

RESEARCH ARTICLE

PTENP1 inhibits the growth of esophageal squamous cell carcinoma by regulating SOCS6 expression and correlates with disease prognosis

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PTEN pseudogene (PTENP1) has a tumor suppressive role in multiple cancers. However, its involvement in esophageal squamous cell carcinoma (ESCC) remains largely unknown. In this study, we set out to identify the role of PTENP1 in the development of ESCC. Gene Expression Omnibus database was employed to investigate the expression of PTENP1 in ESCC. sRNA target Database (StarBase v2.0) was used to query the downstream of PTENP1. Next, both in vitro and in vivo experiments were employed to explore the function. Cell proliferation was evaluated by CCK-8, soft agar, and colony formation assays. Expression of relative genes was assessed by quantitative real-time PCR (qRT-PCR) and Western blotting. 3'UTR luciferase assay was used to confirm the miRNA binding. The clinical significance of PTENP1 was further validated by immunohistochemistry (IHC) and correlation with clinicopathological indicators in additional samples ($n = 93$). We found expression of PTENP1 in ESCC was lower than that in the corresponding adjacent normal tissues ($n = 17$). Overexpression of PTENP1 in Eca109 and TE-1 cells resulted in inhibited proliferation and altered expression of SOCS6-p-STAT3-HIF-1 α pathway both in vitro and in vivo. Subsequent IHC reported a similar trend in human ESCC samples. 3'UTR luciferase assay demonstrated that PTENP1 3'UTR decoyed miR-17-5p from binding to SOCS6. Moreover, PTENP1 expression was correlated with clinicopathological indicators to varying degrees, including histological grade, TNM stage, infiltration depth, lymph node metastasis, and overall survival. Taken together, these results suggested an anti-oncogenic role of PTENP1. Meanwhile, PTENP1 may also serve as a candidate of prognostic indicator for ESCC patients.

KEYWORDS

esophageal squamous cell carcinoma, prognosis, PTENP1, SOCS6

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1 | INTRODUCTION

Esophageal carcinoma is the eighth most common malignant cancer and the sixth leading cause of cancer-related death all around the world.^{1,2} The overall 5-year survival rate for esophageal cancer patients is still dismal, varying from 15% to 25%.^{3,4} The expected increasing possibility of long-term survival of the disease is associated with accurate diagnosis and treatment in the early or locally advanced stages. However, approximately 20-30% patients with esophageal squamous cell carcinoma (ESCC), the predominant histological subtype of esophageal carcinoma, present with nodal or metastatic involvement even in its early stage.⁵ In addition, ESCC patients respond poorly to conventional therapies such as surgical resection, radiation treatment, and chemotherapy.⁶ In spite of the progression in precision oncology, the molecular basis of the ESCC is still largely unknown, impeding further pathogenetic studies of the disease. Exploring the detailed molecular mechanism may help clarify the pathogenesis and find more effective biomarkers for early detection and prognostic analysis of ESCC.

In recent years, mounting evidences have pointed out that long non-coding RNAs (lncRNAs) play crucial roles in the risk and progression of diverse cancers. Multiple studies have indicated that a group of lncRNAs, which could regulate the expression of protein-coding genes at chromatin organization, transcriptional and post-transcriptional levels, are aberrantly expressed in ESCC. Some of those lncRNAs were indicated as promising diagnostic and prognostic indicators of ESCC, such as HOTAIR, POU3F3, and SPRY4-IT1.⁷⁻⁹ However, to the best of our knowledge, how lncRNA PTENP1 functions in ESCC pathogenesis remains unknown.

lncRNA PTEN pseudogene (PTENP1), transcribed by a highly conserved processed PTEN pseudogene, is previously reported to be correlated with multiple cancers, such as clear-cell renal cell carcinoma (ccRCC), gastric carcinoma and melanoma.¹⁰⁻¹² PTENP1, especially its 3' untranslated regions (3'UTR), acts as a competing endogenous RNA (ceRNA) that protects PTEN transcripts from microRNA-mediated downregulation at post-transcriptional level in several cancers, such as colon carcinoma and prostate cancer.¹³ In addition, similar to that HMGA1 pseudogene (HMGA1Ps) regulates the expression of HMGA2, IGF2, and H19, PTENP1 is also capable of upregulating lots of other targets with tumor suppressive activities by functioning as a miRNAs' decoy.^{13,14} Furthermore, Gao et al found that expression of PTENP1 in oral squamous cell carcinoma tissues was negatively associated with patients prognosis.¹⁵ Circulating PTENP1 in sera contributed to maintaining surveillance and forecast prognosis for patients with ccRCC or gastric cancer.^{16,17} This series of evidences suggest that PTENP1 has a tumor suppressive role, which goes beyond the regulation of PTEN alone, and PTENP1 can predict prognosis of cancer patients.

Considering all above, we hypothesized that PTENP1 may also play a crucial role in ESCC progression and could be associated with clinical outcomes of ESCC patients. To address our hypothesis, we first evaluated its expression in ESCC patients by exploring the Gene Expression Omnibus (GEO) database. Then we explored its function in ESCC through overexpressing its 3' UTR *in vitro* and *in vivo*, and further validated its expression in clinical tissues.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human ESCC cell lines Eca109 and TE-1 were purchased from the Cell Bank Type Culture Collection of Chinese Academy of Science (Shanghai, China) where cell lines were characterized by short-tandem-repeat DNA fingerprinting, mycoplasma detection and cell vitality detection. Cells were cultured in Modified RPMI Medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Sijiqing, China), penicillin, and streptomycin (Corning, Shanghai, China). The HEK-293T cells and human normal esophageal epithelial cells Het-1A were kindly provided by the Translational Medicine Center of the First Affiliated Hospital of Xi'an Jiaotong University and grown in Dulbecco's modified Eagle's medium/High glucose (Hyclone) containing 10% fetal bovine serum, penicillin and streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere with 5% CO₂ and passaged for fewer than 6 months in our laboratory after resuscitation.

