

Mammalian Cell Expression of a Fully Functional Recombinant E6 Protein Encoded by HPV16 Isolate from Pakistani Population

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ABSTRACT

Human papillomavirus type 16 (HPV16) accounts for about 65% of the total cervical cancer burden. Viral oncoprotein E6 is an ideal target for immunotherapeutic strategies against HPV induced cervical cancers, as it is constitutively expressed during the disease. Besides it could also serve as an important immunological marker in active cervical infection of any grade. Due to the significance of E6 in cervical malignancies, we proposed to target HPV16 E6 oncoprotein in our study. For this purpose, we isolated E6 gene from local HPV16 isolates that were collected from cervical cancer patients and a full length recombinant HPV16 E6 oncoprotein was synthesized in mammalian expression system. We checked the protein's activity against p53 degradation *in vitro* which showed that the expressed protein is fully functional. This un-mutated full-length functional recombinant E6 protein belongs to Asian HPV16 isolate, being a constantly expressed protein in active HPV infection, can be employed as an ideal serological candidate in early diagnosis, thus helpful in screening the onset and progression of disease in local population. Most importantly, E6 is one of the ideal genes of HPV against which several therapeutic vaccine candidates are being manufactured. So, our study could serve a pivotal role in designing a good inhibitory approach and therapeutic strategies such as therapeutic vaccine against Pakistani HPV 16 isolates.

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Authors' Contribution

SR and MI planned the proposed study. AV, SA, KZ, RM and GZ contributed to the lab work. AV, RM and SA wrote the article. MS helped in manuscript writing. MI and SR have read and approved the final manuscript.

Key words

Human papillomavirus type 16, E6 recombinant protein, UCOE Hu-P vector, HEK293T cells, Therapeutic strategies

INTRODUCTION

Human papillomaviruses (HPVs) belong to an ever-growing family of viruses, the Papillomaviridae family, a family of small viruses with double-stranded DNA that is mainly reported to infect epithelial and mucosal areas of human beings. Recently above 600 discrete papillomaviruses have been reported and around 200 types of HPVs have been recognized and classified up till now (Bzhalava *et al.*, 2014). HPVs are divided into high-risk HPV (HR-HPV) and low-risk HPV (LR-HPV). There are 14 HR-HPV types i.e., 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 23 LR-HPV types i.e., 6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 73, 81, 82,

83, 84, CP6108 and IS39 (Bzhalava *et al.*, 2015). HPV16 related to the alpha-9 category is the most prevalent HR-HPV. It contributes to about 65% of the total cervical cancer and 90% of the total oropharyngeal and head and neck cancer incidence (Gillison *et al.*, 2015).

Cervical cancer (CC) is the 4th most common cancer among females after breast, colorectal and lung cancer and the 2nd most prevalent cancer among females of child bearing age (Arbyn *et al.*, 2020; He *et al.*, 2023; Smith *et al.*, 2007), with more than 0.5 million incident cases and 0.2 million morbidities worldwide each year (Marshall *et al.*, 2022). According to the International Agency for Research on Cancer (IARC), CC is the 3rd most common cancer in Pakistani females, with 5008 cases and 3197 deaths reported in 2020 in Pakistan with incidence of HPV16 is 0.5% in noncancerous patients with respect to 83.4% in cancer patients and there is no data for low grade lesions verses high grade lesion carrying patients (Bruni *et al.*, 2021).

The HPV16 encoding protein E6 is one of the two principal oncogenes involved in disease progression, the other one is E7. Both proteins facilitate each other synergistically and interact with the regulatory proteins of the cell and

