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Original Article

Construction of the Chimeric HSP70 - E7 Vector and Evaluation of its Protein Production

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Abstract

Background and Aims: Since the produced recombinant proteins by molecular genetics techniques commonly have some limitations in the application, chimeric protein are introduced. Chimeric proteins have found widespread application for the study of protein folding, structure stability, function and immunogenisity.

Methods: According to the known Immunomodulatory effect and structure of HSP70 molecule, full length human HSP70 was selected and a hybrid was made with HPV-E7gene.The recombinant plasmid pCDNA3.1/ E7-HSP70 was constructed using sequential PCR and cloning steps. The entire upstream and downstream sequences of the target molecules were synthesized separately. The sequential cloning was performed for cloning of the entire sequences of the target molecule of HPV16-E7 fragment in pcDNA3.1. Target DNA was visualized by staining with ethidium bromide. The Cos-7 cell lines were transfected with fusion proteins in 6 well microtiter plates. After 48 hours of transfection, the target cells were removed and to SDS-PAGE analysis for mRNA detection. Immunoreactivity of the protein product was assayed by Western blotting using monoclonal antibody.

Results: The sequencing analysis showed that E7 gene was fused in frame to the HSP70 gene in pcDNA3.1/E7-HSP70. Western blot analysis of recombinant fusion protein using HSP 70 monoclonal antibody showed desired band as expected. The chimeric structure was expressed in cells, as expected. The resulting E7- HSP70 fusion gene would be a useful construct for future research.

Conclusion: In order to change and enhance of the tropism and immunogenicity of recombinant protein, chimeric E7- HSP70 Vector was constructed. Since monovalent molecule and vaccines were clinically ineffective or poorly immunogenic, so applications of covalently linked product are introduced. The successful expression of the E7- Hsp70 fusion protein can be used as a molecular target for establishment of DNA and recombinant protein vaccine in future research.

Keywords: pcDNA3.1; HSP70; chimeric vector

Introduction

Human papillomaviruses (HPVs) are DNA viruses with small circular genomes that mainly cause infection in epithelial cells, that can progress to hyperplasia ranging from benign papillomas (warts) to premalignant lesions (1).There are over 200 different HPV types, approximately 40 of which specifically infect anogenital tract mucosa (2). HPV is responsible for one of the commonest sexually transmitted infection.

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Genital HPV infection affects 440 million people yearly worldwide and over 80% of these are in developing countries (3). Deaths related to cervical cancer are around 250,000 per year (3).

Actually, the natural history of HPV infections is not clearly understood. It appears that the majority of infections are transient and asymptomatic, but HPV could lead to benign proliferations to invasive malignancy. HPV DNA is detected in approximately 99.7% of cervical cancers (1), being HPV 16 and HPV 18 associated with about 60% and 10% of them, respectively (4).

The high risk HPV early genes E6 and E7 genes are required for the maintenant cellular transformation and are constitutively expressed in the majority of precancer tumor cells. The HPV16 E7 represents a target of choice for the therapeutic vaccination study. In this study, we investigated the production of HPV16 full length E7gene flanking to HSP70 to be used as an adjuvant and for protein production in eukaryotic cells.

Methods

Materials

All restriction enzymes and nucleic acid modifying enzymes and other chemicals were obtained from commercial sources.

Cell Line

The Cos-7 cell line was purchased from the cell bank (Pasteur institute of Iran). The cells were cultured in RPMI 1640 (Gibco) supplemented with 10% of heat-inactivated fetal calf serum, 2mM L-glutamine, 1mM pyruvate, 0.1mM minimal essential medium with nonessential amino acids, 100U of penicillin/ml, 100 µg of streptomycin/ml and incubated at 37°C, 5% CO2.

Construction of Recombinant Plasmids

The HPV16-E7 gene in PUC18\19 was confirmed by sequencing and sub-cloned into the unique EcoRI and XhoI cloning sites of the pcDNA3.1 expression vector (Invitrogen). The expression vector containing human HSP70 was kindly provided by Dr McLean P.J. (Department of Neurology, Mass General Institute for Neurodegenerative Disease, Massachusetts General Hospital, USA).The presence of the HSP gene in the constructed vector (pcDNA3/ HSP 70) was determined using restriction enzyme analysis. The DH5α strain of E.Coli competent cells were transformed with confirmed recombinant vectors in Luria-Bertani medium and the plasmids were extracted using Bioneer commercial Kit. DNA concentrations were determined by measuring the optical density at 260 nm and the absence of the contaminating Ecoli DNA or RNA was checked by agarose gel electrophoresis. Large-scale preparation of the plasmid was performed according to standard methods (5).