2.2 | Downstream molecular prediction

sRNA target Database (StarBase v2.0)^{18,19} (<http://starbase.sysu.edu.cn/>) was used to identify downstream moleculars of PTENP1. StarBase collects interaction networks of miRNA-ceRNA, miRNA-ncRNA and protein-RNA from large-scale CLIP-Seq data.¹⁸ It also provides miR-Function and ceRNA-Function web tools to predict the function of ncRNAs (miRNAs, lncRNAs, pseudogenes) and protein-coding genes from the miRNA-mediated (ceRNA) regulatory networks.¹⁹ StarBase was commonly used in studying lncRNA-miRNA-mRNA network.²⁰⁻²²

2.3 | Overexpression of PTENP1 in ESCC cells

To stably overexpress PTENP1 in ESCC cells, the human 3'UTR of PTENP1 (NR_023917.1) was amplified by PCR and inserted into the BamHI and EcoRI sites of the pGMLV-PA6 lentiviral vector (Genomeditech, China). The primers used for PCR amplification were as follows: forward 5'-GAGGAGCCGTCAAATCCAGAG-3' and reverse 5'-TCGTCAATGTGT-GAGGTTC-3'. Lentiviral vector was packaged into HEK-293T cells and the supernatant was collected for recombinant virus particles according to the manufacturer's instruction. Subsequently, lentiviral particles were infected into Eca109 or TE-1 cells and after infection 48 hours, puromycin (Thermo Scientific, Waltham, MA) (1.5 or 2 µg/ml, MP) was administered to establish the stable cell lines. The final efficiencies for overexpression were evaluated by quantitative real-time PCR (qRT-PCR).

2.4 | RNA isolation, reverse transcription (RT), and qRT-PCR

Total RNA was extracted from ESCC tissue specimens or cell lines using TRIzol reagent (Invitrogen, Shanghai, China) following the manufacturer's protocol. The RT-PCR and qRT-PCR reactions of PTENP1, PTEN, SOCS6, Smad4, FOXO1, Smad5, KLF4, and RUNX3 were performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo) and a

SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) Kit (TaKaRa, Japan). GAPDH served as a quantitative endogenous control to normalize the relative gene expression levels. The sequences were shown as follows:

GAPDH-F GTCTCCTCTGACTTCAACAGCG
GAPDH-R ACCACCCTGTTGCTGTAGCCAA.

The primers for PTENP1, PTEN, SOCS6, Smad4, FOXO1, Smad5, KLF4, RUNX3, and GAPDH were purchased from Sangon Biotech. The annealing temperature was approximately 55°C. The sequences of the primers and the sizes of all the PCR products were shown in Supplementary Table S1.

2.5 | Cell proliferation, soft agar, and colony formation assay

ESCC cells were incubated into 96-well plates at a density of 1000 cells per well. After cultured for 1, 2, 3, and 4 days, the absorbance of each well at 450 nm was measured using a CCK-8 kit (Dojindo, Japan) according to the manufacturer's instruction. For soft agar assay, cell suspension (1×10^4 cells/well) in RPMI rich medium containing 0.3% agar (Dgbio, China) was poured onto 0.5% agar bed in triplicate in the 6-well plate. Colonies were counted and photographed after 14 days. For colony formation assay, 500 cells per well were seeded in 6-well plates. After incubated for 14 days, cells were fixed with methanol (Kelong, China) and stained with 0.1% crystal violet (Sigma, Shanghai, China). The colonies consisting of at least 50 cells were then counted.

2.6 | Western blotting

Cells were harvested and lysed with RIPA lysis and extraction buffer (Pioneer, China) supplemented with protease inhibitors (Roche, Switzerland) and phosphatase inhibitors (Roche). The supernatant of cell lysates was mixed with loading buffer (HEART, China) before boiled for 5 min. Subsequently, the protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membranes (Millipore, Beijing, China), and immunoblotted with the following primary antibodies: anti-SOCS6 (1:1000, Abcam, Shanghai, China), anti-STAT3 (1:1000, CST, Shanghai, China), anti-p-STAT3 (1:1000, CST), anti-HIF-1 α (1:1000, Santa, Shanghai, China). GAPDH (1:1000, CST) was used as a loading internal control. After incubation with recommended secondary antibody coupled to peroxidase, signals were detected using chemiluminescence reagent (Millipore) and ChemiDoc System (Bio-Rad, Hercules, CA). Finally, scanned images were quantified using ImageJ software.

2.7 | 3'UTR luciferase assay

To investigate whether PTENP1 3'UTR could decoy miR-17-5p from binding to SOCS6 mRNA, the plasmid of PTENP1 3'UTR, SOCS6 3'UTR, and SOCS6 3'UTR-MT and miR-17-5p as well as its control were synthesized (Genomeditech, China). The sequences of constructed plasmids and miRNAs were confirmed by DNA sequencing (SangonBiotech, China). HEK393-T cells were seeded in 96-well plates at 4000 per well and cotransfection was performed. Luciferase assay

was performed 24 h after transfection using the Dual-Luciferase Assay System (Promega, China) according to the manufacturer's protocol. Luciferase activity was normalized to Renilla luciferase activity.

2.8 | Subcutaneous tumor xenografts

All animal studies were approved by the Institutional Animal Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University and performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. All male nude mice (4 weeks of age) were purchased from the Experimental Animal Center of School of Medicine (Xi'an Jiaotong University, China) and were randomly divided into experimental or control group. After acclimation about 1 week in special pathogen-free (SPF) condition, the subcutaneous xenograft tumor models were established by subcutaneously injecting approximately 2×10^6 cancer cells in 200 μ L PBS to the posterior back of each mouse. Tumor growth was monitored by measuring the length and width with a caliper every 2 days (tumor volume = $0.5 \times \text{length} \times \text{width}^2$). After 16 days, mice were sacrificed and the tumors were surgically excised.

2.9 | Hematoxylin and eosin staining (H&E) and immunohistochemistry

Tumor tissues were fixed in 10% formalin overnight and embedded in paraffin. Then the specimens were continuously cut into slices with a thickness of 4 μ m. The slices were first stained with H&E to confirm tumor formation. The expression of Ki67, SOCS6, STAT3, p-STAT3, and HIF-1 α protein was detected by two-step immunohistochemical staining with 3,3'-Diaminobenzidine tetrahydrochloride (DAB) according to the standard protocol. The incubated primary antibodies were anti-Ki67 (1:400, CST) and anti-SOCS6 (1:50, Proteintech, China), anti-STAT3(1:200, CST), anti-p-STAT3 (1:300, CST), and anti-HIF-1 α (1:400, Wanlei, China). The results of immunohistochemistry were evaluated independently by two experienced observers in a double blind method.

