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Long non-coding RNA PVT1 regulates TGF-β and promotes the proliferation, migration and invasion of hypopharyngeal carcinoma FaDu cells

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Abstract

Hypopharyngeal carcinoma is one of the malignant tumors of the head and neck with a particularly poor prognosis. Recurrence and metastasis are important reasons for poor prognosis of hypopharyngeal cancer patients, and malignant proliferation, migration, and invasion of tumor cells are important factors for recurrence and metastasis of hypopharyngeal cancer. Therefore, elucidating hypopharyngeal cancer cells' proliferation, migration, and invasion mechanism is essential for improving diagnosis, treatment, and prognosis. Plasmacytoma Variant Translocation 1 (PVT1) is considered a potential diagnostic marker and therapeutic target for tumors. However, it remains unclear whether PVT1 is related to the occurrence and development of hypopharyngeal cancer and its specific mechanism. In this study, the promoting effect of PVT1 on the proliferation, migration, and invasion of hypopharyngeal carcinoma FaDu cells was verified by cell biology experiments and animal studies, and it was found that PVT1 inhibited the expression of TGF-β, suggesting that PVT1 may regulate the occurrence and development of hypopharyngeal carcinoma FaDu cells through TGF-β.

Keywords Hypopharyngeal carcinoma, FaDu cells, Long noncoding RNA PVT1, Transforming growth factor-β

Introduction

Hypopharyngeal carcinoma is a malignant tumor located in the hypopharynx, representing about 5% of all head and neck cancers. The early symptoms of hypopharyngeal carcinoma are not obvious, and many patients have reached stage III or IV at the time of diagnosis and have developed metastasis, so the prognosis of hypopharyngeal carcinoma is poor [1]. Although chemotherapy, radiotherapy, and surgical therapy have significantly developed in recent years [2], the 5-year survival rate of patients with hypopharyngeal cancer is still meager, about 30% [1]. Therefore, it is essential to explore early molecular diagnostic markers for hypopharyngeal carcinoma, improve the early diagnosis rate, explore the mechanism of distant metastasis of hypopharyngeal

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carcinoma, and find the target therapy site for the diagnosis, treatment, and prognosis of hypopharyngeal carcinoma.

LncRNA Plasmacytoma Variant Translocation 1 (PVT1) encodes the human PVT1 gene and is located in human proto-oncogene C-Myc and human chromosome 8q24 [3]. At present, many studies have found that PVT1 is overexpressed in human tumors, including human hepatocellular tumors [4], cervical cell carcinoma [5], renal cell carcinoma [6], and thyroid tumor [7]. Ma et al. [8] found that overexpression of PVT1 could adsorb miR-186 and increase the expression of Atg7 and Beclin1, thus inducing autophagy and promoting the growth, migration, and angiogenesis of vascular endothelial cells in glioma. Wu [9] et al. found that PVT1 can promote the growth and metastasis of pancreatic cancer cells by down-regulating p21. Zhang [10] et al. found that the high expression of PVT1 could adsorb the miR-200b promoter through EZH2 and improve the upstream protein H3K27me3 level of the miR-200b promoter, thus promoting the growth and metastasis of cervical cancer cells and controlling the expression of miR-200b. You [11] et al. showed that PVT1 gene knockout could enhance the sensitivity of human pancreatic cancer cells to gemcitabine [12]. However, whether PVT1 is related to the occurrence and development of hypopharyngeal carcinoma FaDu cells and its specific mechanism has not been studied.

Transforming growth factor β(TGF-β) is a secreted multifunctional cytokine closely related to tumor disease occurrence [13]. Zhang [14] et al. found that PVT1 was upregulated in pancreatic cancer and promoted the occurrence of pancreatic cancer by mediating the TGF-β/Smad signaling pathway. Cao [15] et al. found that PVT1 promoted fibroblast growth, collagen formation, and atrial fibrosis in mice through the miR-128-3p-Sp1-TGF-β1-Smad axis. Wu et al. [3] found that PVT1 activates the TGF-β pathway through the miR-148a-3p/AGO1 axis to promote ovarian cancer progression. This study investigated the role of lncRNA PVT1 in the progression of hypopharyngeal carcinoma using in vitro experiments with FaDu cells and in vivo tumor formation assays in nude mice. Furthermore, it preliminarily explored the regulatory influence of PVT1 on TGF-β and its underlying mechanisms.

Materials and methods

Experimental materials

FaDu cells of human hypopharyngeal squamous cell carcinoma (Shanghai Jikai Kein, China); BALB/c nude mice (Beijing Spafu, China).

Experimental methods

The stable PVT1 knockout FaDu cell line was constructed

After the cells reached the logarithmic growth phase, a suspension was prepared at a density of 5×10^4 cells/ml. A 2 ml aliquot of this suspension was seeded into each well of a six-well plate and incubated at 37 °C in a 5% CO_2 environment. Once the cells reached 80–90% confluence, the medium was replaced with 1 ml of fresh serum-free medium containing 40 μl of HitransGP and an appropriate amount of sh-PVT1 lentivirus suspension (GenePharma, Shanghai). The cells were then incubated under the same conditions. After 16 h, the medium was replaced with fresh normal medium, and the cells were further cultured. Following 72 h of infection, the medium was replaced with fresh medium containing 1 μg/ml puromycin, and the cells were incubated for an additional 48 h to select for stable PVT1 knockdown in FaDu cells. Green fluorescence expression was observed using a fluorescent inverted microscope, and the success of PVT1 knockdown was confirmed via qRT-PCR.

