

ELK4 Promotes Cell Cycle Progression and Stem Cell-like Characteristics in HPV-associated Cervical Cancer by Regulating the FBXO22/PTEN Axis

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Background: Cervical cancer (CC) is a prevalent gynecological carcinoma, and patients infected with human papillomavirus (HPV) have a higher morbidity rate.

Aims: To explore the effects of ETS-like transcription factor 4 (ELK4) in patients with HPV⁺ CC.

Study design: In vitro cell lines and human-sample study.

Methods: The ELK4 levels in human tissue (65 HPV⁺ CC tissue and 25 HPV⁻ normal cervical tissue) and cell lines (human cervical epithelial immortalized cell line H8 and CC cell lines HeLa [HPV18], CaSki [HPV16], and SiHa [HPV⁻]) were quantified using qRT-PCR and western blot assay. ELK4 knockdown transfection was effective and confirmed by western blotting. The MTT and EDU assays were used to evaluate cell viability and proliferation, respectively. Flow cytometry was used to detect the CC cell cycle stage. Stem cell

markers, such as cluster of differentiation 133 (CD133), CD44, and aldehyde dehydrogenase 1, and the cervicospheres formed were measured. ChIP-qPCR and luciferase activity experiments were used to assess the bond between ELK4 and F-box protein 22 (FBXO22).

Results: ELK4 was highly expressed in the HPV⁺ CC tissue. CC cells with ELK4 knockdown had lower viability and proliferation than the control cells. ELK4 knockdown blocked the progression of the cell cycle from G1 to S phase. ELK4 knockdown suppressed the stem cell-like characteristics of the HPV⁺ CC cells. ELK4 bonded with the FBXO22 promoter, inhibiting the levels of phosphatase and tensin homolog (PTEN).

Conclusion: ELK4 facilitated cell cycle progression and stem cell-like characteristics by regulating the FBXO22/PTEN axis. Thus, ELK4 could be a potential therapeutic target to arrest the progress of HPV-associated CC.

INTRODUCTION

Cervical cancer (CC) is the most prevalent gynecological cancer.¹ The annual incidence rates of CC are increasing. It is the leading factor of morbidity in women worldwide, and poses a global challenge.¹ Therefore, it is imperative to discover novel treatment strategies for the management of CC.

Various factors are essential for the development of carcinogenesis, relapse, and resistance to medicine, with a strong emphasis on stem cell-like characteristics and HPV infection.^{2,3} The increased stem cell-like characteristics is associated with biological behavior and an independent prognostic factor in CC. Furthermore, human papillomavirus (HPV) infection is a primary risk factor, which can integrate with the host genome.⁴ Superinfection with HPV, particularly types 16 and 18, plays a crucial role in the etiology of

CC.^{5,6} The development of cancer after an HPV infection can also be affected by changes in the abundance of cellular proteins that play a key role in regulating specific molecular signaling pathways.⁷

ETS-like transcription factor 4 (ELK4), also known as SRF cofactor-1 (SAP-1), is a TCF subfamily of the ETS-domain transcription factors and has been linked to the development of a variety of cancers.^{8,9} The expression levels of ELK4 is substantially elevated in a growing number of malignancies, including gastric carcinoma, prostate cancer, and glioma.⁹⁻¹² ELK4 controls the translation of the lncRNA SNHG22 and influences miR-200c-3p/Notch1 by interacting with EZH2 to facilitate the growth and migration of gastric cancer cells.¹⁰ ELK4 can also modulate the KDM5A-PJA2-KSR1 pathway to initiate macrophage M2 polarization, thereby encouraging gastric cancer cell proliferation,



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migration, invasive potential, and xenograft growth.¹¹ ELK4 is also significantly upregulated in prostate cancer, which act as a potential new androgen receptor target.⁹ Downregulation of the antiapoptotic protein Mcl-1 can also contribute to apoptosis sensitivity in glioblastoma through downregulation of ELK4.¹² These findings suggest that ELK4 is a crucial regulator of the malignant behavior of tumor cells. According to the TCGA database, ELK4 expression is also elevated in patients with CC. However, more studies are needed to establish ELK4's potential role and mechanism in CC. In this study, we aimed to observe an increase in the ELK4 expression in patients with CC relative to that in healthy patients. According to in vitro experiments, silence of ELK4 inhibits tumor cell proliferation, cell cycle progression, and stem cell-like characteristics in HPV-positive CC cells. The inhibitory effect may be exerted by regulating the FBXO22/PTEN signaling pathway. Our findings concluded that ELK4 may be an effective therapeutic target for the management of CC.