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consequently takes control over the regulatory mechanisms of the cell such as the cell cycle and programmed cell death (apoptosis), encourages malignant transformations of cells into the next progeny of cells and in this way allows invasion of the cancerous cells (Doorbar *et al.*, 2015). The HPV16 E6 protein activity lies in its two binding domains, one is the zinc-binding domains at its N-terminus, each with two C-x-x-C motifs which account for the tumorigenic potential of E6 that interacts with a number of cellular target proteins having a highly conserved 'LxxLL' acidic motif. Such proteins are E6AP, p53, Paxillin, MAML1 and IRF3, ARA55, hADA3 where the LxxLL motif ensures its binding into the binding pocket of E6 (Liu *et al.*, 2009; Suarez and Trave, 2018). While the other domain is located at the C-terminus named as PDZ class I binding motif or PBM (Ganti *et al.*, 2015). PDZ-Binding Motif (PBM) of E6 binds to the PDZ motifs of cellular proteins for example MAGI-1, DLG-1 and CAL (CFTR-associated ligand), that are further associated with cell polarity, cell adhesion, and apoptosis (Suarez and Trave, 2018).

E6 had been well characterized for its ability to degrade p53 by its ubiquitination through ubiquitin ligase and subsequent proteasomal mediated degradation. Actually, the Zinc binding domain of E6 interacts with the LXXLL motif of E6AP and forms a heterodimer which further degrades p53, results in the consequent immortalization of transformed infected cells, this is what known as carcinogenicity (He *et al.*, 2023; Martinez-Zapien *et al.*, 2016). The structural association has been studied in X-ray derived ternary E6/E6AP/p53 Complex by Martinez *et al* (Martinez-Zapien *et al.*, 2016). The summarized interaction between full length E6 structure and minimal LxxLL E6AP peptide of 12 mers interact to form E6/E6AP which is efficient enough to interact with p53 core domain (94–292 amino acids), a total of 34 interactions occur between the three proteins are described in crystal structure, previously (Martinez-Zapien *et al.*, 2016). Thus, E6 binding sites such as Zinc binding domains and the PDZ binding motif (PBM) can serve as strong future targets for antiviral therapies (Martinez-Zapien *et al.*, 2016).

Moreover, in our study a new recombinant protein production system i.e., a mammalian expression system, comprising of UCOE 4kb element with the (Guinea pig) gp-CMV strong promoter (Hu-P vector (EMD Millipore) along with 293T adherent cell line (ATCC# CRL-3216) has been introduced for the production of HPV16 E6 recombinant protein. The whole system (Hu-P vector and 293T) is found to be compatible enough for the significant expression of recombinant proteins, as studied in our testimonial study that utilizes 'UCOE element' for the continuous uninterrupted production of recombinant

proteins. The un-mutated, full-length, functional HPV 16 E6 recombinant was further tested in vitro for p53 down-regulation.

MATERIALS AND METHODS

More than 100 vaginal swabs were collected from the Surgery Department of INMOL Hospital, Lahore based on certain criteria. The patients above age 30, with their informed consent and positive for Pap smear test, with symptoms indicative of CC and without any medication were recruited for the study. Samples positive for HPV were processed for HPV type 16 E6 gene amplification using the following primers. Forward primer 5' GCGGC-CGGCCGCGCCCATGCACCAAAAGAGAACTGC 3' with restriction site of *FseI* at 5'. Reverse primer 5' GCGCTAGCTTACAGCTGGGTTTCTCTACGTGTTC 3' with restriction site of *NheI* at 3'.

UCOE Hu-P is a 9kb mammalian expression vector containing the human 4 kb UCOE sequence upstream of a strong (Guinea Pig) gp-CMV promoter, promotes transcriptional activity of proximal genes by altering chromatin structure and an SV40 poly-adenylation site for mRNA stability. The amplified HPV type 16 full-length E6 gene was ligated into the MCR of the vector after restriction digestion with *FseI* and *NheI*. The resultant UCOE-E6 clone (U6) was transformed into *E. coli* top 10 strains with Amp and tetracycline drugs selections. The cloning was confirmed by amplification of the gene with vector-specific primers, designed upstream and downstream of the MCR region and further with restriction digestion analysis also. The right orientation of the gene was confirmed by sequencing.

HEK 293T is a human embryonic kidney cell line with a high transfection efficiency that facilitates the study of ectopically expressed genes. The 293T cell line has high levels of endogenous p53 due to the expression of simian virus 40 (SV40) large T antigen. The cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). The U6 mammalian expression clone (i.e. UCOE-E6) was further transfected in 293T cell lines for protein expression analysis. Liposomal transfection via lipofectamine 3000 was adopted. The procedure was followed according to the manufacturer's protocol. Transfection was run in 6 well cell culture plates in duplication, one plate for mRNA expression and the other one for protein expression analysis. Transfected 293T cells and untransfected as well, were recruited for RNA and protein extraction after 48-72 h of transfection.

