Design, cloning and expression assay of oipA gene in a bicistronic vector harboring mice IL-18 gene: potential implications for Helicobacter pylori vaccine investigations

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Abstract

Introduction: Helicobacter pylori (H. pylori) infection has remained as a global health problem. Animal studies demonstrated the role of H. pylori oipA gene in the development of gastric cancer. The aim of this study was the cloning and expression of Helicobacter pylori oipA gene in a bicistronic vector harboring mice IL-18 gene.
Materials and methods: The target gene encoding oipA was amplified from a codon-optimized clone by PCR, and then double-digested by restriction enzymes. The pIRES-Igk/mIL18/Fc plasmid was simultaneously digested by BstXI/NotI enzymes to elicit the eGFP segment. PCR product of oipA was inserted into pIRES-Igk/mIL18/Fc plasmid using T4 ligase. Transformation into DH5α strain was done. Cloning was confirmed by PCR, enzymatic digestion and sequencing. Expression of the oipA and IL-18 mRNA was assessed by means of TaqMan Real-time PCR.
Results: Electrophoresis of PCR product, enzymatic digestion and sequencing showed that the H. pylori oipA gene was successfully cloned into pIRES-Igk/mIL18/Fc to generate mIL-18-pIRES2-oipA plasmid. The results of Real-time PCR confirmed the successful expression of both oipA and IL-18 in mouse macrophage cell line.
Conclusion: Considering the role of oipA in pathogenesis of H. pylori and potent activity of IL-18 as a molecular adjuvant, the results of the present study showed that the expression of codon-optimized oipA gene in bicistronic vector including mouse IL-18 is successful. So, it could be considered as an appropriate genetic vaccine candidate for H. pylori in future investigations.

Keywords: Cloning, Codon-optimization, oipA gene, Mouse IL-18, Bicistronic vector

Introduction

Helicobacter pylori is a Gram negative, motile, spiral-shaped, and microaerophilic microorganism. This bacterium is one of the most important human pathogens that infect more than half of the population worldwide (1). H. pylori is a most important causative agent in some gastro-duodenal diseases including duodenal ulcer, peptic ulcer, chronic active gastritis,

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gastric adenocarcinoma, and MALT lymphoma (2).

To date, a number of virulence factors including cagA and vacA genes concerned to severe stomach illnesses such as gastric ulcers and gastric cancer have been identified (3). Almost 4% of the H. pylori genome encodes outer membrane proteins (OMPs), some of which act as adhesin (4). OipA (Outer inflammatory protein A) is one of the outer membrane proteins which involve in bacterial adhesion. Several studies showed that the oipA gene is another important virulence factor associated with gastric ulcers and cancer (5). Interleukin 18 (IL-18) is considered as a pro-inflammatory cytokine and inducer factor of interferon γ (IFN-γ) production by NK cells and CTL (6). Moreover it also increases Fas dependent cell death (Fas-Dependent Cytotoxicity) and Perforin in these cells. On the other hand, this cytokine plays an essential role in T helper Lymphocyte activities (T helper 1) (7, 8). Structurally, IL-18 belongs to the super family of IL-1 and is synthesized in inactive and non- secretory precursor form as same as IL-1β (9). The main characteristic of this cytokine in stimulation of CTL and NK cells and following increased cell killing activity has led to be used in the vaccine research and tumor immunotherapy (10). Therefore, this cytokine can be used as adjuvant to increase vaccine efficiency especially in DNA vaccine protocols (11-13). The importance of OipA in pathogenesis of H. pylori from one side and the adjuvant activity of IL-18 ,especially in TH1 shifting, from other side, persuaded us to design a study to construct a genetic material containing H. pylori oipA and mouse IL-18 genes to assess its immunogenicity in the future investigations.

Material and methods

In order to clone the target genes, H. pylori oipA and murine IL-18, the pIRES2-eGFP vector (Clontech, Takara Bio Company, Shiga, Japan) was applied as expression vector. This bicistronic vector allows the expression of two separate protein from the same mRNA by employing IRES (Internal Ribosome Entry Site) element.

