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Construction and characterization of bivalent vaccine candidate expressing HspA and M_r18 000 OMP from *Helicobacter pylori*

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Abstract

AIM: To construct a recombinant vector which can express outer membrane protein (OMP) with M_r18 000 and heat shock protein A (HspA) from *Helicobacter pylori* (*H. pylori*) in *E. coli* BL21, and to exploit the possibility for obtaining the vaccine conferring protection from *H. pylori* infection.

METHODS: The target gene of HspA was amplified from *H. pylori* chromosome by PCR, and then inserted into the prokaryotic expression vector pET32a (+) by restrictive endonuclease enzyme *kpn* I, *Bam*H I simultaneously. The recombinant vector was used to sequence, and then together with pET32a (+)/Omp_{18}, digested by restrictive endonuclease enzyme *Hind* III and *Bam*H I simultaneously. pET32a(+)/HspA and Omp_{18} were recovered from 1 % agarose gel by gel kit, and ligated with T_4 ligase by *Bam*H I digested viscidity end. The recombinant plasmid of pET32a(+)/HspA/Omp_{18} was transformed and expressed in *E. coli* BL21 (DE3) under induction of IPTG. After purification, its antigenicity of the fusion protein was detected by Western blot.

RESULTS: Enzyme digestion analysis and sequencing showed that the target genes were inserted into the recombinant vector, composed of 891 base pairs, encoded objective polypeptides of 297 amino acid residues. Compared with GenBank reported by Tomb *et al,* there were 1.3 % and 1.4 % differences in obtained *H. pylori* nucleotide sequence and amino acid residues, respectively. SDS-PAGE analysis showed that relative molecule mass (M_r) of the expressed product was M_r 51 000, M_r of protein expressed by pET32a (+) was about M_r 20 000, and soluble expression product accounted for 18.96 % of total bacterial protein. After purification with Ni^{+2}-NTA agarose resins, the purification of recombinant fusion protein was about 95 %. Western blot showed that recombinant fusion protein could be recognized by the patients' serum infected with *H. pylori* and anti-Omp_{18} monoclone, suggesting that this protein had good antigenicity.

CONCLUSION: The gene coding for *H. pylori* M_r18 000 OMP and HspA was cloned and expressed successfully. The results obtained lay the foundation for development of *H. pylori* protein vaccine and a quick diagnostic kit.

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INTRODUCTION

Helicobacter pylori (*H.pylori*) is a microaerophilic, spiral and gram-negative bacillus first isolated from human gastric antral epithelium in 1982. It has been recognized as a human-specific gastric pathogen that colonizes the stomaches of at least half the world's population[1], and there are approximately thousands of newly infected people annually. Most infected individuals are asymptomatic. However, in some individuals, their infections are associated with the development of peptic ulcer, gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma[2-20], moreover with extradigestive diseases[21-37]. This organism was recently categorized as a class I carcinogenetic factor by the World Health Organization, and direct evidence of carcinogenesis was recently demonstrated in an animal model[38,39]. Although there are many methods for eradication of *H. pylori* infection, such as bi-, tri- drug therapy, the definitive curative effects were acquired by using a serial of antibiotics, which has led to resistant *H. pylori*. Meantime medical side effects, patients' endurance and compliance were challenged. This has drawn increasing interests of scientists in developing *H. pylori* vaccine so as to reduce and prevent *H. pylori* infection, extinct diseases associated with *H. pylori* infection. Immunization against this bacterium represents a cost-effective strategy to reduce global *H. pylori*-gastric cancer and peptic ulcer rates[40]. To date, *H. pylori* vaccine candidate antigens identified include urease enzyme, VacA, and so on[41-50]. M_r18 000 and HspA are outer membrane proteins of *H. pylori*, and the vaccines prepared with M_r18 000 OMP and HspA respectively were used to inoculate Balb/c mice, 70-80 % of experimental mice were protected. In order to acquire a better immunocompetent effect, some investigators suggested that bi-valent antigen vaccine was possibly superior to single antigen. So in this study, the recombinant plasmid encoding *H. pylori* M_r18 000 OMP and HspA genes was constructed and expressed in BL21 to explore the possibility for obtaining a vaccine conferring protection from *H. pylori* infection.

