In silico analysis for structure, function and T-cell epitopes of a hypothetical conserved (HP-C) protein coded by PVX_092425 in *Plasmodium vivax*

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Objective: *Plasmodium* spp. merozoite glycosylphosphatidylinositol-anchored proteins (GPI-APs) considered as protective immunogen in novel vaccines against malaria. To analyze the structure and function of a hypothetical conserved (HP-C) GPI-AP coded by gene PVX_092425 from *Plasmodium vivax*, and find its potential T-cell epitopes for further vivax malaria vaccine study.

Methods: The structure, function and T-cell epitopes of the HP-C protein named Pvx_092425 were analyzed and predicted by online and offline bioinformatics software.

Results: The bioinformatics data showed that the Pvx_092425 is an 830 amino acid (AA) long polypeptide encoded by five exons gene PVX_092425. It contains a pectin lyase-like superfamily, an AA repeats region, a cys-rich region and a transmembrane domain (TM) in C-terminal region. The alignment analysis drew it has a unique AA repeats region among *Plasmodium* spp. It was located in the cytoplasm, secretory system or cellular nucleus of *P. vivax* merozoite. For the sequence, the fragment of 1823–V829 inserts in the interior side of the membrane, and M1–A812 belongs to the cytoplasmic tail. It has seven protein–protein binding sites. The peptides with the best predicted binding affinities were human leucocyte antigen (HLA) HLA-A*0203, HLA-DRB1*0101 and HLA- DRB1*0701. Among these predicted peptides, 582FLWDKALFD590 epitope interacted with HLA-DRB1*0101 allele showed best binding affinity compared to others. Structural analysis explained that the epitope fits well into the epitope-binding groove of HLA-DRB1*0101.

Conclusions: It proposes that the Pvx_092425 plays a key role during erythrocyte stage and generates information that is useful for development of blood-stage vaccine to block the merozoites invasion.

Keywords: *Plasmodium vivax*, Glycosylphosphatidylinositol, Hypothetical conserved, Human leucocyte antigen, Molecular docking, T-cell epitope

Introduction

As a significant global public health problem, malaria remains one of the leading causes of morbidity and mortality in tropical and subtropical regions.¹ *Plasmodium vivax* is the major malaria agent outside Africa, and half the world's population is estimated to be at risk.^{2,3} Increasing and spreading prevalence of antimalarial drug resistance in *P. vivax* hinder vivax malaria control and elimination. Distribution and emergence of more hazardous varieties of vivax malaria emphasizes the global expectation for development of a logical and rational approach for vaccine against the disease.

Cell surface membrane proteins constitute an important class of biomacromolecules in living cells

as they are in the interface with the surrounding environment.⁴ Numerous mammalian proteins have a special posttranslational modification at their carboxy-terminal known as the glycosylphosphatidylinositol (GPI) anchor, which serves to attach the proteins to the extracellular leaflet of the cell membrane.^{5,6} GPI-anchored proteins (GPI-APs) act as surface coat proteins, receptors, adhesion molecules, ectoenzymes, differentiation antigens and adaptors and may also be involved in intracellular sorting and transmembrane signalling processes.⁴ The GPI-APs represent an interesting amalgamation of the three basic kinds of cellular macromolecules, namely proteins, lipids (phosphatidylinositol group) and carbohydrates moiety (trimannosyl-non-acetylated glucosamine), which in turn is linked through a phosphodiester bond to the carboxy-terminal amino acid (AA) of the mature protein.⁵ Anchoring to the lipid bilayer confers

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the GPI-APs a number of physicochemical properties that are shared with intrinsic plasma membrane proteins. All known GPI-APs contain common features including the lack of transmembrane domains (TMs), a cleavable N-terminal secretion signal peptide (SP) for translocation into the endoplasmic reticulum (ER) and a predominantly hydrophobic region in the C-terminus, which most likely forms a transient TM and functions as a recognition signal for a transamidase.⁴ The enzyme recognizes and processes the C-terminal hydrophobic tail of the nascent protein at the so-called 'omega-site' and transfers the nascent protein to a presynthesized GPI anchor. The cleavage site (between omega and omega + 1 AAs) is a short distance upstream of the hydrophobic region and usually comprised three AAs with small side chains.⁴ Analysis of native GPI-APs and site-directed mutagenesis studies has shown that there are certain sequence constraints for the omega-site. Based on such features, a number of bioinformatic methods for prediction of GPI-APs have been reported.^{4,7-9}

