Long Non-Coding RNA Plasmacytoma Variant Translocation 1 (PVT1) Enhances Proliferation, Migration, and Epithelial-Mesenchymal Transition (EMT) of Pituitary Adenoma Cells by Activating β-Catenin, c-Myc, and Cyclin D1 Expression

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Background: As a kind of benign tumor, pituitary adenomas have attracted increasing attention from researchers. The plasmacytoma variant translocation 1 (PVT1) is a molecule in the IncRNA family protein that has been proven to play critical roles in many cancers; however, no study has explored the special biological roles of PVT1 in pituitary adenoma.

Material/Methods: The qRT-PCR assay was conducted to evaluate PVT1 expressions in various cell lines and tissues. Loss of function assays were carried out to detect the influence of silenced PVT1 on the proliferation, migration, and epithelial-mesenchymal transition (EMT) of pituitary adenoma cells. Western blotting was used to identify correlation between β-catenin and PVT1.

Results: The PVT1 expressions were significantly enhanced in tissues of pituitary adenoma and cancer cells. Cell migration and proliferation were inhibited when the PVT1 gene was knocked down. Knockdown of PVT1 repressed the migration and EMT of pituitary adenoma cells. The PVT1 downregulation obviously blocked Wnt/β-catenin signaling pathway activity. PVT1 aggravated progression of pituitary adenoma through initiating the Wnt/β-catenin signaling pathway.

Conclusions: PVT1 exerts an oncogenic role through activating Wnt/β-catenin signaling in pituitary adenoma cells. The present results may provide a potential therapeutic target or approach for treating pituitary adenomas.

MeSH Keywords: alpha Catenin • Cell Migration Assays • Genes, myc • Pituitary Neoplasms

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Background

In the recent years, pituitary adenoma has become the 3rd most common primary cancer of the human brain. According to a previous report, the average incidence of pituitary adenoma (PA) is approximately 0.01%, and the incidence rate is steadily increasing [1]. Pituitary adenoma results in enormous suffering [2,3]. Some pituitary adenomas can contribute to a large tumor size and can also invade into the paranasal and cavernous sinuses or the parenchyma of the brain [4]. Radiotherapy, chemotherapy, and surgery are 3 most commonly used methods for the treatment of pituitary tumors. However, many shortcomings and limitations, such as bleeding and other adverse effects, still inhibit the curative effect of these treatment methods [5,6].

Because surgery, drugs, and radiation therapy cannot achieve satisfactory results, the treatment of pituitary adenoma has long been a difficult problem to solve in neurosurgery. With the application of multimodal techniques, such as neuro-navigation and intra-operative Doppler, the neuro-endoscopic enlargement of the trans-sphenoidal approach has greatly improved the resection rate and outcome of invasive pituitary adenomas [7]. By investigating the invasion mechanism of tumors, it has been shown that tumor invasion is closely related to pituitary tumor transforming gene (PTTG), vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs). However, these markers cannot accurately predict tumor prognosis in clinical applications [8,9]. In the process of exploring novel therapies, molecular targeted therapy has been used for the treatment of tumors [10–12]. Therefore, more effective molecular targets for pituitary tumors are crucial for clinical applications [13,14].

Long non-coding RNA (lncRNA) plays critical roles in regulating many human cancers, including pituitary adenoma. lncRNA plasmacytoma variant translocation 1 (PVT1) acts as a prognostic factor indicating the radio-resistance of nasopharyngeal carcinoma [15], suppressing cell apoptosis in renal cancer [16]. lncRNAs can modulate tumor cell proliferation and chemo-resistance by regulating the Wnt/β-catenin signaling pathway [17]. A previous study reported that the Wnt/β-catenin signaling pathway plays critical roles in the development and tumorigenesis of pituitary adenoma [18].

Therefore, in this study, we proposed that the PVT1 mediates pituitary gland function by regulating the Wnt/β-catenin pathway, thus affecting tumor cell proliferation and migration, and finally influencing the invasion of pituitary tumors.

Material and Methods

Experimental samples

Eighty-six pairs of pituitary adenoma tissues (86 specimen) and corresponding adjacent non-tumorous tissues (86 specimen) were collected from patients who underwent endoscopic endonasal surgery (EES) at the Department of Neurosurgery, Daping Hospital of the Army Medical University, from November 2017 to July 2018. Tumor tissues and non-tumorous tissues were immediately snap-frozen in liquid nitrogen within 15 min after surgical removal. The mean age of the 86 patients with pituitary adenomas was 51.2 years (range 27–81 years) and there were 41 males and 45 females. None of the included patients had received chemotherapy or radiation therapy before surgery. An informed consent form was signed by all patients.

