RESEARCH ARTICLE

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Suppression of *E6* Oncogene Induces Apoptosis in CaSki Cervical Cancer Cells

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Abstract

Objective: The most important casuse of cervical cancer incidence and high mortality rate is infection to the human papillomavirus (HPV). The aim of the present study was to investigate the effect of silencing HPV *E6* oncogene on cervical cancer cells using specific siRNAs. **Materials and Methods:** CaSki cervical cancer cells, carrying *E6* gene, were cultured and then transfected with *E6* targeting siRNAs. The cell viability through suppression of *E6* expression was explored using MTT assay. Besides, apoptosis induction was investigated by means of flow cytometry using Annexin / PI staining. The changes in the expression of target genes were examined via Real-Time PCR. **Results:** *E6* gene silencing caused a significant decrease in the survival rate of CaSki cells through remarkable enhancement of apoptosis induction. Moreover, *E6* suppression led to significant upregulation of *P53, Bax*, Caspase-3, and Caspase-9 mRNA expression while downregulated *Bcl-2* expression. Interestingly, it was found that suppression of *E6* expression could lead to upregulation of E5 and E7 expression as a compensatory mechanism for *E6* deactivation. **Conclusion:** According to the results of this study, suppression of *E6* expression using specific siRNAs could be considered as a therapeutic approach for cervical cancer.

Keywords: Cervical cancer- *E6*- siRNA- Apoptosis

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Introduction

Human papillomavirus (HPVs) with more than 200 genotypes in the papilloma viridae family has a protein structure and a circular dsDNA genome, with about 800 octahedral, non-enveloped nucleotides (De Villiers et al., 2004; Sohrabi et al., 2014). HPVs are classified into five genera: alpha (α), beta (β), gamma (γ), mu (μ) and nu (ν), of which α and β are the most studied (Bernard et al., 2010). The virus causes malignant and benign tumors in the human mucosa and skin. The cancers associated with this virus are cancers of the cervix, skin, larynx, head, and neck. Among the diseases, we can mention genital warts and conjunctival diseases of the eye (Hoque and Hoque, 2009; Nour, 2009). HPV genotypes are classified into three categories in terms of cancer development: Low Risk, Intermediate Risk, and High Risk, whose presence is necessary for transcription in host cells. The Low-Risk group includes strains such as HPV 6 and 11, which usually only cause genital warts, and the second category, which includes High Risk, includes high-risk strains of the virus, including 31 and 18,16-HPV strains, which commonly cause cervical cancer; which can be seen with moderate to severe cervical dysplasia (Münger et al., 2004; Burk et al., 2009; Blödt et al., 2012; Pappa et al., 2017). The establishment and survival of these viruses are related to the expression of their early proteins (E1 to E7), which are essential for replication and transcription in the host cell (Doorbar et al., 1997; Hemmat and Baghi, 2018). Late gene expression by L1 and L2 proteins occurs in the superficial cervical cells and is released from the cell surface by forming a virus capsid (Laimins, 1996; Buck et al., 2008). E1 and E2 proteins are involved in replication and are the main replication factor in HPV. In addition, transcription in this virus is controlled by E2 protein (Fehrmann and Laimins, 2003; Zheng and Baker, 2006). The main activity of *E6* protein is in high-risk types of viruses, which causes the destruction of P53 protein by

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ubiquitination. Ubiquitination of *E6* to *E6*-AP (cellular *E6*-dependent protein) depends on the binding of *E6* to P53 (Li and Coffino, 1996; Scheffner and Whitaker, 2003). This protein contains 160 amino acids with two zinc fingers bound by CXXC (Kruiswijk et al., 2015; Paek et al., 2016; Tomaić, 2016). P53 acts as a tumor inhibitor. Inhibition of translocation and inhibition of P53 expression by *E6* protein is a key factor in preventing the activity or suppression of genes by P53(Fakhr et al., 2018) (Pim et al., 1994; Elbel et al., 1997). If the *E6* protein is inactive, it increases P53, which in turn causes P53 dependent apoptosis and ultimately kills infected cells. In contrast, the ability of the *E6* protein to modulate P53 levels is a complete factor. *E6* can also increase the regulation of cell telomerase complex activity, which preserves telomeric DNA at the ends of chromosomes (Greider and Blackburn, 1985; Bonab et al., 2021).