Whole cell RNA was extracted by Trizol method from the transfected cells after 48-72 h of transfection. DNase

treated RNA was quantified by nanodrop. And expression was checked qualitatively by reverse transcriptase PCR (RT-PCR) of the extracted RNA.

Candidate recombinant protein was extracted as a crude protein, after lysis with RIPA buffer, from the transfected and untransfected 293T cells (N- control). Cells were treated with lysis buffer for 30 min on ice and the lysate was centrifuged at 14000 rpm for 10 min at 4°C. Protein was quantified by Bradford assay. Extracted protein was further processed for proteomic analysis.

For Dot Blot analysis, crude protein (1 µg) was poured onto the marked portion of the nitrocellulose membrane, labelled untransfected as negative control and transfected ones as E6 1, 2, 3 etc., dried and then blocked with 5% BSA for 1 h. After blocking the membrane was washed with 1 x PBST thrice for 5 min. After washing membrane was incubated with E6-specific mouse monoclonal primary antibody (E6 16/18 (C1P5): sc-460) in 1:500 dilution at 4°C overnight. The membrane was washed with 1 x PBST thrice for 5 min. The membrane was incubated with HRP conjugated secondary antibody (Sigma Aldrich) in 1:10,000 dilution for 2 h at room temperature. Finally, the membrane was treated with '3,3'-Diaminobenzidine (DAB) substrate (Sigmafast) for 30 min at 37 °C, dried and images were captured.

Samples positive for Dot blot were boiled in 4x protein loading dye and were run in replicate on two 12% SDS gels at 100 V, along with a pre-stained protein ladder. For SDS-PAGE the run protein gel was viewed after coomassie blue staining. The other gel used for western was then transferred to a nitrocellulose membrane by sandwiching the membrane between blotting papers soaked in 1 x running buffer. Blotting was done on semi-dry blotting apparatus (Bio-Rad, USA) at 12V for 50 min. Further blotted membrane was blocked and treated with the primary E6 and secondary antibodies just as mentioned in the Dot blot. Required bands were developed by electrochemiluminescence (ECL) method and viewed in Bio-Rad gel doc system using Image Lab software.

RESULTS

A 477 bp fragment of HPV type 16 E6 gene was amplified (Fig. 1) and sequenced as well. The sequence was submitted in NCBI with accession no. MT 955329.1. This length of the gene is antigenic as well, with restriction sites of *FseI* and *NheI* at 5' and 3' sites, respectively.

The amplified *E6* gene was excised, purified and cloned into the UCOE Hu-P expression vector, following after restriction digestion and ligation of the gene into the MCR region of the vector. The cloning was confirmed by colony PCRs of the transformed top-10 bacterial cells, with

vector-specific primers and the double digestion with *FseI* and *NheI* enzymes. UCOE-E6 (U6) as a cloned fragment of 670bp, comprised of 477 bp of E6 gene and 193 bps from vector backbone, upstream and downstream of the gene, was confirmed with UCOE sequence-based primers through PCR, from the mini preps of positive Top10 colonies and diagrammatically viewed through SnapGene software 5.0 (Fig. 2a, b). Further double digestion of the E6 clone with *FseI* and *NheI* enzymes also revealed two bands of 477bp (gene) and 9kb (vector) (Fig. 3). The right orientation of the gene was confirmed by sequencing (Supplementary Data File).

