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A DNA vaccine constructed with human papillomavirus type 16 (HPV16) *E7* and *E6* genes induced specific immune responses

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Abstract

Objective. Cervical cancer is found highly associated with human papillomaviruses type 16 (HPV16). HPV16 E6 and E7 oncogenes are important transforming genes which have become the main focus of anti-cervical cancer therapy. In this study, a recombinant DNA vaccine candidate, termed HPV16-DNA-E6E7, constructed with HPV16 E7 and E6 genes was generated and used to against HPV16-induced tumors.

Methods. We inserted an E7 DNA fragment into E6 gene to produce a recombinant gene (E6E7-DNA). The E6E7-DNA gene was inserted into a mammalian expression vector, pcDNA 3.1+, to construct the DNA vaccine candidate. Animals (C57BL/6 mice) were immunized with the vaccine candidate with various concentrations (50 μg, 100 μg or 200 μg, respectively), and cytotoxicity measurement and tumor protection assay were carried out to examine the immunological effects of the vaccine candidate.

Results. Immunization of with HPV16-E6E7-DNA induced HPV16-specific immune response and also conveyed protection against TC-1 induced tumor in vivo. A survival rate (90%) after 45 days of tumor challenge was observed. The animals injected with a higher dosage of the vaccine (200 μg) exhibited prolonged survival duration of more than 55 days. No transforming activity of the vaccine candidate was detected, as determined by focus formation and degradation of endogenous p53.

Conclusion. Our results demonstrated that the HPV16-E6E7-DNA compound might become a candidate for HPV16 precautionary and immunotherapy.

Keywords: Human papillomavirus type 16 (HPV16); *E6* oncogene; *E7* oncogene; Recombinant DNA vaccine; Tumor vaccine

Introduction

Cervical cancer is the second leading cause of cancer mortality in the world. The cancer remains one of the most common malignancies worldwide, with 470,000 new cases diagnosed each year [1]. Strong epidemiological and molecular biological evidences indicate that the origin of cervical cancer is closely linked to genital infection with oncogenic types of human papillomavirus (HPV) [2], and HPV type 16 (HPV16) is the most common one associated with severe cervical dysplasia and cancer [3].

The HPV16 oncogenes, *E6* and *E7*, are important in the induction and maintenance of cellular transformation and are co-expressed in most HPV16-containing cervical cancers [4]. Some evidences suggest cell-mediated immunity is important in controlling both HPV infection and HPV-associated neoplasms [5]. Therefore, *E6* and *E7* oncogenes become attractive targets for T-cell-based immunotherapy of cervical cancer.

Since HPV16 E6 and E7 are transforming oncoproteins, one of the dangerous side effects might be the transformation that is not anticipated with vaccine. In our study, we constructed a recombinant gene, which contains the HPV16 *E6* and *E7* gene. In order to eliminate the chance of transformation, we used only part of the *E7* gene that encodes a 21-amino-acid peptide from aa 38 to aa 57. HPV16 *E6* gene was divided into two parts at the 73rd amino acid position to destroy the encoding protein's natural structure. The *E7* gene fragment was inserted the middle of *E6* gene to construct a recombinant gene named *E6E7*-DNA gene. The recombinant gene was inserted into a mammalian

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expression plasmid, pcDNA3.1+, which was subsequently expressed as a DNA vaccine candidate, termed HPV16-*E6E7*-DNA.

A good vaccine should always have several essential elements including safety and low cost in manufacture, storage, and administration [6]. Protein-based vaccines are heat-sensitive and require low temperature conditions during manufacturing and storage to maintain efficacy. Conventional vaccines have raised safety concerns, as inactivated virus vaccines have been found some fatalness caused by the release of incompletely inactivated virus. DNA vaccines provide a safe and efficient alternative. The chemical nature of DNA contributes to the safety and convenience in manufacturing, storage, and administration of DNA vaccines. Immunization with plasmid DNA is able to elicit both cell-mediated and humoral immune responses, a fact that has contributed much to its appeal as a vaccine [7–9]. DNA-encoded antigens can induce $CD8^+$ T cell through the endogenous antigen pathway. The plasmid mimics virus behavior and produces antigen inside the cell, leading to the formation of peptide-MHC class I complexes [10]. In the absence of antibodies, cytolytic T lymphocyte (CTL) elicited by DNA immunization has been proven to be able to provide protection against virus infection [11,12]. DNA vaccine can also induce $CD4^+$ T cell and neutralizing antibody; the secretory proteins encoded by the plasmid and the antigen-presenting cells (APCs) play an important role in this process [13–16].

