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Characterization of a novel merozoite surface protein of *Plasmodium vivax*, Pv41

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ABSTRACT

Since the genome of *Plasmodium vivax* was sequenced, few proteins have been characterized as highly immunogenic and candidates for inclusion in a vivax malaria vaccine. The *P. vivax* 41 (Pv41) protein has a signal peptide, one glutamate-rich domain in its central region, and two sexual stage s48/45 domains, and is characterized as a gametocyte surface protein; however, this protein may be expressed principally on the merozoite surface of parasites. The previous study reported the transcription, blood-stage expression, and subcellular localization of Pv41 within the parasite. In this study, the recombinant Pv41 protein was expressed as a soluble form, of a molecular mass ~44 kDa, by a cell-free expression system and was specifically recognized by animal immune sera and vivax patient sera. Evaluation of the human humoral immune response to Pv41 indicated a high immunogenicity, with 62.5% sensitivity and 95% specificity, by protein array. Immunofluorescence assays (IFA) using polyclonal anti-Pv41 antibodies showed that Pv41 was localized on the merozoite surface. The high immunogenicity of Pv41 indicates its potential as a vivax malaria vaccine candidate antigen, particularly in light of its location on the merozoite surface of the parasite.

1. Introduction

Plasmodium vivax is a neglected tropical disease (Carlton et al., 2011), and only two *P. vivax* vaccines are at present in preliminary (phase I) clinical trials (Herrera et al., 2007). Understanding of the functions of *Plasmodium* spp. proteins and their detailed characterization are considered effective ways to develop a malaria vaccine. Complete genome sequencing and profiling of the transcriptomes and proteomes of the various developmental stages of *Plasmodium* spp., might lead to a greater understanding of the parasite–erythrocyte interaction. With the development of bioinformatics tools and updating of the PlasmoDB database, potential antigenic *P. vivax* target proteins have been identified based on orthologous *Plasmodium falciparum* stage-specific vaccine candidates (Chen et al., 2010).

To date, 12 distinct members of the s48/45 protein family (Pfs230, Pfs48/45, Pfs230p, Pfs47, P52, P36, Pf41, Pf38, Pf12, P12p, Pf92, and sequestrin) have been found to possess in *P. falciparum* parasites. These were identified in studies of the antigens coating the surface of merozoites (the asexual stage parasite for invasion of erythrocytes) and gametocytes (the sexual stage parasite in mosquitoes) (Gerloff et al., 2005; Sanders et al., 2007). Two major proteins (Pfs230 and Pfs48/45) containing s48/45 domains are expressed by *Plasmodium* male or female gametes, and specific antibodies against the s48/45 domain including 6-cysteine (Cys) residues, inhibited parasite development (Eksi et al., 2006; Gilson et al., 2006; Kumar, 1987; van Dijk et al., 2001). Thus Pfs48/45 and Pfs230 are current targets of malaria transmission-blocking vaccine development (Chowdhury et al., 2009; Williamson, 2003).

The unique 6-Cys protein family members are present on the parasite surface during each extracellular life-cycle stage, and those on the surface of liver-infective and sexual-stage parasites, have been also shown to play important roles in hepatocyte growth and fertilization, respectively (van Dijk et al., 2001). About half of the 6-Cys family members (Pf12, Pf38, Pf47, Pf48/45, Pf52, and PFF0620c) characterized to-date possesses glycosylphosphatidylinositol (GPI) moieties that anchor them to the plasma membrane of the merozoite surface. Among them, Pf41 is not GPI-anchored, but antibodies generated to the relatively long spacer region between its s48/45

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domains (as well as to its 6-Cys domains) indicated its surface expression and potential for inhibiting erythrocyte invasion (Garcia et al., 2009; Gilson et al., 2006). Furthermore, Pf41 is strongly recognized by immune sera from naturally infected patients (Sanders et al., 2005). The 6-Cys domain proteins are generating particular interest as vaccine candidates because of their presence on the surfaces of the various stages of parasites.

Antibodies are likely to play a key role in protection through several mechanisms, including inhibition of parasite invasion, intra-erythrocytic parasite blockage, and mononuclear cell-mediated inhibition (Richie and Saul, 2002). Previously, based on the sequences of the signal peptide (SP), transmembrane domain (TM), or orthologs of well-known *P. falciparum* proteins, we screened 89 blood-stage candidate proteins of *P. vivax* parasites using 20 vivax patients sera samples (Chen et al., 2010). We identified several immunoreactive proteins, one of which was Pv41.

The previous study reported the basic characteristics of Pv41 (Angel et al., 2008); however, its immunogenicity and exact localization remained unclear since it was not compared to a known candidate protein. In this study, we successfully expressed and purified a soluble form of recombinant Pv41 (rPv41) using a wheat germ cell-free (WGCF) protein expression system for both antigenic and immunogenic characterization and determination of its localization. Evaluation of the human immune response against this purified rPv41 using sera from 112 vivax patients and 80 healthy subjects from the Republic of Korea (ROK) showed that Pv41 possessed high immunogenicity. Animal immune serum generated against this protein exhibited a high titer and interacted specifically with both the recombinant and native proteins, confirming Pv41's merozoite surface localization and suggesting its role in the invasion of target cells.