2.10 | Patients and tumor samples

The study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from all patients. The clinical specimens were obtained from ESCC patients underwent surgical procedures from April 2011 to May 2013. All patients were histologically examined. Patients that received preoperative adjuvant therapy (chemotherapy or radiotherapy) were excluded. Once collected after resection, all samples were snapped frozen in liquid nitrogen and stored at -80°C in order to prevent the degradation of RNA or protein. Clinical information of each patient was carefully retrieved from the medical records by two co-authors independently.

2.11 | Statistical analysis

All statistical analysis was performed using SPSS 19.0 software. All quantitative results were presented as the mean \pm standard deviation from at least three independent experiments, unless indicated

otherwise. The differences between two groups were analyzed using Student's *t*-test. The Kaplan-Meier method was used to calculate survival, and significance was determined by log-rank test. Multivariate logistic regression was performed to identify the independent factors related to ESCC prognosis. The relationship between PTENP1 expression levels and clinical parameters was assessed with χ^2 test. Correlations were analyzed by Pearson rank correlation. Tests with two-sided *P* values <0.05 were considered to be statistically significant.

3 | RESULTS

3.1 | PTENP1 expression was down-regulated in ESCC tumor tissues and ESCC cell lines

The PTENP1 was down-expressed in various tumor types, which was suggested to contribute to cancer development. To investigate the

expression of PTENP1 in ESCC patients, we first investigated its expression by employing the GEO database. The expression profile of 17 pairs of ESCC tissues and corresponding adjacent normal tissues was downloaded from the GEO database (GSE20347).²³ After statistical analysis, we found that the expression of PTENP1 was significantly lower in 88.235% ESCC samples comparing to the corresponding adjacent normal ones ($t = 4.415$, $P < 0.001$, Supplementary Figure S1A). Moreover, as shown in Figure 1A, the expression of PTENP1 and PTEN were reduced in both Eca109 and TE-1 cells.

3.2 | Effects of PTENP1 on cell proliferation in vitro

Several studies have reported the tumor suppressive role of PTENP1 in various tumors. However, no study has been conducted on the effects of PTENP1 in ESCC progression. To elucidate the functions of PTENP1 in ESCC, PTENP1 3'UTR was transfected into Eca109 and TE-1 cells,

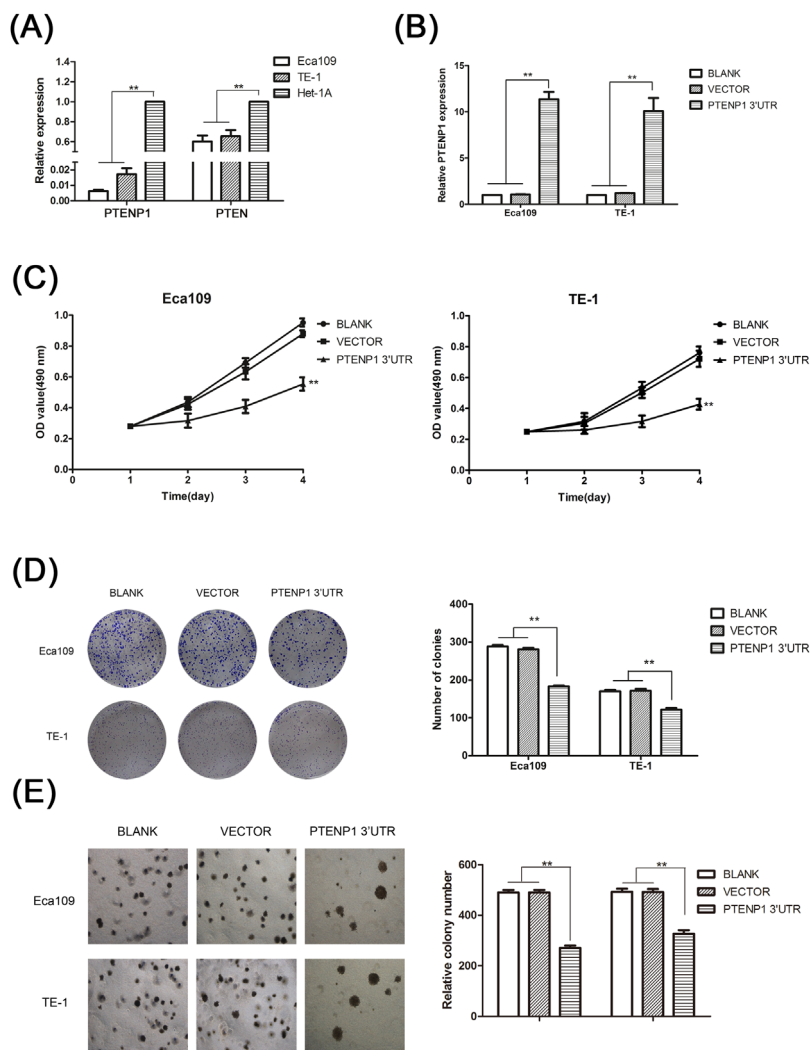


FIGURE 1 Effects of PTENP1 on cell proliferation in vitro. (A) The basal levels of PTENP1 and PTEN expression in Eca109 and TE-1 cells detected by qRT-PCR; (B) The relative expression of PTENP1 was determined by qRT-PCR in Eca109 and TE-1 cells after transfection with PTENP1 3'UTR; (C) Relative cell viability in Eca109 and TE-1 cells was measured by CCK-8 assay; (D) Colony formation assay was performed to detect the proliferation of Eca109 and TE-1 cells; (E) Soft agar growth assay of Eca109 and TE-1 cells after overexpressing PTENP1 (** $P < 0.01$)

respectively. After lentivirus stable transfection by puromycin selection, we confirmed the transfection efficiency by real-time PCR assay. As expected, after transfection PTENP1 expression was significantly increased in both Eca109 and TE-1 cells, respectively (Figure 1B).

