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Developing Michigan Cancer Foundation 7 Cells with Stable Expression of E7 Gene of Human Papillomavirus Type 16

Faezeh Ghasemi[1], Sina Rostami[2], Maryam Sadat Nabavinia[3], *Zahra Meshkat[4]

1. Dept. of New Sciences and Technology, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

2. Dept. of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

3. Dept. of Biotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

4. Antimicrobial Resistance Research Center, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

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ABSTRACT

Background: Human papillomavirus (HPV) is responsible for the development of cervical neoplasia. Infection with human papillomavirus type 16 (HPV-16) is a major risk factor for the development of cervical cancer. The virus encodes three oncoproteins (E5, E6 and E7), of which, the E7 oncoprotein is the major protein involved in cell immortalization and transformation of the infected cells. The aim of the current study was to develop Michigan Cancer Foundation 7 (MCF7) cells, which could stably express E7 protein of HPV type 16.

Methods: E7 gene of HPV type 16 was introduced into MCF7 cells by Lipofectamine 2000 reagent and the transfected cells were treated with G418 antibiotic. After antibiotic selection of the transfected cells, stable expression of E7 gene of HPV16 was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR).

Results: Antibiotic selections of transfected cells were performed and transfected cells were alive in cytotoxic concentration of the antibiotic. RNA was extracted from transfected cells and E7 gene of HPV16 was amplified by RT-PCR method and a 350-bp band corresponds to E7 was observed.

Conclusion: Results confirmed the stable transfection of cells. The stably transfected cells can be used as a useful tool in future studies on HPV16 and cancers caused by this virus.

Corresponding Information: Antimicrobial Resistance Research Center, Mashhad University of Medical Sciences, Mashhad, IR Iran. P.O Box: 9196773117, Tel: +98-511- 8012453, Fax: +98-511-8002287, Email: meshkatz@mums.ac.ir

Introduction

Papillomaviruses lack an envelope. Its nucleocapsid has icosahedral symmetry and is 52-55 nm in size (1). Papillomaviruses are very specific and show tropism for squamous epithelial cells (2). These viruses produce epithelial squamous and fibroepithelial tumor in their natural host (2, 3). Several types of HPVs may infect female genital tract and are capable of inducing cervical dysplasia in infected women (4).

More than 100 types of HPV have been found. Based on their ability to cause cancers, they have been classified as high-risk and low-risk HPVs (5).

HPV type 16 is considered to be in high-risk group and is responsible for more than half of cases of cervical cancers (6). HPV has a small circular genome of 8 kb in length; it contains two structural glycoproteins and six nonstructural proteins (7). E7 is a small nuclear protein. The ORF of E7 of HPV type 16 encodes a 98-amino acid phosphorylated protein (8). The encoded protein, with its oncogenic properties, could be involved in the induction of cancer (9). E7, with its stable expression in HPV or in cells infected with HPV could infect cells moreover, through its interactions with intracellular proteins and binding to tumor suppressor protein, pRB is critical in the control of cell cycle. Functions of E7 includes making cells eternal activation of Cyc E-A proteins, inactivation of Rb, induction of apoptosis and inhibition of several CDKs, increasing the permeability of cells to external DNA, inducing mutagenesis and resolution or inactivation of tyrosine kinas enzyme (10). MCF7 is derived from human breast cancer cells. It was firstly extracted from malignant breast tumor of a 69-year old woman in 1970. It grows attached to the surface (11).

The aim of the current study was to develop MCF7 cells with stably expression of E7 gene of HPV type 16.

Materials and Methods

Vector: Eukaryotic expression plasmid containing E7 gene of HPV type 16 was evaluated in the present study.

Cell line: MCF7 cell line was used. This cell line was purchased from Pasteur Institute of Iran. Cells were cultured with Dulbecco's modified Eagle's medium DMEM (high glucose) containing 10% bovine serum albumin, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100µg/ml of streptomycin and 2 mM glutamine at 37 ºC under 5% CO2 condition.

Determination of cytotoxicity of G418 antibiotic: concentrations higher than 400 µg/ml resulted in the death of all cells. Unviable cells were detected by their globular and granulated appearance and their faint plasma membrane.