CCK-8 cell proliferation assay

After the cells grew to the logarithmic phase, the cell suspension at a density of 8×104/ml was prepared, and 200ul of the cell suspension was evenly seeded in 96-well plates. Each group was set up with six multiple Wells and cultured in an incubator at 37℃ with 5% CO_2. At 4 h, 24 h, 48 h, and 72 h of culture, 10 μl CCK-8 solution was added to the cells, respectively, and the cells were incubated with 5% CO_2 and 37℃ for 2 h. The 96-well plate was shaken and mixed on the excellent shaker. The absorbance value at 450 nm was measured using a microplate reader.

Clone formation assay

After the cells were grown to logarithmic phase and made into cell suspension, 800 cells/well were seeded uniformly in six-well plates containing 2 ml medium and cultured in an incubator at 37℃ with 5% CO_2. When cell mass could be seen after 2–3 weeks of cell culture, the culture was terminated. The supernatant was discarded, washed 3 times with PBS buffer salt solution, and stained with crystal violet staining solution for 15 min, and then the crystal violet staining solution on the surface was cleared, the colony formation area was scanned, and the number of cell clones was counted.

Cell scratch assay

Draw lines on the back of the 6-well plate so that the same field of view can be located when taking photos; After the cells grew to a logarithmic phase, the cell suspension at a density of 5×105/ml was prepared, and 2 ml cell suspension was seeded in a six-well plate and cultured in an incubator at 37℃ with 5% CO_2. When the cell

density increased to more than 90% confluence, the cells were immediately replaced with serum-free medium, and the wound was scratched with 10 μl of the gun tip perpendicular to the marker line. Pictures were taken at 0 h, 6 h, 12 h, 24 h, and 48 h to observe cell migration.

Transwell assay

The cells in the logarithmic growth phase were prepared with serum-free medium for cell suspension, and 200 μl cell suspension (1×105 cells) was uniformly seeded in the upper chamber of the Transwell chamber of a 24-well plate. Then 800 μl medium containing 10% fetal bovine serum was added to the lower chamber of a 24-well plate and cultured in an incubator at 5% CO_2 and 37℃ for 24 h. The cells in the upper chamber were gently wiped off with a cotton swab, washed with PBS buffer salt solution, and stained with crystal violet staining solution for 15 min to clean the surface crystal violet staining solution. Photos were taken and counted in six randomly selected fields of view for microscopic observation.

Matrigel was coated in the upper Transwell chamber, diluted with a serum-free medium at a ratio of 1:4, and then 40 μl diluted Matrigel was evenly spread on the bottom membrane of the upper chamber and incubated in the incubator at 37℃ for 4 h. The remaining experimental procedures were the same as the cell migration experiment.

Real-time quantitative PCR (qRT-PCR)

The primer sequence of qRT-PCR is shown in Table 1.

ELISA

Human TGF-β1 ELISA Kit (BOSTER, Wuhan, China) was used to detect the expression level of TGF-β1 in cells according to the instructions. The absorbance (OD value) at 450 nm was determined by enzymoleter, and the standard curve was drawn by ELISA Calc software to calculate the sample concentration.

Tumor formation experiment in nude mice

The cells in the logarithmic growth phase were resuspended in PBS buffer salt solution at a cell density of 1×107 cells /ml. Four-week-old female nude mice were selected and acclimatized for 1 week, with 6 mice in each group. After the nude mice were anesthetized by ether inhalation, 100 μl cell suspension was thoroughly mixed and injected subcutaneously into the middle and posterior part of the right axilla of nude mice with a syringe. After the injection, the injection point was pressed for 60 s with a cotton swab. After the tumor was visible to the naked eye, the length and width were measured every 3 days, and the volume V= (length × width^2) ×0.5 was calculated. After 6 weeks, all the mice were sacrificed, the tumors were removed, and then the net weight of the tumors was measured.

Table 1 The primer sequence of qRT-PCR

Gene	Sequence
PVT1	Forward: 5′- TGAGAACTGTCCTTACGTGACC-3′
	Reverse: 5′- AGAGCACCAAGACTGGCTCT-3′
TGF-β	Forward: 5′- GGGACTATCCACCTGCAAGA-3′
	Reverse: 5′- CCTCCTTGGCGTAGTAGTCG-3′
β-actin	Forward: 5′- CACCATGTACCCAGGCATTG-3′
	Reverse: 5′- CCTGCTTGCTGATCCACATC-3′

TGF-β 3′-UTR reporter vector construction, transfection, and dual luciferase assay

The TGF-β 3'UTR gene sequence was synthesized, and SacI/HindIII restriction enzyme sites were added at both ends of the sequence according to the polyclonal sites of the pMIR-REPORT plasmid. TGF-β 3'UTR gene sequence and pMIR-REPORT plasmid were digested with SacI/HindIII restriction enzyme. DNA fragments were recovered by DNA purification kit (Guangzhou Megi, China) according to the manufacturer's instructions, and an ultramicro ultraviolet spectrophotometer detected the concentration. The 3'UTR fragment recovered by restriction enzyme digestion was ligated with pMIR-REPORT plasmid fragment at a volume of 3:1. Plasmids were extracted with a plasmid extraction kit (Axygen, USA) according to the manufacturer's instructions, and plasmid concentrations were measured with an ultramicro ultraviolet spectrophotometer. The extracted plasmids were sequenced to verify the successful construction of the TGF-β 3'UTR reporter vector.