Subsequently, we measured the effects of PTENP1 overexpression on cell proliferation. The CCK-8 assay showed that in comparison with the controls, overexpression of PTENP1 significantly decreased the vitality of both two cell lines (Figure 1C). Consistently, the overexpression of PTENP1 significantly suppressed the colony numbers of both Eca109 and TE-1 cells measured by soft agar and colony formation assay (Figures 1D and 1E).

3.3 | PTENP1 overexpression promotes the suppressor of cytokine signaling 6 (SOCS6) expression

Given that PTENP1 was the pseudogene of PTEN, we first detected the expression of PTEN in Eca109 and TE-1 cells after transfection with PTENP1 3'UTR. However, we observed the elevated expression of PTEN only in Eca109 cells, but not in TE-1 cells (Figures 2A and 2B). Pseudogenes can regulate the expression of many other genes in addition to their cognate gene. Therefore, we investigated possible candidate targets of PTENP1 using starBase v2.0. We found several downstream molecules, including SOCS6, RUNX3, Smad4, Smad5, FOXO1, and KLF4.^{13,14,18,19} The expression of these targets in Eca109 and TE-1 cells

was evaluated by qRT-PCR. As shown in Figures 2A and 2B, overexpression of PTENP1 in Eca109 and TE-1 cells remarkably increased the expression of SOCS6, while the expression of FOXO1 was slightly elevated. Therefore, we selected SOCS6 for subsequent experiments.

3.4 | PTENP1 may act through the SOCS6-p-STAT3-HIF-1 α signal pathway

Overexpression of SOCS6, a well-known negative regulator of cytokine receptor signaling, could reduce p-STAT3 and HIF-1 α levels in hepatocellular cancer and breast cancer.^{24,25} We speculated that in ESCC PTENP1 may also function as an upstream of SOCS6-p-STAT3-HIF-1 α signal pathway. In order to verify our hypothesis, the expressions of SOCS6 and downstream key proteins p-STAT3 and HIF-1 α were investigated by Western blotting. The results showed that overexpression of PTENP1 resulted in elevated levels of SOCS6 protein and suppressed levels of p-STAT3 and HIF-1 α in both Eca109 and TE-1 cells, while the level of STAT3 protein was not affected, as shown in Figures 2c and 2D.

3.5 | PTENP1 3'UTR decoyed miR-17-5p from binding to SOCS6

PTENP1, especially its 3'UTR, was previously reported to be a competing endogenous RNA (ceRNA) of PTEN.^{13,26} By functioning

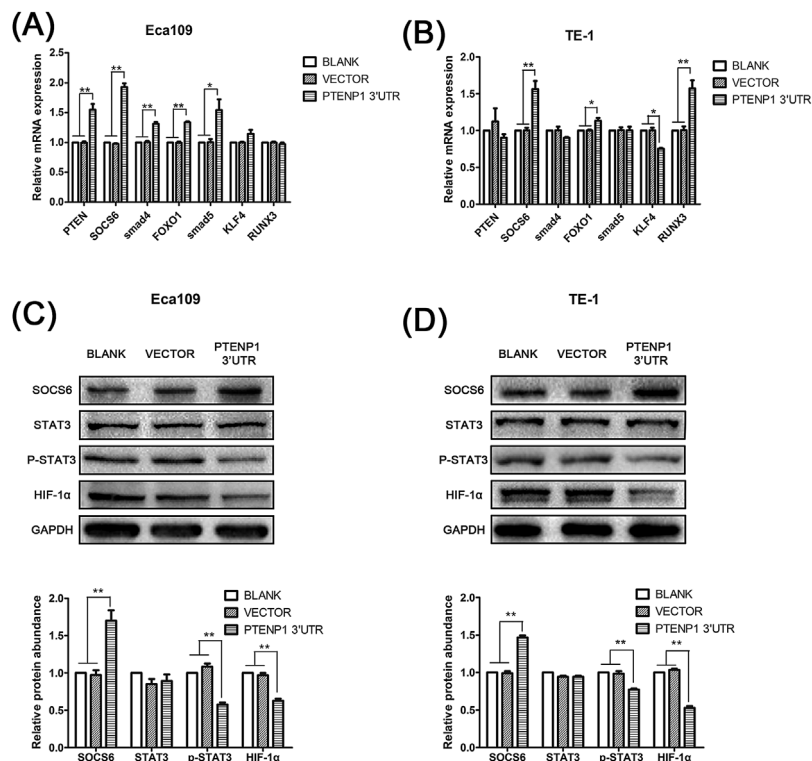


FIGURE 2 PTENP1 overexpression promotes the suppressor of cytokine signaling 6 (SOCS6) expression. (A and B) mRNA expression of PTEN, SOCS6, RUNX3, Smad4, Smad5, FOXO1, and KLF4 in Eca109 and TE-1 cells; (C and D) Protein expression of SOCS6-p-STAT3-HIF-1 α signal pathway in Eca109 and TE-1 cells (* P < 0.05, ** P < 0.01)

as a miRNAs' decoy, pseudogenes like HMGA1 or PTENP1 were capable of upregulating multiple targets with tumor suppressive activities.^{13,14} Such effect of PTENP1 may protect SOCS6 transcripts from microRNA-mediated downregulation at post-transcriptional level.²⁷⁻²⁹

To investigate the involved miRNAs, all shared miRNAs were first predicted by starBase v2.0, resulted in miR-17/17-5p/20ab/20b-5p/93/106ab/427/518a-3p/519d, miR-204/204b/211, miR-382, miR-494, miR-544/544ab/544-3p, and miR-590-3p.

Further literature review suggested miR-17 and miR-93 may involve in the development of ESCC.^{30,31} In 3'UTR luciferase assay, ectopic expression of miR-17-5p mimics significantly diminished the luciferase activity of the reporter containing the wild-type 3'UTR of SOCS6, while PTENP1 3'UTR decoyed miR-17-5p from binding to SOCS6 (Figures 3A and 3B).