MATERIALS AND METHODS

Tumor and normal tissues

At random, 65 samples of HPV⁺ CC tissue and 25 samples of HPV⁻ normal cervical tissue were taken from patients at Cangzhou People's Hospital. The age of the patients in the HPV⁺ group ranged from 26 to 68 years, with a mean age of 47 ± 21 years. The pathological grades of the CC tissue were I (FIGO stage, n = 20) and II & III (FIGO stage, n = 45). The age of the patients in the HPV⁻ group ranged from 30 to 54 years, with a mean age of 42 ± 12 years. The clinical data of the patients included in the study are listed in Table 1. The study was approved by the Ethics Committee of Cangzhou People's Hospital.

TABLE 1. Clinical information of HPV⁺ group and HPV⁻ group.

Categories	N	Mean \pm SD
HPV⁺ group		
Age (y)		47 ± 12
< 40	32	
> 40	33	
FIGO stage		
I	20	
II & III	45	
Tumor size (cm)		
≤ 4	38	
> 4	27	
Distant metastasis		
No	34	
Yes	31	
HPV⁻ group		
Age (y)		42 ± 8
≤ 40	11	
> 40	14	
SD, Standard deviation, HPV, human papillomavirus		

Cell culture and transfection

The human cervical epithelial immortalized cell line H8 (PCS-480-011) and cervical carcinoma cell lines such as HeLa (HPV18, CRM-CCL-2), CaSkI (HPV16, CRM-CRL-1550), and SiHa (HPV⁻, HTB-35) (USA) were obtained from the American Type Culture Collection. The cell lines were characterized by epithelioid adherent growth. Cells were grown in the DMEM media (Gibco, CA, USA) and supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Cells were maintained in a humidified incubator with 5% CO₂ at room temperature.

After the cells were grown to 70% confluence, 2×10^5 cells were planted in 12-well plates for at least 12 h. Subsequently, shELK4-1# and shELK4-2#, or NC were transfected into cells using Lipofectamine 2000 reagent (0.5 μ l, Invitrogen) in line with the manufacturer's instructions. For ELK4 siRNA, the subsequent primers were applied for transfection: shELK4-1#: 5'-CCGGGCC CAAGTATTCTCCATCTCTCGAGAACGATGGAGAAACTACT TGGGCTTTTG-3'; shELK4-2#: 5'-GCCGCAACGACTATTT CACCAAGTATGGAGAACATACTCTCATTAGAGGCACTTGT ACCG-3'; and NC: 5'-GGCGCCGAACATTTCACCAGTATGG CGCGCAACGACTATTGGCACGGTTGCCGTAG-3'.

qRT-PCR

Total RNA was extracted and the mRNA expression was relatively quantified using the $2^{-\Delta\Delta CT}$ method. Subsequently, the concentration and purity of RNA were detected using a spectrophotometer (Thermo Fisher Scientific, Inc. MA, USA). Thereafter, using the Step One PCR amplifier (Applied Biosystems, USA) and SYBR Premix Ex Taq II PCR kit (Takara Biotechnology Co., Ltd.) appropriate primer-coated RNA was reverse transcribed into cDNA. The ELK4 primer were: F: 5'-GGGTTAGAACTGGCACCCAC-3'; R: 5'-GCTGGACTTAGGGAGCAAC-3'. The GAPDH primer were: F, 5'-AATGGGCAGCCGTTAGGAAA-3'; R, 5'-GCGCCAATACGACCAAATC-3'.