After 293T cells were grown to the logarithmic phase, the cell suspension was prepared, seeded in 12-well plates, and cultured in an incubator at 37℃ with 5% CO_2. When the cell density reached more than 60% confluence, PMIR-TGF-β reporter Gene and CV045 plasmid (Renilla Luciferase) (Jikai, Shanghai) were transfected according to the instructions of X-treme GENE 9 DNA transfection reagent. Dual luciferase assays were performed 24 h to 48 h after transfection. According to the instructions of the dual luciferase reporter gene detection system kit (Promega, USA), the fluorescence values of firefly luciferase and renilla luciferase were detected by GloMax Navigator microplate luminescence detector, respectively, and the data were analyzed.

Statistical analysis

SPSS 26.0 and GraphPad Prism 8.0 software were used for data analysis and drawing. Quantitative data were expressed as mean±standard deviation. One-way analysis of variance (ANOVA) was used to compare the data between multiple groups of samples, and a two-tailed unpaired t-test was used to compare the data between two groups of samples.

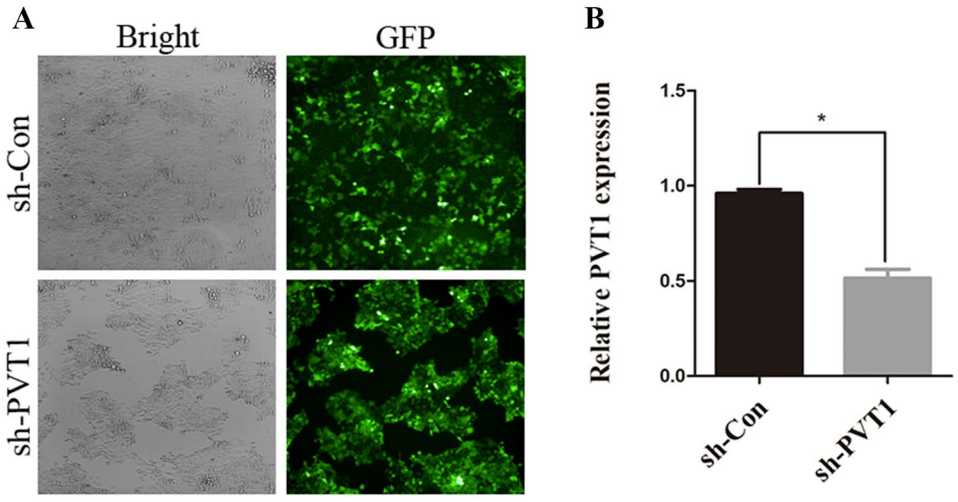

Fig. 1 The stable PVT1 knockout FaDu cell line was constructed. (**A**) Green fluorescence indicates successful infection of cells with lentivirus. (**B**) The expression level of PVT1 in the Lv-shPVT1 group was significantly lower than in the Lv-Con group. (*$P<0.05$, mean ± SD, one-way ANOVA, double-tailed unpaired t-test, $n=6$/group)

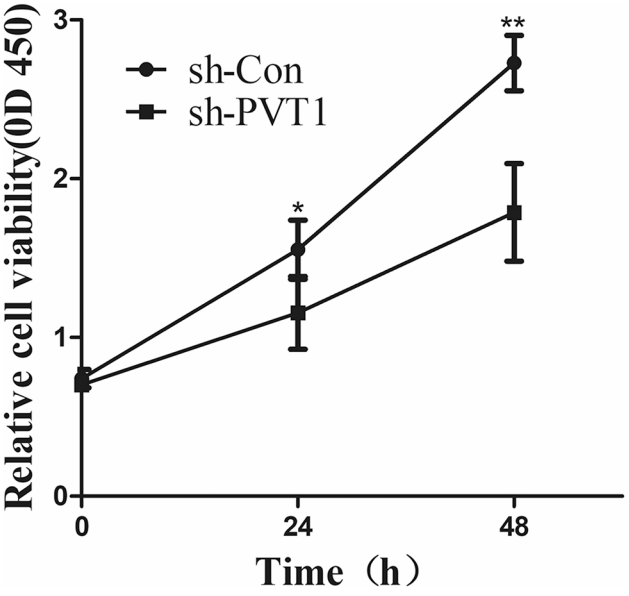

Fig. 2 PVT1 knockout inhibited FaDu cell proliferation (*$P<0.05$, **$P<0.01$, mean ± SD, one-way ANOVA, double-tailed unpaired t-test, $n=6$/group)

Results

PVT1 promotes the proliferation, migration, and invasion of hypopharyngeal carcinoma FaDu cells

The stable PVT1 knockout FaDu cell line was constructed

In order to investigate the effect of PVT1 on the biological behavior of FaDu cells, we used lentivirus gene knockout technology to construct a stable PVT1 knockout cell line FaDu. FaDu cells were infected with lentivirus Lv-shPVT1 to knock out PVT1, and FaDu cells were infected with lentivirus Lv-shCon as control. As observed under fluorescence microscopy, green fluorescence indicated that the cells were successfully infected with the lentivirus (Fig. 1A). The expression of PVT1 in the two groups of cells was detected by qRT-PCR, and the results showed that the expression level of PVT1 in the Lv-shPVT1 group was significantly lower than that in the Lv-Con group, and the difference was statistically significant ($P<0.05$) (Fig. 1B). In summary, we successfully established PVT1 stable knockout in hypopharyngeal carcinoma FaDu cells.