3.6 | PTENP1 inhibited tumorigenesis of ESCC in vivo

To investigate the effects of PTENP1 overexpression on the growth capacity of ESCC cells in vivo, the ESCC Eca109 cells transfected with PTENP1 3'UTR were injected subcutaneously into male nude mice. As shown by Figures 4A and 4B, xenografts derived from

transfected group were significantly smaller compared with those derived from control mice in the checked time points. Subsequent immunohistochemistry analysis revealed that compared to control group, there were significantly elevated expression of SOCS6 ($P = 0.0266$) and depressed expression of Ki67, p-STAT3 ($P = 0.0492$), and HIF-1 α ($P = 0.0003$), consistent with the result in vitro (Figures 4C and 4D).

3.7 | Correlation between PTENP1 and SOCS6 expression in ESCC patients

We investigated the expression of SOCS6 in GEO database (GSE20347) and found that similar to PTENP1, SOCS6 expression was also significantly lower in ESCC samples ($t = 3.972$, $P = 0.001$, Supplementary Figure S1B). However, there was no correlation between PTENP1 and SOCS6 expression ($r = 0.240$, $P = 0.353$, Supplementary Figure S1C).

We also evaluated the expression of SOCS6 in an independent cohort of 93 ESCC patients with stages II or III. The results showed that the expression of PTENP1 was positively correlated with the expression of SOCS6 in both mRNA ($r = 0.255$, $P = 0.014$) and protein level ($r = 0.351$, $P = 0.001$) (Supplementary Figure S2). Further analysis also identified correlation of positive rates of the molecules in SOCS6-p-STAT3-HIF-1 α signal pathway in ESCC clinical samples (Supplementary Figure S3).

3.8 | Lower expression of PTENP1 was correlated with poor prognosis of ESCC patients

According to the mean value of PTENP1 expression in these tumor tissues, patients were divided into a high expression group ($n = 44$) and a low expression group ($n = 49$). We then examined the potential associations between PTENP1 expression level and the clinicopathological characteristics of ESCC patients. As demonstrated in Table 1, patients with lower PTENP1 expression tended to get a poorer histological grade ($P = 0.037$), more advanced TNM stage ($P = 0.015$), deeper infiltration depth ($P = 0.045$) and lymph node metastasis ($P = 0.013$) in ESCC tissues. Furthermore, Kaplan-Meier survival analysis indicated that ESCC patients with lower PTENP1 expression levels had a poorer overall survival (OS) than those with higher PTENP1 expression levels ($P = 0.009$, Figure 5). Further multivariate analysis also showed that PTENP1 expression (Hazard ratio = 0.216, $P = 0.044$) and TNM stage (Hazard ratio = 6.520, $P = 0.001$) were independent factors that affected the OS of ESCC patients after radical esophagectomy.

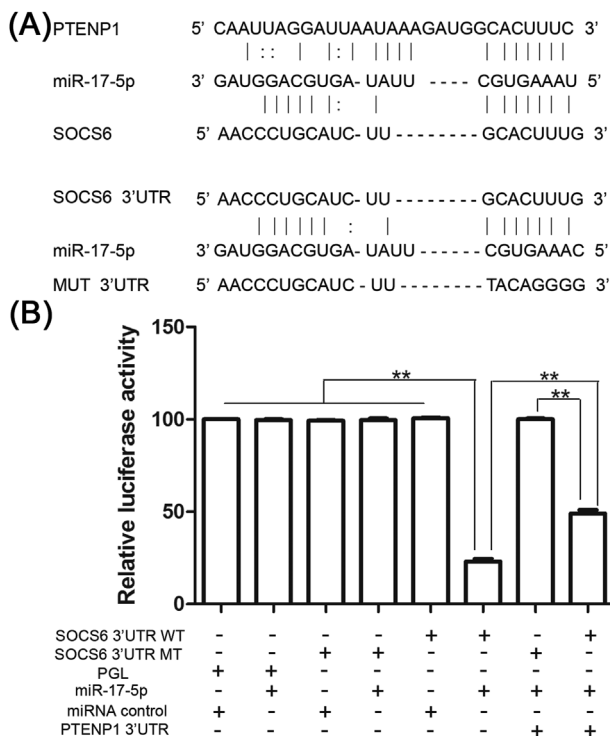


FIGURE 3 PTENP1 modulated SOCS6 level by competitively binding to miR-17-5p. (A) Complementary sequences of PTENP1 3'UTR, SOCS6 3'UTR, miR-17-5p, and mutant SOCS6 3'UTR; (B) PGL3-SOCS6/PGL3-mutSOCS6 and miR-17-5p mimics were co-transfected into HEK-293T cells with or without PTENP1. Luciferase signals were detected 24 h after the transfection (** $P < 0.05$)

4 | DISCUSSION

LncRNA PTENP1 was reported to have a tumor suppressive function and presented lower expression in several cancers such as gastric carcinoma, ccRCC, colon carcinoma, and prostate tumor.^{10,11,13} However, the expression of PTENP1 and the involvement of PTENP1