MATERIALS AND METHODS

Material

A well-characterized strain of *H. pylori* was afforded by the Department of Microbiology, Chongqing University of Medical Sciences. Top10, BL21 *E.coli* strains and pET32a (+), pET32a(+)/Omp_{18} plasmid, anti-Omp_{18} antibody were provided by the Institute of Viral Hepatitis of Chongqing University of Medical Sciences. Restriction endonuclease enzymes (*Kpn* I, *Hind* III, *Bam*HI) and T_4DNA ligase were purchased from Promega, *Tag*DNA polymerase was produced by the Immunology Department of Beijing University of Medical Sciences. Isopropyl-β-D-thiogalactopyranoside (IPTG), dNTP and oligonucleotide primers were obtained from Sigma.

Cloning of H.pylori HspA gene

Oligonucleotide primers were designed to amplify *H. pylori* open reading frame (ORFs) of HspA based on GenBank. The primers had a *KpnI* site incorporated into the 5' end and a *Bam*HI site at the 3' end and their sequences were as follows (5'-3'): CCGGTACCATGAAGTTTCAACCATTAGG (forward) and CCGGATCCGTGTTTTTTGTGATCATGAC (reverse). The reverse 5' end stop codon TAA was banned. Genomic DNA prepared from Chinese *H. pylori* strains was used as the template in PCR. The PCR consisted of 30 cycles of denaturation at 94 ℃ for 60 s, annealing at 52 ℃ for 50 s, and an extension step at 72 ℃ for 50 s. The products were visualized on 10 g· L^{-1} agarose gel and purified using a PCR purification kit. After digestion with the restriction endonuclease enzymes *Bam*HI and *KpnI* simultaneously, the purified products were cloned into the compatible sites of the expression vector pET32a (+) by using T_4 DNA ligase at a molar ratio of 4:1 at 4 ℃ overnight.

Construction of recombinant plamids

After the above connected products were transfected into Top10, pET32a(+)/HspA was selected and identified by the methods reported by Jiang *et al*[51]. After pET32a(+)/HspA and pET32a(+)/Omp_{18} were digested by restrictive endonuclease enzymes *Bam*HI and *Hind* III simultaneously, the segments of Omp_{18} and pET32a(+)/HspA were recycled by gel extract kit, and ligated by using T_4 DNA ligase at a molar ratio of 4:1 at 4 ℃ overnight. pET32a(+)/HspA /Omp_{18} was selected, appraised by PCR or enzyme digestion (Figure 1).

