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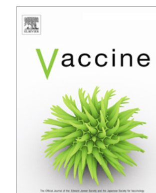

Development of a human papillomavirus type 6/11 vaccine candidate for the prevention of condyloma acuminatum

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ABSTRACT

Condyloma acuminatum (CA) represents a significant human papillomavirus (HPV) disease burden worldwide, resulting in substantial healthcare costs and loss of life quality in both genders. To address this problem, we tried to develop a bivalent HPV6/11 virus-like particle (VLP) vaccine targeting CA. HPV6/11 VLPs were generated in *Hansenula polymorpha*, and a disassembly and reassembly (D/R) treatment was further conducted to improve the stability and monodispersity of the VLPs. The HPV6/11 VLPs were identified by transmission electron microscopy (TEM), high performance liquid chromatography (HPLC), mass spectrum (MS) and dynamic light scattering (DLS), and were evaluated for their immunogenicity in both mice and cynomolgus monkeys. The results showed that the HPV6/11 L1 proteins were correctly expressed and assembled into HPV6/11 VLPs, and the HPV6/11 VLPs formulated with aluminum phosphate induced vigorous production of specific neutralizing antibodies against HPV6/11 VLPs in mice and cynomolgus monkeys. These data indicated that the *Hansenula polymorpha*-derived HPV6/11 VLPs could be formulated into a bivalent vaccine used in prevention of CA.

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1. Introduction

Condyloma acuminatum is one of the most widespread sexually transmitted diseases caused by human papillomavirus [1,2]. About 40 distinct types of HPV related to CA have been identified, while HPV types 6 and 11 account for more than 90% of the cases [3]. At present, CA represents a significant HPV disease burden worldwide, resulting in substantial healthcare costs and deteriorating quality of life. The overall reported annual incidence of CA is ranged from 160 to 289 per 100,000, with a median of 194.5 per 100,000 [4]. About 1 million new cases of CA are reported each year and the cost of treatment is increased due to the high recurrence rate after initial remission [5]. In view of over 90% of CA are associated with HPV6 and HPV11, introduction of a HPV vaccine that includes HPV types 6 and 11 has the potential to greatly reduce the current burden of CA [1].

Primary prevention through vaccination has the ambitious objective to greatly reduce the incidence of HPV-related diseases [6]. Currently, two licensed HPV vaccines (Gardasil® and Gardasil9®) have been demonstrated with high efficacy not only in preventing precancerous and cancerous condition of the uterine cervix, vulva, oropharynx, vagina, and anal canal, but also in reducing the burden of CA, as both including non-oncogenic HPV types 6 and 11 [7–12]. Although Gardasil® and Gardasil9® have been reported to provide protection against CA associated with HPV6 and HPV11 [13,14], some barriers might limit their further applications to the specific prevention of CA, especially in males. Firstly, additional VLPs of other HPV types, HPV types 16/18 in Gardasil® and types 16/18/31/33/45/52/58 in Gardasil9®, may cause immune interference that reduces the immunogenicity of VLPs of HPV6 and HPV11. VLPs of HPV 16, 18 and 58 formulated a trivalent vaccine containing HPV 16, 18, and obviously interfered HPV 58 L1 specific neutralizing antibody levels [15]. Secondly, the overall reported annual incidence of CA in males is higher than that in females [4], whereas current vaccines are more beneficial to females as their excellent effects in prevention of cervical cancer [8,13], leading the vaccination coverage in males significantly lower than the current females vaccination rate [16–18]. Besides, from the economic considerations, gender-neutral vaccination is unlikely to be cost-effective if the coverage in girls is more than 50% [19–21]. Take above concerns into consideration, development of

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a bivalent HPV6/11 vaccine is attractively required for the specific prevention of CA, especially in males.

A suitable expression system is essential for the large-scale production of recombinant antigens for HPV6/11 vaccines. It needs not only to generate high yield of the required proteins, but also to satisfy stringent regulatory requirements [22]. GSK produces their HPV VLPs in insect cells via infection with baculoviruses carrying major capsid protein (L1) encoding genes of HPV. However, the insect cells expression systems have the disadvantage of low expression levels, complex growth requirements and slow growth rate, leading to high production costs [23]. Merck uses a stable L1 recombinant *Saccharomyces cerevisiae* expression system for the production of HPV VLPs as yeasts are economical, can be rapidly grown to high cell densities, and therefore produce big amount recombinant protein without pathogens, pyrogens or viral inclusions [24]. Alternatively, other types of yeasts such as *Pichia pastoris* and *Hansenula polymorpha* have been demonstrated their advantages and potential to produce recombinant protein antigens as they have tunable promoters, rapid growth rate, efficient protein production and capability of assembling VLPs [23–25]. The *Pichia pastoris* has been employed to express L1 proteins of HPV16 or HPV18, with satisfied yields and qualities of the recombinant proteins [23,26,27]. *Hansenula polymorpha* has been successfully used to produce commercialized recombinant interferonalpha-2a, hirudin, insulin, phytase and hepatitis B surface antigens [28–30], showing a great potential to produce other recombinant proteins, such as L1 proteins. Up to now, to our knowledge, the *Hansenula polymorpha* has not been reported to be adopted to produce L1 proteins of HPV6 and HPV11.