For *Plasmodium falciparum* parasite, the surfaces of the various extracellular forms of the merozoite, gamete, ookinete and sporozoite are coated by different proteins that are either known or predicted to be GPI-APs, which consider as protective immunogen in novel vaccines against malaria.¹⁰ Available information points out that the GPIs of *P. falciparum* are the specific and dominant parasite-associated molecular patterns recognized by the host innate immune system.¹¹ The parasite GPIs appear to be mainly responsible for the ability of parasite to induce potent proinflammatory responses in monocytes and macrophages, thereby play a key role in malaria pathogenesis and thus constitute promising vaccine candidates.¹¹ The GPI-APs also can elicit strong immune responses that appear to play a critical role in acquired and/or vaccine-induced immunity.¹⁰ For these reasons, antimalarial vaccines incorporating recombinant GPI-APs are presently being developed to protect against malaria.

A number of GPI-APs have been characterized and their functions and potential as vaccines are currently being explored. The GPI-APs of *P. falciparum* have been found to participate in erythrocyte invasion by *Plasmodium* merozoites, such as merozoite surface protein 1 (PfMSP-1).¹² The proteomic analysis of merozoite surface proteins of *P. falciparum* signals that only 11 proteins represent approximately 94% of the GPI-anchored proteomes.¹⁰ Several GPI-APs including PfMSP-1, PfMSP-4, PfMSP-5, PfMSP-8, PfMSP-10, Pf34, Pf38, Pfs25, Pfs28 and PfCSP are candidates for inclusion in a blood-stage malaria vaccine.¹³⁻¹⁵ When the whole genome of the *P. vivax* Salvador I (*Sal I*) strain was sequenced, 31 putative GPI-APs of *P. vivax* were identified as GPI-Hidden Markov Model, which had comparable similarity with predicted 30 members of GPI-APs in

P. falciparum.¹⁶ Among them, 12 GPI-APs (PvMSP-1, PvMSP-4, PvMSP-5, PvMSP-8, PvMSP-10, Pv12, Pv34, Pv38, Pvs25, Pvs28, PvCSP and PvRAMA) have been characterized and some of them have been considered as potential vaccine candidates.¹⁷⁻²⁵ However, the other *P. vivax* GPI-APs including a hypothetical conserved (HP-C) protein named Pvx_092425 coded by gene PVX_092425 still remain uncharacterized and their precise role also stay unclear. To find novel vaccine candidates of *P. vivax*, the identification and characterization of new antigens among GPI-APs of *P. vivax* are considered to be one of the promising strategies.

In our previous study, four GPI-APs including MSP8, MSP10, Pv12 and Pvx_092425 were recognized by IgG antibody in *P. vivax* infected patient sera by protein array.²⁴⁻²⁶ MSP8, MSP10 and Pv12 were recently identified, and the recombinant proteins were highly recognized by *P. vivax* infected patient sera.^{24,27,28} In contrast to the apical and peripheral classes of blood-stage antigens, the GPI-APs appear to be essential to blood-stage growth, together with considerable data highlighting their potential as targets of protection antibodies, places the merozoite GPI-APs among the most highly validated blood-stage vaccine targets.²⁹ This study aims to design epitope-based peptides of HP-C protein Pvx_092425 for the utility of vivax malaria vaccine development. Therefore, the structure, function and T-cell epitopes in human beings of Pvx_092425 were analysed by online and offline bioinformatics methods.

Materials and Methods

Bioinformatics analysis

Nucleotide and amino acid sequences used in the present study have been reported in PlasmoDB (<http://plasmodb.org>) under Gene ID PVX_092425. Once the entire putative open reading frame was assembled, searches for similar protein sequences were carried out by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with default search parameters.

Multiple sequence alignments and phylogenetic analysis were conducted by ClustalW programme.^{30,31} The physiological-biochemical property, SP, TM, subcellular localization and secondary structure of the protein were predicted based on <http://www.expasy.org/tools/protparam.html> <http://www.cbs.dtu.dk/services/SignalP/>, <http://www.cbs.dtu.dk/services/TMHMM/>, <http://psort.nibb.ac.jp/form2.html> and <http://www.predictprotein.org/>, respectively. The Simple Modular Architecture Research Tool (SMART)^{32,33} was used to identify conserved protein domains in Pvx_092425.

Homology modelling

Search for sequential homologues to Pvx_092425 was executed by using crystal structure reported on PDB/BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).³⁴

Three-dimensional (3D) model was constructed and analyzed with SPDBV programme.

