Original Article

Efficacy of HPV-16 E7 Based Vaccine in a TC-1 Tumoric Animal Model of Cervical Cancer

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Abstract

Objective: The human papillomavirus as an etiological agent of cervical cancer does not grow adequately in tissue culture systems. The tumor cell line TC-1 continuously expresses the E6 and E7 oncogenic proteins of HPV, and is considered a suitable tool in laboratory investigations and vaccine researches against cervical cancer.

Materials and Methods: The TC-1 cell line was grown in RPMI 1650 supplemented with 10% FBS, glutamine and antibiotics, and was used for tumor development in mice. Six to seven week-old tumor bearing C57BL/6 mice were divided into 3 groups consisting of 7 mice per group. The first group received pcDNA-E7, the second group received pcDNA3, and the third group received phosphate buffered saline (PBS). The treated animals were monitored for their tumor size progression and survival. At last, the tumoric tissues from autopsied animals were fixed and examined with Mayer's hematoxylin and eosin (H&E). All experiments were done in accordance with guidelines of the Laboratory Animal Ethical Commission of Tarbiat Modares University. Data analysis was performed using the one-way ANOVA followed by Tukey's test in both experimental and control groups. A p-value <0.05 was considered significant.

Results: There were significant decreases in tumor growth; there were also improvements in survival among mice in the treated groups ($p<0.041$). H&E stained sections from untreated mice were studied independently in a blinded fashion by two observers and showed malignant neoplasms composed of severely pleomorphic tumor cells with nuclear enlargement, high nuclear-cytoplasmic (N/C) ratios, and prominent nucleoli in solid and fascicular patterns of growth. High mitotic activity with extensive necrosis was also noted in both test and control groups.

Conclusion: The TC-1 lung metastatic model can be used to test the efficacy of various E7-based therapeutic cancer vaccine strategies for cervical cancer and the prevention of HPV-related neoplasia.

Keywords: TC-1, C57BL Mice, DNA Vaccine

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Introduction

Human papillomavirus (HPV) infections have been implicated in the development of epithelial malignancies, especially cervical cancer, and are considered the most frequent cause of sexually transmitted infections (STIs) (1-3). Each year, genital HPV infection affects 440 million people worldwide; it causes about 200,000 deaths, 80% of which occur in developing countries (3, 4). The high risk HPV types 16 and 18 have particularly been constantly implicated in causing cervical cancer (3, 5).

Since cervical cancer related case-control studies and clinical trials are not ethically permitted, a tumor model in C57BL/6 mice which expressed the HPV-16 tumor associated antigens E6 and E7 was used to establish an HPV-16 positive cervical cancer model (6). A well-characterized transplantable mouse tumor model which expresses E6 and E7 is the TC-1 tumor cell line. This cell line was derived from the primary lung epithelial cells of C57BL/6 mice; it was immortalized with the amphotropic retrovirus vector containing E6 and E7 genes and was subsequently transformed with the plasmid expressing the activated human c-Ha-ras oncogene (6-8). TC-1 cells are poorly immunogenic in vivo and form solid tumors when injected subcutaneously (8, 9).

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In this study, TC-1 cells were used to develop a tumor model in C57BL/6 mice for investigation of tumor production and evaluation of tumor features. This model can be used to evaluate the effectiveness of the HPV vaccine.
It is shown that HPV vaccinations potentially can protect against cervical cancer, indicating that immunoreactivity of HPV-16 E7 based vaccine against HPV plays a key role in cancer prevention and therapy (10).

Materials and Methods

Cell line

The TC-1 cells were purchased from a cell bank (Pasteur Institute of Iran). They were cultured in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum, insulin, growth factor, 2 mM L-glutamine, 1 mM pyruvate, 0.1mM minimal essential medium with nonessential amino acids, 100U penicillin/ml and 100 µg streptomycin/ml and was incubated at 37°C in 5% CO_2 (8).