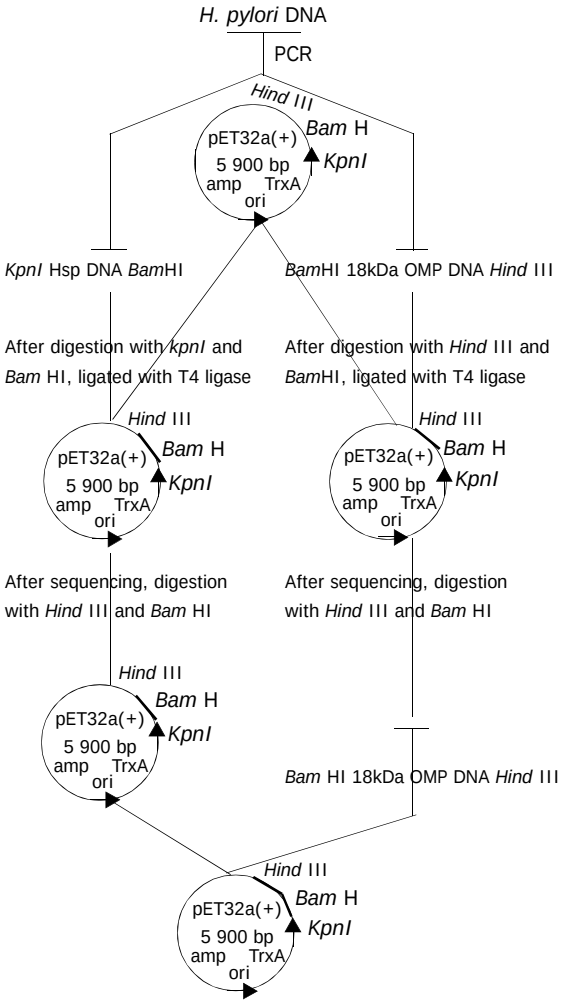

Figure 1 Schematic construction of plasmid pET32a(+)/HspA-Omp_{18}.

Extraction and expression of recombinant plasmid

The single bacterial colony (Top10/pET32a (+)/HspA/Omp_{18}) was picked, and cultivated in 2 ml LB broth containing 100 mg· L^{-1} of ampicillin, at 300 r· min^{-1} at 37 ℃ overnight, then recombinant plasmids were extracted according to the manufacturer's instructions, in the meantime, identified by PCR and restriction endonuclease enzyme digestion. The recombinant plasmid was transfected into competent BL21 (DE3) *E.coli* strains by using standard procedures reported by Jiang *et al*. BL21 *E.coli* strains containing recombinant plasmid were grown until mid-log phase (optical density at 600 nm=0.5 to 1.0), and then induced to express recombinant fusion protein by adding 1 mmol· L^{-1} IPTG for 4 h. Following induction, bacteria were harvested by centrifugation at 12 000 r· min^{-1} for 2 min, resuspended in protein-buffer and seethed for 5 min. Total proteins were electrophoresed on 150 g· L^{-1} SDS-PAGE gel and stained with Coomassie. The rate of recombinant fusion protein to total protein was deduced by Image Master Totallab v1.11 software.

Immunoblotting analysis of the recombinant fusion protein

Due to C end of recombinant fusion antigen with six histidines, the recombinant fusion antigen was purified by using Ni^{2+}-NTA agarose resin. Briefly, 500 ml of bacteria cultivated suspension was prepared, centrifugated, resuspended with the buffer liquid (50 mmol· L^{-1} phosphate, 300 mmol· L^{-1} NaCl, pH 7.0), and sonicated by ultrasonic wave with the energy of 600W×35 % for 40 min, and ultracentrifugated for 15 min at 10 000 r· min^{-1} at 4 ℃. The sonicated recombinant fusion antigen was purified by using Ni^{2+}-NTA agarose resin with abluent (50 mmol· L^{-1} phosphate, 300 mmol· L^{-1} NaCl, 20 mmol· L^{-1} imidazole, pH 7.80) and lavation (50 mmol· L^{-1} phosphate, 300 mmol· L^{-1} NaCl, 250 mmol· L^{-1} imidazole, pH 7.80) respectively, and quantified. The antigenicity of expressed recombinant fusion protein was determined by immunoblotting. Following electrophoretic transfer of SDS-PAGE-separated (150 g· L^{-1} acrylamide) recombinant fusion protein to 0.45 μm pore size PVDF membrane, and after a 30-min wash in tris-saline blotting buffer, antigen-impregated PVDF strips were incubated with the sera of patients infected with *H. pylori* and anti-Omp_{18} antibody for 2 h at RT. After a washing, the protein was detected by incubating the strips in alkaline phosphatase-conjugated goat anti-man IgG antibody for 1 h at RT.

RESULTS

PCR amplification of H.pylori HspA gene

According to literature[55], *H. pylori* HspA ORF was amplified by PCR with Chinese *H. pylori* strain's chromosomal DNA as the templates. The cloning products were electrophoresed and visualized by10 g· L^{-1} agarose gel (Figure 2). It revealed that the size of HspA DNA fragment amplified by PCR was between 250-500 base pairs, and contained a gene of approximately 357 nucleotides, and was compatible with the previous reports[55].