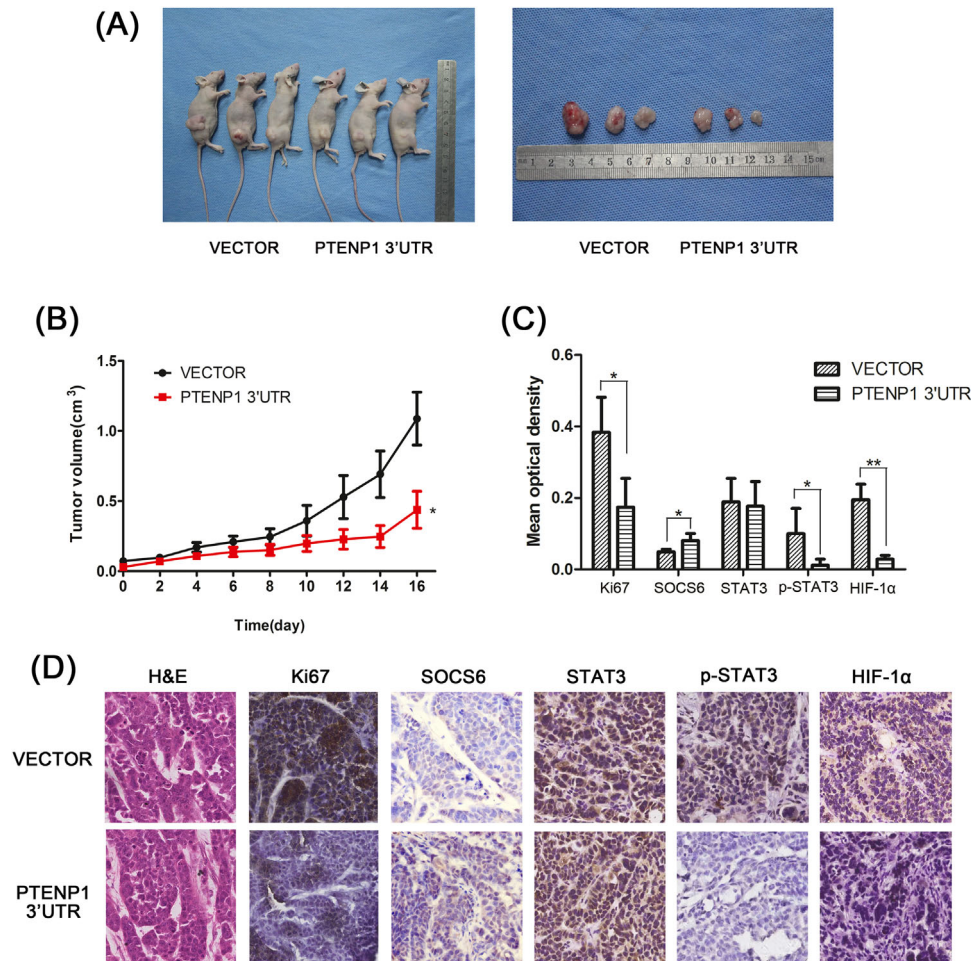


FIGURE 4 PTENP1 inhibited tumorigenesis of ESCC in vivo. (A) Subcutaneous tumors of nude mice in each group after sacrifice; (B) Xenografts derived from transfected group grew significantly slowly compared with those derived from control mice; (C and D) Significantly elevated expression of SOCS6 and depressed expression of Ki67 p-STAT3 and HIF-1 α , in transfected group were observed by immunohistochemistry analysis (* $P < 0.05$, ** $P < 0.01$)

in the development of ESCC have yet not been well characterized. In the present study, we have conducted an investigation to identify the correlation between PTENP1 and clinicopathological characteristics of ESCC. We have also explored the possible mechanism by in vitro and in vivo experiment. To the best of our knowledge, this is the first study about PTENP1 in ESCC.

We found that the expression of PTENP1 was significantly down-regulated in ESCC tissues compared with the corresponding adjacent normal ones. Moreover, low expression levels of PTENP1 in ESCC patients were significantly associated with numerous clinicopathological characteristics, including TNM stage, tumor infiltration depth, histological grade, and lymph node metastasis. We also found that PTENP1 expression level was an independent risk factor for OS after esophagectomy. These results suggested that PTENP1 could be a candidate to indicate prognosis of ESCC.

PTENP1 is reported to be hyper-methylated in multiple cancers cells and cell lines.^{10,32–34} For example, Marsit CJ identified PTENP1, rather than PTEN, was more likely to be hypermethylated in squamous cell carcinoma.³⁴ The result was further validated in colorectal, breast

and haematological cancer cell lines.³³ Moreover, Yu et al identified lower expression of PTENP1 mRNA and hyper-methylation of PTENP1 promoter in clear-cell renal cell carcinoma cell lines and tissues. Further experiment in demethylation agent treated cancer cells confirmed that the lower expression of PTENP1 was due to hyper-methylation.¹⁰ Considering these facts, it is reasonable to infer the down-regulation of PTENP1 in ESCC may also due to the hyper-methylation of its DNA.

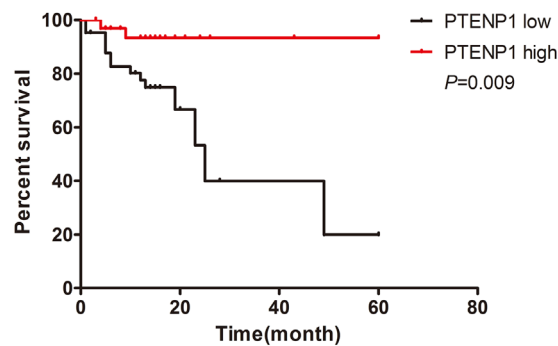
Previous studies have provided evidence that PTENP1, especially the 3'UTR of PTENP1, exerts tumor suppressive function in multiple cancers.^{10,12,13,35} In the present study, we found that the over-expression of lentiviral-mediated PTENP1 3'UTR significantly inhibited the proliferation of Eca109 and TE-1 cells. It is known that PTENP1 expression could regulate the expression of PTEN.^{11,13,15,36} However, we only observed elevated expression of PTEN in Eca109 whereas no significant alteration was found in TE-1 cell line. This may be explained by the variation of the PTENP1 abundance and subcellular localization in different cell lines, which remains to be further validated.

TABLE 1 Correlation between PTENP1 expression and ESCC clinicopathological characteristics in 93 patients

Characteristics	PTENP1 expression levels		χ^2 value	P-value
	High expression	Low expression		
Gender				
Male	33	39	0.280	0.597
Female	11	10		
Age (years)				
≤58	24	30	0.425	0.515
>58	20	19		
Smoking history				
Yes	27	32	0.155	0.693
No	17	17		
Drinking history				
Yes	24	26	0.021	0.886
No	20	23		
Tumor differentiation				
G1	13	8	6.612	0.037
G2	22	19		
G3	9	22		
Primary tumor location				
Proximal	3	3	0.574	0.750
Middle	22	21		
Distal	19	25		
TNM stage				
II	34	26	5.936	0.015
III	10	23		
Infiltration depth				
T1 + T2	28	21	4.016	0.045
T3 + T4	16	28		
Lymph node metastasis				
Yes	13	27	6.177	0.013
No	31	22		

G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated.

