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Human papillomavirus L1 protein expressed in *Escherichia coli* self-assembles into virus-like particles that are highly immunogenic

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ABSTRACT

HPV vaccines based on L1 virus-like particles (VLPs) provided a high degree of protection against HPVs infection. In this study, the codon optimized HPV16 L1 gene were sub-cloned into five procaryotic expression vectors (pET-28a, pET-32a, pGEX-4T-2, pE-sumo and pHSIE), and fused with different protein tags. No recombinant proteins were expressed in pET-28a-L1 and pHSIE-L1, and the proteins expressed by pET-32a-L1 plasmid with TRX-tag were in the form of inclusion body. Only SUMO-tagged and GST-tagged L1 proteins expressed by pE-Sumo-L1 or pGEX-4T-L1 were soluble. The yield of SUMO-L1 protein reached 260 mg/L fermentation medium in shake flask. After SUMO tags were eliminated, a 90% purity of L1 proteins was generated by ion-exchange and Ni-NTA affinity chromatography. The purified HPV16 L1 protein self-assembled into virus-like particles (VLPs) and showed a haemagglutination activity. High titers specific and neutralizing antibodies were detected in HPV 16 L1VLPs vaccinated mice. Cytokines such as IFN-γ and IL-2 showed significant higher in VLPs vaccinated mice compared with negative control ($p < 0.05$, $p = 0.055$). Thus, the expression of recombinant HPV16 L1 VLPs in *Escherichia coli* was feasible, which could potentially be used for a VLP-based HPV vaccine.

1. Introduction

Human papillomavirus (HPV) is an epithelial cell specific virus, which is correlated with human pathogens (de Villiers, 2013). Until now, more than 200 subtypes of HPV have been identified. Some of them classified as high-risk HPV types are related to cancers (especially cervical cancer), while low-risk types are widely detected in sexually transmitted disease worldwide, such as genital wart cases in both man and woman (Anhang et al., 2004). HPV 16, a high-risk type, has been detected in about 54% of malignant cells of cervical cancers (Munoz et al., 2003; Tommasino, 2014). Recent studies have shown that HPV16 may associate with other epithelial cancers, such as nasopharyngeal carcinoma and head and neck cancer (Maxwell et al., 2015).

HPV is a non-enveloped, double stranded circular DNA virus with a virion particle of 55–60 nm in diameter, which present as T = 7 icosahedral symmetry (Munger et al., 2004). The capsid of HPV contains 72 copies of pentamers which consisted by 5 L1 proteins, and finally arrange in an icosahedral particle with L2 protein together (Buck et al., 2008). The HPV L1 protein can self-assemble into virus-like particles (VLPs), which exhibited morphologically and immunologically features like native virions (Buck et al., 2013). Since the absent of infectious genome, the VLP could not infect and lead to disease. Therefore, HPV VLPs have been approved by FDA (USA Food and Drug Administration) as safe and effective vaccine for cervical cancers and other related diseases control (McKee et al., 2015; Shaw, 2013).

Although yeast and baculovirus expression systems can produce HPV16 VLPs with high immunogenicity (Hanumantha Rao et al., 2011; Zhang et al., 2010), some defects, such as big technical difficulties, high cost and low yield, limit the widely use of these systems, especially in poor countries and regions. Therefore, looking for a new and more efficient approach to prepare HPV L1 protein is very important to reduce the cost of HPV vaccine and expand the availability of the vaccine. Bacterial expression system might be a reasonable choice to solve this problem, due to its high level on protein expression and rapid growth in relatively inexpensive culture medium (Zhang et al., 2010). How-

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Table 1
Primers used in this article.

Primers	Sequences (5′–3′)[a]
P1	TT*GAATTC*ATGTCTCTGTGGCTGCCGTCTGAG
P2	AAT*GCGGCCGC*TTACAGTTTACGTTTTTTACG
P3	TT*GGTCTCTAGGT*ATGTCTCTGTGGCTGCCG
P4	AAT*CTCGAG*TTACAGTTTACGTTTTTTACG
P5	TT*GGATCC*ATGTCTCTGTGGCTGCCGTCTGAG

[a] The sequence underline were the restriction enzyme sites used for inserting amplified fragments into the expression vector.

ever, the production of HPV L1 protein in *bacteria* also has a few technical matters, like mainly in existed as inclusion body, low immunogenicity, etc. (Chen et al., 2001; Ma et al., 2007). Fusion protein approach has been shown to be an effective method to solve the protein inclusion body in *Escherichia coli* expression system. Proteins, such as thioredoxin (TRX), glutathione-s-transferase (GST), maltose binding protein (MBP) and small ubiquitin like modifier protein (SUMO), are commonly used to promote the correct folding and stability of recombinant protein in *E. coli* (Xu et al., 2014; Zhang et al., 2015).

In this study, we produced HPV L1 protein from *E. coli* expression system and VLPs were self-assembled. The VLPs shown equivalent biological activity and immunogenicity as generated from *eukaryotic* expression system. The results indicated the VLPs generated from *E. coli* felicitously present the antigen epitopes of HPV 16 capsid and have a potential as a cost-effective vaccine candidate for HPV prevention.