Peptide predication

Computation analysis of available Pvx_092425 was performed to find potential T-cell epitopes using MHCpred version 2.0 (<http://www.ddg-pharmfac.net/mhcpred/MHCPred/>).³⁵ The selected alleles for binding affinity prediction were human leukocyte antigen (HLA) including major histocompatibility complex (MHC) class I alleles: HLA-A*0101, HLA-A*0201, HLA-A*0202, HLA-A*0203, HLA-A*0206, HLA-A*0301, HLA-A*1101, HLA-A*3101, HLA-A*6801, HLA-A*6802 and HLA-B*3501 and MHC class II alleles: HLA-DRB1*0101, HLA-DRB1*0401 and HLA-DRB1*0701. The peptides with best confidence of prediction (Maximum=1) and high binding affinities for each allele were listed.

HLA-epitope-binding prediction

The online software PEP-SiteFinder 1.0 (<http://mobile.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=PEP-FOLD#forms:PEP-SiteFinder>) was employed for docking purpose. For the docking study, the 3D structure of selected epitopes were designed using the PEP-FOLD server: *De novo* peptide structure prediction (<http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD/>).^{36–38} The crystallographic structure of a rheumatoid arthritis MHC susceptibility allele HLA-DR1 (DRB1*0101) (PDB ID: 2FSE) was downloaded from the Protein Data Bank Database (www.rcsb.org/) and then uploaded to the website. All parameters are set to the default value in the online software PEP-SiteFinder 1.0.

Results

Physiological and biochemical characters of Pvx_092425

For Pvx_092425, the accession number of AA in NCBI Reference Sequence was XP_001615477.1. Using PyEMP3 (PlasmoDB: PY01227) as a query in BLAST analysis, it was identified that the Pvx_092425 gene in *P. vivax* (PVX_092425), as well as *Plasmodium knowlesi* (PKH_093410), *P. falciparum* (PF3D7_1136200), *Plasmodium chabaudi* (PCHAS_071370), *Plasmodium yoelii* (PY01227) and *Plasmodium berghei* (PBANKA_091240) with 77.4, 38.6, 36.9, 35.8 and 34.7% of AA identity, respectively.

P. vivax *SalI* protein coding gene on PviS_CM000450 is from 1 360 536 to 1 363 665 (Chromosome: 9). The full-length Pvx_092425 protein consists of 830 AA residues with predicted molecular mass of 82.26 kDa and isoelectric point (pI) of 6.72. It has a putative N-terminal SP sequence (AA1 – 24, length 24), C-terminal TM domain (AA811 – 829, length 19) and a pectin lyase-like superfamily (AA194 – 410, length 217) with

E-value (6e–11) (Fig. 1). The AA repeats and cys-rich region were also predicted by SMART¹⁶ (Fig. 1).

The phylogenetic tree showed that Pvx_092425 and Pkh_093410 are closer than others (Fig. 2). Alignment of the deduced AA sequences demonstrated that 24 Asparagine (Asn) residues conserve among the *Plasmodium* spp. (Fig. 3). For AA composition, Asn (N) and Gly (G) account for 26.1% (217/830) and 11.1% (92/830), respectively. However, Pyl (O) and Sec (U) are negative in the composition. The conserved AA sequences including KII, YCG, WDK, CPLN and YQRLF are also discovered (Fig. 3). Several unique insertions consisting repeat peptide sequences are positioned at AA244–442 and AA766–783. For AA244–442, it includes NNN (V/M) NG, NN VNGG and N (N/S) (V/P) NG, for which the number is 13, 6 and 14, respectively (Fig. 3). For AA766–783, the sequence of NNNG (A/V) Q is triple repeats (Fig. 3). The estimated half-life of protein predicted in mammals reticulocytes (*in vivo*), yeast (*in vivo*) and *Escherichia coli* (*in vivo*) were 30, >20 and >10 hours, respectively, and its instability coefficient was 26.15. Thus, the Pvx_092425 was predicted as a stable protein. The grand average of hydropathicity (GRAVY) and aliphatic index were –0.976 and 57.41, respectively.

Subcellular localization and structure of Pvx_092425

Based on the prediction, the possibility subcellular localizations of Pvx_092425 are 21.7% in cytoplasm, 17.4% in vesicles of secretory system and 17.4% in cell nucleus, with I823–V829 in setting the interior side of the membrane, and M1–A 812 belonging to the cytoplasmic tail. For the secondary structure of Pvx_092425, the numbers of helix and strand are 8 and 11, respectively (Fig. 1).