Identification of recombinant vector by PCR or restriction enzyme digestion

pET32a(+)/HspA identification by PCR After the single colony of Top10 *E. coli* /recombinant pET32a(+)/HspA was picked and incubated in 2 ml LB broth containing 100 mg· L^{-1} of ampicillin at 300 r· min^{-1} at 37 ℃ overnight, then 50 μl was incubated and seethed for 10 min, with the genomes of supernate and recombinant vector as templates respectively, products were amplified by PCR under the condition as mentioned above. The PCR products were visualized by 10

g· L^{-1} agarose gel electrophoresis (Figure 3). It indicated that recombinant plasmid contained the objective gene. At the same time, it was successful in transfecting recombinant plasmid into Top10 *E. coli*.

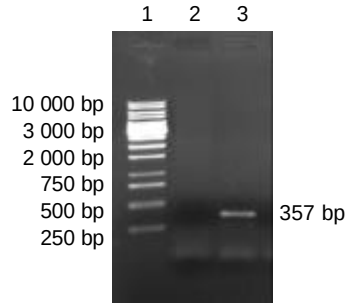

Figure 2 10 g·L^{-1} agarose gel electrophoresis of HspA DNA fragment amplified by PCR from Helicobacter pylori. Lane 1. PCR marker, Lane 2. Negative control, Lane 3. PCR products.

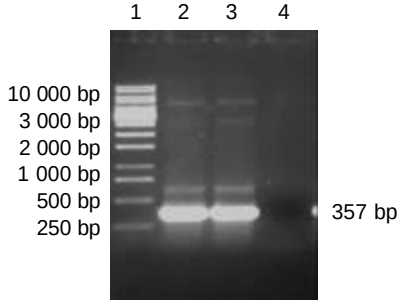

Figure 3 The identification of recombinant vector by PCR. Lane 1. DNA Marker, Lane 2, 3. PCR products with the template of Top10/recombinant vector, and recombinant vector respectively, Lane 4. Negative control.

pET32a(+)/HspA-Omp$_{18}$ identification by restriction enzyme digestion Recombinant plasmids pET32a(+)/HspA-Omp$_{18}$ were digested by single, bi-, tri-enzyme digestion with *Hind*III, *KpnI* and *Bam*HI, respectively, then digestive products were visualized on 10 g· L^{-1} agarose gel (Figure 4). It demonstrated that recombinant plasmids were digested to 357 bp, 528 bp DNA fragment, and contained the objective gene.

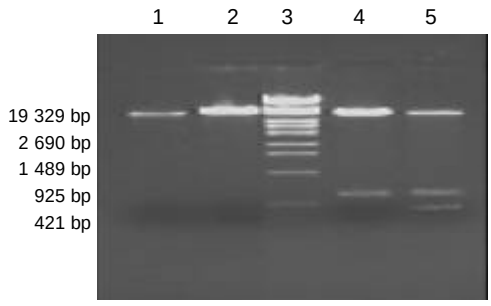

Figure 4 Identification of recombinant plasmid by restriction enzyme digestion. Lane 1. pET32a(+)/HspA digested by *Bam*HI, Lane 2. pET32a(+)/HspA-Omp$_{18}$ digested by *Bam*HI, Lane 3. DNA marker, Lane 4. pET32a(+)/HspA-Omp$_{18}$ digested by *Bam*HI and *Hind* III simultaneously, Lane 5. pET32a(+)/HspA-Omp$_{18}$ digested by kpnI, *Bam*HI and *Hind* III simultaneously.

