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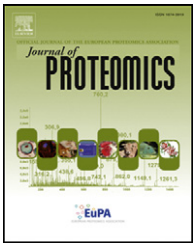

Systematic analysis of natural antibody responses to *P. falciparum* merozoite antigens by protein arrays

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ABSTRACT

With the completion of the functional genome, merozoite proteome and stage-specific transcriptomes of the intraerythrocytic developmental cycle of *Plasmoidum falciparum*, the development of new vaccine candidates targeting *Plasmodium* merozoites is now possible. Here we report using protein array technology to detect antibody responses to *Plasmodium* merozoite proteins by screening the serum of *Plasmodium*-exposed individuals. A total of 138 genes encoding *P. falciparum* merozoite proteins were cloned using the In-Fusion cloning method and expressed using a wheat germ cell-free system (WGCF). These proteins were then screened with serum from *Plasmodium*-exposed individuals and unexposed subjects using protein arrays. A total of 30 highly immunoreactive merozoite antigens were identified (21.7% of 138 target proteins), including 10 well-characterized blood-stage vaccine candidates for *P. falciparum*. In addition, we report for the first time 7 proteins (MSP3.5, MRSP2, ETRAMP11.2, ETRAMP14.1 and RALP1, and two hypothetical proteins PFA0210c and PF14_0572) as being immunologically reactive. These novel *Plasmodium* merozoite antigens may be potential vaccine candidates for blood-stage malaria, and warrant further study.

1. Introduction

Malaria caused by *Plasmodium falciparum* is an extremely important global disease, causing high levels of mortality and morbidity worldwide, particularly amongst young children [1]. After repeated exposure to malaria, individuals develop effective humoral and cellular immunity that controls blood-stage parasitaemia, thereby reducing clinical symptoms and life-threatening complications [2]. The most direct evidence that antibodies are important mediators of immunity to malaria comes from passive transfer studies, in which antibodies from malaria-immune adults were successfully used to treat patients with severe malaria [3].

Protective antibodies produced during the blood-stage of *P. falciparum* are thought to target merozoite surface antigens and erythrocyte invasion ligands [3]. Humoral immune responses against blood-stage antigens are therefore an important component of naturally acquired immunity to malaria [4]. A number of merozoite antigens have established roles in erythrocyte invasion and some have been identified as targets of human invasion inhibition antibodies, intraerythrocytic parasite blockage or antibody dependent cellular inhibition [5–7]. Antigens on the *Plasmodium* merozoite surface, such as merozoite surface protein 1 (MSP1), are thought to interact with heparin-like molecules on the surface of red blood cells (RBCs) and mediate initial attachment during erythrocyte invasion [8]. Apical membrane

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antigen 1 (AMA-1) has been implicated in apical reorientation of the merozoite prior to invasion, and in the formation of a moving junction between the merozoite and the RBCs, which is conducted by the interaction of two parasite proteins, AMA1 and Rhoptry neck protein 2 (RON2) [9]. Two invasion ligand families present in the apical organelles of merozoite, the Duffy binding-like (DBL) family and the *P. falciparum* reticulocyte binding protein homolog (RBL) family, are also required for invasion. DBL and RBL interact with their receptors on the surface of the RBCs, such as EBA-175 binding to glycophorin A, EBL-1 binding to glycophorin B, EBA-140 binding to glycophorin C, PfRH4 binding to complement receptor 1 and PfRH5 binding to basigin [10–13]. There are still a large number of merozoite antigens with unknown function, including the 6-cys family, the MSP3 family, the MSP7 family and glycosylphosphatidylinositol (GPI) anchored membrane proteins [14,15].

Many of merozoite antigens are currently being evaluated or developed as candidates for blood-stage malaria vaccines. However, the investigation of merozoite antigens had been restricted to a limited number of antigens until the recent application of high-throughput screening assays, namely, the protein microarray platform, for the study of malaria [16]. Even so, only a small number of *Plasmodium* blood-stage proteins have been investigated [17]. With the completion of the functional genome, merozoite proteome and stage-specific transcriptomes of the intraerythrocytic developmental cycle of *P. falciparum*, a post-genomic era in the study of *P. falciparum* has begun, and enabled the rapid discovery of new vaccine candidates targeting *Plasmodium* merozoites [18–22]. The recent expression of circa 100 malaria genes using wheat germ cell-free system (WGCF) demonstrated that the recombinant malaria proteins synthesized by WGCF are of great value in the discovery of malarial vaccine candidates [23].