As described in previous study, the plasmid containing mouse IL-18 gene fused to murine IgG1 Fc (Fcy2a) and signal sequence of immunoglobulin kappa (IgK) (pIRES-Igk/mIL-18/Fc) was constructed after implementing several cloning and sub-cloning process. As a summary, total RNA of stimulated spleenocytes was extracted and reverse transcribed to produce cDNA of murine IL-18 and Fcy2a. mIL-18/Fc mixed segment was constructed by insertion of Fc fragment and mIL-18 into pSL1180 plasmid. In the following, addition of kappa sign was accomplished by sub-cloning of mIL-18/Fc into pSectag2a plasmid. Finally, Igk/mIL-18/Fc was cloned into pIRES2-eGFP vector using NheI/XmaI enzymes to construct pIRES-Igk/mIL-18/Fc plasmid. Identification and confirmation of cloned mIL-18 was assessed by enzymatic digestion, sequencing and ELISA (14).

Codon-optimization of oipA gene: The objective of the present study was cloning of H. pylori oipA gene into previously constructed pIRES-Igk/mIL-18/Fc plasmid. For this purpose, the eGFP coding sequence must be replaced by the desired sequence just after IRES element, using BstXI and NotI restriction enzymes. Regarding to the existence of BstXI restriction site within H. pylori oipA gene, codon optimization strategies was used to eliminate it and improvement of translational efficiency based on Mus-musculus codon usage. Modified sequence accuracy was analyzed by NEBcutter V2.0 (New England Biolabs, U.S.A.), SnapGene (GSL Biotech LLC.), BlastX, and Mega 6.0 softwares. The codon-optimized oipA sequence was constructed in BioNeer Co. (Daejeon, Korea).

oipA PCR: The plasmid harboring modified oipA gene was used as template

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for amplification and cloning of this gene into previously constructed pIRES-Igk/mIL-18/Fc plasmid. The primers containing restriction sites for BstXI and NotI enzymes were designed using AlleleID v6.0 (PREMIER Biosoft International, USA) and OLIGO 7 (Molecular Biology Insights, Inc., CO, USA) and constructed in BioNeer Co. (Daejeon, Korea) (Table 1). The oipA gene was amplified using Pfu DNA polymerase by PCR with initial denaturation at 94 ˚C for 4 min, followed by 25 cycles of denaturation at 94 ˚C for 20s, annealing at 60 ˚C for 20s, elongation at 72 ˚C for 2 min and final extension at 72 ˚C for 10 min. The final concentrations of reagents in 50 µl PCR reaction were as follows: 1X PCR buffer, 2.5mM MgCl2, 1mM dNTP, 50 pmol primers and 15 ng plasmid DNA as template. PCR product was electrophoresed on a 0.8% low melting agarose gel at 70V for 2 h. and purified using QIAquick gel extraction kit (Qiagen, Valencia, CA) according to manufacturer's protocol .

Cloning of oipA gene: Amplified oipA gene and pIRES-Igk/mIL-18/Fc plasmid were double-digested by FastDigest BstXI and fastDigest NotI (Thermo Fisher Scientific, U.S.A). Digested PCR product and plasmid were purified by QIAquick gel extraction kit. Ligation reaction was performed using DNA Ligation kit (Takara Bio Company, Shiga, Japan). The considered molar ratio of vector to insert was 1:3. The recombinant plasmid was transformed into E. coli DH5α by heat-shock protocol and calcium chloride treatment. Finally transformants were selected on LB agar containing 50 µg/ml kanamycin (Sigma-Aldrich, USA) .

Confirmation of Cloned DNA: Colony PCR was conducted using specific primers to evaluate the existence of insert in bacterial colonies. Positive colonies according to PCR results were selected and inoculated into LB broth medium containing 50 µg/ml kanamycin. QIAprep Spin Miniprep kit (Qiagen, Valencia, CA) was utilized for extraction of plasmids. The accuracy of the cloned oipA gene was verified by enzymatic digestion and bidirectional sequencing (BioNeer Company, Korea). The sequence of used primers in colony PCR and bidirectional sequencing were shown in table 1.

Table 1. List of primer sequences used for cloning of oipA gene in this study.

The oipA cloning PCR primers sequences (Amplicon: 954 bp)	
oipA F (BstXI)	5' CTAACCACAACCCGTGGATGAAGAAGGCCCTGTTG 3'
oipA R (NotI)	5' CGGCGCGGCCGCTAATGTTTGTTTTTAAAG 3'
The sequences of *oipA* colony PCR primers (Amplicon: 459 bp)	
oipA-con F	5' CAACCGTGGATGAAGAAG 3'
oipA-con R	5' GGTATTGAAGCCGTATGG 3'
The sequences of *oipA* bidirectional sequencing primers	
oipA-seq F	5' GCTTTACATGTGTTTAGTCGAGGTT 3'
oipA-seq R	5' CCTCTACAAATGTGGTATGGCTGATT 3'

PCR, polymerase chain reaction. F, forward. R, reverse.