Construction of DNA expression vector

As shown previously, the HPV16-E7 gene was isolated using PCR, cloned into pTZ57R/T-E7 and sequenced. The target gene was sub-cloned into the unique EcoRI and XbaI cloning sites of the pcDNA3 expression vector (Invitrogen, Canada). The competent DH5α strain of *E. Coli* cells were transformed using a confirmed recombinant pcDNA3-E7 vector in Luria-Bertani medium (Fig 1).
The presence of the HPV16-E7 gene in the constructed vector (pcDNA3-E7) was determined using restriction enzyme analysis (11). Large-scale plasmid preparation was performed according to the standard polyethylene glycol (PEG) precipitation method (12).

Mice

Six to seven week-old female C57BL/6 mice were obtained from the Pasteur Institute of Iran. Given free access to food and water, the mice were housed as of one week before the experiment, and were maintained in a good standard condition. All experiments were done in accordance with the Animal Care and Use Protocol of Tarbiat Modares University.

Optimization of tumor formation

Inoculation of HPV-16 E6 and E7 expressing TC-1 tumor cells caused rapid tumor formation. TC-1 cells were subcutaneously injected into the left flanks of the C57BL/6 mice at various dosages (4×10^5, 5×10^5, 10^6 cells/mouse), suspended in 100µl phosphate buffered saline (PBS).

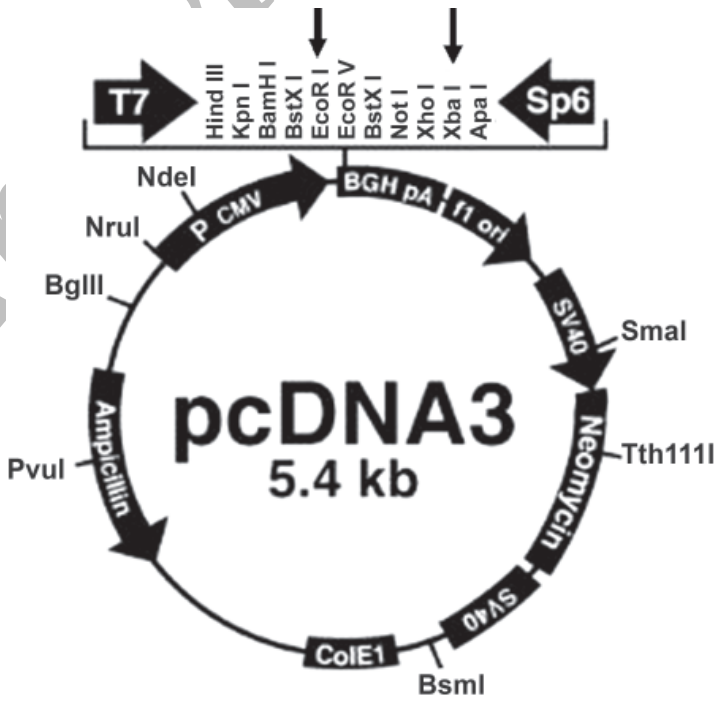

Fig 1: pcDNA3 as an expression vector. Restriction enzymes used are shown with two arrows.

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Monitoring of tumor model

The C57BL/6 mice were challenged by subcutaneous injections of TC-1 cells (5×10^5) suspended in 100 µl PBS to their left flanks. After 2 weeks, the resulting tumors were obvious and palpable in the mice. Tumor sizes (in millimeters) were reported as the average of all measured dimensions. Each tumor's smallest diameter (a) and biggest (b) diameters were measured and its volume was calculated using the formula: $V= (a^2b)/2$ (13).

Immunization of mice

Two weeks after the initial inoculation of the mice with TC 1 cells, the animals were grouped into 3 cages and each group was intramuscularly vaccinated 3 times in two-week intervals with 100µl PBS (negative control), 100µg pcDNA3 (negative control), and 100µg pcDNA3-E7 (test). They were then monitored every other day for survival as well as infection symptoms, and their tumor diameter sizes were measured and their means were calculated and recorded.

Histological study

The mice were euthanized and histologically studied. Autopsied tissues from control and test animals were fixed in 10% phosphate-buffered formalin. They were imbedded in paraffin and were sectioned at 4-6 µm; they were then stained with Mayer's hematoxylin and eosin (H&E). The slides were mounted and microscopically evaluated (×40) (Fig 2).