Western blot assay

The cells were lysed using the RIPA buffer (Beyotime, Shanghai, China). The lysed cells were centrifuged to remove the protein debris, and the protein was quantified using the bicinchoninic acid kit (BCA; Beyotime, Shanghai, China). Equal amounts of the protein were divided by electrophoresis based on molecular weight. Subsequently, the protein strips were transferred onto PVDF membranes and blocked with 5% milk for one hour at 37 °C. The protein bands were detected using the ECL detection method (GE Healthcare, Piscataway, NJ, USA) after being incubated with the respective primary antibodies at 4 °C overnight, followed by secondary antibodies at 37 °C for one hour. The specific primary antibodies of ELK4 (#ab86002, 1:1000), CD133 (#ab222782, 1:1000), CD44 (#ab254530, 1:1000), ALDH1 (#ab52492, 1:1000), FBXO22 (#ab230395, 1:1000), PTEN (#ab267787, 1:1000), and β-actin (#ab8226, 1:1000) were supplied by Abcam. The anti-mouse (#4410, 1:10000) and anti-rabbit (#4414, 1:10000) peroxidase-conjugated secondary antibodies were acquired from Cell Signaling Technologies.

Cell proliferation and viability

The MTT and EDU tests were used to measure cell growth and viability. Initially, 2×10^3 cells/ml were seeded into 96 well plates and subjected to several conditions. The cells were grown at 37 °C for four hours after the MTT solution (0.5 mg/mL, Beyotime) was added. After discarding the medium, 100 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added. Subsequently, a microplate reader (BioTek, Winooski, VT, USA) was used to measure the samples' optical density at 490 nm.

After two hours of cultivating the 2×10^3 cells/ml in 96 well plates, EDU (10 µM, Beyotime) was added. Following fixation of the specimen with 4% paraformaldehyde and permeabilization using 0.3% Triton X-100, the cells were stained with reaction solution for 30 min at 37 °C in the dark. Finally, the EDU-stained cells were used to determine the cell proliferation activity.

Cell cycle analysis

Flow cytometry was used to examine the cell cycle. Approximately 1×10^5 cells were placed in ice-cold 70% ethanol, which was maintained at 20°C overnight, and dyed for 15 minutes at room temperature with PI/RNase solution (BD Biosciences). Using the FACSAria III cell sorter, a cell-cycle analysis was conducted, and the FlowJo program was used to analyze the proportion of cells at various stages of division.

Cervicosphere formation

Six scored well plates were precoated with 1.2% poly-HEMA (Sigma-Aldrich), and 1×10^4 cells/well were seeded in a predefined medium (DCM) consisting of K-SFM (Invitrogen) and supplemented with 10 ng/ml of basic fibroblast growth factor (BD Biosciences), 10 ng/ml of EGF (Sigma-Aldrich), and B27 (Invitrogen). This was used to determine the sphere-forming efficiency. Subsequently, the cells were cultured in 1.2% poly-HEMA-coated six-well plates to produce secondary and tertiary cervicospheres.

ChIP-qPCR

ChIP was carried out as instructed by the manufacturer. The samples were separated into two groups: one with only a nonspecific IgG and no additional antibody and the other with anti-ELK4 antibody (#ab86002, Abcam). The DNA fragments were purified after the cross-links were broken to perform PCR, whose results were analyzed by gel electrophoresis. The input was obtained from DNA samples acquired before precipitation. The PCR test alone consumed 10% of the total input.

Luciferase activity

Primers and DNA polymerase were used to amplify regions of the FBXO22 promoter containing the ELK4 binding site (Genestar, Florham Park, NJ, USA). In addition, the ELK4 coding area was isolated as DNA fragments using restriction enzymes and PCR amplification. A pGL4.70-FBXO22 and pcDNA3.1-ELK4 vector were created. We utilized the Renilla luciferase-expressing

pcDNA3.1 vector as a reference point. Using a dual-luciferase assay method, the firefly and Renilla luciferase activity were measured in triplicate (E1910, Promega). The ratio of firefly to Renilla luciferase activity was used to standardize the results.