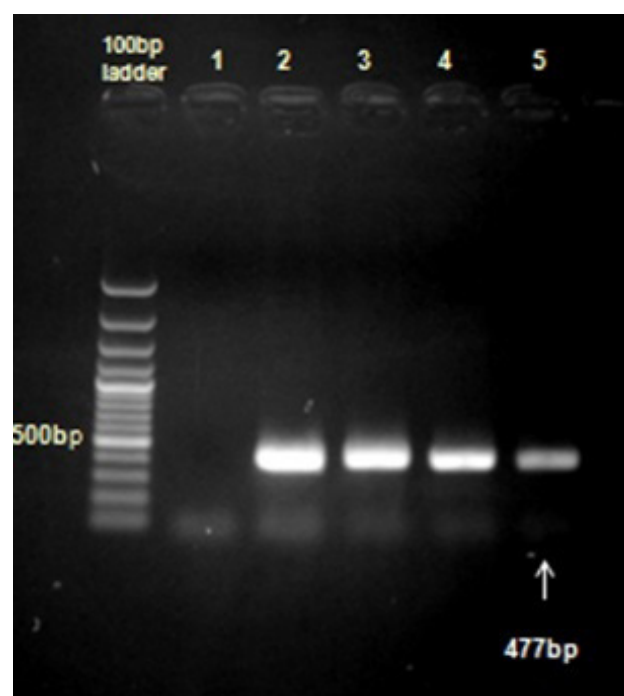


Fig. 1. PCR amplification of HPV 16 E6 gene fragment, Marker: Thermo scientific GeneRuler 100 bp Plus DNA Ladder (SM0321). Lane 1, Negative control; Lanes 2-5, HPV DNA positive for 477bp amplified *E6* gene.

The UCOE-E6 clone was transfected into a 293T cell line for expression analysis. Transfection was done with lipofectamine 3000 as per the manufacturer's recommendation. Expression was analyzed by mRNA expression via RT-PCR and protein expression via the dot-blot method and western blotting.

The exogenous E6 protein synthesis was analyzed by a qualitative estimation of mRNA synthesis by RT-PCR of the whole cell extracted RNA and reciprocal amplification of E6 DNA mediated by cDNA synthesis. Reverse-transcriptase mediated DNA amplification was differentiated from the false DNA

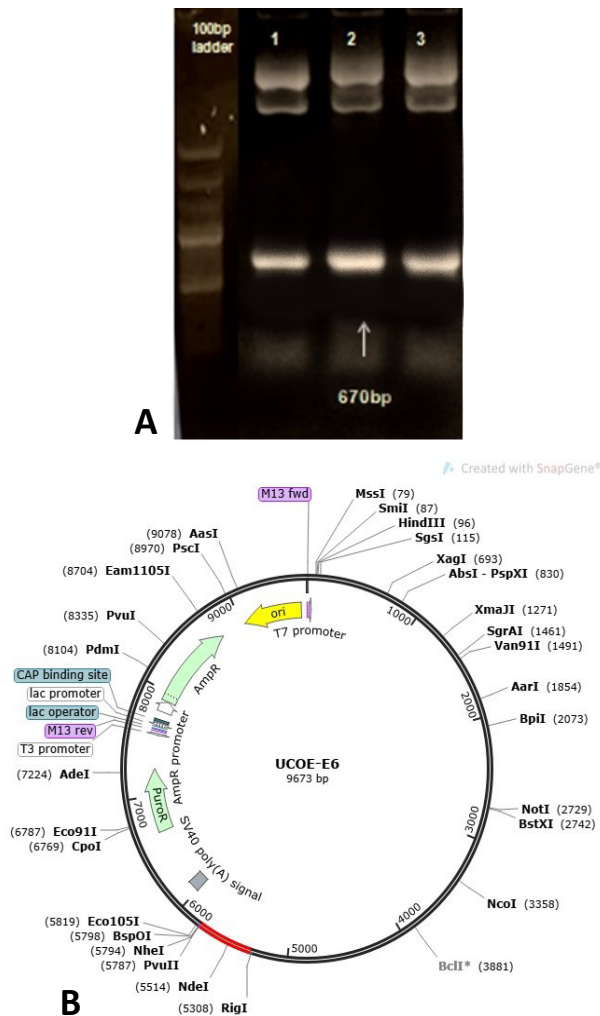


Fig. 2. (A) A 670bp fragment of cloned gene *E6* amplified with vector backbone-based primers UCOE 1 and UCOE 2. Thermo scientific GeneRuler 100 bp Plus DNA Ladder (SM0321) is in the first lane. Lane 1-3, Bands of 670bp are visible. (B) Diagrammatic view of UCOE-E6 (U6) construct, drawn with Snap Gene software 5.0.

amplification which may be possible due to the presence of crude trace DNA, left untreated even after DNase treatment, represented as RT +ve (RT+) and RT-ve (RT-), respectively (Fig. 4).