Statistical analysis

To compare results between the different groups, the univariate analysis of variance (ANOVA) was performed using the SPSS 11.0 statistical software. Differences were considered statistically significant when p-value <0.05.

Results

Rapid tumor formation

Fig 2 shows the confluent monolayer of TC-1 cells. These adherent cells have a fibroblastic appearance and continuously express E6 and E7 oncogenic proteins of HPV. As shown in fig.3, rapid tumor growth appeared in mice inoculated with 5×10^5 TC-1 cells.

Cancer prevention and therapy in the immunized mice

The mice were inoculated as described in material and methods and monitored for evidence of tumor growth by palpation and inspection of two of their perpendicular tumor diameters every other day. Data analysis was performed using the one-way ANOVA followed by Tukeys' test in the experimental and control groups. A p-value <0.05 was considered significant. There were significant decreases in tumors sizes and improvements in survival among the treated mice ($p<0.041$). The mean tumor volumes and tumor sizes were not significantly different between the two control groups ($p=0.9$), whereas significant differences were observed in the DNA vaccinated group ($p=0.00$) (Tables 1, 2).

Fig 2: Confluent monolayer of TC-1 cells (×40)

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Table 1: Comparison of tumor size between test and control groups. The difference between the sizes in pcDNA3 and pcDNA3/E7 shows a meaningful reduction and is statistically significant.

Groups	P value
PBS & pcDNA3-E7	0.00
pcDNA3 & pcDNA3-E7	0.041
PBS & pcDNA3	0.90

P-values were calculated using univariate analysis of variance. The E7 groups had significant differences of p= 0.04 and 0.00 in comparison to the pcDNA3 and PBS control groups.

A neoformed tumor lesion is composed of undifferentiated polyhedral, round and plump spindle cells with high N/C ratios, eosinophilic cytoplasms with indistinct borders, highly pleomorphic nuclei with coarse granular chromatin, and many giant cells with multiple nuclei. Furthermore, such a lesion displays a high mitotic rate with atypical features, and is packed together by a cellular sheath with large necrotic areas.
It also invades its peripheral skeletal muscle fibers.

In our specimens, none of these special cytoplasmic and nuclear features of a viral infection, which are common in the highly undifferentiated HPV-induced tumor lesions, were identified (Fig 3).

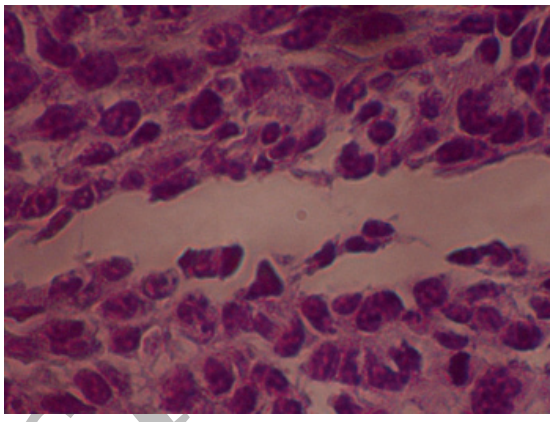

Fig 4: A high grade intraepithelial neoplastic lesion. This figure shows cells of tumoric tissue cells after injection with TC-1 cells injection (×40). TC-1 tumoric model showing many tumoric giant cells with multiple nuclei; no differentiation was detectable on the surface.