PVT1 knockout inhibited FaDu cell proliferation

This study, the CCK-8 assay was used to determine the proliferation ability of FaDu cells in the Lv-shCon group and Lv-shPVT1 group. The results showed that the proliferation rate of FaDu cells in the sh-PVT1 group was significantly slower than that in the sh-Con group, and the difference was statistically significant ($P<0.05$) (Fig. 2). PVT1 knockout inhibited FaDu cell proliferation.

PVT1 knockout inhibited the colony formation ability of FaDu cells

Cell clone formation assays were also used to examine cell proliferation and tumorigenic ability. The cell colony formation assay results showed that the colony formation rate of the sh-PVT1 group (23.55%±6.57%) was significantly lower than that of the sh-Con group (8.86%±5.38%). The difference was statistically significant ($P<0.01$) (Fig. 3A). The area of single clone formation in the sh-PVT1 group (8.94±2.75) was significantly lower than that in the sh-Con group (3.44±1.0). The difference was statistically significant ($P<0.05$) (Fig. 3B). It was confirmed that the proliferation and tumorigenic ability of FaDu cells were weakened after knocking out PVT1.

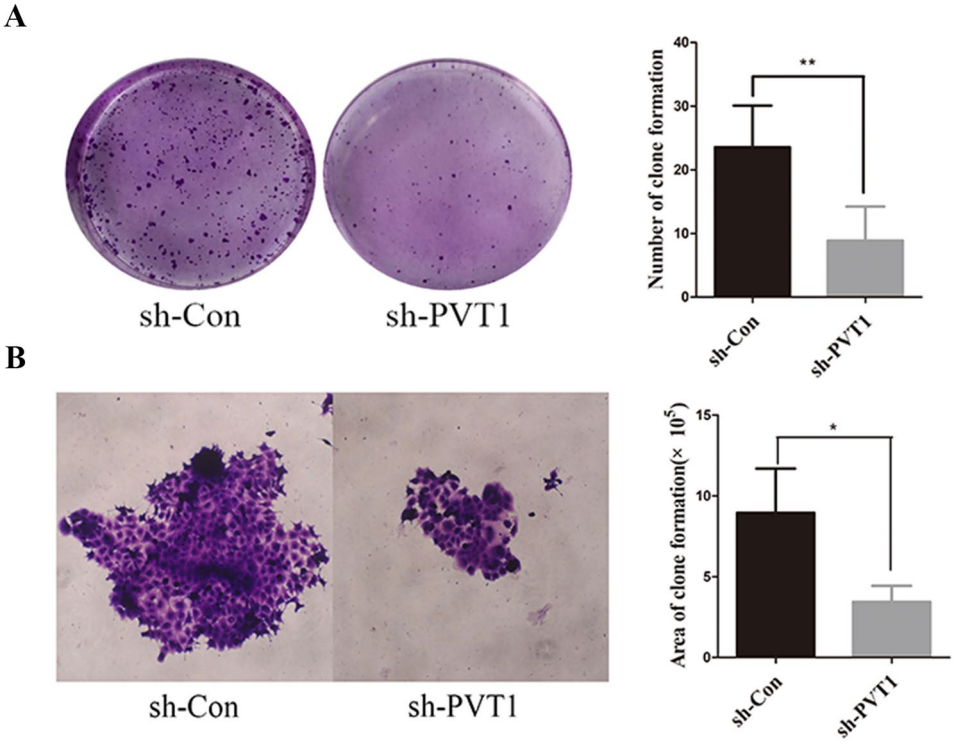

Fig. 3 PVT1 knockout inhibited the colony formation ability of FaDu cells. (**A**) Clone formation rate. (**B**) Area of single clone formation. (*$P < 0.05$, **$P < 0.01$, mean ± SD, one-way ANOVA, double-tailed unpaired t-test, $n = 6$/group)

PVT1 knockout inhibited the migration and invasion of FaDu cells

The ability of cell migration and invasion can reflect the metastatic potential of tumor cells. This study determined the effect of PVT1 on the migration and invasion of FaDu cells using cell scratch and Transwell assays. The results of the cell scratch experiment showed that the scratch width of the sh-PVT1 group was 46.49%±5.14% of the initial width, and the scratch width of the sh-Con group was 33.17%±6.18%; the difference was statistically significant ($P<0.05$). This indicated that the scratch healing speed of the sh-PVT1 group was significantly lower than that of the sh-Con group (Fig. 4A). The results of the Transwell migration assay showed that the number of transmembrane cells in the sh-PVT1 group (392±69.20) was significantly lower than that in the sh-Con group (120.33±28.36). The difference was statistically significant ($P<0.01$) (Fig. 4B). Scratch assay and Transwell migration assay confirmed that PVT1 knockout inhibited the migration ability of FaDu cells. The results of the Transwell invasion assay showed that the number of transmembrane cells in the sh-PVT1 group (164.33±2.08) was significantly lower than that in the sh-Con group (84.67±68.62), and the difference was statistically significant ($P<0.001$) (Fig. 4C). PVT1 knockout inhibited the invasion of FaDu cells.