The exogenous recombinant E6 protein was further evaluated by direct protein detection methods i.e., dot-blot. Protein size estimation was done by SDS-PAGE and western blotting as well. The E6 18kDa protein was isolated on SDS-PAGE and detected by mouse monoclonal primary antibody (C1P5): sc-460 by Santa-Cruz on western blot and dot-blot (Fig. 5).

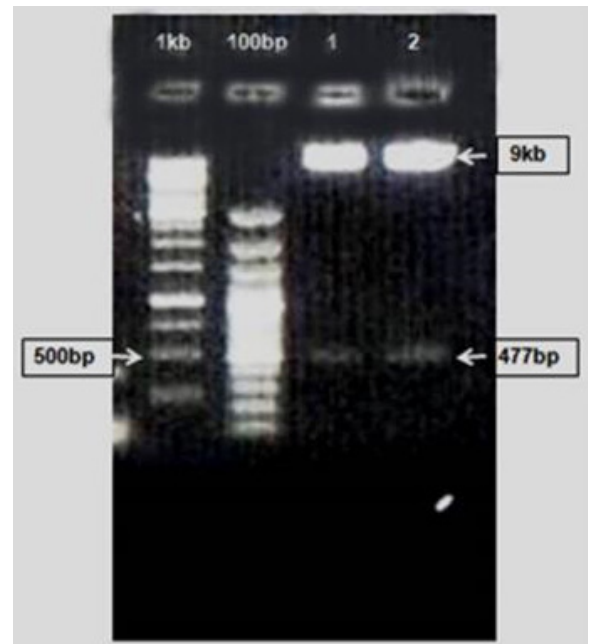


Fig. 3. Clonal confirmation of U6 clone in 9kb UCOE Hu-P vector digested with *FseI* and *NheI*. Markers: Thermo Scientific O' GeneRuler 1 Kb DNA Ladder (SM1163). Thermo scientific GeneRuler 100 bp Plus DNA Ladder (SM0321). Lane 1-2: Represent the digested E6 gene of 477bp size excised from 9kb vector.

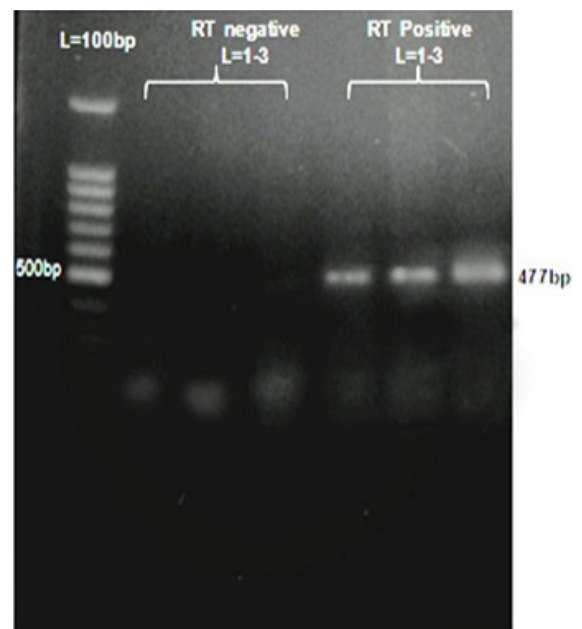


Fig. 4. mRNA expression checked by RT PCR, shows 7 lanes - left to right 100bp ladder. Lanes 1-3, there is no E6 expression in RT-ve PCR; Lanes 4-6, E6 expression in RT+ve PCR.