Construction of MCF7 cells with stable expression of E7 gene

First, MCF7 cells were cultured in two wells of a 6-well microplate containing rich medium. Following that, cells of one well were transfected with pcDNA3/E7 by Lipofectamine 2000 reagent, according to the manufacturer's recommendations. The second well was chosen as a control (all steps were also carried out on the minus control well). After 48 hours of transfection, 20% of cells in each well were transferred to the corresponding adjacent wells and growth medium containing G418 (the minimum concentration needed for kill this cell line) was added. Cells were passaged each two days to a new medium containing antibiotic so that the non-transfected cells (lacking antibiotic resistant gene) were killed and only those with expression vectors remained. In other words, cell focuses were formed. At the time all cells in the minus control completely were killed, concentration of the antibiotic was doubled in order to kill non-transfected cells in test well. Cells were then transferred to cell culture flask and replacement of culture with the new culture containing G418 antibiotic was performed to produce the needed amount of cells.

RT-PCR to assess the stable expression of E7

Following construction of E7 cells, their stable expression were confirmed by RT-PCR. Briefly, total RNA was extracted using RNX-Plus (Cinnagen, Tehran, Iran). DNA contamination was removed by *DNase I*. After that, RT-PCR

was performed. Complementary DNA (cDNA) was synthesized for 1µg of RNA by using oligo-dT primers at 37 ºC. In the final step, PCR was carried out using E7 specific primers, as described previously (12).

Results

Confirming the expression of the E7 of HPV type 16 by RT-PCR

In order to determine the fatal concentration

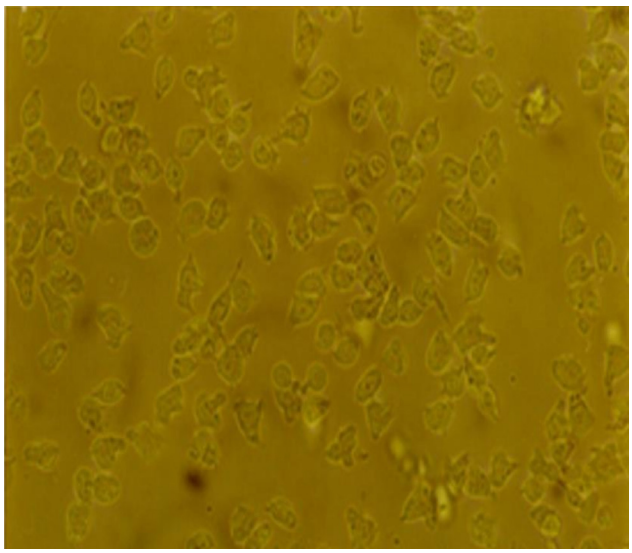

Fig. 1
Dead MCF7 cells due to high concentration of antibiotic

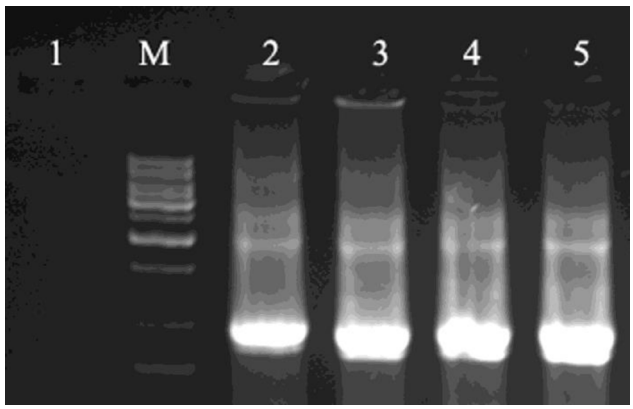

Fig. 3
Gel electrophoresis of RT-PCR products. RT-PCR was performed for amplifying 350 bp fragment using HPV 16 E7 specific primers. Lanes 1 and 2 are negative and positive controls in RT-PCR, respectively; Lanes 3, 4, and 5 correspond to HPV 16 E7 stable transfected cells; M: 100 bp DNA size marker