Sequence analysis of cloned HspA, M_r18 000 OMP nucleotide

The nucleotide sequence of the cloned genes M_r18 000 OMP and HspA inserted into pET32a (+) was analyzed by automated sequencing across the cloning junction, using the universal primer T_7. The results showed that the cloned genes M_r18 000 OMP and HspA were connected by *Bam*H I enzyme digestion adhesion end. The cloned HspA genes contained 357 nucleotides with a promoter codon coding a putative protein of 119 amino acid residues with a calculated molecular mass of M_r13 000, and provided a putative signal peptide. Compared with previous reports, 5 base pairs of the cloned gene and 2 amino acid residues (G→D, A→S) encoded were changed. The cloned gene M_r18 000 OMP and its encoding protein sequences were published in GenBank (AF374387).

Analysis of the recombinant fusion protein

After pET32a(+)/HspA-Omp$_{18}$ was transfected into BL21 *E. coli* strains, the strains with high expressions of fusion proteins were selected. BL21 (DE3) *E.coli* strains containing recombinant plasmid were grown until mid-log phase (optical density at 600 nm=0.4 to 0.6), and then induced to express recombinant fusion protein by adding of 1 mmol· L^{-1} IPTG for 4 h. Following induction, bacteria were harvested by centrifugation at 12 000 r· min^{-1} for 5 min, resuspended in protein-buffer and seethed for 5 min. Total protein was electrophoresed on 150 g· L^{-1} SDS-PAGE gel and stained with Coomassie. Its molecular mass was M_r 51 000 by 150 g· L^{-1} SDS-PAGE gel analysis. After the recombinant bacteria were sonicated by ultrasonic wave and ultracentrifuged (10 000 r· min^{-1}, 15 min, 4 ℃), the level of soluble fusion protein in the supernate was about 18.96 % of total cellular protein. After purification by Ni^{2+}-NTA agarose resin columniation, the purity of recombinant fusion protein was about 95 % (Figure 5).

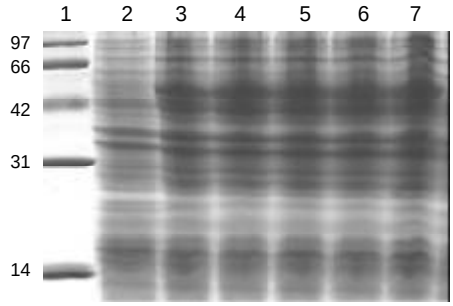

Figure 5 150 g·L^{-1} SDS-PAGE of total protein in recombinant vector expressed in BL21 *E.coli*. Lane 1. Standard protein marker (M_r 14; 31; 42; 66; 97×10^3), Lane 2. Bacterial protein expressed in BL21 after induction for 4 hours with IPTG, Lane 3-7. Expression of recombinant vector in BL21 after induction for 4 h with IPTG.

Antigenicity of recombinant fusion protein

After bacteria BL21/pET32a(+)/HspA-Omp$_{18}$, BL21/pET32a (+), BL21/pET32a(+)/HspA were induced to cultivate by adding of 1 mmol· L^{-1} IPTG for 4 h respectively, 1 ml of cultivated medium was respectively ultracentrifuged, resuspended in protein-buffer and seethed for 5 min. Total protein was electrophoresed on 150 g· L^{-1} SDS-PAGE gel, and then the proteins of SDS-PAGE-separated (150 g· L^{-1} acrylamide) were transferred to 0.45 μm pore size PVDF membrane at 14V, at 4 ℃ overnight. Following a 30-min wash in tris-saline blotting buffer, antigen-impregated PVDF strips were incubated with the sera of patients infected with *H. pylori* and anti-Omp$_{18}$ antibody for 2 h at RT. After a washing, the proteins were detected by incubating the strips in alkaline phosphatase-conjugated goat anti-man IgG antibody for 1 h at RT. In this study, antigen-impregated PVDF strips of BL21/pET32a(+)/HspA-Omp$_{18}$ were recognized by anti-Omp$_{18}$ antibody, showing brown strip corresponding to the site of the

recombinant fusion protein. Antigen-impregated PVDF strips of BL21/pET32a(+)/HspA were recognized by patient' s sera infected with *H. pylori*, also showing brown strip corresponding to the site of the fusion protein, while antigen-impregated PVDF strips of BL21/pET32a(+) were not recognized by patient' s sera infected with *H. pylori* (Figure 6).