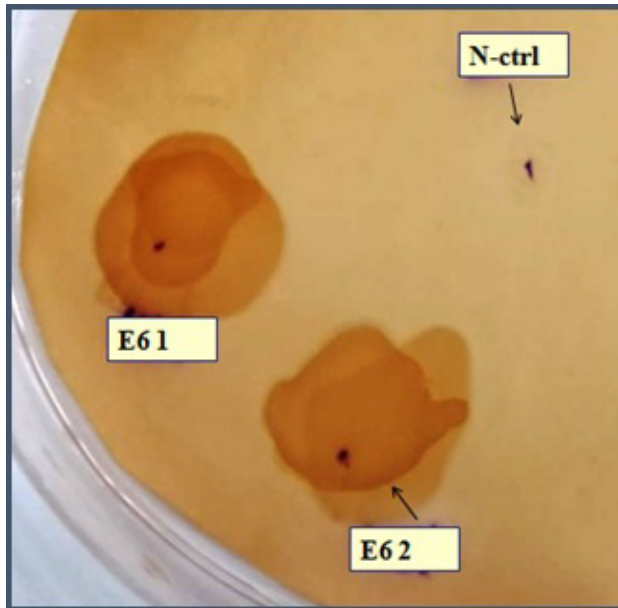


Fig. 5. Crude E6 protein was expressed on Dot Blot along with negative control and the positive samples were labeled as E61 and E62, antibody used was (C1P5): sc-460 [Santa-Cruz].

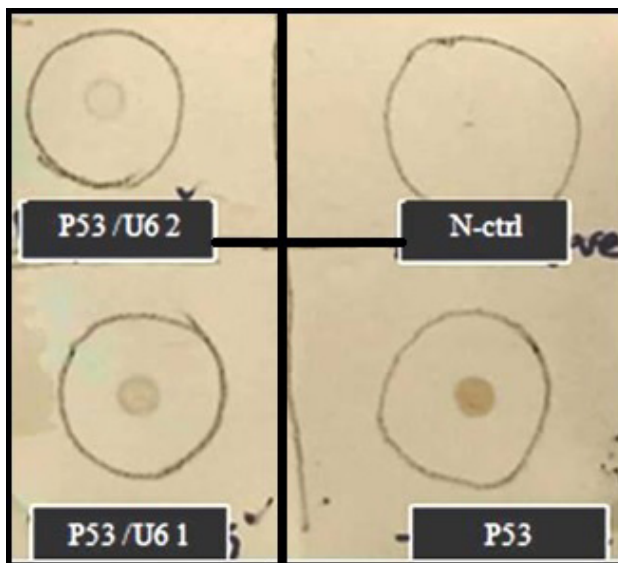


Fig. 6. Dot blot for the qualitative description of p53 down-regulation when induced with E6 protein as U61 and U62 samples, in the first quadrant is a negative control, in 4th quadrant expression of crude p53 protein is visible while in the 2nd and 3rd quadrant lowest expression of p53 in the same cell line is visible.

E6-mediated down-regulation of p53 was also estimated qualitatively. A decline in endogenous p53

expression was recorded as per the increased induction of the E6 gene. Since p53 and E6 are nuclear proteins, anti-histone H3 mouse monoclonal antibody (AHO1432) was used against the expression of histone H3 as a control, a nuclear protein of size 17 kDa (Figs. 6, 7). The primary antibody used for p53 detection was sc-47698.

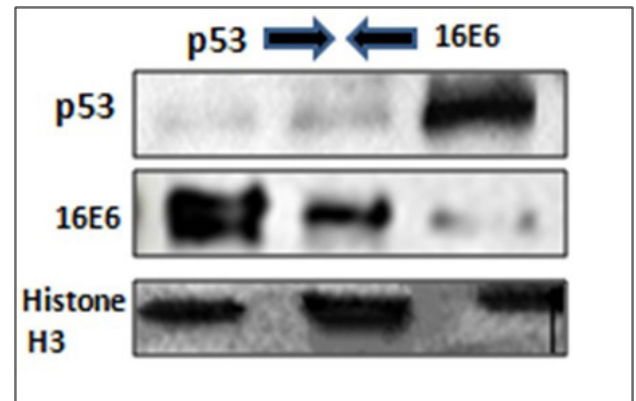


Fig. 7. A western blot depicting the expression of p53 (53kda), E6 (18kda) protein, a nuclear protein Histone H3 nuclear protein (17kda) as an internal control. The arrows indicate that with the increase in concentrations of E6, expression of p53 is decreased gradually. Figure clearly explains that p53 is progressively down-regulated as the induction of E6 protein is increased.