PVT1 knockout inhibited the tumorigenic ability of FaDu cells in vivo

The effect of PVT1 on the tumorigenic ability of FaDu cells in vivo was detected by tumor formation assay in nude mice. The above two groups of cells were subcutaneously inoculated into BALB/c nude mice. The results showed that the tumor growth rate of the sh-PVT1 group was significantly lower than that of the sh-Con group. The difference was statistically significant ($P<0.05$) (Fig. 5A). The tumor weight of the sh-PVT1 group (0.71±0.35) was less than that of the sh-Con group (0.24±0.15), and the difference was statistically significant ($P<0.01$) (Fig. 5B). Knockout of PVT1 inhibited the tumorigenic ability of FaDu cells in vivo.

PVT1 knockout inhibited TGF-β expression

Using the qRT-PCR method, TGF-β mRNA expression was compared between the sh-PVT1 group and the sh-Con group, and it was found that the degree of TGF-β mRNA expression was significantly reduced after knocking down PVT1. The difference was statistically significant ($P<0.001$) (Fig. 6A). We further extracted proteins from the above two groups of cells and determined the protein level of TGF-β by ELISA. The results showed that the protein level of TGF-β in the sh-PVT1 group was significantly lower than that in the sh-Con group, and the difference was statistically significant ($P<0.01$) (Fig. 6B)

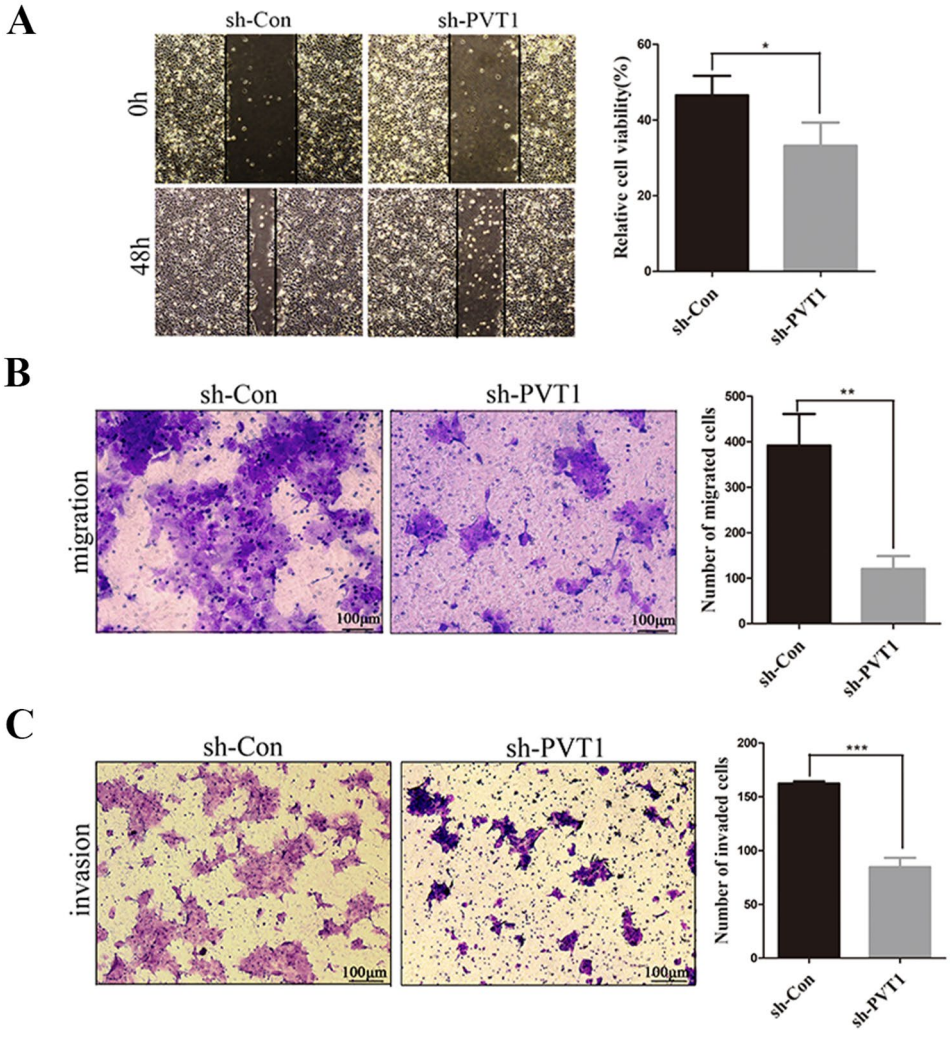

Fig. 4 PVT1 knockout inhibited the migration and invasion of FaDu cells (**A**) sh-PVT1 inhibited the scratch healing rate of FaDu cells. (**B**) sh-PVT1 inhibited FaDu cell migration. (**C**) sh-PVT1 inhibited FaDu cell invasion. (*$P<0.05$, **$P<0.01$, ***$P<0.001$, mean ± SD, one-way ANOVA, double-tailed unpaired t-test, $n=6$/group)

The above experiments indicated that knockout of PVT1 inhibited TGF-β expression.