Given that Human papillomavirus is the cause of many cancers, including the "main cause of cervical cancer" and also considering that the main cause of carcinogenicity of this virus is the function of its *E5*, *E6* and *E7* oncoproteins, nowadays, using gene silencing methods, including small interfering RNAs (siRNAs) to inhibit expression of these genes can be an effective strategy for cancer therapy (Fire et al., 1998; Hemmat et al., 2020; Nahand et al., 2021). These synthetic 21 or 22 nucleotides siRNAs mimic the functions of microRNAs and suppress the gene expression through interacting with their 3' UTR regions (Zarredar et al., 2018; Zarredar et al., 2019a).

Considering the significance of *E6* HPV gene in cervical cancer progression, the current research was performed to evaluate the therapeutic effect of siRNA-mediated suppression of *E6* expression in CaSki cervical cancer cells. The obtained results illustrated that suppressing *E6* expression could lead to the elimination of cervical cancer cells through increasing apoptosis induction.

Materials and Methods

Cell culture

CaSki, human cervical cancer cell line, carrying *E6* gene prepared from the National Cell Bank of Iran, was cultured in T25 cell culture flasks with RPMI-1640 medium (Gibco, USA) containing antibiotics containing streptomycin (100 µg/mL), penicillin (100 IU/mL) and FBS (10%, Gibco). The incubation condition for cells was an atmosphere providing 37°C heat, 5% CO2, and humidity. Trypsin EDTA (25%, Gibco) was used for harvesting and sub-culturing the cells, as they reached 70% confluence.

siRNA transfection

FITC-conjugated controls (Gene PharmaCo, Shanghai) and specific siRNAs targeting *E6*, using Gene Pulser electroporation system (Bio-Rad), in the amount of 100 pmol were transfected into CaSki cells at the density of 1×10^6 cells suspended in 500 µL electroporation buffer in a 0.2 cm cuvette, according to supplied protocols as the following: Volts=160v and TC=12.5 ms. 2×10^5 of *E6* siRNA-transfected cells were seeded into six-well culture plates, and after 24,48 and 72 hours of cultivation, they were subjected to flow cytometry and real-time PCR, as explained in the following section to evaluate the efficiency of *E6* suppression using siRNA.

qRT-PCR

GeneAll Trizol RNA extraction kit (Korea) was used to extract total RNA according to provided instructures. Then, the evaluation of RNA concentration and quality was perforemd regarding optical density of samples at 260 nm and 280 nm wavelengths using the The DeNovix DS-11 Spectrophotometer (Wilmington, USA). Besides, the extracted RNA was visualized on agarose gel (1% w/v in TBE buffer) to check its integrity. Afterward, 1µg of qaulified RNA samples were subjected to complementary DNA (cDNA) synthesis by using RT Master Mix (Takara Prime Script). Finally, the expression levels of target genes, including Bax, Bcl-2, Caspase 3,9 and 10, *E6*, *E5*, *E7, P53* were relatively quantified using a Bio FACT™ 2X Real-Time PCR Master Mix (Korea) by means of the Applied Biosystems Step One Plus Real-Time PCR System (USA). The endogenous control gene of GAPDH was used to normalization of gene expression in samples. The relevant sequences for used oligonucleotides are shown in Table 1.

MTT assay

To find out the effect of *E6* suppression on CaSki cells viability, MTT assay (3-(4,5-dimethylthiazol- 2-yl) -2,5-diphenyltetrazolium bromide) was performed. A total number of 1.2×10^4 of CaSki cells transfected with *E6* siRNA were cultured in each well of 96- well plates. Following 24,48, and 72 hours of incubation, the cells were exposed to 50 µl MTT solution (2 mg/mL) for 4 hours. Then, the formed formazan crystals were solubilized by substitution of the medium by dimethyl sulfoxide (150 µL). After a 30 min incubation, the cells were subjected to a microplate reader (Tecan, Switzerland) to evaluate cell viability rate according to the optical density (OD) at 570 nm wavelength.