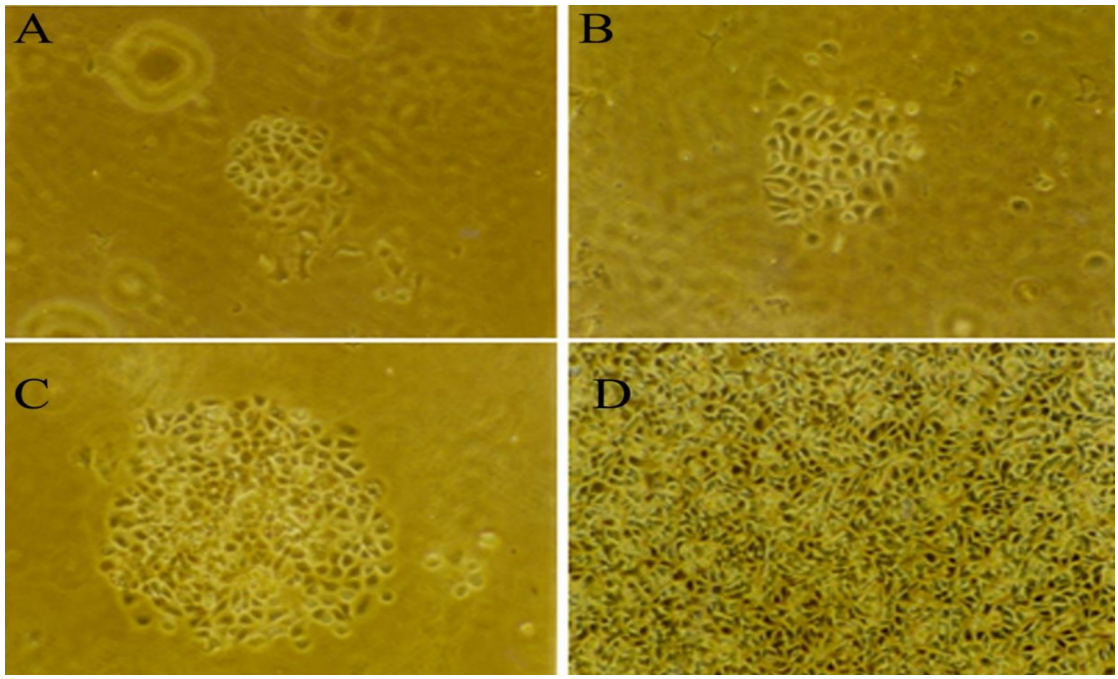

Fig. 2
Antibiotic selection of MCF7 cell clones. A: Primary stages of clone formation. B and C: growth of cells. D: formation of monolayer

of antibiotic for MCF7 cells, after 24 hours, cell membranes were destroyed and no isolated cells were observed (Fig. 1). The fatal concentration of G418 antibiotic was 400µg/ml for MCF7 cells.

After transfection of MCF7 cells with E7 expression vector of HPV, antibiotic selection was performed using fatal concentration of G418. Antibiotic treatment was performed by 400 µg/ml antibiotic for 14 days to obtain a stable clone of the cells. After antibiotic selection, the resulted clone was treated by 2x fatal concentration of G418 (800 µg/ml) for more 14 days to confirm the strong expression of the E7 gene. The resulted clone after antibiotic selection is indicated in Figure 2 at stages A, B and C. Stage D indicates the final monolayer. In order to evaluate the expression of E7 in final monolayer cells, RT-PCR was performed by oligo-dT, and 350-bp band corresponds to E7 (Fig. 3).

Discussion

More than 100 types of HPVs have been suggested, among which, type 16 is the most common (13). Type 16 is associated with severe and lethal cervical cancers. In primary genital lesions, HPV-DNA is found mostly in its episomal form (14).

However, in cell lines of cervical cancers and malignant tumors, viral DNA is found in its integrated form (15). Integration induces a mechanism in which pre-malignant lesions develop to cervical cancer transformation and its development to malignancy (16).