Statistical analysis

The data were statistically analyzed using SPSS (version 22.0; IBM, Armonk, NY, USA). Student t-test and one-way ANOVA (and Dunnett's test as the posthoc test) were used to identify statistically significant differences. Results from the three experiments are expressed as means and standard deviations (SD). A $p < 0.05$ was considered statistically significant.

RESULTS

ELK4 is highly expressed in patients with HPV⁺ CC

Initially, the mRNA and protein expression levels of ELK4 were measured using qRT-PCR and western blot assays, respectively, in 65 samples of HPV⁺ CC tissue and 25 samples of HPV⁻ cervical tissue. ELK4 mRNA (Figure 1a) and protein (Figure 1b) levels were higher in the HPV⁺ CC tissues than in the HPV⁻ cervical tissues. Following this, western blotting was used to analyze ELK4 protein expression levels in the human cervical epithelial immortalized cell line H8 and the CC cell lines HeLa (HPV18), CaSki (HPV16), and SiHa (HPV⁻). Compared to the H8 cell line, the HeLa (HPV18) and CaSki (HPV16) cell lines showed considerably higher protein expression levels of ELK4. The SiHa (HPV⁻) cells showed only a small rise in number (Figure 1c). Collectively, these results indicate that ELK4 is significantly upregulated in HPV⁺ CC.

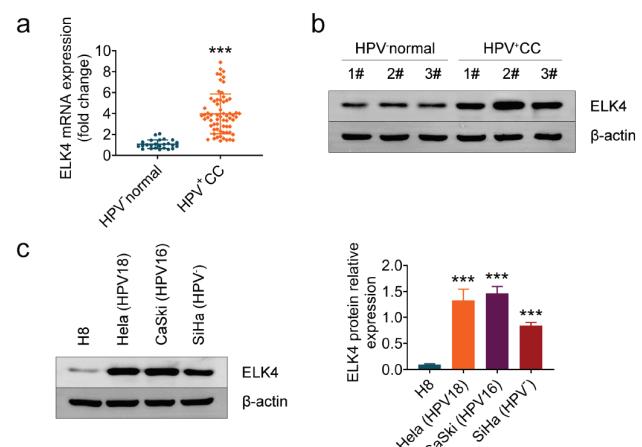


FIG. 1. ELK4 is highly expressed in HPV+ CC patients. (a) The mRNA expression of ELK4 in cervical tissues was compared with cervical cancer tissues by qRT-PCR. (b) Western blot assay showed the protein expression of ELK4 in cervical tissues and cervical cancer tissues. β -actin was normalized control. (c) ELK4 protein levels in human cervical epithelial immortalized cell line H8 and Cervix cancer cell lines (HeLa, CaSki, and SiHa) were detected by western blot. β -actin was normalized. The data are expressed as the mean \pm SD. *** $p < 0.001$ vs normal tissues or cell line H8.
SD, Standard deviation

ELK4 knockdown inhibits the proliferation and cell cycle progression of HPV⁺ CC cells

The HeLa (HPV18) and CaSki (HPV16) cell lines, which express significant levels of ELK4, were employed in the subsequent tests. Stable ELK4-knockdown cell lines were produced for both HeLa and CaSki cell lines, and the transfection effectiveness of ELK4 knockdown was confirmed by western blot analysis. In both the HeLa (HPV18) and CaSki (HPV16) cell lines, the expression level of ELK4 was considerably lower in the shELK4-1# and shELK4-2# groups than in the shNC group (Fig. 2a). Cell viability and cell proliferation were reduced in the shELK4-1# and shELK4-2# groups than in the shNC group, as demonstrated by the MTT and EDU tests (Fig. 2b, c). A flow cytometry test revealed that knocking down ELK4 prevented the transition of the HeLa and CaSki cell lines from the G1 to S phase of the cell cycle (Figure 2d). Together, our data showed that knocking down ELK4 slowed the passage of HPV⁺ CC cells through the cell cycle.