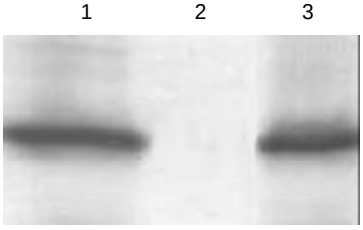

Figure 6 Antigenic analysis of the expression of recombinant vector by Western blot. Lane 1. BL21 / pET32a(+) / HspA-Omp$_{18}$, Lane 2. BL21 / pET32a(+), Lane 3. BL21 / pET32a(+) / HspA.

DISCUSSION

Heat shock phenomena were found by a geneticist Ritossa in studying on change of drosophila cell chromosome stimulated by heat in 1962. Heat shock protein, found by Tissiers *et al* in 1974, indwells in man, microbe, foliage, and animal. It belongs to secretory protein, and accounts for 5 % of total cellular proteins. When cells are stimulated by environment, Hsps are induced to synthesize. A series of heat shock proteins, such as HspA, HspB, Hsp60, and Hsp70, synthesized by *H. pylori*, play a significant role in *H. pylori* pathogenesis, for example, taking part in regulation of cell immunization, initiating auto-immunoreaction of gastric epithelial cell, serving as a promotor in development of chronic gastric pathological changes, and mediating recognition and adhesion of pathogens with host. HspA and HspB genes encode 118, 545 amino acid residues respectively, corresponding to calculated molecular mass of M_r 13 000, 58 200. HspA is consisted of two domains: N domain is conservative sequence, associated with immune appearance; C domain is composed of 27 amino acid residues, including 8 histidines, and 4 cysteines. So the frame is a nickel combinative region, and plays a role in nickel ion translation and presentation. The experiments suggested that HspA, B were important factors in *H. pylori* conglutination and *H. pylori* active proteins stabilization under the extremely unfavorable conditions, meantime they increase urease activity. Others suggested that Hsp60 and HopZ (*H. pylori* outer membrane protein Z) of *H. pylori* were associated with *H. pylori* adhesion. HspA and HspB were homologous with *E. coli* GroEs and GroEI, associated with auto-immunoreaction, which might lead to the production of damaging auto-antibody. Animal experiments demonstrated that mice fed with HspA, B resulted in coronary artery sclerosis[52].

The outer membrane is a continuous structure on the surface of gram-negative bacteria and an asymmetric bilayer with phospholipids in the inner monolayer and the bulky glycolipid lipopolysaccharide (LPS). In the outer monolayer, *H. pylori* as bacterial pathogens, has particular significance as a potential target for inducing host protective immunity and escaping from the host' s immune system. Outer membrane vaccines have been used with considerable success to induce protection against a number of organisms, including the heat shock protein in *H. pylori*, urease A, and B. M_r18 000 OMP is a lipoprotein (Lpp20) belonging to other outer membrane protein[53]. In an earlier study, immunoreactive species-specific M_r19 500 *H. pylori* OMP actually was Lpp20-M_r18 000 OMP. The Lpp20 antigen appears to be commonly expressed in all *H. pylori* strains examined so far. Furthermore, no cross-reaction was shown when antibodies (polyclonal and monoclonal) to *H. pylori* Lpp20 were used to immunoscreen closely related species of helicobacter, campylobacter, or a diverse range of other bacteria. It shows that the Lpp20 gene to be unique to *H. pylori*[54]. Keenan *et al* demonstrated the protein was expressed on the surface of the bacteria by immunolabeling of *H. pylori* with gold-labeled anti-Lpp20 antibodies. Bacterial lipoproteins have been well described, not only as vaccine target candidates, but also as immunostimulatory molecules.