DISCUSSION

Early diagnosis and screening of a disease relies on the robust, reliable and economical diagnostic methods and therapeutic interventions. Biotechnology and biomedical research has helped a lot by proficient, economical and bulk production of large quantities of protein in less time. In the present study, we aimed to synthesize a full-length unmodified functional recombinant HPV16 E6 oncoprotein in mammalian cells, in almost an equivalent amount comparable to bacterial expression systems. In spite of a few limitations of mammalian expression system as compared to the bacterial expression system such as costly, slow, and a tedious production of recombinant protein, we have been able to produce a significant expression of our protein by utilizing a versatile expression system that is based on the use of a ubiquitous chromatin opening element technology-based vector (UCOE Hu-P vector) from EMD Millipore in 293T adherent cell line (ATCC# CRL-3216). The whole system is found to be fair enough for the significant expression of exogenous full length functional recombinant HPV 16 E6 protein. The strength of the study is versatile expression system itself which is more promising if expressed in suspension cultures such

as CHO-S and HEK 293S cells, capable of producing up to milligrams of protein (Bandaranayake *et al.*, 2011; Brendel *et al.*, 2012). Advancement in the expression vectors and cell lines has overcome these limitations with the passage of time (Antoniou *et al.*, 2003; Boscolo *et al.*, 2012; Williams *et al.*, 2005).

The antigenic recombinant E6 protein, obtained from our local Asian isolates, has its potential significances. Since HPV E6 protein is constitutively expressed in the HPV infected cells, thus it could be an important ideal target for therapeutic purposes. Therefore, a therapeutic strategy based on HPV E6 recombinant protein represents an efficient approach to resolve HPV-associated tumors such as in a study, anti-tumor efficacy of HPV16 E6 and E7 fusion protein has been demonstrated well in a mouse model (Zhou *et al.*, 2004). Moreover, it can be used as an ideal serological marker in patients with HPV16-associated malignancies in the form of ELISA, western blot and other immune precipitation assays (Singini *et al.*, 2023). The second conclusive scope of our study, is the pivotal role of antigenic recombinant E6 protein that can be used to raise antibodies against E6 in animal models so as to study the innate and humoral immune responses of host, therefore, it could possibly lead to the intervention of therapeutic vaccine against HPV 16 (Illiano *et al.*, 2016; Zhou *et al.*, 2004). Several therapeutic vaccines based on E6 full-length or partial lengths epitopes are in different phases of study trials. The classical examples are peptide vaccines (PepCan and PDS0101) (Rumfield *et al.*, 2020; Coleman *et al.*, 2016).

CONCLUSION

HPV -16 E6 is an ideal target for designing inhibitory and immunotherapeutic strategies against HPV16 active infection, which is the major etiological agent in cervical cancer. Since it is constitutively expressed during the course of active disease, therefore in-vitro synthesized full length E6 protein from local Asian Pakistani HPV16 isolates can be a potential screening marker for early diagnosis and disease screening. So, our protein could be beneficial in the development of cheap serology diagnostic kits which could replace expensive screening tests such as qPCR for HR-HPVs. Furthermore, antibodies can be raised in animal models against the recombinant HPV16 E6 protein for several inhibitory and therapeutic purposes, thus making E6 as an ideal candidate for therapeutic vaccine development.

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IRB approval

The study has been over viewed and approved by the Institutional Review Board (IRB), whose approval has been provided.

Ethics statement

The work has been done by the approval of Ethical Review Board of the University of Punjab.

Supplementary material

There is a revised supplementary material attached to this article and can be viewed at <https://dx.doi.org/10.17582/journal.pjz/20221116071159>.

Statement of conflict of interest

The authors have declared no conflict of interest.

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