To investigate the mechanism of PVT1 regulating TGF-β expression

lncRNA can regulate TGF-β expression. We further explored the mechanism by which PVT1 regulates TGF-β expression. We constructed a TGF-β promoter activity reporter gene designated as PGL3-TGF-β (Fig. 7A) to verify whether lncRNA regulated TGF-β expression at the transcriptional level. The results showed that there was no statistically significant difference in fluorescence intensity between the sh-PVT1 group and the sh-Con group ($P>0.05$) (Fig. 7B). We constructed a TGF-β 3'-UTR reporter gene, named pMIR-TGF-β (Fig. 7C), and transfected it into Fadu cells with or without PVT1 knockout. The results of the dual luciferase assay showed that the fluorescence intensity of the sh-PVT1 group was higher than that of the sh-Con group ($P<0.01$) (Fig. 7D).

Discussion

LncRNA refers to a class of noncoding RNA genomic transcripts, which are more than 200 nucleotides in length. Many scientific studies have proved that lncRNA plays a tumor suppressor or tumor-promoting role in the development and progression of tumors [16] and plays an essential regulatory role in tumor proliferation, invasion and migration, immune escape, and tumor metabolism [18.19]. Therefore, lncRNA can be used as a tumor diagnostic marker and potential therapeutic target [19]. In hypopharyngeal carcinoma, several lncRNA has also been found to play important regulatory roles in the occurrence and development of tumors by regulating cell proliferation, migration and invasion, angiogenesis, tumor

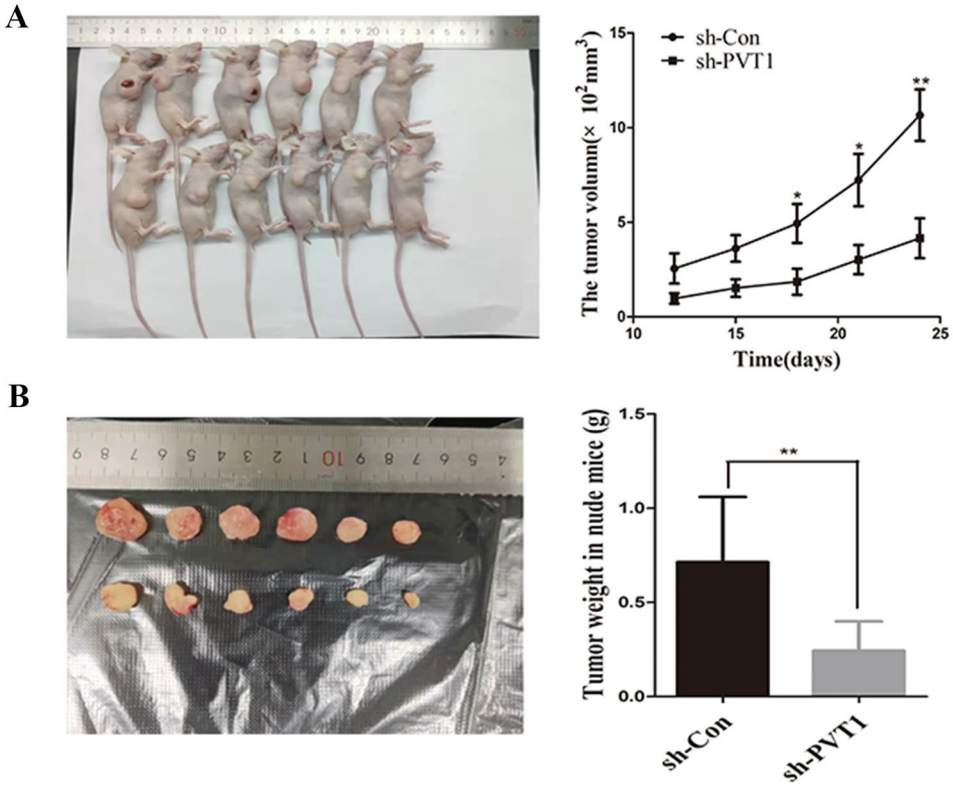

Fig. 5 PVT1 knockout inhibited the tumorigenic ability of FaDu cells in vivo (**A**) Tumor growth rate. (**B**) Tumor weight. (*$P < 0.05$, **$P < 0.01$, mean ± SD, one-way ANOVA, double-tailed unpaired t-test, $n = 6$/group)

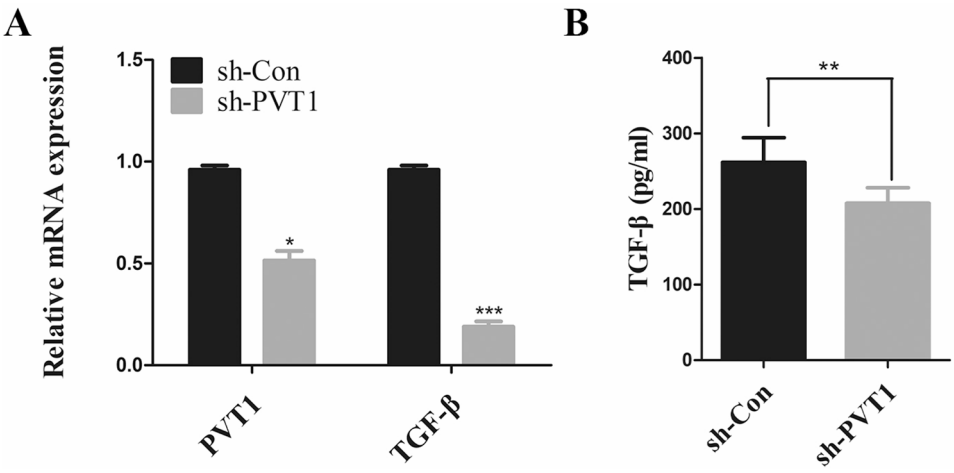

Fig. 6 PVT1 knockout inhibited TGF-β expression (**A**) mRNA expression of TGF-β in FaDu cells. (**B**) Protein expression of TGF-β in FaDu cells. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, mean ± SD, one-way ANOVA, double-tailed unpaired t-test, $n = 6$/group)