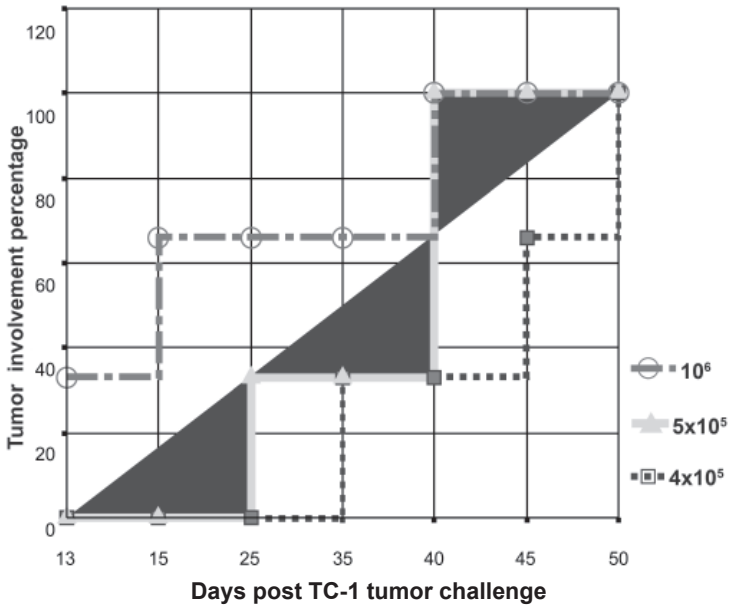

Fig 3: Tumor growth kinetic of TC-1. The cells were subcutaneously injected into C57BL/6 mice subcutaneously with various doses (4×10^5, 5×10^5, 10^6 cells/mouse). The mice were then monitored for tumor appearance.

Table 2: Measurement of tumor size in mice from the PBS, pcDNA3 and pcDNA3- E7 groups on different days

Days p.i	PBS mean ± SD	pcDNA3 mean ± SD	pcDNA3/E7 mean ± SD
34	607 ± 25.7	600 ± 24.3	305 ± 5.5
55	4123.5 ± 20.4	4469 ± 21.1	1860.2 ± 3.4
66	4998 ± 8.2	5482.1 ± 20	1492.3 ± 1.5

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Discussion

HPV is an epithelium tropic DNA virus and is responsible for one of the most widespread sexually transmitted infections (4).

High-risk HPV infections are the major cause of cervical cancer with a high mortality rate in women. (10, 14).

Tumor models in C57BL/6 mice have been used in cervical cancer studies since clinical trials are not ethically allowed. Therefore, TC-1 cells are produced for experimental purposes because they continuously express the E6 and E7 oncoproteins (15). These cells have gained an appropriate importance status for vaccine researches against cervical carcinoma.

Administration of HPV vaccines plays a central role in preventing human papilloma virus (HPV) infections in people. The majority of animal studies using viral vectors for the HPV16 E7 DNA vaccine have focused on prophylaxis to generate immune responses which could reject a subsequently injected tumor challenge. The only available vaccine approved by the U.S. food and drug administration (FDA) for human use is Gardasil, a quadrivalent recombinant vaccine manufactured and marketed by Merck (16). Several studies have shown that such purified recombinant HPV proteins and HPV DNA vaccines have been successfully used to generate cell-mediated immune responses in experimental animal models (17, 18).

We conducted therapeutic vaccination trials in mice with already established tumors. Thru a number of immune measure assessments, Meshkat et al. showed that immunization of C57BL/6 mice induced efficient immune responses and generated HPV16 E7-specific cytotoxic T lymphocytes (CTLs) (11). In this study we used the same construct and therapeutic strategies which are capable of generating an antitumor response in tumor bearing mice.

In this study, tissues were perused independently and showed malignant neoplasms composed of severely pleomorphic tumor cells with nuclear enlargement, high N/C ratios and prominent nucleoli in solid and fascicular patterns of growth. High mitotic activity with extensive necrosis was also noted in both test and control groups. It seems that immunization with this construct alone could not improve the mitotic activity. Therefore, the linkage of different genes or gene fragments to a DNA vaccine represents a prospective approach for increasing the potency of DNA vaccines.

With continued attempts in the development of HPV therapeutic vaccines, HPV therapeutic DNA vaccines will appear as a significant approach which can be combined with existing forms of therapy such as chemo and radiation therapies. Therapeutic strategies such as a DNA vaccine capable of generating an antitumor response, should be explored as a way to treat infected patients.

Conclusion

Currently, there is a major thrust to develop vaccines in an effort to prevent infectious diseases. In order to test the efficacy of these vaccines, suitable animal models are needed. Normally, absolute protection against disease is the ultimate goal, but in cases such as cervical cancer, it may take years for HPV-associated lesions to appear; thus, protection against the initial infection would be a faster way to determine efficacy. In conclusion, the results of this study might improve our knowledge in an attempt to treat the TC-1 tumor-cell challenged animals as the first step in a successful preventive vaccine development.

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There is no conflict of interest in this article.

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