In keratinocyte cell line, E6/E7 of type 16 makes cells immortal eternal and inhibits final differentiation of cells. Expression of E6/E7 of type 16 was performed with B-actin promoter in P1321plasmid. This was important in making keratinocyte cells immortal eternal (17). Considering the crucial role of pRb in cell cycle, inhibiting the action of this protein by mutations and its further interactions with oncogenic genes of HPV, would result in the reduction of its level in cancer cells (18). E7 binds to hypophosphsrylated pRb and inhibits its action. This promoter the cell cycle to the S phase and triggers the synthesis of DNA (19).

In low risk types of HPV such as types 6-11, the affinity of protein E7 to pRb is ten times less than that of the high-risk types of HPV (20). Low risk types of HPV have less efficiency in cell transformation (21).

Expression of E6-E7 in both cell lines (BT20-MCF7) is accompanied by an increase in the expression of a transcription factor (Id-1). Recent studies showed that Id-1 is a regulatory factor of breast cancer cells in terms of metastasis (22).

Oncoprotein E6-E7 increases the expression of Id-7 in MCF7 and BT20 cell lines. Furthermore, HPV type 16 was present in all breast cells (23).

In another study, coupled receptors and ErbB-2 oncoprotein E6, E7 HPV type 16 tumor geneses of breast cancer cells was observed. ErbB-2 receptor is over-expressed in 30% of human breast cancers and 50% of breast cancers are positive for HPV type 16 and 18 (24).

Oncoprotein ErB-2 and E4-E6 of HPV type 16 cause malignant changes in epithelial cells of the mouth. Moreover, E6 and E7 of HPV type 16 transform non-invasive breast cells to invasive. In order to evaluate the association of ErB-2 and E6-E7 with tumorgenesis, transgenic mice, which had ErB-2 and E4-E6 of HPV type 16 and human keratin 14 as promoter were designed. After six months, these mice have developed more severe breast cancer. In Syria, high associations between HPV infection and breast cancer have been proved (25).

In a cohort study on 113 patients with breast cancer, HPV types 16, 18, 31, 33 and 35 was observed in 8.84, 9.73, 0.o7, 55, 75 % and 37.16% of samples with invasive breast cancer, respectively. Totally, 69 (61.6%) of 113 samples were positive for HPV. Among which, 24 (37.78%) were infected with more than one type of HPV. Besides, expression of E6 oncoprotein in high – risk HPV was associated with the expression of

Id-1 in almost all invasive breast cancer samples. Another study showed the association of ErbB-2 receptor and the oncoprotein E6 and E7 of HPV type 16 cause tumorgenesis of breast cells (25).

Survivin is a member of IAP apoptotic inhibiters, which controls cell cycle. The expression of this gene is low in normal cells (26). This gene is mostly expressed in embryological and tumor tissues. Temporary expression of E6, E7 of HPV type 16 with surviving gene promoter was studied in vitro (27). P53 reduces the expression of survivin .Transformation of E6, E7 of HPV in fibroblast cell lines increased endogenous expression of mRNA of survivin. This was performed in MCF7, HeLa and Soas2 cell lines (28).

In another study, E6, E7 of HPV type 16 inactivated CDKp27 and CDKp21 inhibiters. Thus, direct relationship between this protein and regulatory factors of cell cycle exist considering the association of E6 and E7 of HPV type16 and intracellular factors such as S4 subunit of 265 proteosome, the association of this protein and cell tumor genesis was suggested (27).

In the present study, MCF7 cells with stable expression of HPV 16 E7 protein were developed. MCF7 cells originated from human breast cancer and the designed model can use as a HPV positive breast cancer model in in vitro studies. In addition, the interaction between HPV E7 oncoprotein and activated human oncogenes of MCF7 cells can be studied in this model in future.

Conclusion

Considering there is no animal model for HPV, MCF7 cells stably expressing HPV type 16 E7 protein will be valuable in HPV studies to develop a tumoral model for HPV16 in laboratory animals and to evaluate their immunologic responses in animal model as antigen candidates for vaccine production. In addition, it can be used as a model for HPV 16 positive human breast cancers in future studies.

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