Apoptosis assay

To follow apoptosis induction in treatment groups, CaSki cells after transfection with *E6* siRNA, at a density of 2×10^5 cells per well, were incubated in six-well plates for 48 hours. After that, the cells were detached with trypsin/EDTA, harvested and washed with PBS. Afterward, the harvested cells were incubated by annexin V (5 µl) and DAPI (5 µl) dissolved in 200 µL binding buffer (Exbio - Czech). Then, the cells were rewashed with PBS and the subjected to the flow cytometry device (Milteny Biotec™FACSQuant 10; Germany) to evaluate the portion of apoptotic, necrotic, and live cells.

Statistical analysis

Statistical analysis and designing of graphs were done by using GraphPad Prism version 6.0 software (San Diego, CA). Flow cytometric data were analyzed using FlowJo software. All data was represented as means ± standard errors. The T-test and one-way ANOVA test were used to specify the statistical significance

of intergroup differences. P values less than 0.5 were regarded to be statistically significant.

Results

E6 gene expression was sufficiently suppressed using designed siRNAs

To find out whether designed siRNA can efficiently suppress *E6* expression, flow cytometry and real-time PCR analysis were performed. The results obtained from flow cytometry revealed that 97.2 percent of FITC-conjugated control sequences were successfully transfected into CaSki cells. Moreover, qPCR results evidenced that transfection of *E6* siRNA into CaSki cells was able to efficiently suppress its mRNA expression. The significant suppression of *E6* gene expression using specific siRNAs was stable till 48 hours after transfection (Figure 1). Then, according to the obtained results, 48 hours of incubation after transfection were applied for all following experiments.

E6 suppression led to a decrease in CaSki cell survival

MTT assay was performed to find out the effect of *E6* suppression using siRNAs on CaSki cell viability. After transfection of cells with *E6* siRNA, the survival rate of CaSki cells was significantly reduced after 24 h ($p<0.001$), 48 h ($p<0.0001$), and 72 h ($p<0.01$) of incubation compared to the control group. As presented in Figure 2, the lowest proliferation rate was observed at 48 hours, and the highest proliferation rate was at 72 hours.

E6 suppression induced apoptosis in CaSki cells

To investigate whether *E6* suppression exerts its anti-proliferative effect through apoptosis induction, flow cytometry analysis was performed. As illustrated in Figure 3, the obtained results evidenced that suppressing *E6* expression remarkably ($p<0.0001$) increased apoptosis rate from 0.31% to 33.48% in CaSki cells, confirming *E6* anti-apoptotic role through tumorigenesis. Then, qPCR was performed to evaluate the modulation of major apoptosis regulators through suppression *E6* in CaSki cells. Following the transfection of cells with *E6* siRNA, the expression levels of *Bax* and *Bcl-2* genes were significantly ($p<0.001$ and $p<0.0001$) increased and decreased in comparison with control, respectively (Figure 4). As previously established, Bax acts as a key proapoptotic gene induced by various factors in the internal apoptosis pathway, and *Bcl-2* has an anti-apoptotic effect in response to various apoptotic stimuli by preventing the release of cytochrome C from mitochondria. Besides, qPCR results evidenced that *E6* knockdown led to significant upregulation of caspase-3 ($p<0.0001$), caspase-9 ($p<0.01$), and P53 ($p<0.0001$), as the key effectors in apoptosis induction, in CaSki cells. However, no significant difference was observed in the expression of levels of caspase-10 following the transfection of CaSki cells with *E6* siRNA compared to the control group.

