HPV 16 E6 promotes growth and metastasis of esophageal squamous cell carcinoma cells in vitro

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Abstract
Background Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies worldwide. Increasing evidence suggests that human papillomavirus (HPV) infection may be associated with the etiology of ESCC. However, the precise role of HPV in ESCC remains unclear.

Methods and results Proliferation and apoptosis of ESCC cells upon infection with HPV16 E6 were detected using CCK-8 assays and Western blot analyses. The migration rate was measured with a wound healing assay, and a Transwell Matrigel invasion assay was used to detect the invasive ability. RT-qPCR was performed to detect the expression of E6AP, p53, and miR-34a. The proliferation rates were significantly higher in HPV16E6-transfected cell groups compared with the negative control groups. Bax protein expression was downregulated in HPV16E6-treated groups compared to the controls. The wound healing and Transwell Matrigel invasion assays indicated that HPV16 E6 infection could increase ESCC cell migration and invasion. Furthermore, E6AP, p53 and miR-34a expression were decreased in HPV16 E6-transfected cell lines.

Conclusion Our results not only provide evidence that HPV16 E6 promotes cell proliferation, migration, and invasion in ESCC, but also suggests a correlation between HPV infection and E6AP, p53 and miR-34a expression. Consequently, HPV16 E6 may play an important role in ESCC development.

Keywords Proliferation · Apoptosis · Migration · Invasion · Esophageal squamous cell carcinoma · Human papillomavirus

Introduction
Esophageal cancer (EC) is a leading cause of digestive tract malignancy-associated mortalities, and ranks seventh in terms of incidence and sixth in overall mortality [1, 2]. EC has two major histological subtypes: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). EAC is the major histological type of esophageal cancer in Western countries, and is closely associated with gastroesophageal reflux [3]. Whereas the incidence of ESCC is particularly common in Asia, especially in China [4, 5]. Nevertheless, the causes and the mechanisms underlying ESCC remain to be elucidated. Several studies have reported that HPV infection might be a potential risk factor for developing ESCC [6–8].

Human papillomavirus (HPV) is a double-stranded, circular DNA virus with specific tropism for squamous epithelium, which can cause persistent growth of squamous epithelium, leading to tumorigenesis [9]. HPV was first detected in cervical cancer and had a positivity rate of approximately 96% in cervical squamous cell carcinoma [10]. Persistent infection
with HPV is closely associated with development of cervical cancer [11, 12]. Interestingly, recent studies reported that HPV infection was involved in the etiology of non-genital cancers [13–15]. For example, HPV16 infection can accelerate the occurrence and course of head and neck tumors [16]. Our previous study found that HPV16 infection was a potent risk factor for ESCC [14, 17–19]. It had been shown that the infection rate of HPV16 was 30–66.67% in kazak esophageal cancer in Xinjiang [20–23]. However, little is known about the effects of HPV16 on the pathogenesis of ESCC.

The high-risk HPV16 E6 protein inhibits miR-34a expression through the p53 pathway, which is closely related to the high invasiveness of the tumor and poor patient prognosis [24]. As a transcription factor, p53 suppresses various malignant processes by inducing the expression of micro-RNAs. miR-34a is a miRNA that is directly regulated by p53, and has been extensively studied in tumor growth, migration, and invasion [25, 26].

miRNAs are highly conserved, 22–24 nucleotide non-coding RNAs that negatively regulate gene expression at the post-transcriptional level [27]. Emerging evidence has demonstrated that altered miRNA expression is closely associated with several human diseases, including cancer [27, 28]. Recently, miR-34a has been implicated as a tumor suppressor gene that plays a key role in leukemia [29], hepatocellular carcinoma [30], pancreatic cancer [31], lung cancer [32] and colon cancer [33]. Moreover, ectopic expression of miR-34a induces apoptosis and senescence, and inhibits migration and invasion in various cancer cells by targeting oncogenes [34–37]. It was observed that miR-34a could also inhibit ESCC cell migration and invasion [28]. Reports from several laboratories showed that miR-34a was regulated by p53 and exhibited tumor-suppressing effects [38]. Some studies have suggested that in cervical cancer, ubiquitination of p53 by HPV16 E6 could inhibit miR-34a expression [39], thereby promoting cell proliferation and inhibiting cell apoptosis [40]. However, the correlations between HPV16 E6, p53, and miR-34a in ESCC have not been reported.