In order to overcome the weak antigenicity of a single antigen, *H. pylori* M_r18 000 OMP and HspA gene were amplified by PCR, and inserted into pET32a(+) vector simultaneously in our study. The pET32a(+) vector was designed for cloning and high-level expression of peptide sequences with the 109aa Trx.· Tag™ thioredoxin protein. Cloning sites were available to produce objective proteins also containing cleavable His· Tag and s· Tag™ sequences for detection and purification. The expressed protein of pET32a(+) vector had a putative molecular mass of M_r 20 000, so the expression of recombinant vector was a fusion protein with a calculated molecular mass of M_r 51 000, consistent with our results. Compared with the reports, 1.8 % of the cloned genes was mutated, and 1.7 % of amino acid residues was changed. The reasons for the discrepancy might be as follows: (1) *H. pylori* chromosomal DNA as templates was different, (2) there were heterogeneity among *H. pylori* strains, and (3) *H. pylori* was provided with the ability of transformation, which could lead to *H. pylori* variation and genome reset[54]. But there was much homogeneity between them.

Todoroki *et al*[55] investigated the effect of DNA vaccines encoding *H. pylori*-heat shock proteins A and B (pcDNA3.1-hspA and -hspB) on inducing immune responses against *H. pylori* in mice. C57BL/six mice aged 5 weeks were immunized by a single injection of 10microg of pcDNA3.1-hspA and pcDNA3.1-hspB into intracutaneous tissue. Plasmid DNA lacking the inserted Hsp was injected as a control. The results demonstrated that DNA vaccines encoding *H. pylori*-Hsp induced significant immune response against *H. pylori*, decreased gastric mucosal inflammation, indicating that a pcDNA3.1-hspA or -hspB DNA vaccine can be a new approach against *H. pylori* in human. Jiang *et al*[51] reported that recombinant fusion OMP$_{18}$ protein also had good antigenicity. While being an immunogenic marker, the patient sera infected with *H. pylori* M_r 18 000 Omp antigen showed high sensitivity and specificity[56]. Moreover, a significant association was found between the serologic response to M_r 18 000 Omp antigen and malignant outcome of *H. pylori* infection[57]. So the serum test for detecting antibody with lower-molecular-weight proteins of *H. pylori* could be used to identify *H. pylori*-infected patients at risk of peptic ulcer or malignancy. A recently published study also identified M_r 18 000 Omp as a candidate following the successful immunization of mice with purified recombinant antigen. In our study, the purified M_r 51 000 recombinant fusion HspA-Omp$_{18}$ protein could be recognized by patients' sera infected with *H. pylori* and anti-Omp$_{18}$ monoclonal antibody, and the purified M_r 33 000 recombinant fusion HspA protein could also be recognized by patients' sera. The results demonstrated that recombinant fusion protein had good antigenicity. These showed that M_r 51 000 recombinant fusion HspA-Omp$_{18}$ protein would not only provide HspA characteristics, but possess M_r 18 000 Omp specialty. Meantime, M_r 51 000 recombinant fusion HspA-Omp$_{18}$ protein was suggested to be a true vaccine candidate and not merely an immunogenic marker for *H. pylori* infection.

In addition to construction of the recombinant vector, looking for living carriers would be a key step. Immunization via the mucosal or intracutaneous-inoculated route offers the advantage that has the potential to stimulate both mucosal and systemic immunity. It is simple, safe and can be used for the

immunization of a large population. Bacillus Calmette-Guerin (BCG), the attenuated strain of mycobacterium bovid and the current vaccine against tuberculosis, has been widely used as a living, innately immunogenic vehicle for multiple protective recombinant antigens for vaccines against pathogenic microorganisms. With the aim of developing a recombinant vaccine, vaccines against human immunodeficiency virus, diphtheria, pertussis, tetanus (DPT), and parasite[58-68] have been investigated. We are developing a living carrier-BCG to provide a mucosal or intracutaneous-inoculated vaccine vector to deliver M_r 51 000 recombinant fusion HspA-Omp_{18} protein to antigen-presenting cells on mucosal surface. We believe that before long, *H. pylori* vaccine could be constructed successfully for eradicating *H. pylori* infection and *H. pylori* associated diseases.

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