2. Materials and methods

2.1. *P. vivax* infected blood samples and serum preparation

A total 112 blood samples were collected from vivax malaria patients (mean age, 24.5 years; range, 18–42 years) who experienced symptoms and were positive for vivax malaria by microscopy (mean parasitemia, 0.117%; range, 0.010–0.46%) at local health centers and clinics in Gyeonggi and Gangwon Provinces, endemic areas of the ROK. Eighty blood samples from healthy individuals; *i.e.*, those negative by microscopy, were collected in non-endemic areas of ROK. Serum samples were purified whole blood and used for this study. This study was approved by the Institutional Review Board at Kangwon National University Hospital.

2.2. Enrichment of parasite infected erythrocytes for parasite antigen

P. vivax infected blood samples were collected from patients and parasite infected erythrocyte were purified by previous study (Fernandez, 2008). Infected patients samples were used for removal of white blood cells by Plasmodifur filter (Euro-diagnostica, Arnhem, The Netherlands) and resuspended in RPMI-1640 medium to make a 10% suspension of erythrocytes. Parasite infected erythrocytes were purified by 60% Percoll-gradient centrifugation and used for parasite antigens for Western blot analysis.

2.3. Sequence alignment

Pv41 gene sequence data and gene expression profiles were analyzed using the resources provided by the PlasmoDB website (http://PlasmoDB.org; accession no. PVX_000995). Predicted protein domains were further analyzed using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/) and SOSUI signal (http://bp.nuap.nagoya-u.ac.jp/sosui/).

P. vivax genomic DNA was extracted from one of the *P. vivax* Korean isolates using a QIAamp DNA blood kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and used for PCR amplification of the Pv41 gene. The open reading frame (ORF) encoding the *pv41* gene, PVX_000995 (http://www.plasmoDB.org), was amplified by PCR from genomic DNA and cloned into the pEU-E01-His-Tev-N2 plasmid vector (CellFree Sciences, Matsuyama, Japan), which is an expression plasmid with a N-terminal hexahistidine (His)-tag followed by a tobacco etch virus (TEV) protease cleavage site. This plasmid was designed for the WGCF protein expression system. The primers used for In-Fusion cloning (Clontech, Palo Alto, CA) were as follows: primers Pv41-F (5′-gggcggatat*CTCGAG*GAACACATCTGCGATTTTACGA-3′) and Pv41-R (3′-gcggtacccg*GGATCC*CTACTCCTGGAAGGACTTGGC-5′). The vector sequences were lower case letters, and the *Xho*I and *Bam*HI sites were italicized with underline. The PCR reaction system contained 0.5 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA), 0.2 μM of each sense and antisense primer, 50 ng/μl of genomic DNA, 200 μM deoxyribonucleotide triphosphates (dNTPs), and $MgSO_4$ to a final concentration of 2.0 mM. The gene targets were amplified in parallel, beginning with an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 3 min, then a final extension at 68 °C for 10 min. The inserted nucleotide sequence was confirmed using the ABI PRISM 310 Genetic Analyzer and a Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA). Sequence alignments were carried out using the Lasergene software (DNASTAR, Madison, WI, USA), and protein sequence alignments and analysis with the MegAlign option.

2.4. Expression and purification of recombinant Pv41

We expressed and purified recombinant Pv41, which lacked the signal peptide (ΔSP), using a WGCF expression system (Tsuboi et al., 2010). Highly purified plasmid DNA is required for *in vitro* transcription and subsequent translation. Plasmid DNA was then prepared using the Maxi Plus™ Ultrapure plasmid extraction system (Viogene, Taipei, Taiwan) according to the manufacturer's instructions. Purified plasmid DNA was eluted in 0.1× TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and used for *in vitro* transcription for recombinant protein expression with the WGCF translation system. The rPv41 proteins were expressed using a WGCF system and purified using a Ni-Sepharose column under non-denaturing conditions as described elsewhere (Arumugam et al., 2011).

2.5. SDS-PAGE and Western-blot analysis

P. vivax parasites from patient blood samples were harvested and the parasite proteins were then extracted in SDS-PAGE loading buffer. The recombinant Pv41 protein and parasite proteins were separated by 12% SDS-PAGE under reducing conditions. The separated protein was transferred onto a 0.45 μm PVDF membrane (Millipore, Billerica, MA) in a semi-dry transfer buffer (50 mM Tris, 190 mM glycine, 3.5 mM SDS, 20% methanol) at a constant 400 mA for 40 min using a semi-dry blotting system (ATTO Corp., Tokyo, Japan). After blocking, anti-penta His antibody, mouse immune sera, or mixed patient sera diluted 1:200 into PBS/T and secondary IRDye® goat anti-mouse (1:10,000 dilution) or IRDye® goat anti-human (1:20,000) (LI-COR® Bioscience, Lincoln, NE, USA) antibodies were used to detect His-tagged recombinant protein and thus determine immune serum specificity. Data were obtained with