In this study, we aim to determine the effects of HPV16 E6 on the proliferation, migration and invasion of ESCC cells, as well as explore the correlations between E6AP, p53, and miR-34a in HPV-infected ESCC cell lines. This will help elucidate the role of HPV16 in the development of esophageal cancer.

Materials and methods

Cell lines culture and transfection

The ESCC cell line Ec109 was obtained from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The ESCC cell line Ec9706 was obtained from Fuxiang Biotechnology (Shanghai, China). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco), supplemented with 10% fetal bovine serum (Gibco), 100 units of penicillin/ml (Solarbio), and 100 mg of streptomycin (Solarbio), at 37 °C in humidified air with 5% CO2. We constructed an HPV16 E6 transfection plasmid. HPV16 E6 coding sequence insertion was confirmed via sequencing, and the plasmid was transferred into competent bacteria (plasmid construction commissioned from Shanghai Heyuan Company). Ec109 and Ec9706 cells were evenly plated onto a 6-well plate in Opti-MEM (Corning) and cultured at 37 °C. Once cell fusion reached 70–80%, Lipofectamine 2000 (QIAGEN) was used as the transfection reagent to transfect the plasmids (10 µg) containing HPV16 E6 genes into esophageal cancer cells, and were named Ec109-HPV16 E6 and Ec9706-HPV16 E6. Esophageal cancer cells transfected with nonsense segments were named Ec109-PLNCX and Ec9706-PLNCX and used as negative controls. Untreated esophageal cancer cells were named Ec109-NC and Ec9706-NC and used as blank controls.

RNA extraction and quantitative real-time PCR (RT-qPCR)

Total DNA from HPV16E6-transfected esophageal cancer cells was extracted using a miRNA extraction kit (QIAGEN). A one-step miScript reverse transcription kit (QIAGEN) was used to synthesize cDNA according to the manufacturer’s instructions. An ultraviolet spectrophotometer (Nanodrop 2000) was used to assess the concentration and purity of the RNA, and each sample was tested at least three times. The RT-qPCR assay was carried out using the miScript SYBR Green PCR Kit (QIAGEN) and a 7500FAST real-time PCR system (ABI). The thermocycler settings were as follows: p53 and E6AP were added to 2 µl cDNA in each well at 95 °C for 5 min, 95 °C for 10 s and 60 °C for 30 s; and miR-34a was added to 1 µl cDNA per well at 95 °C for 5 min, 94 °C for 15 s, 55 °C for 15 s, and 70 °C for 30 s. The reaction cycle was repeated 40 times and the assays were performed in triplicate. The following primers were used in the experiment: HPV16 E6 forward 5‘-GACCCAGAAATTTACAC AG-3’ and reverse 5‘-CACAACGTGGTTGTAGTGATG-3’; p53 forward 5‘-TCAACAAAGATGTGTGCAACTG-3’ and reverse 5‘-ATGCTGCTGTGACTGCT GTGATAGT-3’; miR-34a forward 5‘-CTGTCCTTTTGGA AACG-3’ and reverse 5‘-GGACATTTAGGTAGTTGCTCTT-3’; and E6AP forward 5‘-GAGGCTTGGTACAGGTTTCT-3’ and reverse 5‘-CTGTTGAGTATCAGTATCCTC-3’. U6 and β-actin were used as internal controls for the standardization and quantification of miRNA and mRNA, respectively. Relative gene expression was analyzed using the log2−ΔΔCT method.
Western blot analysis