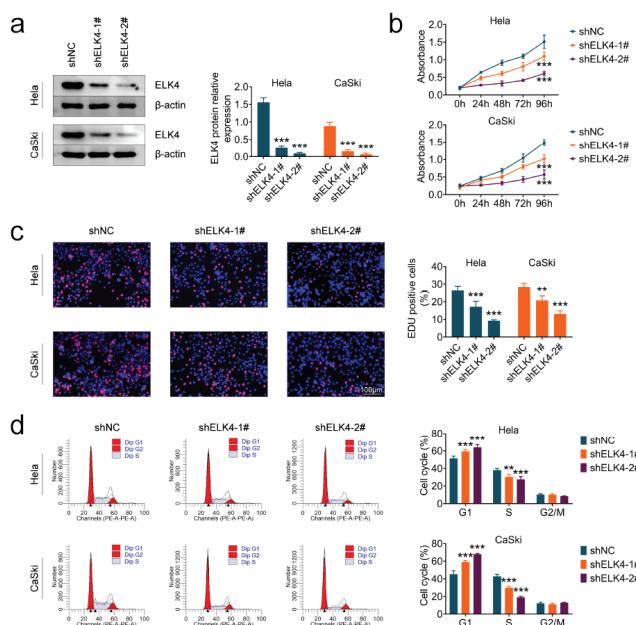


FIG. 2. ELK4 knockdown inhibited the proliferation and cell cycle progression of HPV⁺ CC cells. (a) Western blot analysis showed the transfection efficiency of ELK4 knockdown in HeLa (HPV18) and CaSki (HPV16) cell lines. β-actin was normalized control. (b) MTT assay showed the viability of HeLa (HPV18) and CaSki (HPV16) cell lines in shNC, shELK4-1#, and shELK4-2# groups. (c) EDU assay detected HeLa (HPV18) and CaSki (HPV16) cell proliferation in shNC, shELK4-1#, and shELK4-2# groups. (d) Flow cytometry showed the cell cycle of HeLa (HPV18) and CaSki (HPV16) cell lines in shNC, shELK4-1#, and shELK4-2# groups. The data are expressed as the mean ± SD. ***p*<0.01, ****p*<0.001 vs the shNC group.

SD, Standard deviation, HPV, human papillomavirus

ELK4 knockdown suppressed the stem cell-like characteristics in HPV⁺ CC cells

The levels of the stem cell markers CD133, CD44, and ALDH1 were much lower in the shELK4-1# and shELK4-2# groups than in the shNC group (Figure 3a). The in vitro sphere-forming abilities of HeLa and CaSki cells in the shNC, shELK4-1#, and shELK4-2# groups was assessed and the cervicospheres formed were counted (Figure 3b). Cells in the control group formed several primary cervicospheres (Figure 3b). However, neither shELK4-1# nor shELK4-2# groups had cervicosphere formation, with more cells growing slowly as adherent monolayer cells (Figure 3b). Compared to the shELK4-1# and shELK4-2# cells, the spheres generated by the control group's HeLa and CaSki cells were noticeably bigger (Figure 3b). These findings suggested that knockdown of ELK4 suppressed the stem cell-like characteristics of HPV⁺ CC cells.

ELK4 inhibited the expression of PTEN by promoting FBXO22 transcription

The hTFtarget prediction revealed that ELK4 could bind to the FBXO22 promoter. Subsequently, the correlation between them was predicted via the GEPIA website data. ELK4 and FBXO22 expression are positively correlated in CC. Thus, we speculated that ELK4 could promote the transcription of FBXO22 and increase its expression.

Finally, RT-qPCR and western blot assays were applied to examine the mRNA and protein levels of ELK4 and FBXO22 in the NC,