Homology modelling of Pvx_092425

For homology modelling analysis, there is no satisfying 3D structure for partial or entire AA sequence of HP-C protein Pvx_092425.

Predicted peptides of Pvx_092425

The results of the computational analysis included peptides and their corresponding 50% inhibitory concentration (IC₅₀) value. The peptides with the best predicted binding affinities listed in Table 1. The HLA-DRB1*0101, HLA-A*0203 and HLA-A*0101 showed significantly lower IC₅₀ values than other alleles (Table 1). Meanwhile, the HLA-A*6801 showed the highest IC₅₀ values than other alleles (Table 1). The peptide of 7FALFKIIAL15 was found both in HLA-A*0203 and HLA-B*3501. Similarly, the 782VQVNPPGNV790 was also found in HLA-A*0202 and HLA-DRB1*0701, respectively (Table 1).

Figure 1 Schematic representation of Pvx_092425. The predicted N-terminal signal peptide (SP), amino acid (AA) repeats region, Cys-rich region and C-terminal glycosylphosphatidylinositol-anchor (GPI anchor) are indicated by black, pink, light and dark blue box, respectively. Vertical red bars indicate conserved Cys residues in orthologues among *Plasmodium* spp. Vertical black bars with red triangle indicate protein binding site (AA1, 144, 146, 191, 613, 665 and 797). The helix and strand showed red and blue curve in secondary structure of Pvx_092425. Amino acid represents AA residue.

Figure 2 Cladogram of orthologues of Pvx_092425 among *Plasmodium* spp. pber, pyoe, pcha, pviv, pkno and pfal represent *Plasmodium berghei* str. ANKA, *Plasmodium yoelii* yoelii str. 17XNL, *Plasmodium chabaudi* chabaudi, *Plasmodium vivax* Sal-I, *Plasmodium knowlesi* strain H and *Plasmodium falciparum* 3D7.

Figure 3 Amino acid (AA) sequence alignment of orthologues of Pvx_092425 among *Plasmodium* spp. AA sequence alignment of Pvx_092425 and its orthologous proteins. Deduced AA sequences were aligned using Clustal W2. Asterisks, dots and colons under the alignment indicate identical, conserved and semiconserved substitutions, respectively.

Molecular docking study of HLA-epitope interaction

For the docking study, the 582FLWDKALFD590 epitope that showed the highest affinity with HLA-DRB1*0101 was chosen. Docking predicted more than 90 possible binding models for HLA-DRB1*0101 (PDB: 2FSE) and 582FLWDKALFD590 interaction.

On the basis of higher binding probability with HLA-DRB1*0101, the top 10 output models for the epitope were displayed. For these top 10 models, the predicted epitope-binding sites were generally divided into three domains and each domain shared three or four epitope-binding sites. The interacting and binding of HLA-epitope is illustrated in Fig. 4.

Table 1 Peptides with the best predicted binding affinities for each allele

Allele	Peptide	IC ₅₀ (nM)	Confidence
HLA-A*0101	526 LTDCTNVTY 534	5.53	1.00
HLA-A*0201	587 ALFDFSKCV 595	25.41	1.00
HLA-A*0202	782 VQVNPPGNV 790	36.31	1.00
HLA-A*0203	7 FALFKIAL 15	5.19	1.00
HLA-A*0206	13 IALENMFHI 21	76.03	1.00
HLA-A*0301	619 HLFRLANFH 627	12.68	1.00
HLA-A*1101	624 ANFHIYGTK 632	8.09	1.00
HLA-A*3101	818 LLLPIIMYF 826	124.17	0.89
HLA-A*6801	598 VNKVQASK 606	916.22	1.00
HLA-A*6802	458 STNAPNANT 466	158.85	1.00
HLA-B*3501	7 FALFKIAL 15	118.03	1.00
HLA-DRB1*0101	582 FLWDKALFD 590	0.37	1.00
HLA-DRB1*0401	58 WGSSNTLLG 66	56.49	1.00
HLA-DRB1*0701	782 VQVNPPGNV 790	13.52	1.00

HLA represents human leucocyte antigen; number before and after peptide represent amino acid residue position in the hypothetical conserved (HP-C) protein Pvx_092425.