The study was approved by the Ethics Committee of Daping Hospital of Army Medical Center of PLA. All samples were stored at –80°C.

Cell culture and transfection

The human pituitary adenoma cell lines GH3 and HP75 and the human neuroepithelial cell line HNPG were obtained from the Cell Bank of CAS (Shanghai, China). Pituitary adenoma cell lines and the HNPG cells were cultured and grown in DMEM (HyClone, UT, USA) containing 10% FBS (Gibco, Thermo Fisher Scientific, Inc., CA, USA), streptomycin (100 μg/ml), and penicillin (100 units/ml). The above cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Cell transfection was conducted for subsequent experiments. To interfere with the expression of PVT1, PVT1-specific shRNAs and negative control shRNA were synthesized. Then, the PVT1-specific shRNAs and negative control shRNA were inserted into pcDNA3.1 expression plasmid vector to form sh-PVT1#1, sh-PVT1#2, and NC vectors. The above expression plasmids were transfected into HP75 and GH3 cells with Lipofectamine 3000 (Thermo Fisher Scientific, Inc., CA, USA). In this study, we used 0.2 g shRNAs as the optimal concentration for the transfection. After approximately 2 days, cells were collected for functional assays.

RNA extraction and quantitative real-time PCR (qRT-PCR)

TRizol™ reagent (Invitrogen, Thermo Fisher Scientific, USA) was used to isolate total RNA from tissues or cultured cells. Reverse transcription was accomplished by using a specific kit (Promega, Madison, WI, USA). To detect the expression of PVT1, real-time PCR was carried out with the Step One Plus™ PCR System (Applied Biosystems, Foster City, CA, USA). According to the user guide, the GoTaq® qPCR Master Mix of Power SYBR® Green (Promega) was employed to conduct qRT-PCR. Prior to the qRT-PCR assay, the qRT-PCR efficiency was performed and
illustrated higher efficiency (data not shown). The data of targeting genes was normalized to the GAPDH expression. PCR primers for PVT1 and GAPDH were:
PVT1: 5’-TGGAGACTGTCCCTACGTGACC-3’ (forward) and 5’-AGGCCACAAAGACTGCTCT-3’ (reverse);
GAPDH: 5’-GGAGCGAGATCCCTCCAAAAT-3’ (forward) and 5’-GGCGTTGTCCTACACTTCTCAATG-3’ (reverse).
The qRT-PCR assay in this study was conducted for at least 6 biological replicates to assure accuracy. The results of the relative fold-change differences were calculated by $2^{-\Delta \Delta Ct}$ method.

Western blot analysis

Cells were lysed in RIPA buffer (Beyotime Biotechnology, Beijing, China). Then, the protein lysates were segregated by SDS-PAGE and transferred onto PVDF membranes (Sangon Biotechnology Co., Shanghai, China). The membranes were sealed and incubated with rabbit anti-human Cyclin D1 monoclonal antibody (Cat. No. ab16663, 1: 3000), rabbit anti-human c-Myc polyclonal antibody (Cat. No. ab168727, 1: 2000), rabbit anti-human $\beta$-catenin monoclonal antibody (Cat. No. ab32572, 1: 3000), rabbit anti-human E-cadherin polyclonal antibody (Cat. No. ab15148, 1: 2000), rabbit anti-human N-cadherin polyclonal antibody (Cat. No. ab18203, 1: 2000), and rabbit anti-human GAPDH (Cat. No. ab9485) at 4°C overnight. Then, PVDF membranes were treated using the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cat. No. 6721, 1: 3000), rabbit anti-human Cyclin D1 monoclonal antibody (Cat. No. ab16663, 1: 3000), rabbit anti-human N-cadherin polyclonal antibody (Cat. No. ab15148, 1: 2000), rabbit anti-human $\beta$-catenin monoclonal antibody (Cat. No. ab32572, 1: 3000), rabbit anti-human E-cadherin polyclonal antibody (Cat. No. ab15148, 1: 2000), rabbit anti-human N-cadherin polyclonal antibody (Cat. No. ab18203, 1: 2000), and rabbit anti-human GAPDH (Cat. No. ab9485) at 4°C overnight. Then, PVDF membranes were treated using the horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cat. No. 6721, 1: 1000) for 2 h at room temperature. All of the above antibodies were purchased from Abcam Biotech (Cambridge, MA, USA). Finally, the blots were measured by an ECL system (Amer sham Pharmacia, Piscataway, NJ).