Construction of Recombinant pcDNA3.1/ E7-HSP70 chimer Plasmid

The fragment bearing the full region of the E7 gene from PUC18\19 was purified and ligated into plasmid pcDNA3.1 which was partially digested with the same enzymes. A 300bp sticky ended Kpn1 and BamHI fragment, which contained the entire E7gene without stop codon was amplified by PCR and cloned in pcDNA3.1.

The resultant clone was digested with BamHI and XhoI for insertion of the full length HPS70 gene fragment in frame with initial start codon of HPV-E7 gene. The final full length PCR product of modified HSP70 using specific primer set containing designed restriction enzyme and linker fragment was excised from the agarose gel, purified by Bioneer extraction kit (Korea) and applied for cloning in the vector by digestion with BamHI and XhoI enzymes. The confirmed sequenced HSP70 fragment was applied and ligated into plasmid pcDNA3.1/E7 at the 3' end of the E7gene, which had been digested with BamHI, and XhoI.

The sequence analysis showed that E7 gene was fused in frame to the HSP70 gene in pcDNA3.1, which could produce target fusion protein under the control of the CMV promoter. The final schema of amplification gene product is illustrated in Figure 1 with their expected base pair (bp) size.

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In Vitro evaluation of transient expression in Cos7 Cells

In order to monitor the expression of E7-HSP70 recombinant protein, 10^6 Cos7 cells were seeded into a 6-well microtiter plate and incubated overnight in complete medium without any antibiotics. The cells were used for transfection at 70% confluency. The cells were transfected with pcDNA3.1/E7-HSP70 or pcDNA3.1 using Lipofectamine2000 (Invitrogen) according to the manufacturer's structures. At 48 h post transfection, the cells were collected by scrapping and the total cell lysates were prepared by 4 times freezing and thawing followed by sonication (60 HZ, 0.5 Amplitude for 60 sec). PMSF (1mM, Phenyl Methane Sulfony Fluoride) was added to the cell lysates to inactive the cellular proteinase. The lysates were centrifuged (800g), and the protein was quantitated by Bradford assay.

RT-PCR for confirming expression of E7-HSP70

To determine the presence of E7-HSP70 mRNA in the Cos7 transfected cells, RT-PCR was performed at 48 h after transfection. Total cellular RNA was purified from the cells using RNX Plus kit (Cinagen, Iran). The contaminating DNA was removed by DNase treatment and the extracted RNA was used in RT-PCR. In brief, cDNA synthesis was performed on 1 µg total RNA with specific primers at 37ºC. PCR was performed on cDNA using specific primers.

Gel Electrophoresis and Western Blot Detection of recombinant fusion protein

A binary fusion protein was constructed as described above. The target protein was harvested and prepared by heating at 90 ^{o} C in SDS sample buffer for2 min. SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described elsewhere (6). Immunoblotting was performed by electro-phoretic transfer of the proteins from gel on to nitrocellulose paper for 18-24 h at 25 mV at 4^{o}C. Unoccupied sites on the nitrocellulose papers were blocked using 5% nonfat dried milk in phosphate buffered saline (21 mM KH,PO, 11 mM Na2HP04.7H20, 0.138 M NaCl, 2.5 mM KCl,pH 7.4). The paper was incubated with mouse anti-HSP antibody for 1 h and washed three times for 10 min each with 15 ml of phosphate buffered saline containing 5% dried milk. To detect the antigen-antibody complex, the membrane was washed and incubated with horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin G antibody (1:1000). Each antibody solution was in contact with the membrane for 1 h at room temperature. The membranes were washed 3×20 min with TBS-T, and the bonds were detected using diaminobenzidine (DAB) substrate (Biogen).

Results

These results indicate that linkage of HSP70 to DNA construct may significantly increase the yield of protein production in one step transfection. It was shown that HSP complexes isolated from tumor or virus infected cells were able to induce potent anti-tumor or antiviral immunity (7-9). To facilitate the presentation of antigenic peptides, the antigen or peptide must be associated with HSPs in the form of covalent HSP-peptide hybrid, fusion protein, or naturally binding in cells (10-13).