Discussion

In the study, we have developed a bioinformatics platform for the analysis of PVX_092425. For alignment and phylogenetic analysis of Pvx_092425, it shows that the HP-C proteins were only found in *Plasmodium* spp. and its homology genes in bacterium, plant, insects and even vertebrates was not discovered. The gene ontology (GO) analysis showed that Pvx_092425 belongs to the group OG5_165059, which only has six sequences. All the proteins in the group are conserved *Plasmodium* protein, and their function remains a secret. These unique proteins in the *Plasmodium* spp. mark that they are essential for the malaria parasite. The SMART results demonstrated that Pvx_092425 belongs to pectin lyase-like

superfamily, and the precise role remains largely unknown. The AA repeat region of the HP-C protein is also unique during the *Plasmodium* spp. It indicated that the Pvx_092425 plays a vital role during the protein-protein interaction according to the seven protein binding sites. Based on these bioinformatics, the role of Pvx_092425 will be dissected by physical and biochemical methods.

Based on the *in silico* analysis, the subcellular localization of Pvx_092425 was predicted. However, the exact localization still needs to be updated and confirmed by immunofluorescent assay of immunoelectron microscope. For tertiary structure, the crystallographic structure of the Pvx_092425 is still unclear. Thus, a computer-assisted homology

Figure 4 Docking to predict the binding of predicted epitope 582 FLWDKALFD 590 to major histocompatibility complex (MHC) class II molecule, HLA-DRB1*0101. Binding of 582FLWDKALFD590 to the different binding grooves of the retrieved structure of HLA-DRB1*0101. The red, dark green and dark blue bar represent X axis, Y axis and Z axis, respectively. The white with blue coloured portion, green portion represent HLA-DRB1*0101 molecule and 582FLWDKALFD590 epitope, respectively.

modelling study was conducted to build its 3D structure based on the known sequence of AAs of Pvx_092425. However, no match is found for Pvx_092425. For future research, crystal structure of the entire or partial Pvx_092425 will be determined, and then structure-based drug design, T-cell and B-cell epitopes prediction will be done.

For prediction of potential T-cell epitopes including MHC I and MHC II epitopes, the MHCPre software was used.³⁵ According to the corresponding IC₅₀ values, which indicate the binding affinity, all the predicted peptides were potential immunodominant epitopes. These peptides are useful markers for further vaccine study and minimized the number of tests for malaria vaccine development. It illustrated that epitope of 582FLWDKALFD590 shows the highest binding affinity with the HLA-DRB1*0101 allele. More attractive thing is that both the peptide 7FALFKIHAL15 and 782VQVNP-PGNV790 is corresponding to two alleles, respectively. It indicates that these two peptides are also potential T-cell epitopes for vaccine development. Besides, the peptide of 526LTDCTNVTY534 was also considered as a potential T-cell epitope for vaccine development. For molecular docking study, it predicted more than 90 binding models for HLA-DRB1*0101 allele and the peptide 582FLWDKALFD590. The top 10 models were selected for further analysis. During these 10 models, the binding sites are generally distributed into three regions and these regions were considered as the potential T-cell conformational epitope-binding groove.

Furthermore, for further confirmation of the claim that these predicted peptides of Pvx_092425 are immunogenic, it is indispensable to study humoral immune response to Pvx_092425 in larger population of vivax malaria cases in endemic countries and cell-mediated immunity basing the current bioinformatics approach. The humoral immune response to Pvx_092425 may be partially associated with protection from *P. vivax* parasites infection. Analysis of serum reactivity profiles using protein array supplies a chance to evaluate human response to malarial antigens and helps us to find new vaccine candidates for malaria control. Due to difficulty *in vitro* culture of *P. vivax*, and lack of animal model including the small New World monkeys, the genetically modified *Plasmodium* parasites from *P. bergheri* and *P. yoelii* are alternatively the ideal tool for evaluation of the human *Plasmodium* parasites. The transgenic Pvx_092425 parasite of *P. berghei* will provide convenient model to evaluate the capability of Pvx_092425 as blood-stage vaccine candidate of vivax malaria. Further studies aimed at establishing the immunogenicity and protection-inducing ability of the protein or its products in the genetically

modified experimental model of *P. bergheri* parasites are proposed.

In conclusion, it hypothesized that the Pvx_092425 is essential in the process of erythrocyte stage. It will be possible to understand biology of *Plasmodium* merozoite and, as a result, generate information useful for the development of tools such as an erythrocyte stage vaccine to block the *Plasmodium* merozoites penetration of reticulocyte.

Disclaimer Statements

Contributors LJ designed the study; MJY and LJ were responsible for bioinformatics analysis; All authors contributed to data analysis, writing the manuscript, read and approved the final version. LJ is guarantor of the paper.

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Conflicts of interest We declare that we have no conflicts of interest.

Ethics approval Ethical approval was not necessary.

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