E6 gene suppression increased E5 and E7 expression levels

E5 HPV protein stimulates cancer cells to proliferate by forming a complex with epidermal growth factor receptor, platelet-derived growth factor receptor, and clonal stimulus receptor. Also, *E7* HPV was shown to interact with Rb and lead to activation of E2F transcription factor through separation from Rb. High *E7* activity may lead to apoptosis in E7 expressing cells. Then, *E5* and *E7* gene expression in transfected CaSki cells was analyzed by RT-PCR. Following the transfection of CaSki cells using *E6* siRNA, the expression of *E5* and *E7* genes were significantly ($p<0.01$) increased compared to the control

Table 1. Primer Sequences

Gene	Type	Primer sequence
P53	Forward	CCCGGACGATATTGAACAATGG
	Reverse	CAGAATGCAAGAAGCCCAGAC
E5	Forward	AAGGCGGCCGCTATGACAAATCTTGATACTGC
	Reverse	ATGCTCTAGACATTATGTAATTAAAAAGCG
E6	Forward	AGGGAGTAACCGAAAACG
	Reverse	CATAAAACCAGCCGTTAC
E7	Forward	AGCGCGGCCGCTATGCACCAAAAGAGAACTGC
	Reverse	ATGCTCTAGAGATTATGGTTTCTGAGAACAG
BAX	Forward	TTTGCTTCAGGGTTTCATCC
	Reverse	CAGCTCCATGTTACTGTCCA
BCL-2	Forward	CTGTGGATGACTGAGTACCTG
	Reverse	GAGACAGCCAGGAGAAATCA
Caspase 3	Forward	GTGGAACTGACGATGATATGGC
	Reverse	CGCAAAGTGACTGGATGAACC
Caspase 9	Forward	GCAGGCTCTGGATCTCGGC
	Reverse	GCTGCTTGCCTGTTAGTTCGC
Caspase 10	Forward	GGAGTGGCTCTGTAAGGACG
	Reverse	AGCAGGTTGTTCACATCCCC

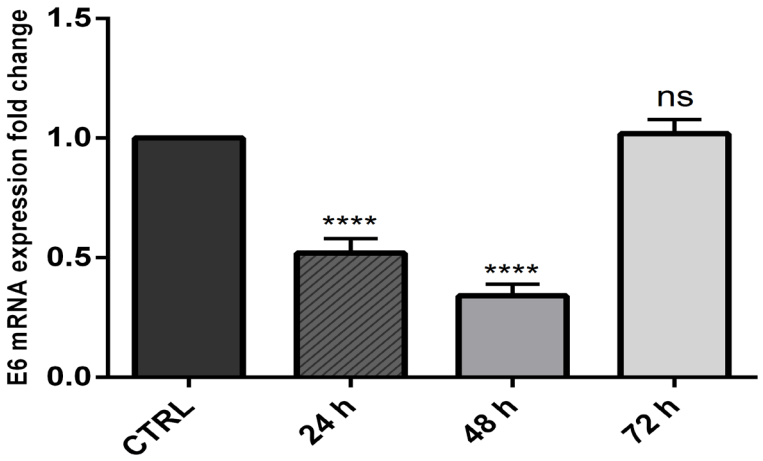

Figure 1. The Significant Suppression of *E6* Gene Expression in CaSki Cells after Specific E6 siRNA Transfection; ****p<0.0001.

group (Figure 5).

Discussion

In the current study, we investigated the therapeutic effects of inhibiting the human papillomavirus (HPV) *E6* gene expression using specific siRNA through the modulating cellular apoptosis on CaSki cervical cancer cells. This was examined by comparing the expression of cancer-related genes. This experimental approach demonstrated the benefits of using siRNA, which is predicted to be associated with changes in *p53* expression.