2. Materials and methods

2.1. Construction of HPV16 L1 expression plasmid

The gene encoding HPV16 L1 protein (GenBank No. AACO9292) was optimized according to the codon usage in *E. coli,* allowing some deviations and synthesized by GenScript (Nanjing, China) (Grote et al., 2005; Wu et al., 2007). As shown in Fig. 1, five primers (Table 1) were synthesized to subclone L1 gene into *procaryotic* expression vectors. Briefly, Primer P1 and P2 were used to subclone the L1 gene into the pHSIE vector downstream of intein coding sequence (Wang et al., 2012). Primers P3 and P4 were used to generate a SUMO fusion protein and the P4 and P5 primer were used to insert the L1 encoding gene into pET-28a, pET-32a and pGEX-4T-2, respectively. The recombinant plasmids, named as pE-SUMO-L1, pHSIE-L1, pET-32a-L1, pET-28a-L1 and pGEX-4T-L1, were transformed into *E. coli* BL21 (DE3), respectively and further confirmed by restriction analysis and sequencing. Single positive colony was selected and inoculated to Luria–Bertani (LB) medium with 50 μg/ml kanamycin or 80 μg/ml ampicillin. With shaking at 37 °C until OD_{600} reached 0.6, the culture were induced by adding 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated at 20 °C for 12 h. Then, cells were collected by centrifugation at 12000*g* and resuspended in 50 mM phosphate buffer (PB, pH 7.0). All the cells were lysed by ultrasonic (pulses of 5 s with 10 s off during 8 min) at 30% intensity in iced water bath and clarified by centrifugation at 12000*g* for 20 min. The supernatant and precipitation fractions were analyzed, respectively, by SDS-PAGE and Western Blot (using anti-HIS_6 tag monoclonal antibody) as previously described (Xu et al., 2014).

2.2. Expression and purification of HPV16 L1 protein

The single clone of E. *coli* BL21(DE3) carrying plasmid pESumo-L1 was picked from LB agar plate supplemented with 100 μg/ml ampicillin, and cultured overnight in LB broth (10 ml) at 37 °C. The overnight starter cultures (10 ml) were then inoculated into a flask containing fresh LB broth (1 l) and cultured under 37 °C with shaking (220 rpm). Until the optical absorbance OD_{600} reached 1.0, 0.3 mM IPTG was used to induce target protein for 20 h at 20 °C. After centrifugation, cell pellets were harvested and disrupted by high pressure homogenizer. SP Sepharose™ Fast Flow column (GE, China) was equilibrated with PB buffer containing 0.2 M NaCl. The clarified cell lysates were pumped onto the columns at a flow rate of 0.5 ml/min. After washing with 20 beds of PB buffer (0.4 M NaCl), SUMO-L1 proteins were eluted by PB buffer with 0.4–1.0 M NaCl. All the fractions were collected and analyzed by SDS-PAGE and Western Blot. Then, the SUMO tag was eliminated by incubating the purified product with SUMO protease (Solarbio, Shanghai) at 30 °C for 4 h. Then, all the samples were loaded onto a Ni-NTA Sepharose FF (GE, China) and the flow through samples were collected. Besides, endotoxin was cleared away by using the affinity matrix of modified polymyxin B (PMB) (GenScript, China) and determined by LAL based colorimetric assay (GenScript, China). The concentration of purified fraction was calculated using Micro BCA™ protein assay kit (Thermo Scientific, USA) following the manufacturer's protocol.

2.3. Assembly and characterization of HPV 16 VLPs

After dialyzing against PB buffer containing 0.5 M NaCl for 24 h, HPV 16 L1 protein was placed onto a 200 mesh carbon-coated copper grid for negatively stained with 1% phosphotungstic acid (PTA). Observation was carried out by using a transmission electron microscope (TEM, JEM-1400) at 80 KV. Size distribution of HPV VLPs was analyzed by dynamic light scattering (DLS) and a computerized inspection system (Malvern, UK). Hemagglutination (HA) and hemagglutination inhibition (HAI) assays were performed as previously described (Bazan et al., 2009).

2.4. Immunization of animals

Animals care and study procedure were following the guideline of the Animal Research Ethics Board of Zhengzhou University. HPV16 L1 VLPs were adsorbed on alhydrogel by mixing them for 30 min at 25 °C. Four-week-old female Kunming mice were randomly assigned to three groups each consisting of four mice. One group was subcutaneously inoculated with PBS as a negative control; the other two groups were immunized with 5 μg or 20 μg VLPs mixture, respectively. The boost was performed at 14 days after the first vaccination. Blood samples were collected at 0, 7, 14, 21, 28, 37 and 60 days post first immunization and sera were prepared for detection.

2.5. ELISA for antibody detection

The antiserum titer was detected by using indirect ELISA (Xu et al., 2014). ELISA plates were coated with 1 μg of HPV L1 VLPs per well in 0.1 M CBS at 4 °C overnight. After blocked with 5% non-fat milk, serum samples were added to the plate and incubated at 37 °C for 1 h. HRP-conjugated goat anti-mouse IgG diluted in 5% non-fat milk (1:5000) was incubated in the plate at 37 °C for 30 min followed by six washes with PBST. The colorimetric reaction was performed by incubating all plates with 70 μl substrate solutions for 8 min at room temperature. Then, after stopping by 70 μl of 2 N H_2SO_4, absorbance was read at 450 nm.

2.6. Pseudovirus neutralization assay

HPV16 pseudovirus was produced according to previous reports (Buck et al., 2004; Day et al., 2013). A plasmid encoding green fluorescent protein (GFP) was packaged in the pseudovirus as a reporter gene. Pseudovirus neutralization assays were performed