Being inspired by the success of using the *Hansenula polymorpha*, we expressed HPV 6/11 L1 proteins using the yeasts. The L1 proteins were assembled into typical VLPs, and used to prepare bivalent HPV6/11 VLP vaccines formulated with aluminum phosphate. The immunogenicity of bivalent HPV6/11 VLP vaccines was evaluated in mice and cynomolgus monkeys. The data provided here suggest that bivalent HPV6/11 VLP vaccines using *Hansenula polymorpha*-derived VLPs as antigens could be developed as a candidate vaccine for the prevention of CA in males.

2. Materials and methods

2.1. Preparation of HPV6 VLPs and HPV11 VLPs

Full-length HPV6 L1 protein and HPV11 L1 protein were expressed in the *Hansenula polymorpha* expression system and purified up to 95% purity with microfiltration and cation exchange chromatography.

Briefly, comparatively analysis of HPV L1 gene sequences was used to select protein sequences in GenBank and the consistent sequence with highest percentage of amino acid sites was chosen. The sequences of the genes encoding HPV6 L1 protein and HPV11 L1 protein, as shown in Fig. S1, were adjusted with yeast preferential codons for improving the yield of the L1 proteins in *Hansenula polymorpha*. The target gene sequence was combined with plasmid vector to construct recombinant expression plasmid pRMHP2.2-HPV L1 and the schematic diagram was shown in Fig. S2. We got the genetic stability of recombinant *Hansenula polymorpha* with serial passage and induced expression was used to select the optimal recombinant strain.

The initially expressed HPV VLPs were purified by POROS 50HS chromatography with ÄKTA Avant 150 protein chromatography system (Gelifesciences, USA). Further purification was conducted by Ceramic Hydroxyapatite chromatography with ÄKTA Avant 150 protein chromatography system, successively. In order to improve the stability and monodispersity of HPV6 and HPV11 VLPs, D/R treatment was prepared by treating the untreated VLPs with an ultra-filtration system containing dithiothreitol (DTT) under pH 8.0–9.0 and low concentration of salt at room temperature (RT), the samples were then reassembled by removing DTT using ultrafiltration at 4 °C.

2.2. Characterization of HPV6 VLPs and HPV11 VLPs

2.2.1. Electron microscopy

TEM was performed using negative staining. Samples were fixed on a 500-mesh copper grid, stained with phosphotungstic acid, and examined using a Tecnai 12 electron microscope (Philips Electron Optics Co., Eindhoven, Netherlands). Three fields were randomly selected for each sample at a magnification between 50,000× and 150,000×.

2.2.2. Size exclusion chromatography

SEC-HPLC was performed using Agilent 1260 (Agilent Technologies, Santa Clara, USA) high performance liquid chromatogram system. A TSKgel G6000PWXL-CP column (7.8 mm × 30 cm; Tosoh Corporation, Tokyo, Japan) was used at a flow rate of 0.5 mL/min using 0.5 M NaCl, 20 mM histidine and 0.02% Tween-80 elution buffer and adjust the pH to 6.2.

2.2.3. Dynamic light scattering

DLS was performed using a Malvern 3600 Light Scattering System (Malvern Instruments Ltd, Worcestershire, UK). The apparent hydrodynamic size of antigen particles was recorded as Z-average hydrodynamic diameter (d.nm). The Z-average hydrodynamic diameter value was obtained based on average diffusion coefficients by using the Stokes–Einstein equation. Each sample was measured in triplicate. The Z-average diameter was calculated from the correlation function using the ZETA size-software.

2.2.4. SDS-PAGE gel electrophoresis

Samples are separated according to their molecular weight using denaturing polyacrylamide gel electrophoresis (PAGE). The NuPAGE LDS Sample Buffer (4×, Life Technologies) contains lithium dodecyl sulfate (LDS) and the sample reducing agent (10×, Life Technologies) contains 500 mM DTT which would lead the sample in a denatured state once heated at 70 °C for 10 min. Then load the sample onto NuPAGE Novex 10% Bis-Tris gels (Life Technologies) at 180 V for 60 min, which was conducted with NuPAGE MES SDS running buffer on ice containing 1 × antioxidant (Life Technologies). The gel was dye-labeled by coomassie staining.

2.2.5. Ultra performance liquid chromatography – tandem mass spectrometer

Test samples were separated using acquity UPLC I-Class system (Waters Corp., Massachusetts, USA) and then analyzed by XevoG2-XS QTof mass spectrometer (Waters Corp., Massachusetts, USA). The analysis time was 20 min with positive ions detection method, precursor ion scan range was 500–4000 *m*/*z*. The original data were processed by UNIFI software (1.8.2, Waters).

2.3. Fabrication of the bivalent HPV6/11 vaccine

The bivalent HPV6/11 VLP vaccine was formulated with 20 μg HPV6 and 40 μg HPV 11 VLPs/dose and stabilized with 0.005–0.05% (w/v) Tween 80, 0.15–0.5 M NaCl and 2–20 mM histidine during purification and storage and adsorbed to 0.7–1.25 mg/ml aluminium phosphate.

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