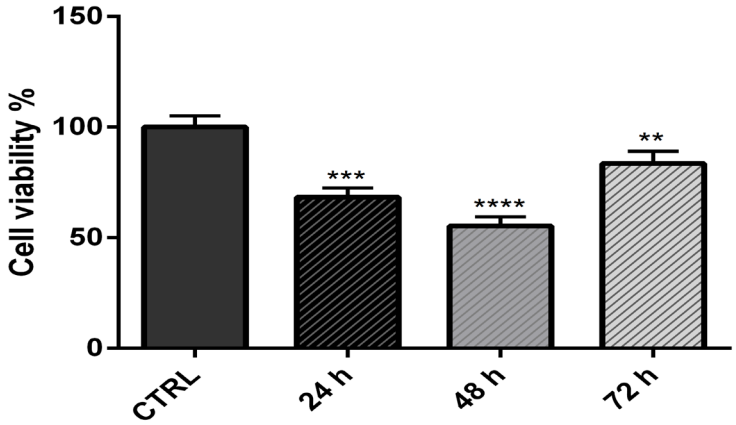

Figure 2. MTT Assay was Performed to Find Out the Effect of *E6* Suppression Using siRNAs on CaSki Cell Viability. After transfection of cells with E6 siRNA, the survival rate of CaSki cells was significantly reduced after 24 h (p<0.001), 48 h (p<0.0001), and 72 h (p<0.01) of incubation compared to the control group.

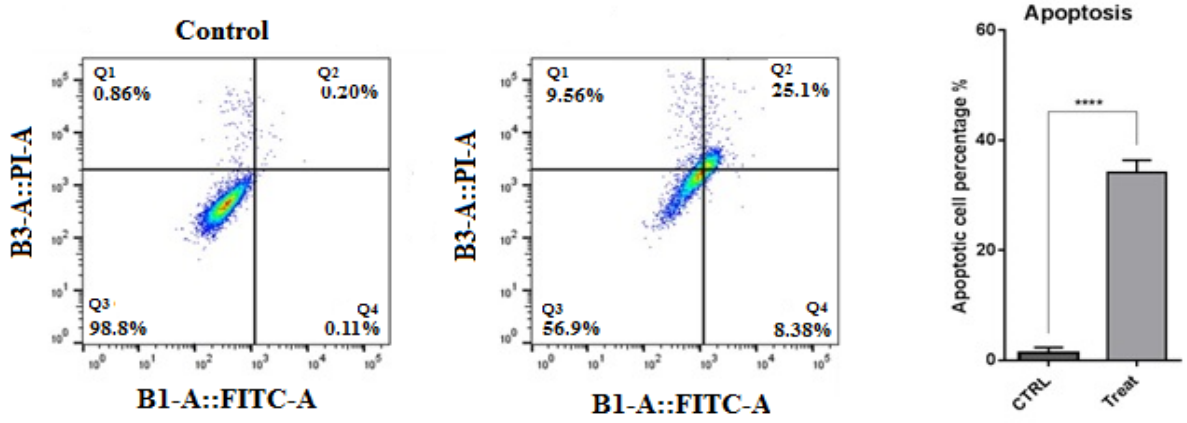

Figure 3. Annexin V / PI Staining Results. The obtained results from flowcytometry, shown that suppressing of the *E6* expression remarkably (p<0.0001) increased apoptosis rate from 0.31% to 33.48% in CaSki cells, confirming *E6* anti-apoptotic role through tumorigenesis ****p<0.0001.