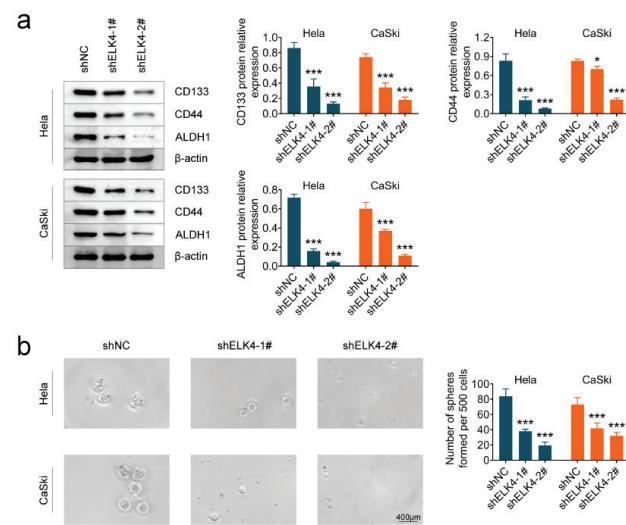


FIG. 3. ELK4 knockdown suppressed the stem cell-like characteristics in HPV⁺ CC cells. (a) Western blot assay showed the protein expressions of CD133, CD44, and ALDH1 in shNC, shELK4-1#, and shELK4-2# groups in HeLa (HPV18) and CaSki (HPV16) cell lines. (b) Assessment of the cervicosphere-forming ability of HeLa (HPV18) and CaSki (HPV16) cells in shNC, shELK4-1#, and shELK4-2# groups, respectively. Representative photomicrograph of cervicosphere formation with HeLa (HPV18) and CaSki (HPV16) cells (magnification, 100x). The data are expressed as the mean ± SD. ****p*<0.001 vs the shNC group.

SD, Standard deviation, HPV, human papillomavirus

ELK4, shNC, and shELK4-2# groups in the HeLa (HPV18) and CaSki (HPV16) cell lines, respectively. Compared to the ELK4 group, the mRNA and protein expression of FBXO22 in the HeLa and CaSki cells were downregulated by the knockdown of ELK4 (Figure 4a, b). ChIP-qPCR (Figure 4c) and luciferase (Figure 4d) activity tests demonstrated that ELK4 bonded with the promoter of FBXO22, thereby regulating the levels of FBXO22. Furthermore, the PTEN mRNA and protein expression levels in the HeLa and CaSki cells in the shNC, shELK4-2#, and shELK4-2# + FBXO22 groups, using RT-qPCR and western blot, indicated that ELK4 inhibited the expression of PTEN by promoting FBXO22 transcription (Figure 4e).

DISCUSSION

Despite significant progress in cancer research, the molecular mechanism of CC remains poorly understood. Several studies have reported that ELK4 expression is involved in the formation

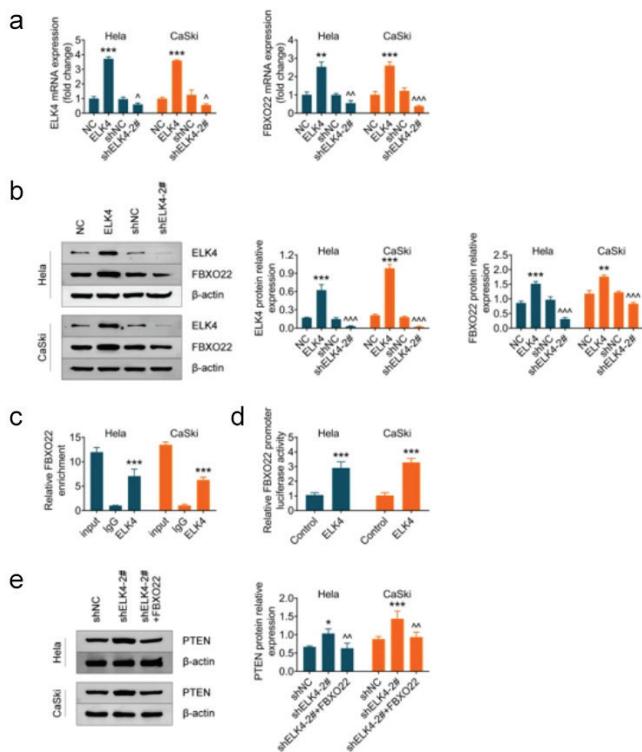


FIG. 4. ELK4 inhibited the expression of PTEN by promoting FBXO22 transcription. (a) The mRNA expression of ELK4 and FBXO22 in NC, ELK4, shNC, and shELK4-2# groups in HeLa (HPV18) and CaSki (HPV16) cells were measured by qRT-PCR. (b) Protein expression of ELK4 and FBXO22 in NC, ELK4, shNC, and shELK4-2# groups in HeLa (HPV18) and CaSki (HPV16) cells were measured. (c) ChIP-qPCR was applied to detect the bind status between ELK4 and FBXO22. (d) Luciferase activity indicated ELK4 bonded with FBXO22 promoter. (e) Western blot was performed to measure the level of PTEN protein. The data are expressed as the mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs the NC, shNC, or control group. ^ $p < 0.05$, ^ $p < 0.01$, ^ $p < 0.001$ vs the ELK4 group.