In the present study, *in silico* data mining by comparative genomics combined with In-Fusion cloning methods, a WGCF expression system, and protein arrays, were applied to the high-throughput profiling of antibody responses to *P. falciparum* merozoite antigens. In addition to a set of well-characterized blood-stage vaccine candidates, 7 merozoite proteins (MSP3.5, MRSP2, ETRAMP11.2, ETRAMP14.1, RALP1, PFA0210c and PF14_0572) have been identified as immunologically reactive for first time.

2. Materials and methods

2.1. Samples collection and ethics statement

Plasmodium falciparum malaria positive serum samples were collected from 16 patients (mean age, 33 years; range 15–52 years) in Yunnan province, an area with low endemic malaria levels in the P.R. China. Only 0.23/100,000 indigenous falciparum incidents (97 indigenous falciparum cases from 42.36 million of population) were reported in Yunnan province in 2010 and only 0.84% (9/1075) of *Anopheles* mosquitoes were found to be infected by falciparum sporozoites in 2008 [24,25]. All patients were experiencing fever (>37.5 °C), and serum samples were microscopically examined and found to be positive for *P. falciparum* (mean parasitemia, 0.469%; range 0.004–3.703%). Serum samples from unexposed individuals used as the control in the study were collected in Hangzhou, Zhejiang province, a area where malaria is not endemic.

The study was approved by the Ethics Committee of the National Institute of Parasitic Diseases (NIPD), China CDC. The study protocol, potential risks, and potential benefits were explained to the villagers. After informed consent to participate in the study was give, field workers visiting the enrolled families provided detailed information to all participants, and answered any questions participants had. All participants in a given household provided written informed consent.

2.2. PCR amplification and In-Fusion cloning

The *P. falciparum* merozoite proteins (Table 1) were selected according to specific sets of criteria, including (1) evidence for schizont stage-specific expression, (2) proteins implicated in *P. falciparum* merozoite invasion, (3) the presence of a signal peptide, and (4) the presence of one or more transmembrane domains. A total of 112 unique genes encoding *P. falciparum* merozoite proteins were selected for PCR amplification and In-Fusion cloning. The major categories ($n=21$) were gene families encoding merozoite surface proteins (MSP1, MSP2, MSP3 family, MSP4, MSP5, MSP7 family, MSP8, MSP9 and MSP10) [6,20]. The other categories included the 6-cys gene family ($n=5$), GPI anchored proteins ($n=14$), the early transcribed membrane proteins (ETRAMP) family ($n=7$), the Duffy binding-like (DBL) family ($n=5$), the *P. falciparum* reticulocyte binding protein homolog (RBL) family ($n=5$), well-known genes/proteins (known antigenicity or immunogenicity, $n=22$), and other unknown genes/proteins ($n=33$) [6,7]. Because the reliability of producing desired PCR products decreases as the length of the genomic DNA fragment increases, genes longer than 1800 bp were divided into multiple overlapping sections (112 genes, divided into 192 ORFs), with 45 nucleotide overlaps. Sequence information for *P. falciparum* was derived from the *Plasmodium* database (PlasmoDB, http://www.plasmodb.org/plasmo/home.jsp) [26].

Gene-specific primers were designed with Invitrogen OligoPerfect Designer (http://tools.invitrogen.com/content.cfm?pageid=9716) [23]. The nucleotide sequences for the signal peptide and the GPI anchor were excluded from the gene expression constructs [27,28]. Each gene-specific sense and antisense primers were converted into In-Fusion PCR primers for extension at the 5′ terminus with the sequence 5′- GGG CGG ATA TCT CGA G -3′ and 5′- GCG GTA CCC GGG ATC CTT A -3′, respectively. Genomic DNA of *P. falciparum* was used for PCR amplification of target genes as described in the previous report [23].

The pEU-His vector (derived from pEU, CellFree Sciences, Matsuyama, Japan) was used for In-Fusion cloning. The vector was first linearized by double digestion with restriction enzymes *Xho* I and *Bam*H I (Takara, Japan) and cloned with PCR products in a high-throughput manner as described previously [23]. A target colony was selected for plasmid preparation using the Midi Plus™ Ultrapure plasmid extraction system (Viogene, Taipei, Taiwan) according to the manufacturer's instructions. High purity DNA was eluted in 50 μl 0.1×TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and the concentration was determined by a NanoVue Plus Spectrophotometer (GE Healthcare).