Methods and materials

Construction of HPV16 E6E7 DNA vaccine candidate

A plasmid containing the HPV16 *E6* N-terminal gene, the HPV16 *E6* C-terminal gene and the epitope of *E7* gene, was inserted into a stable mammalian vector pcDNA 3.1+(Invitrogen) and containing the markers of *ampR* for selection in bacterial cells and a CMV promoter for expression in mammalian cells. For cloning the *E6* N-terminal, a sense primer with the sequence: AGAGGATCCATGCACCAAA AGAG, and an antisense primer with the sequence: ACGAATTCTGGATTCCCATCTC were used, and a 231-bp PCR fragment was produced. For cloning the *E6* C-terminal, a sense primer with the sequence: GCAAGCTTCCATATGCTGTATG and an antisense primer with the sequence: GCACTCGAGCAGCTGGGTTTC were used, and a 280-bp PCR fragment was generated. As for the *E7* epitope, a sense primer with the sequence: GAGGAATTCATGATAGATGGTCC and an antisense primer with the sequence: GCGAAGCTTAAAGGTTACAATATTG were used to generate a 78-bp PCR fragment. The plasmid and PCR fragments were cut with various restriction enzymes including, *Bam*HI, *Eco*R I, *Hin*dIII and *Xho*I, and the three digested fragment were subsequently inserted into the plasmid to form a new plasmid named HPV16E6E7-pcDNA3.1+(Fig. 1) For large-scale plasmid DNA preparations, the plasmid was transformed into *E. coli* DH5α, and the bacteria were grown in culture medium supplemented with ampicillin. The plasmid obtained namely HPV16-*E6E7*-DNA, was extracted and the DNA concentration was determined by measuring the optical density at 260 nm, and the integrity of the plasmid as well as the absence of contaminating *E. coli* DNA or RNA were examined by agarose gel electrophoresis. DNA was stored at −20°C in a buffer (Tris–Cl buffer containing 20 μg/ml RNase). For animal injection, DNA was diluted in PBS to a final concentration of 2 μg/μl.

Identification of the DNA vaccine candidate expression

To determine whether the HPV16-*E6E7*-DNA could be expressed in mammalian cell line, we transfected the plasmid into a Cos-7 cell line (African green monkey kidney cells, ATCC) and determined the protein expression of protein by Western blot. Cos-7 cells were either transfected with HPV16-*E6E7*-DNA or pcDNA3.1+only (as a control), using a Lipofecin Reagent Kit (Invitrogen) according to the manufacturer's recommendations. The Cos-7 cells were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 5% antibiotics and antimycotic solution (Gibco). At 4 h after transfection, fresh medium was added instead of the transfection agent. After 12 h, the cells were harvest and washed with PBS containing ALLN. The expression protein was confirmed by Western blotting analysis using a HPV16 E6 C-terminus monoclonal antibody as a primary antibody.

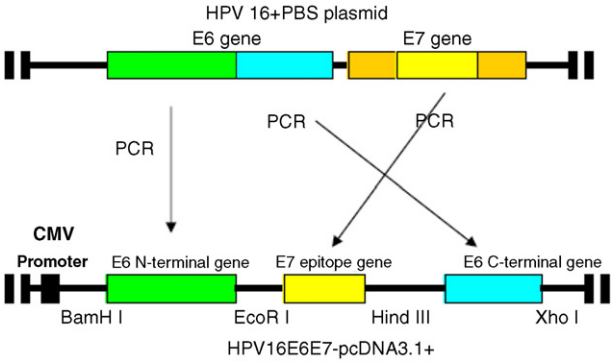

Fig. 1. DNA plasmid, HPV16 E6E7, was constructed by inserting three PCR fragments deriving from pHPV-16 plasmid. PCR was carried out and produced an E6 N-terminal gene fragment with *Bam*HI and *Eco*RI sites, an E6 C-terminal gene fragment with *Hin*dIII and *Xho*I sites, and an E7-epitope gene fragment with *Eco*RI and *Hin*dIII sites. The three PCR fragments were cloned into a mammalian vector, pcDNA3.1+, to produce a new mammalian plasmid termed HPV16E6E7-pcDNA3.1+.

In order to confirm whether the DNA vaccine candidate could be expressed in mice muscle tissues. C57BL/6 mice were immunized with the vaccine candidate and the tissues were isolated from the immunized locations at 5 days after the first immunization. Muscle tissues were frozen in liquid nitrogen as soon as it was taken out and lyzed with a lysis buffer (10 mM Tris–HCl, 100 mM EDTA, 0.5% sodium dodecyl sulfate, and ALLN, pH 8.0). After centrifugation, the expression protein in the supernatant was confirmed by Western blotting analysis using an anti-HPV16 E6 C-terminus monoclonal antibody.

Retroviral expression of the E6E7 gene

The *E6E7* gene was amplified by PCR using the appropriate primers. The PCR products were cloned into a retroviral expression vector, pBabe-puro (kindly given by from Dr. M. Tommasino). The BOSC23 cells were transfected with 10 μg recombinant pBabe-puro vector by a Lipofecin Reagent Kit (Invitrogen). After 48 h, the retrovirus-containing supernatant was harvested. Balb/c 3T3 cells were infected with retrovirus supernatant. After 2 days selection was started by adding 2 μg/ml of puromycin for 2 days. The puromycin concentration was reduced to 1 μg/ml, and was kept at this concentration during the experiment in order to maintain the selection pressure.

Soft agar transformation assay

Balb/c 3T3 cells (5×10^4 cells) infected with recombinant pBabe-puro retrovirus were resuspended in 10 ml of DMEM supplemented with 10% FBS, 1% melted agar and 1 μg/ml puromycin. Of this suspension, 2 ml (1×10^4 cells) were poured onto a pre-cast base layer consisting of 5 ml of DMEM supplemented with 10% FBS, 1% soft agar and 1 μg/ml of puromycin in Petri dishes (60 mm in diameter, Falcon). Soft agar cultures were incubated at 37°C (with 5% CO_2), and 0.5 ml of complete DMEM supplemented with 10% FBS (without puromycin) was added to the culture once per week. After 4 weeks, all foci located within a 9 cm^2 field of the soft agar plate were counted. Data are given as mean of three independent infection experiments. As a standard, the average number of foci caused by infection with the E6 wild type encoding