immune response, and other activities [20–23]. LncRNA PVT1 is encoded by the human PVT1 gene, located in human proto-oncogene C-Myc and human chromosome 8q24. Existing research results have confirmed that it plays an essential role in the occurrence and development of cancer cells in many tumors, such as gastric cancer and hepatocellular carcinoma, and can be used as a diagnostic marker and prevention target [3]. However, whether PVT1 is related to the occurrence and development of hypopharyngeal carcinoma and its specific mechanism has not been confirmed. In this study, we successfully established PVT1 stable knockout in hypopharyngeal carcinoma FaDu cells. We verified that PVT1 knockout inhibited the cell proliferation, colony formation, migration, and invasion ability of FaDu cells in vitro by CCK-8, colony formation, cell scratch, and Transwell

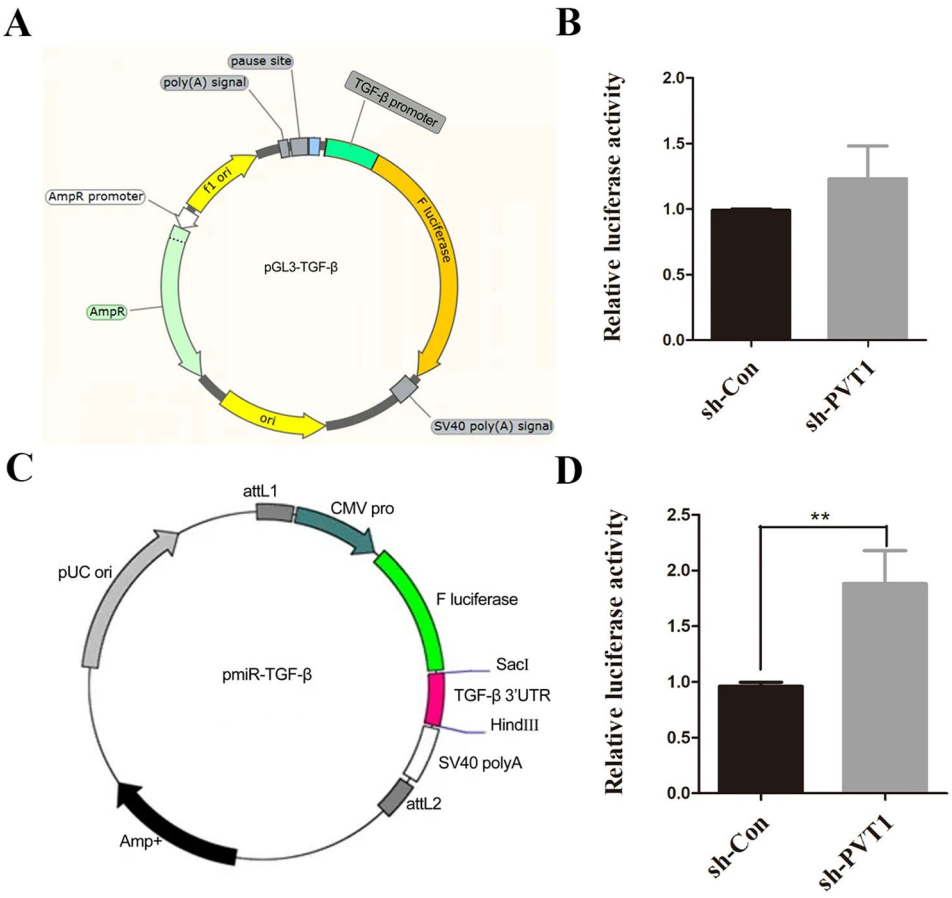

Fig. 7 To investigate the mechanism of PVT1 regulating TGF-β expression (**A**) Knockout of PVT1 down-regulated the expression of TGF-β in FaDu cells. (**B**) The structural pattern of TGF-β promoter activity reporter (pGL3-TGF-β). (**C**) Knockout of PVT1 did not affect TGF-β promoter activity. (**D**) The structural pattern of TGF-β 3'UTR reporter gene (pmiR-TGF-β). (ns means $P > 0.05$, $^{**}P < 0.01$, mean ± SD, one-way ANOVA, double-tailed unpaired t-test, $n = 6$/group)

assays. Subsequently, the tumor formation experiment in nude mice showed that PVT1 knockout inhibited the tumor formation ability of FaDu cells in vivo, suggesting that PVT1 promotes the occurrence and development of hypopharyngeal cancer.