ESCC cells were lysed at 4 °C in radio immunoprecipitation assay buffer (Solarbio) mixed with protease and phosphatase inhibitors. The total protein concentration was assessed using an ultra-micro ultraviolet spectrophotometer (Thermo, USA). Protein lysates were resolved with sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to Immun-Blot PVDF membranes (Solarbio) before being immersed in a blocking solution containing 5% non-fat milk and 0.1% Tween-20 for 1 h. After blocking, membranes were incubated with specific primary antibodies against Bcl-2 (Beyotime Biotechnology), Bax (Abcam), MMP-9 (Abcam), MMP-2 (Santa Cruz), and β-actin (Abcam) at 4 °C overnight and then with secondary antibodies for 2 h at room temperature. After washing, the blots were developed with ECL reagents (Thermo), quantified by densitometry, analyzed with the ImageJ software (BIO-RAD Company, USA), and normalized to the corresponding β-actin bands. The experiment was repeated three times.

Cell proliferation assay

A Cell Counting kit-8 (CCK-8, Solarbio) assay was used to assess cell proliferation according to the manufacturer’s protocol. At 0 h, 24 h, 48 h, and 72 h after transfection, the absorbance of HPV16 E6-transfected Ec109 and Ec9706 cells at 450 nm was evaluated using an XMARK microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were independently repeated three times.

Wound healing assay

ESCC cells were cultured in a 6-well culture plate (~7 × 10^5 cells/well) after being transfected with HPV16 E6 plasmid for 24 h. The cells were cultured until they reached full confluence. A wound was created in each well by scraping the well with a 20 µl pipette tip. A wash with serum-free medium was performed to remove dislodged cells. The cells that migrated into the wound or protruded from the wound border were imaged using an inverted microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) after 0 h, 12 h, and 24 h of incubation at 37 °C with 5% CO_2. Cell migration distances were quantified by subtracting the distance between the wound edges at 24 h from the distance measured at 0 h.

Cell migration and invasion assays

ESCC cells were infected with HPV16 E6 plasmid for 24 h and then seeded onto a synthetic basement membrane in the inset of a 24-well culture plate. A 24-well Transwell plate (Corning, USA) was used to measure the migratory and invasive abilities of each cell line. For the migration assay, 4 × 10^4 cells were transfected for 24 h and seeded in the upper chamber. For the invasion assay, the membrane was coated with Matrigel to form a matrix barrier and the cells were placed in the upper chamber after being transfected for 24 h. Cells in 100 µL of serum-free DMEM were carefully loaded onto each filter insert in the upper chamber, and 600 µL of DMEM with 10% FBS was added to each of the lower chambers. After incubation at 37 °C in 5% CO_2 for 24 h, filters were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 15 min. Five photographs were obtained from each group and analyzed using an inverted light microscope at 5 high-power fields of vision magnification. This experiment was independently performed three times.

Quality control

The required experimental materials were sterilized with ultraviolet light for 30 min, and all operation processes were strictly aseptic to avoid cell contamination before the experiments. The cell culture cycle was kept relatively short to prevent cell aging. The culture medium was replaced according to the cell growth status. Disposable sterile gloves and masks were worn to prevent RNA contamination during each experiment.

Statistical analysis

Statistical analyses were performed using SPSS software (version 17.0). Each experiment was independently repeated three times. Data were expressed as mean ± SD and evaluated using unpaired t tests. p < 0.05 was considered statistically significant.