In this study, full length HPV 16 E7 DNA was selected and fused to human HSP70 gene by cloning and PCR whenever required. The chimeric expression vector was sequenced and subsequently used for transfection. Protein production was confirmed by mRNA detection and western blot analysis.

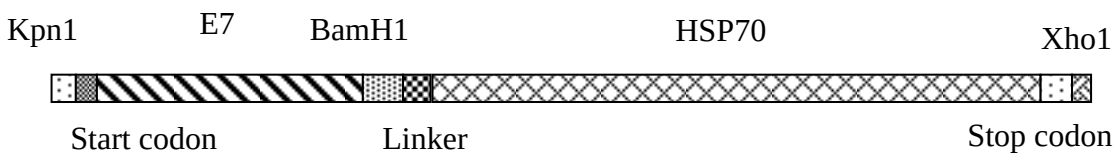

Fig. 1. The diagram of the recombinant gene.

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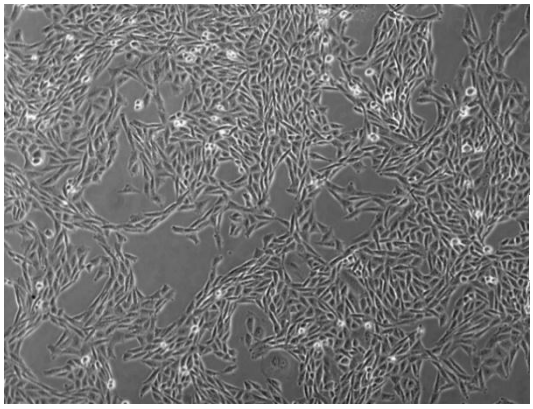

Fig. 2. Confluent monolayer of Cos7 cells.

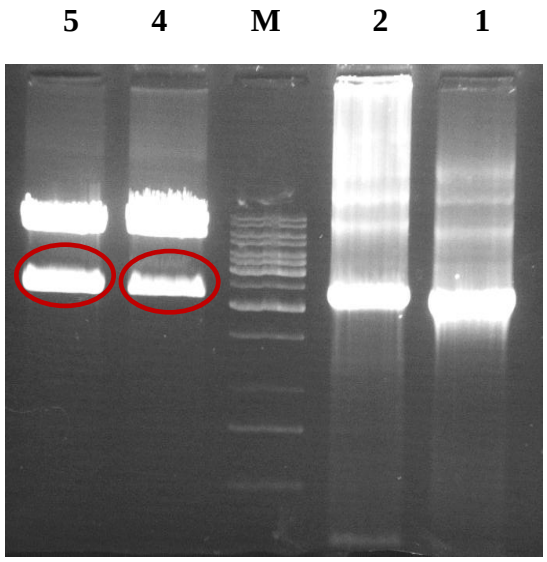

Fig. 3. The recombinant plasmid pcDNA3/ E7-Hsp70 was identified by colony-PCR (lane 1, 2) and enzyme digestion (Kpn1 and Xho1) (lane 4, 5), lane M is DNA ladder 1kb.

Confirmation of Recombinant pcDNA3.1/ E7-HSP70 chimer Plasmid production

The recombinant plasmid pc DNA3/E7-HSP70 was identified by enzyme digestion as shown in Figure3. The sequencing test confirmed that HPV-E7 gene fragment (335bp) and human HSP70 gene (1800bp) had been inserted into the pcDNA3.1 vector and the gene had the same sequence as that had been reported (14).

Expression of Hsp70-E7 fusion protein by RT-PCR

Transcription experiment in transfected cells was confirmed by PCR which was performed on cDNA by the specific primers belonging to the fusion gene, which was shown in Figure 4.

Gel Electrophoresis and Western Blot analysis

The transfected cell with pc DNA3/E7-HSP70 along with cells transfected with the control plasmid pc DNA3.1 were analyzed by SDS-PAGE under reducing conditions, which showed a new band with an apparent molecular mass (~82 kd). Taking the molecular weight of the HSP70 protein (70 kd) and that of the target wild-type E7protein (12 kd) into account, the size of the new band fit with (82 kd), what was expected for the protein shown in Figure 5a.