Transfection of mIL-18-pIRES2-oipA plasmid: The expression of cloned genes were assessed in eukaryotic cell lines by transfection of constructed mIL-18-pIRES2-oipA plasmid into murine macrophage-lineage, RAW 264.7 cell lines. The cells were transfected at 70-90% confluence using Lipofectamine 2000 (Invitrogen, U.S.A.) with 100 ng of three different plasmids including mIL-18-pIRES2-oipA, pIRES-Igk/mIL-18/Fc (As negative control for oipA gene) and pIRES2-eGFP (As positive control of transfection) according to manufacturer's protocol. The efficiency of transfection was analyzed by epifluorescence microscopy.

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Assessment of mIL-18 expression and bioactivity: As previously described by Pouriayevali et al,(14) the expression and bioactivity of cloned mIL-18 from constructed plasmid were evaluated by mouse IL-18 ELISA kit (Bender MedSystems, Austria) and Mouse IFN-γ ELISA (U-cytech bioscience, Netherlands) after harvesting the supernatants of transfected RAW 264.7 cell line according to recommendation of manufacturers.

Assessment of oipA expression: To evaluate the expression of cloned oipA gene in transfected cells, TaqMan Real-time PCR technique was used. Total RNA was extracted from transfected RAW 264.7 cells using the RNeasy Mini Kit (Qiagen) and reverse transcription of RNA into cDNA was carried out by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, U.S.A.). TaqMan Real-time PCR assay for evaluation of codon-optimized oipA and mIL-18 genes expression was accomplished by designing specific primer and probe using AlleleID software (Table 2). TaqMan Universal PCR Master Mix (Applied Biosystem, U.S.A.) and StepOnePlus Real-time PCR system (Applied Biosystem, U.S.A.) were utilized for running the reaction. The selected method was End-point detection Real-time PCR. The housekeeping mGAPDH (murine glyceraldehyde-3-phosphate dehydrogenase) reference gene was considered as Internal Positive Control (IPC). The Real-time PCR reactions were carried out in total volume of 20 µl using 10 pmol of specific primer and probes for oipA and mIL-18 separately under the following conditions: 60°C for 30s (Pre-PCR plate read), 50°C for 2 min and 95°C for 10 min (Holding Stage), followed by 45 cycles of 95°C for 15s, 60°C for 1 min (Cycling stage) and 60°C for 30s (Post-PCR plate read). mIL-18-pIRES2-eGFP and pIRES2-eGFP were used as positive and negative control respectively.

Table 2. The designed primers and probes sequences for Real-time PCR reaction

mIL-18 -F	5' AAATGCCCAGCACCTAACCTC 3'
mIL-18-R	5' GGTCATCCTCGCTCACATCC 3'
mIL-18-TaqMan	5' FAM/ACCATCCGTCTTCAT/NFQ-MGB 3'
mGAPDH-F	5' ATGTTCCAGTATGACTCCACTCAC 3'
mGAPDH-R	5' GCTCCTGGAAGATGGTGATGG 3'
mGAPDH-TaqMan	5' VIC/ATTCAACGGCACAGTC/NFQ-MGB 3'
Mod-oipA-F	5' GAAGGCTTCTGCACAGAATGC 3'
Mod-oipA-R	5' CGGTTAGCGATTTTGTTTGAATCC 3'
Mod-oipA-TaqMan	5' FAM/CCAAGCCATCAACAA/NFQ-MGB 3’

PCR, polymerase chain reaction. F, forward. R, reverse.

Results

Codon-optimization of oipA: The sequencing analysis of ordered plasmid containing modified oipA gene demonstrated the deletion of BstXI restriction site in codon-optimized oipA sequence. Nonetheless, BlastX results confirmed complete homology of codon-optimized and native oipA in coding protein level.

oipA PCR: The electrophoresis of oipA PCR product confirmed the presence of desired 954 bp fragment concerned to this gene (Figure 1).