SD, Standard deviation, HPV, human papillomavirus

of a tumor. ELK4 acts as a prognostic marker, and it is highly expressed in various cancers, such as glioblastoma,¹³ prostate cancer,¹⁴ esophageal squamous cell carcinoma,¹⁵ and breast cancer.¹⁶ ELK4 promotes cell proliferation and its presence indicated a poor prognosis in patients. ELK1 belongs to the ELK family, and it exhibits an increase in the HPV-18-induced transcriptional activity during CC development.¹⁷ In this study, the ELK4 was elevated in the HPV+ CC tissue samples and HPV+ cell lines, indicating that ELK4 may operate as a molecular target that governs the development of CC. This is the first investigation into the potential functional involvement of ELK4 in CC development.

In this study, cells infected with shNC, shELK4-1# and shELK4-2# were examined. Our study findings indicated that the viability and cell cycle progression of HPV+ CC cells were inhibited with ELK4 knockdown. Changlin, et al. also reported that β -Klotho downregulated the expression of ELK4, inducing apoptosis and inhibition of cell proliferation in prostate cancer cells.¹⁴ Furthermore, a persistent HPV infection may accelerate the progression to CC. For instance, CaCxSLCs exhibited highly tumorigenic abilities through selectively overexpressing the HPV oncogene E6 in the CC stem cells.¹⁸ Weilei Dong revealed that TGF- β induced EMT and cancer stem cell-like characteristic properties, resulting in human CC.¹⁹ Given the critical pathogenicity of stem cell-characteristics in CC, our study also observed that the protein expressions of stem cell markers such as CD133, CD44, and ALDH1 and cervicosphere-forming ability. We determined that the expression of stem cell markers were dramatically suppressed in the HeLa (HPV18) and CaSki (HPV16) cell line following ELK4 knockdown than in the shNC group. The ELK4 could induce stem cell-like characteristic activation and accelerate the deterioration of CC.

Finally, ChIP-qPCR was performed and luciferase activity was evaluated to detect the bond between ELK4 and FBXO22. Our study results indicate that ELK4 promoted FBXO22 expression by bonding to the FBXO22 promoter. Our study data indicated that FBXO22 degraded the protein level of PTEN and promoted the development of CC.²⁰ Therefore, in this study, the PTEN expression was measured in the shELK4-2# CC cells with or without FBXO22. The results indicated that ELK4 inhibited the expression of PTEN by promoting FBXO22 transcription.

In conclusion, there is a statistically significant increase in ELK4 expression in patients with HPV+ CC. The stem-like properties of HPV+ CC cells were downregulated and cell proliferation was stifled by knocking down ELK4. Furthermore, the study results showed that ELK4 controlled the FBXO22/PTEN axis. Our findings provide further evidence that ELK4 regulates the FBXO22/PTEN axis in patients with HPV-associated CC. This will promote cell proliferation and maintain stem cell-like properties. This may offer a fresh target for medical therapy in CC and other types of malignancies.

Ethics Committee Approval: The study was approved by the Ethics Committee of Cangzhou People's Hospital.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- F.G., C.W., X.B., J.J., X.H.; Design- F.G., C.W., X.B., J.J., X.H.; Data Collection or Processing- F.G., C.W., X.B., J.J., X.H.; Analysis or Interpretation- F.G., C.W., X.B., J.J., X.H.; Literature Search- F.G., C.W., X.B., J.J., X.H.; Writing- F.G., C.W., X.B., J.J., X.H.

Conflict of Interest: No conflict of interest was declared by the authors.

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