Tumor tissue contains many cancer cells, fibroblasts, endothelial cells, infiltrating inflammatory cells, T lymphocytes, tumor-associated dendritic cells, etc. These cells can produce a variety of cytokines, chemokines, and growth factors and change the local tumor microenvironment by regulating the immune response, inhibiting the expression of vascular cell adhesion protein, or inducing angiogenesis. It plays a role in promoting and suppressing cancer. For example, TNF-α, IL-6, and IL-17 can promote cancer, while some cytokines, such as IFN-γ, IL-2, and IL-15, can inhibit cancer [24]. TGF-β is a cytokine, a member of the transforming growth factor superfamily, with multiple effects on inflammation, angiogenesis, fibrosis, and tumor progression. In cancer, TGF-β is involved in cell proliferation, angiogenesis, epithelial-mesenchymal transition, immune infiltration, metastatic dissemination, and tumor drug resistance [3, 25–29]. Li [26] et al. reported that HER2/EGFR regulates Smad3 through AKT, which promotes TGF-β to enhance cell invasion and metastasis, thus promoting the development of breast cancer. Mingfang Ao [27] et al. found that TGF-β induced EMT invasion ability by inhibiting Akt to promote prostate cancer. Studies have verified the role of TGF-β in regulating tumor cell proliferation, migration, and invasion by PVT1 in glioma and other tumors [32.33]. We found that knockout of PVT1 inhibited TGF-β mRNA expression by qRT-PCR and further showed that knockout of PVT1 also inhibited TGF-β protein expression by ELISA. Therefore, we hypothesized that PVT1 might regulate the proliferation, migration, and invasion of hypopharyngeal carcinoma FaDu cells through TGF-β.

Further studies are needed to confirm this speculation. We aimed to verify whether blocking TGF-β can block the effect of PVT1 on the proliferation, migration, invasion, and tumorigenesis of FaDu cells by overexpressing PVT1 in FaDu cells and blocking TGF-β with a TGF-β inhibitor. In addition, PVT1 has been confirmed to regulate the occurrence and development of tumors by promoting tumor cell proliferation, inhibiting apoptosis,

inducing tumor cell metastasis, mediating drug resistance [31], and other mechanisms. Whether the above mechanisms also play a role in the progression of hypopharyngeal carcinoma and the importance of TGF-β in the various mechanisms of PVT1-regulated progression of hypopharyngeal carcinoma need to be further explored.

PVT1 can regulate target gene expression through a variety of mechanisms. PVT1 can participate in DNA rearrangement, thereby interfering with the regulation of various oncogenes and tumor suppressor genes to promote the occurrence of tumors [32]. One study found that PVT1 rearrangement was detected in multiple myeloma, and two highly expressed chimeric genes were detected: the PVT1-NBEA chimera and the PVT1-WWOX chimera. NBEA and WWOX were abnormally highly expressed in the two chimeras, among which NBEA and WWOX were tumor suppressors in multiple myeloma [33]. As a ceRNA, PVT1 interacts with miRNA to regulate miRNA expression and thus regulate downstream target genes [32]. Wu [3] et al. found that PVT1 upregulates AGO1 expression by adsorption of miR-148a-3p and thus promotes ovarian cancer progression. PVT1 can also regulate the promoter activity of target genes. Wan [34] et al. demonstrated that PVT1 promoted EZH2 to bind to the LATS2 promoter and inhibited LATS2 transcription. PVT1 can regulate the expression of target genes by affecting the DNA methylation level. Xin [35] et al. found that knocking out lncRNA PVT1 could reduce the DNA methylation level and promoter activity of BNIP3. The mechanism by which PVT1 regulates TGF-β in hypopharyngeal carcinoma FaDu cells was preliminarily explored. Because PVT1 can regulate TGF-β mRNA expression, we hypothesized that PVT1 might affect TGF-β expression by regulating mRNA synthesis or degradation.

We constructed a TGF-β promoter-reporter gene to verify whether PVT1 plays a regulatory role at the transcriptional level, affecting TGF-β mRNA biosynthesis and its expression. However, the results showed that knocking down PVT1 did not affect TGF-β promoter activity, denying that PVT1 regulates TGF-β expression at the transcriptional level. We constructed a TGF-β 3'-UTR activity reporter gene and found that knockout of PVT1 only slightly upregulated TGF-β 3'-UTR activity, which could not explain our finding that knockout of PVT1 significantly downregulated TGF-β mRNA and protein expression. These results suggest that PVT1 may regulate TGF-β expression through other mechanisms. In addition, previous literature reported that PVT1 regulates TGF-β expression through the miR-148a-3p/AGO1 axis in ovarian cancer [3], which is inconsistent with our finding that PVT1 has only a weak regulatory effect on TGF-β 3'-UTR, which may be due to the differences in different tumor types. In conclusion, the mechanism by which PVT1 regulates TGF-β expression in hypopharyngeal carcinoma FaDu cells requires further investigation.

Conclusions

In this study, we verified the promotion effect of PVT1 on the proliferation, migration, and invasion of hypopharyngeal squamous cell carcinoma cells by cell biological experiments. We found that PVT1 inhibited the expression of TGF-β by analytical and biological experiments, suggesting that PVT1 may regulate the occurrence and development of hypopharyngeal carcinoma FaDu cells through TGF-β.

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Author contributions
Y Z and L Z conducted the entire experiment, with Z Y also writing the article. Z M guided the experimental design. MC L provided the experiment idea. S W and J L provided experimental guidance. LL G and LQ L supervised the entire study and reviewed the manuscript.

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None.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All experimental procedures were approved by the Animal Care and Use Committee of Liaocheng People's Hospital.

Consent for publication
This publication does not contain any individual person's data in any form.

Competing interests
The authors declare no competing interests.

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