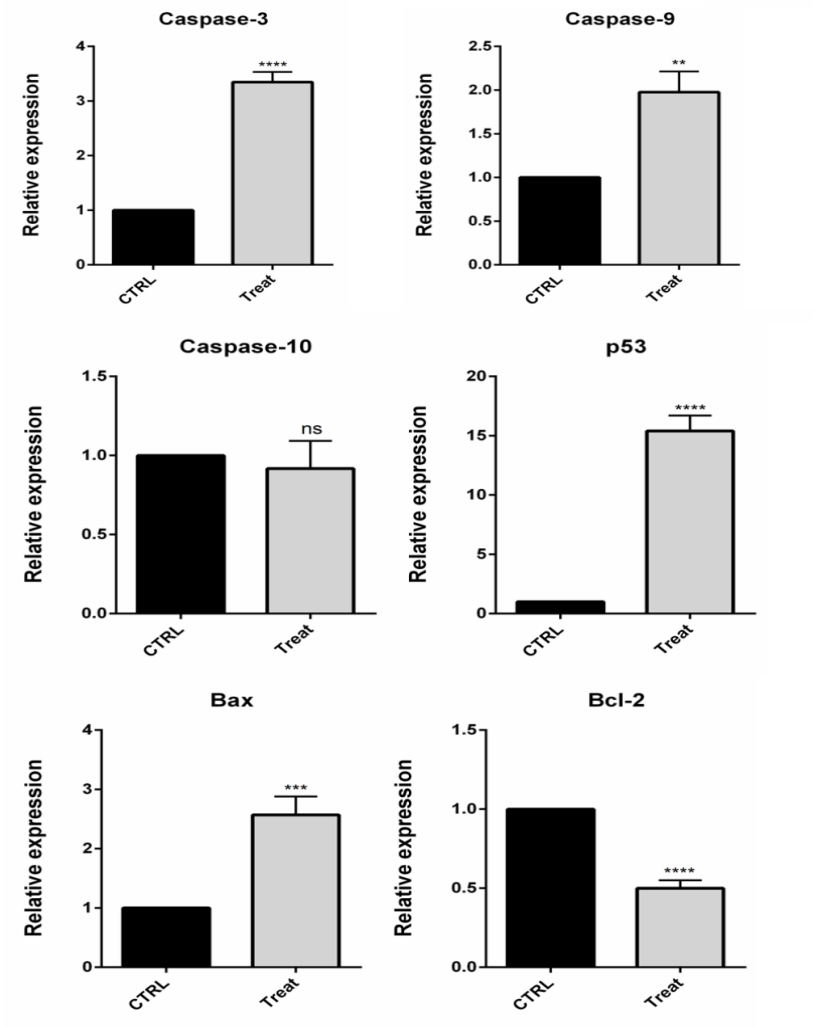

Figure 4. The Changes in the Expression Levels of Cell Survival and Apoptosis-Related Genes were Determined Using qPCR through Transfecting CaSki Cells with E6 siRNA; ****p<0.0001, ***p<0.001 and **p<0.01. qPCR results shown that E6 knockdown led to significant upregulation of caspase-3 (p<0.0001), caspase-9 (p<0.01), Bax (p<0.001) and P53 (p<0.0001), as the key effectors in apoptosis induction, in CaSki cells. Also there is no significant difference was observed in the expression of levels of caspase-10 following the transfection of CaSki cells with E6 siRNA compared to the control group. Also, there is significant low expression in Bcl-2 expression in treated cell (p<0.0001).

Interestingly, we observed that reducing the expression of *E6* led to downregulation of *Bcl-2* while increasing the expression levels of *Bax, E5, E7, P53,* caspase-3, caspase-9 in the CaSki cells. The main activity of *E6* protein is in high-risk types of viruses, which causes the destruction of P53 proteins by ubiquitination. P53, along with Bax, caspase-3, caspase-9, is the imperative modulator of the apoptosis signaling pathway. When

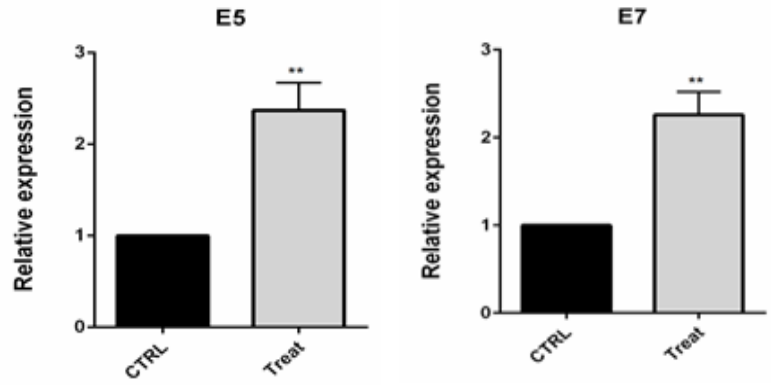

Figure 5. qPCR Results Evidenced the Upregulation of E5 and E7 HPV Gene Expression through E6 Knockdown in CaSki cells; **p<0.01.

