful tool for epidemiological surveillance, drug resistance studies and vaccine assessment[20,21]. We can believe that the method of genotyping will facilitate efficient control of malaria in China.

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REFERENCES

1. Borre, M. B., Dziegiel, M., Hogh B., Petersen, E., Rieneck, K., Riley, E., Meis, J. F., Aikawa, M., Nakamura, K., Harada, M., Wind, A., Jakobsen, P. H., Cowland, J., Jepsen, S., Axelsen, N. H. and Vuust, J. (1991). Primary structure and

localization of a conserved immunogenic *Plasmodium falciparum* glutamate rich protein (GLURP) expressed in both the preerythrocytic and erythrocytic stages of the vertebrate life cycle. *Mol. Biochem. Parasitol.* **49**, 119-131.
2. Theisen, M., Cox, G., Hogh, B., Jepsen, S., and Vuust, J. (1994). Immunogenicity of the *Plasmodium falciparum* glutamate-rich protein expressed by vaccinia virus. *Infect. Immun.* **62** (8), 3270-3275.
3. Theisen, M., Vuust, J., Gottschau, A., Jepsen, S. and Hogh, B. (1995). Antigenicity and immunogenicity of recombinant glutamate-rich protein of *Plasmodium. falciparum* expressed in *Escherichia coli. Clin. Diagn. Lab. Immunol.* **2** (1), 30-34.
4. Jakobsen, P. H., Theander, T. G., Hviid, L., Morris, J. S., Jensen, J. B., Bayoumi, R. A., Greenwood, B. M., Bygbjerg, I. C. and Heegaard, P. M. (1996). Antibody reactivities to glutamate-rich peptides of *Plasmodium falciparum* parasites in humans from areas of different malaria endemicity. *APMIS.* **104** (10), 734-740.
5. Dziegiel, M., Rowe, P., Bennett, S., Allen, S. J., Olerup, O., Gottschau, A., Borre, M. and Riley, E. M. (1993). Immunoglobulin M and G antibody responses to *Plasmodium falciparum* glutamate-rich protein: correlation with clinical immunity in Gambian children. *Infect. Immu.* **61** (1), 103-108.
6. Oeuvray, C., Theisen, M., Rogier, C., Trape, J. F., Jepsen, S., and Druilhe, P. (2000). Cytophilic immunnoglobulin responses to *Plasmodium falciparum* glutamate-rich protein are correlated with protection against clinical malaria in Dielmo, Senegal. *Infect. Immun.* **68** (5), 2617-2620.
7. Viriyakosol, S., Siripoon, N., Petcharapirat, C., Petcharpirat, P., Jarra, W., Thaithong, S., Brown, K. N. and Snounou, G. (1995). Genotyping of *Plasmodium falciparum* isolates by the polymerase chain reaction and potential uses in epidemiological studies. *Bulletin of the World Health Organization* **73**, 85-95.
8. Zhu, X. P., Liu, Q., Zhou. L., Jiang, H. J., Niu, C. (1999). Detection of *Plasmodium falciparum* and mixed infection by polymerase chain reaction. *Chinese J. Parasitic Disease Control* **12** (1), 16-19 (in Chinese).
9. Snounou, G., Viriyakosol, S., Jarra, W., Thaithong, S., and Brown, K. N. (1993). Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infection. *Mol. Biochem. Parasitol.* **58**, 283-292.
10. Holder, A. A. (1988). The precursor to major merozoite surface antigens: structure and role in immunity. *Prog. Allergy* **41**, 72-97.
11. Clark, J. T., Donachie, S., Anand, R., and Wilson, C. F. (1989). 46-53 kilodalton glycoprotein from the surface of *Plasmodium. falciparum* merozoites. *Mol. Biochem. Parasitol.* **32**, 15-24.
12. Nussenzweig, V. and Nussenzweig R. S. (1985). Circumsporozoite proteins of malaria parasites. *Cell* **42**, 401-404.
13. Theisen, M., Soe, S., Oeuvray, C., Thomas, A. W., Vuust, J., Danielsen, S., Jepsen, S., and Druilhe, P. (1998). The glutamate-rich protein (GLURP) of *Plasmodium. falciparum* is a target for antibody-dependent monocyte-mediated inhibition of parasite growth *in vitro*. *Infec. Immun.* **66** (1), 11-17.
14. Bouharoun, T. H. and Druilhe, P. (1992). *Plasmodium falciparum* malaria: evidence for an isotype imbalence which may be responsible for delayed acquisition of protective immunity. *Infec. Immun.* **60**, 1473-1481.
15. Hogh, B., Petersen, E., Dziegiel, M., David, K., Hanson, A., Holm, A., Vuust, J., and Jepsen, S. (1992). Antobodies to a recombinant glutamate-rich *Plasmodium falciparum* protein: evidence for protection of individuals living in a holoendemic area of Liberia. *Am. J. Trop. Med. Hyg.* **46** (3), 307-313.
16. Boudin, C., Chumpitazi, B., Dziegiel, M., Peyron, F., Picot, S., Hogh, B. and Ambroise-Thomas, P. (1993). Possible role of specific immunoglobin M antibodies to Plasmodium falciparum antigen in immunoprotection of humans living in a hyperendemic area, Burkina Faso. *J. Clin. Microbiol.* **31**, 636-641.
17. Zhu, X. P., Snounou, G., Jarra, W. Thaithong, S., and Brown, K. N. (1998). Genotyping of *Plasmodium falciparum* isolates by amplification of glutamate-rich protein gene using polymerase chain reaction. *Chinese Journal of Parasitology and Parasitic Diseases* **16** (5), 331-334 (In Chinese).
18. Dodoo, D., Theisen, M., Kurtzhals, J. A. L., Akanmori, K. A. K., Jepsen. S., Nkrumah, F. K., Theander, T. G., and Hviid, L. (2000). Naturally acquired antibodies to the glutamate-rich protein are associated with protection against *Plasmodium falciparum* malaria. *J. Infect. Dis.* **181** (3), 1202-1205.
19. Snounou, G., Zhu., XP., Siripoon, N., Jarra, W., Thaithong, S., Brown, KN., and Viriyakosol, S. (1999). Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans. Roy. Soc. Trop. Med. & Hyg.* **93**, 369-374,
20. Jelinek, T., Kilian, A. H., Westermeier, A., Proll, S., Kabagambe, G., Nothdurft, H. D., von Sonnenburg, F., and Loscher, T. (1999). Population structure of recrudescent *Plasmodium falciparum* isolates from western Uganda. *Trop. Med. Int. Health* **4** (7), 476-480.
21. Haywood, M., conway, D. J., Weiss. H., Metzger, W., D'Alessandro, U., Snounou, G., Targett, G., and Greenwood, B. (1999). Reduction on the mean number of *Plasmodium falciparum* genotypes in Gambian children immunized with the malaria vaccine SPf66. *Trans. R. Trop. Med. Hyg.* **93** (Suppl. 1), 65-68.

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