Results

HPV16 E6 promotes ESCC cells proliferation

To determine the effects of HPV16 E6 on ESCC cell proliferation, we transfected the HPV16 E6 plasmid into Ec109 and Ec9706 cells. RT-qPCR indicated that E6 mRNA expression levels were significantly higher in the Ec109-HPV16 E6 and Ec9706 groups compared with the control groups (Fig. 1a–b). The CCK-8 assay revealed that HPV16 E6 treatment significantly increased the proliferation of Ec109 (Fig. 1c–d) and Ec9706 cells (Fig. 1e–f) compared to control cells. Furthermore, we conducted Western blotting to determine the expression of apoptosis-related proteins, and found that HPV16 E6 treatment inhibited Bax expression (Fig. 1a–c), while Bcl-2 expression increased compared to the control groups (Fig. 1g–h). These results
HPV is associated with proliferation and apoptosis of ESCC cells. (a–b) The expression of HPV16 E6 mRNA in Ec109 (a) and Ec9706 (b) cells was measured using real-time PCR. (c–f) Proliferation ability of HPV16 E6-transfected ESCC cell lines as revealed by CCK-8 assay: (c–d) Ec109 and (e–f) Ec9706. *P < 0.05 vs. control NC. PLNCX (Student’s t-test). (g–h) Western blot analysis was used to compare the expression levels of apoptosis-related proteins in Ec109 and Ec9706 cells transfected with HPV16 E6 plasmid with those in negative control (*P < 0.05). NC, normal control; PLNCX, vector control.

suggest that HPV16 E6 inhibits apoptosis and promotes proliferation in ESCC cells.

**HPV16 E6 promotes ESCC cells migration and invasion**

To determine the effect of HPV16 E6 on migration in ESCC cells, wound healing analysis was performed. HPV16 E6 treatment significantly promoted the migration of Ec109 (Fig. 2a,c) and Ec9706 cells (Fig. 2b,d) compared to control groups. In addition, the cell invasion assay showed that the transfection group exhibited a significantly higher invasion capacity compared with the control group (Fig. 2e–f). Furthermore, we investigated the expression of MMP-2 and MMP-9 proteins (associated with cell invasion) in HPV16 E6-treated cells. We found that HPV16 E6 treatment increased the expression of MMP-2 and MMP-9 proteins compared to the respective controls (Fig. 2g–h). These results demonstrate that HPV16 E6 infection can effectively promote the migration and invasion capabilities of ESCC cells.

**HPV16 E6 affects E6AP, p53, and miR-34a expression in ESCC cells**

Previous studies have reported that HPV16 E6 promotes proliferation and migration through the p53/miR-34a pathway in cervical cancer cells [40]. We detected the expression of E6AP, p53, and miR-34a in HPV16 E6-treated cells via RT-qPCR. The results revealed that in Ec109 cells, the expression of E6AP, p53, and miR-34a were significantly decreased in the HPV16 E6-transfected group compared to the control groups (Fig. 3a, c, e). Similar results were obtained with Ec9706 cells (Fig. 3b, d, f). These results suggest that HPV16 E6, p53, and miR-34a expression may be related in ESCC cells.

**Discussion**

HPV infection has been suggested to be associated with various types of cancer [12]. Previous studies on oncogenes have identified the critical function of HPV16 in carcinogenesis [41]. Researchers have found that high-risk HPV infections promote proliferation and migration in cervical cancer cells [42]. Increasing evidence has indicated that HPV16 may be a major cause of ESCC [8]. However, the impacts of HPV infection on ESCC progression remain poorly understood.