Cell proliferation assay

The MTT assay was used to measure cell viability. GH3 and HP75 cells were seeded in 96-well plates. After the requisite incubation, cells were treated with MTT (5 mg/ml, Merck KGaA, Darmstadt, Germany) for 4 h at 37°C. Then, we removed the medium and added dimethyl sulfoxide to each well. The optical density values of the cells were assessed on a micro-plate reader at 490 nm. Colony formation assay was conducted as follows: The transfected cancer cells were cultured in the 6-well plates (Corning-Costar, Corning, NY, USA) and maintained in the medium containing 10% FBS (Gibco BRL Co., Grand Island, NY, USA) for 2 weeks. Medium was replaced every 4 days. Finally, colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma, San Francisco, CA, USA). The colonies were manually counted in this study.

Cell migration assay

Transwell insert chambers coated with Matrigel (BD Biosciences, NJ, USA) were used according to the user guide to detect the migration ability of the cells. After 1-day incubation, migrated cells were treated with 4% polyoxymethylene (Beyotime Biotechnology, Beijing, China). Finally, 0.1% crystal violet was utilized for staining cells on the bottom. The migrating cells were counted with a microscope at 100× magnification.

Statistical analysis

Data are presented as mean ±SD. All experiments or tests were conducted at least 3 times. Statistical analysis was conducted using GraphPad Prism Version 6.0 software. The differences between groups were analyzed by using the t test or a one-way ANOVA with the LSD post hoc test. Kaplan-Meier analysis was used to assess the overall survival probability of pituitary adenoma patients. The valuation was conducted with log-rank test. Differences were considered statistically significant when the p value was less than 0.05.

Results

PVT1 is a potential oncogenic biomarker in pituitary adenoma

To investigate the regulatory role of PVT1 in pituitary adenoma, qRT-PCR analysis was conducted to examine the relative expression of PVT1 in tumorous tissues and non-tumor tissues, as well as in pituitary adenoma cells and normal cells (Figure 1A, 1B). The results indicated that expression of PVT1 was significantly higher in the tumorous tissues compared to the normal tissues (Figure 1A, p<0.05). Expressions of PVT1 were also significantly higher in the cancer cells (GH3 cells and HP75 cells) compared to the normal cells (HNPG cells) (Figure 1B, p<0.05). These results suggest a positive relationship between the expression levels of PVT1 and tumor occurrence. Therefore, we hypothesized that PVT1 is a promising oncogenic biomarker in pituitary adenoma.

Effects of sh-PVT1 on the proliferation ability of pituitary adenoma cells

Our preliminary findings showed that the above shRNAs transfection had higher transfection efficiency (Figure 2A); therefore, these shRNAs were used for the following experiments. To verify the oncogenic role of PVT1 in pituitary adenoma progression, functional assays were conducted. The high PVT1 expression was first decreased by transfection with sh-PVT1 (Figure 2B). Control shRNA (sh-NC) was used as the negative control. After transfecting with the optimal concentration of shRNAs for 48 h, the effects of PVT1 silencing on pituitary adenoma cell proliferation were evaluated using colony formation assay and MTT assay, respectively (Figure 2C, 2D), and both demonstrated decreased proliferation in PVT1-underexpressing tumorous cells.
Knockdown of PVT1 repressed migration and EMT of pituitary adenoma cells

The migration abilities of GH3 and HP75 cells were examined after PVT1 expression was knocked down. The results of the Transwell assay verified that the migration of GH3 and HP75 cells was inhibited by transfection with sh-PVT1 (Figure 3A, 3B). The level of EMT-related protein was detected in GH3 and HP 75 cells under the same conditions as in the experiment described above. EMT was notably reversed by the interference of PVT1 (Figure 3C).

PVT1 knockdown negatively regulated Wnt/β-catenin pathway-associated molecules

To verify that the Wnt/β-catenin signaling pathway is initiated and activated in pituitary adenoma cells or tissues, the Wnt/β-catenin signaling pathway-associated molecules, including β-catenin, cyclin D1, and c-Myc, were evaluated in pituitary adenoma cells or tissues. The Western blotting results illustrated that expressions of these molecules were remarkably increased in the pituitary adenoma cells or tissues compared to that in the normal cells or tissues (Figure 4A, 4B, p<0.05). Then, we detected the protein levels of β-catenin, c-Myc and cyclin D1 in 2 pituitary adenoma cell lines in which PVT1 was downregulated. We found that the protein levels of these 3 factors were greatly reduced (Figure 4C). After analyzing the above data, we concluded that PVT1 positively modulates Wnt/β-catenin signaling in pituitary adenoma. Summarizing the abovementioned results, we posit that decreased expression of PVT1 drives a reduction in the proliferation, migration, and EMT of pituitary adenoma cells by inactivating Wnt/β-catenin signaling.