caspases are upregulated and activated, the apoptotic pathway begins and induces cell death (Greider and Blackburn, 1985). *Bcl-2* is an anti-apoptotic protein that can compete with Bax for its anti-apoptotic function (Igney and Krammer, 2002). Also, our test was based on the assumption that *E6* knockdown using siRNA could exert its effects through modulating caspase 10. Contrary to the hypothesis, no association was found between *E6* suppression and caspase-10 expression in the present study.

In a similar study by Jiang and Milner (2002), *E6* and E7 human papillomavirus genes were suppressed by specific siRNAs in SiHa and CaSki cell lines infected with HPV-16. *E6* and E7 knockdown increases p53 and p21 protein levels in infected cell lines and inhibits cell growth through induction of apoptosis. Yoshinouchi et al., (2003) also evidenced that suppression of the expression of HPV-16 oncogenes caused the re-accumulation of tumor-inhibiting proteins in infected SiHa cervical cancer cells. The study also showed that SiHa cells transfected with *E6* siRNA in the NOD / SCID mice animal model produced significantly smaller tumors than the animal model receiving SiHa cells transfected with negative control siRNAs. Besides, Yamato et al., (2006) conducted an experiment showing that in HeLa cells carrying HPV-18 genome, suppression of *E6* gene expression using siRNAs could inhibit tumorigenicity of HeLa cells better than suppressing *E6* and E7 genes.

Qin and Cheng, (2010) Tested siRNA user IKKε to show that siRNA could provide a new treatment strategy for breast cancer by silencing IKKε. IκB kinase ε (IKKε) is a member of the IKK family that plays an important role in NF-κB activation. IKKε is expressed in more than 30% of breast cancers and has recently been identified as a potential oncogen for breast cancer (Zarredar et al., 2019b). In 2017 Aletaha et al., (2017) Examined siRNA for MDA-MB-468 cancer cells and showed that siRNA transfection was effective in breast adenocarcinoma cells and inhibited migration, proliferation and induction of apoptosis.

Wechsler et al., (2018) examined the inhibitory function of siRNA on the expression of the Human papillomavirus *E5* gene in *AKC2* cells and showed that inhibiting the expression of this gene reduces the expression level of *EGFR* protein. In addition, this inhibition will inhibit the expression of two other Human papillomavirus oncogenes, *E6* and E7.

Finally, Salguero-Aranda et al., (2019) Showed that the *STAT6 siRNA* sequence is capable of inhibiting the proliferation and induction of apoptosis of colorectal cancer cells *HT-29* and breast cancer cells *ZR-75-1*, and halves the number of cancer cells in a short time.

In conclusion, to sum up, our finding, alongside previous reports, implied that using of RNA interference strategy, including siRNA, could be an effective way to suppress the oncogenic activity of HPV through targeting *E6* gene in cervical cancer cells. We also illustrated that *E6* knockdown diminishes CaSki cervical cancer cell viability through apoptosis induction by modulating the expression of Bcl2, Bax, P53, caspase-3, and caspase-9. Having considered that *E6* suppression could be an effective strategy, however, there is a need for further confirmatory experiments in case of in vivo experiments and clinical trials as well as illustrating further underlying mechanisms.

Author Contribution Statement

F.R. performed the majority of experiments and data analysis. M.A. and H.Z. contributed to carry out the experiments and interpreted the results; H.O., K.D., M.J. and L.V. contributed to perform experiment and analyse the results. A.M., B.B. and H.B.B. revised the manuscript, designed and conducted the project..

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Ethics statement

This study involving human participants was reviewed and approved by Tabriz University of medical sciences and under the ethical approval code of IR.TBZMED.VCR.REC.1398.147.

Availability of data and material

All data generated in this study are available in the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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