We determined that HPV16 E6 increased Bcl-2 protein expression, which regulates apoptosis in ESCC cells. In addition, we showed that Bax protein expression was decreased. Bcl-2 is an anti-apoptotic factor, and increased Bcl-2 can inhibit tumor cell apoptosis [43]. Most EC cell lines show decreased Bcl-2 [44]. Bax is a pro-apoptotic factor which promotes apoptosis in a variety of neoplasms, including ESCC [45, 46]. Our results suggest that HPV infection may inhibit ESCC cell apoptosis. The results of the CCK8 assay showed that HPV16 E6 treatment could enhance the proliferative capability of ESCC cells. We also showed that the migratory capacity was higher in the HPV16 E6-transfected groups compared to the control groups. Similarly, in the cell invasion assay, the number of membrane-crossing cells was significantly higher in the transfected groups compared with the negative controls, and the results showed that ESCC cells transfected with E6 displayed increased migratory and invasive abilities. Furthermore, we also revealed that HPV16 E6 treatment increased expression of the MMP-2 and MMP-9 proteins which are associated with ESCC cell invasion. These results suggested that expression of HPV16 E6 promoted ESCC cell proliferation, invasion, and migration. This is consistent with the role of HPV infection in cervical cancer cells [47], whereas Jun et al. found that HPV infection inhibited the invasion and metastasis of OSCC cells [16]. Metastasis is a major cause of death in patients with malignant cancers [48]. Previous studies have reported that HPV infection was correlated with distant metastasis and poor prognosis in cervical cancer [49], head and neck squamous cell carcinoma [50], and laryngeal squamous cell carcinoma [51]. Our previous study showed that the presence of HPV16 E6 DNA in EC tissues was significantly higher than in normal esophageal tissues [52]. The results of this study revealed that HPV infection may play a critical role in ESCC development and prognosis.

Our previous studies showed that HPV infection was significantly associated with ESCC [53], and that there was a positive correlation between p53 and miR-34a in ESCC [54]. In this study, we found that p53 expression in HPV-infected ESCC cells decreased with miR-34a expression. Furthermore, we only studied the implications of HPV E6 on p53 at mRNA level, but accumulating evidence suggests that p53 expression in patients with HPV-positive esophageal cancer was decreased [55]. These results indicate that there might be a close relationship between p53 and miR-34a expression in HPV-infected ESCC cells. Studies have suggested that the combination of p53 and E6 is E6AP dependent, and the level of E6AP protein was found to be reduced in the presence...
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of HPV E6 [56–58]. In HPV-induced cancers, many of the E6 functions that affect the global transcriptional program of cancer cells involve interactions with E6AP [59]. E6 binds to a short leucine (L)-rich LxxLL consensus sequence within the cellular ubiquitin ligase E6AP, and subsequently induces proteasome-dependent p53 degradation [58, 60]. Our results showed that the expression of E6AP in HPV-infected ESCC cells decreased with p53 expression. This

Fig. 2 HPV16 E6 promotes ESCC cells migration and invasion in vitro. (a–d) Ec109 cells (a, c) and Ec9706 cells (b, d) were transfected with HPV16 E6 plasmid and negative controls and were assessed for migration by wound-healing assay at 0 and 24 h. (scale bar = 200 μm) (*P < 0.05). (e–f) Effects of HPV16 E6 plasmid on Ec109 cells and Ec9706 cells invasion were obtained by Transwell Matrigel invasion assay (scale bar = 200 μm) (*P < 0.05). (g–h) Western blot analysis was used to detect the expression of invasion-related molecules MMP-2 and MMP-9 in Ec109 and Ec9706 cells transfected with HPV16 E6 plasmid. β-actin was used as an internal control. Representative images of three independent reproducible experiments are shown.
implies a correlation among HPV16E6, p53, and E6AP in ESCC. In this study, decreased expression of miR-34a leads to a decline in the expression of E6AP and p53 in HPV-infected ESCC cells. These results imply an additional relationship between E6AP, p53, and miR-34a in HPV-infected ESCC cells. miR-34a overexpression, as a downstream target of p53 [27, 61], can suppress cell proliferation, migration, and invasion [62, 63]. Studies have shown that reduced p53 and miR-34a expression (mediated by HPV16 E6) contributes to cervical cancer cell proliferation and transformation [64, 65]. However, further analysis is required to prove that whether there is a regulatory relationship between E6AP, p53, and miR-34a in HPV-infected ESCC cells and whether they are involved in ESCC progression.

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Author contributions YL, XBC, JMH and FL conceived of and supervised this study. JJH, YJ and TTM performed experiments. SYZ analysed the data. JIH, and YJ wrote the manuscript with input from all authors.

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Data availability The datasets obtained and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.
Declarations

Conflict of interest The authors have no conflicts of interest or other disclosures to report.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication Not applicable.

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