The results demonstrate that the E7-HSP70 protein can interact with mouse anti-HSP antibody as shown in Figure5b. No HSP70-E7 protein reactivity was seen in the cells transfected with the control plasmid (fig. 5.a/b).

SDS PAGE and Western blot detection of chimericic protein expression in Cos7 cells in vitro

Cos7 cells were transfected with pc DNA3.1/E7-HSP70 or pc DNA3.1 at a concentration of 50 µg/ml. Seventy two hours after transfection, medium was removed and cellular pellet were collected and prepared to run on a 12.5% sodium dodecyl phosphate-polyacrylamidc gel electrophoresis and transferred overnight to a polyvinylidcne difluoride membrane. The expressed protein was immunoprecipitated with monoclonal antibody.

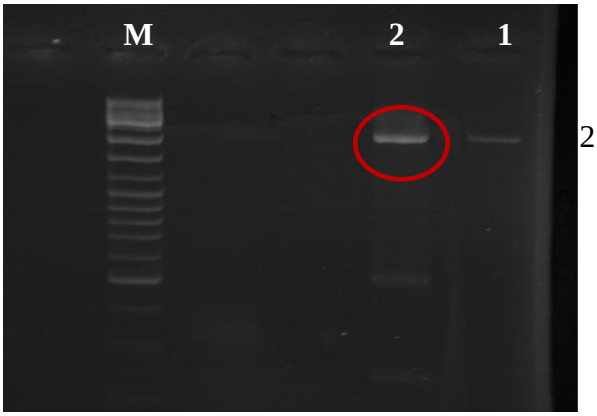

Fig. 4. RT-PCR analysis result (lane 2) was compared with PCR product (lane 1) and DNA ladder (lane M).

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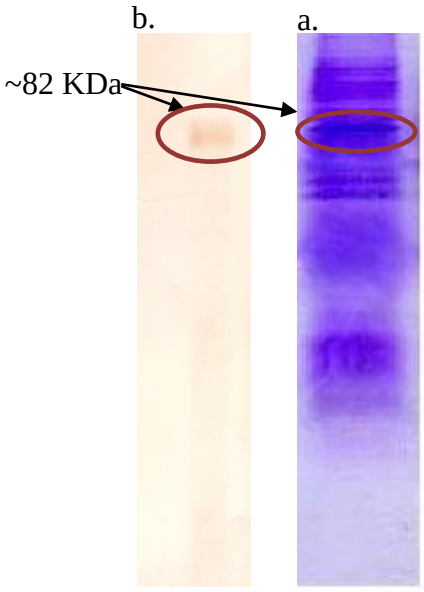

Fig. 5. (a) Conformation of fusion protein production in cos-7 cells by SDS page (a) and western blot analysis (b) using monoclonal HSP70 Ab.

Discussion

HPV is one of the major cause of sexually transmitted infection worldwide and induces significant physical and psychosocial morbidity and mortality (15). There are numerous gene products of biological interest that cannot be obtained from the natural sources in quantities sufficient for detailed biochemical and biological analysis. HSPs are families of extremely conserved and abundant intracellular proteins involved in chaperoning, cross-presentation, and activation of antigen presenting cells (16). Studies have reported that fusion of HSP70 to a candidate antigen can be a suitable approach to enhance the immunogenicity of vaccines (8, 16).

The E7 products are unique antigens and can be ideally used as tumor vaccines (13, 17, 18).

The HPV16 E7 is currently a leading candidate for vaccine development against HPV causing genital infections (19).

It is suggested that HSP70 is a favored immunogenic target and can function as an immunogenic carrier for peptide epitopes. In conclusion, the constructed vector can be used in experimental study, because it offers reliable data and makes a base for E7- HSP70 fusion protein to clinical application as a kind of vaccine.

In summary, in this study, we demonstrated the particular importance of the fusion protein production. Although fusion DNA construct act as a DNA vaccine but, there are many questions which needs to be answered: Is the constructed fusion DNA vaccine able to induce the immune responses in animal model?

How are the HSP70 and E7 genes, effects on each other in combination using the desired vaccine and how strong is their immune responses? More other complicated problems are important in this regards, which must be dissolved in future animal study.

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