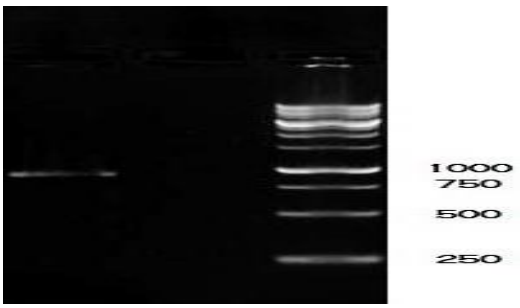


Figure 1. Electrophoresisi of oipA PCR product.

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Confirmation of cloned DNA: The authenticity of cloned DNA was verified by enzymatic digestion, PCR and sequencing. As shown in Figure 2 the inserted 954 bp fragment was brought out after double digestion with BastXI and NotI. Also 459 bp amplicon was produced by plasmid while added as a template in PCR reaction. Analysis of bidirectional sequencing proved complete accuracy of cloned gene.

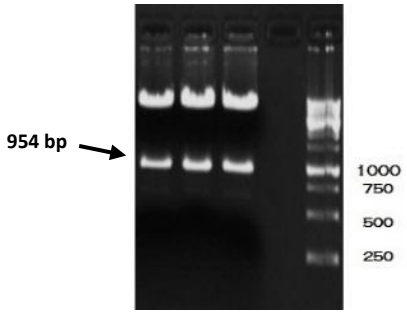


Figure 2. Electrophoresis of confirmatory double digestion.

Recombinant mIL-18 expression and bioactivity: Assessment of murine IL-18-Fcy2a fusion protein expression and biological activity in supernatant of RAW 264.7 cells showed the production of mIL-18 near 250 pg/ml and IFN-γ (as a bioactivity indicator) almost 20 pg/ml.

Codon-optimized oipA expression: Endpoint TaqMan Real-time PCR assay results showed the successful expression of oipA and mIL-18 genes from mIL-18-pIRES2-oipA plasmid in transfected RAW 264.7 cells in comparison to pIRES2-eGFP (as negative control).

Discussion

Despite great progress in H. pylori vaccine design, the mechanisms of mucosal immunity against this bacterium is unclear up to now. In fact, it is surprising that proteins such as urease and heat shock protein homologues which are present in the cytoplasm of other intestinal pathogens may be serves as vaccines against H. pylori. Successful H. pylori vaccination strategies were evaluated in different animal models by oral administration of H. pylori antigens in combination with mucosal adjuvants such as cholera toxin and E. coli LT enterotoxin. Various antigens have been used as prophylactic or therapeutic H. pylori vaccines in animal models. Chen et al in 2012 reported that injection of the recombinant OipA protein in combination with IL-2 molecular adjuvant and pLTB genes could be effective against H. pylori by reducing the bacterial load in immunized mice (16). In another study, Chen and colleagues showed that injection of genetic vaccine expressing oipA loaded on attenuated Salmonella typhimurium strain in C57 mice can effectively stimulate cellular and humoral immunity (18). Falivene et al in 2012 showed the importance of IL-18 cytokine in effective immunization strategies against antigens carried by MVA vector (Modified Vaccinia Ankara) by deleting IL-18 binding protein gene in this vector (17). Due to the ability of IL-18 in stimulation of innate and adaptive immune responses and Th1 shifting, DNA vaccine containing IL-18 molecular adjuvant sounds appealing strategies in preventing of classic and re-emerging diseases (19). Several advantages have been cited for fusion of murine Fcy2a to cytokines including increased avidity, prolonged circulating half-life due to molecular size increase and reduced renal clearance. Furthermore, fusion with Fc results in alteration of complement-mediated and antibody dependent cell-mediated cytotoxicity, enhancing the effects of IL-18 as a molecular adjuvant (14).

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Considering OipA virulence and carcinogenesis, the aim of present study was to build a bicistronic plasmid containing codon-optimized H. pylori oipA and murine IL-18 genes to assess immunogenicity of this plasmid in animal models in future studies. Selection of a bicistronic vector enabled us to express both oipA and IL-18 genes from the same construct. Applying two plasmids in DNA vaccine projects can hamper the reproducibility of experiments, so bicistronic vector was used to ensure equal molar ratio of genes (Equimolar) in DNA vaccine formulation in ongoing study. The results of sequencing, transfection and RT-PCR showed that H. pylori optimized oipA gene as well as murine IL-18 were expressed successfully from constructed mIL-18-pIRES2-oipA plasmid in monocytes / macrophages cell line. Moreover, the application of this construct is possible as a promising H. pylori DNA vaccine candidate.

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