Intriguingly, it has been demonstrated that pseudogenes' regulation upon target gene expression is sometimes more than functioning through their cognate genes. For instance, pseudogene-HMGA1P7 can regulate the expression of H19 and IGF2 as well as its cognate gene-HMGA1.¹⁴ In the present study, we found that overexpression of PTENP1 resulted in the upregulation of SOCS6 expression in both mRNA and protein levels. SOCS6 has previously been found downregulated in multiple human cancers.³⁷⁻⁴² We found the expression of SOCS6 was positively correlated with the expression of PTENP1 both in cell lines and in clinical samples. A similar trend was seen in data from GEO database despite it failed to



Independent factors related to overall survival rate of ESCC patients

Characteristics	Hazard ratio	95% CI	P value
PTENP1	0.216	0.049-0.957	0.044
TNM stage	6.520	2.054-20.697	0.001

FIGURE 5 Kaplan-Meier analysis of survival based on PTENP1 expression levels. Multivariate analysis identified two independent factors related to overall survival rate of ESCC patients (n = 93)

reach the significance threshold, probably owing to the smaller sample size. Association between suppressed tumorigenesis and SOCS6 expression was also supported by our results of experiments *in vivo* and *in vitro*.

Moreover, SOCS6 is an important regulator of survival signaling.^{37,43} In the present study, PTENP1 3'UTR overexpression significantly inhibited the proliferation of ESCC cells. Our subsequent western blotting analysis identified that up-regulated PTENP1 expression in ESCC cell lines resulted in elevated levels of SOCS6 protein and reduced levels of p-STAT3 and HIF-1 α . Further 3'UTR luciferase assay identified that PTENP1 could regulate the expression of SOCS6 by miR-17-5p in ESCC. Together these evidences suggested that PTENP1 may also affect SOCS6-p-STAT3-HIF-1 α signal pathway in the pathogenesis of ESCC. SOCS6-p-STAT3-HIF-1 α was previously reported to involve in modulating multi-drug resistance of hepatocellular cancer cells and TAM resistance of breast cancer cells.^{24,25} In this case, PTENP1 may also be involved in regulating multi-drug resistance of ESCC.

In conclusion, our study has preliminarily clarified the role of PTENP1 in ESCC, presenting the promising clinical value of this molecule. PTENP1 correlates with tumor progression in ESCC patients. PTENP1 is also a promising candidate of prognostic indicator for ESCC patients. We hope our discoveries could provide basic evidence for subsequent molecular studies of ESCC.

ACKNOWLEDGMENTS

We thank the Translational Medicine Center of the First Affiliated Hospital of Xi'an Jiaotong University for their kind donation of HEK-293T cells. We further thank Mr WY Wang, Miss YY Guo, and Mr Y Liu for their support in bioinformatics and language.

REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69–90.
2. Schweigert M, Dubecz A, Stein HJ. Oesophageal cancer—an overview. *Nat Rev Gastroenterol Hepatol.* 2013;10:230–244.
3. Pennathur A, Gibson MK, Jobe BA, Luketich JD. Oesophageal carcinoma. *Lancet.* 2013;381:400–412.
4. Pickens A, Orringer MB. Geographical distribution and racial disparity in esophageal cancer. *Ann Thorac Surg.* 2003;76:S1367–S1369.
5. Enzinger PC, Mayer RJ. Esophageal cancer. *N Engl J Med.* 2003;349:2241–2252.
6. Lagergren J. Oesophageal cancer in 2014: advances in curatively intended treatment. *Nat Rev Gastroenterol Hepatol.* 2015;12:74–75.
7. Song W, Zou SB. Prognostic role of lncRNA HOTAIR in esophageal squamous cell carcinoma. *Clin Chim Acta.* 2016;463:169–173.
8. Tong YS, Wang XW, Zhou XL, et al. Identification of the long non-coding RNA POU3F3 in plasma as a novel biomarker for diagnosis of esophageal squamous cell carcinoma. *Mol Cancer.* 2015;14:3.
9. Xie HW, Wu QQ, Zhu B, et al. Long noncoding RNA SPRY4-IT1 is upregulated in esophageal squamous cell carcinoma and associated with poor prognosis. *Tumour Biol.* 2014;35:7743–7754.
10. Yu G, Yao W, Gumireddy K, et al. Pseudogene PTENP1 functions as a competing endogenous RNA to suppress clear-cell renal cell carcinoma progression. *Mol Cancer Ther.* 2014;13:3086–3097.
11. Guo X, Deng L, Deng K, et al. Pseudogene PTENP1 suppresses gastric cancer progression by modulating PTEN. *Anticancer Agents Med Chem.* 2016;16:456–464.
12. Polisenio L, Haimovic A, Christos PJ, et al. Deletion of PTENP1 pseudogene in human melanoma. *J Invest Dermatol.* 2011;131:2497–2500.
13. Polisenio L, Salmena L, Zhang J, Carver B, Haveman WJ, Pandolfi PP. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. *Nature.* 2010;465:1033–1038.
14. De Martino M, Forzati F, Marfella M, et al. HMGA1P7-pseudogene regulates H19 and Igf2 expression by a competitive endogenous RNA mechanism. *Sci Rep.* 2016;6:37622.
15. Gao L, Ren W, Zhang L, et al. PTENp1, a natural sponge of miR-21, mediates PTEN expression to inhibit the proliferation of oral squamous cell carcinoma. *Mol Carcinog.* 2017;56:1322–1334.
16. Wu Y, Wang YQ, Weng WW, et al. A serum-circulating long noncoding RNA signature can discriminate between patients with clear cell renal cell carcinoma and healthy controls. *Oncogenesis.* 2016;5:e192.
17. Dong L, Qi P, Xu MD, et al. Circulating CUDR, LSINCT-5 and PTENP1 long noncoding RNAs in sera distinguish patients with gastric cancer from healthy controls. *Int J Cancer.* 2015;137:1128–1135.
18. Yang JH, Li JH, Shao P, Zhou H, Chen YQ, Qu LH. starBase: a database for exploring microRNA-mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data. *Nucleic Acids Res.* 2011;39:D202–D209.
19. Li JH, Liu S, Zhou H, Qu LH, Yang JH. StarBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res.* 2014;42:D92–D97.
20. Song C, Zhang J, Liu Y, et al. Construction and analysis of cardiac hypertrophy-associated lncRNA-mRNA network based on competitive endogenous RNA reveal functional lncRNAs in cardiac hypertrophy. *Oncotarget.* 2016;7:10827–10840.
21. Tong GF, Qin N, Sun LW, Xu XL. Long noncoding RNA MEG3 suppresses glioma cell proliferation, migration, and invasion by acting as competing endogenous RNA of MiR-19a. *Oncol Res.* 2017. [Epub ahead of print]. <https://doi.org/10.3727/096504017X14886689179993>
22. Han Y, Xu H, Cheng J, et al. Downregulation of long non-coding RNA H19 promotes P19CL6 cells proliferation and inhibits apoptosis during late-stage cardiac differentiation via miR-19b-modulated Sox6. *Cell Biosci.* 2016;6:58.
23. Hu N, Clifford RJ, Yang HH, et al. Genome wide analysis of DNA copy number neutral loss of heterozygosity (CNNLOH) and its relation to gene expression in esophageal squamous cell carcinoma. *BMC Genomics.* 2010;11:576.
24. Wang XJ, Zhang DL, Fu C, Wei BZ, Li GJ. MiR-183 modulates multi-drug resistance in hepatocellular cancer (HCC) cells via miR-183-IDH2/SOCS6-HIF-1alpha feedback loop. *Eur Rev Med Pharmacol Sci.* 2016;20:2020–2027.
25. Shen R, Wang Y, Wang CX, et al. MiRNA-155 mediates TAM resistance by modulating SOCS6-STAT3 signalling pathway in breast cancer. *Am J Transl Res.* 2015;7:2115–2126.
26. Gao L, Ren W, Zhang L, et al. PTENp1, a natural sponge of miR-21, mediates PTEN expression to inhibit the proliferation of oral squamous cell carcinoma. *Mol Carcinog.* 2017;56:1322–1334.
27. Salmena L, Polisenio L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta stone of a hidden RNA language? *Cell.* 2011;146:353–358.
28. Tay Y, Rinn J, Pandolfi PP. The multilayered complexity of ceRNA crosstalk and competition. *Nature.* 2014;505:344–352.
29. Qi X, Zhang DH, Wu N, Xiao JH, Wang X, Ma W. CeRNA in cancer: possible functions and clinical implications. *J Med Genet.* 2015;52:710–718.
30. Xu XL, Jiang YH, Feng JG, Su D, Chen PC, Mao WM. MicroRNA-17, microRNA-18a, and microRNA-19a are prognostic indicators in esophageal squamous cell carcinoma. *Ann Thorac Surg.* 2014;97:1037–1045.
31. Ansari MH, Irani S, Edalat H, Amin R, Mohammadi Roushandeh A. Deregulation of miR-93 and miR-143 in human esophageal cancer. *Tumour Biol.* 2016;37:3097–3103.
32. Masunaga A, Omatsu M, Kunimura T, et al. Expression of PTEN and its pseudogene PTENP1, and promoter methylation of PTEN in non-tumorous thymus and thymic tumours. *J Clin Pathol.* 2017;70:690–696.
33. Hesson LB, Packham D, Pontzer E, Funchain P, Eng C, Ward RL. A reinvestigation of somatic hypermethylation at the PTEN CpG island in cancer cell lines. *Biol Proced Online.* 2012;14:5.
34. Marsit CJ, Zheng S, Aldape K, et al. PTEN expression in non-small-cell lung cancer: evaluating its relation to tumor characteristics, allelic loss, and epigenetic alteration. *Hum Pathol.* 2005;36:768–776.
35. Chen CL, Tseng YW, Wu JC, et al. Suppression of hepatocellular carcinoma by baculovirus-mediated expression of long non-coding RNA PTENP1 and MicroRNA regulation. *Biomaterials.* 2015;44:71–81.
36. Wang L, Zhang N, Wang Z, Ai DM, Cao ZY, Pan HP. Pseudogene PTENP1 functions as a competing endogenous RNA (ceRNA) to regulate PTEN expression by sponging miR-499-5p. *Biochemistry (Mosc).* 2016;81:739–747.
37. Kabir NN, Sun J, Ronnstrand L, Kazi JU. SOCS6 is a selective suppressor of receptor tyrosine kinase signaling. *Tumour Biol.* 2014;35:10581–10589.
38. Letellier E, Schmitz M, Baig K, et al. Identification of SOCS2 and SOCS6 as biomarkers in human colorectal cancer. *Br J Cancer.* 2014;111:726–735.
39. Wu K, Hu G, He X, et al. MicroRNA-424-5p suppresses the expression of SOCS6 in pancreatic cancer. *Pathol Oncol Res.* 2013;19:739–748.
40. Wu Q, Luo G, Yang Z, et al. MiR-17-5p promotes proliferation by targeting SOCS6 in gastric cancer cells. *FEBS Lett.* 2014;588:2055–2062.
41. Li ZB, Li ZZ, Li L, Chu HT, Jia M. MiR-21 and miR-183 can simultaneously target SOCS6 and modulate growth and invasion of hepatocellular carcinoma (HCC) cells. *Eur Rev Med Pharmacol Sci.* 2015;19:3208–3217.
42. Qi X, Li J, Zhou C, Lv C, Tian M. MiR-142-3p suppresses SOCS6 expression and promotes cell proliferation in nasopharyngeal carcinoma. *Cell Physiol Biochem.* 2015;36:1743–1752.

43. Xue X, Liu Y, Wang Y, et al. MiR-21 and MiR-155 promote non-small cell lung cancer progression by downregulating SOCS1, SOCS6, and PTEN. *Oncotarget*. 2016;7:84508–84519.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Gong T, Zheng S, Huang S, et al. PTENP1 inhibits the growth of esophageal squamous cell carcinoma by regulating SOCS6 expression and correlates with disease prognosis. *Molecular Carcinogenesis*. 2017; 56:2610–2619. <https://doi.org/10.1002/mc.22705>