Discussion

Invasive pituitary adenoma (IPA) is a type of pituitary adenoma that invades the dura mater, bone, saddle, and cavernous sinus, as well as the surrounding nerves and blood vessels [19,20]. Although the 2017 version of the WHO classification noted that invasive behavior is an important factor predicting tumor prognosis, IPA has not been directly included in classification as a pathological diagnosis, mainly because the IPA diagnostic criteria are not accurate enough, and its pathological characteristics make it difficult to make a separate diagnosis. Therefore, the present study investigated the special biological roles of PVT1 in pituitary adenoma and also explored the possibility of PVT1 as diagnostic biomarker for pituitary adenoma. Many previous studies [21–27] have proven that IncRNAs can be promoters of tumorigenesis; however, the special role of IncRNAs in pituitary adenoma is still unclear. In recent years, an increasing number of studies have found that IncRNAs have important regulatory effects on tumor cell proliferation, apoptosis, invasion, and metastasis [28,29]. Therefore, in this study, we analyzed the association between pituitary adenoma and PVT1.

Our results showed that the PVT1 was highly expressed in the cancer tissues of pituitary adenoma patients and in the pituitary adenoma cell lines GH3 and HP75, consistent with previous studies in other cancers [30,31]. We also transfected shRNA-PVT1 and -PVT2 to silence the PVT1 expression in the pituitary adenoma cells and observed their effects on the cell viability of HP75 and GH3 cells. The findings indicated that the sh-PVT1 significantly reduced the cell viability of both HP75 and GH3 cells. Furthermore, sh-PVT1 transfection also inhibited the invasion and migration of both HP75 and GH3 cells. All of the above results demonstrated the specific characteristics of PVT1 in tumor cells, as previous studies demonstrated.
Figure 2. The effects of sh-PVT1 on the proliferation ability of pituitary adenoma cells. (A) Statistical analysis for plasmid transfection efficiency. (B) Higher PVT1 expressions in pituitary adenoma cell lines (HP75 and GH3) were downregulated by the transfection of specific shRNAs. (C, D) MTT and colony formation assays were used to examine the proliferation ability of pituitary adenoma cells after PVT1 was knocked down. Means ±SDs of results obtained from at least 3 experiments are shown. ** p<0.05 vs. Normal control (NC) group.
have described [32,33]. Therefore, these results suggest that the PVT1 in cancer tissues is associated with the invasive characteristics of invasive pituitary adenomas.

A previous study [34] reported that metastasis is associated with epithelial-to-mesenchymal transition (EMT), which includes 2 main biomarkers, E-cadherin and N-cadherin. Our results showed that E-cadherin expression was significantly increased and N-cadherin expression was significantly decreased in HP75 and GH3 cells undergoing sh-PVT1 treatments. There results suggest that the silence of PVT1 in cells inhibits the EMT process of the pituitary adenoma cell lines, consistent with the effects of PVT1 in other tumors [35,36].

The PVT1 has been proven to be widely expressed in cancers downstream of the proto-oncogene Myc and can modulate the proto-oncogene Myc and enhance tumorigenesis [37,38]. A previous study [39] also demonstrated that Cyclin D1 regulates the progression of many cancer. The Wnt/β-catenin signaling pathway is a common pathway that plays critical roles in the progression and development for many tumors [40]. β-catenin is considered to be an important molecule involving in the Wnt molecule signaling in the extra-cellular ligands of cancer cells [40]. In the present study, our results demonstrated that expressions of β-catenin, c-Myc, and cyclin D1 in human pituitary tumor cells were also significantly higher compared to that in normal pituitary cells. These findings suggest that PVT1

**Figure 3.** Knockdown of PVT1 represses the migration and EMT of pituitary adenoma cells. (A, B) Transwell assays tested the migration ability of 2 pituitary adenoma cell lines in which PVT1 was silenced. (C) Levels or expressions of EMT-associated molecules were measured using Western blot analysis. Means ±SDs of results obtained from at least 3 experiments are shown. **p<0.05 vs. Normal control (NC) group.
plays critical roles in enhancing tumorigenesis and suppressing tumor invasion and migration by reducing expression of β-catenin, c-Myc, and cyclin D1 in human pituitary tumor cells.

Conclusions

Long non-coding RNA PVT1 enhanced proliferation, migration, and EMT of pituitary adenoma cells by activating β-catenin, c-Myc, and cyclin D1 expression. Our results provide preliminary evidence of the relationship between PVT1 and the Wnt/β-catenin signaling pathway. These findings need to be expanded by future investigations that include more pathway components. Our findings provide a new experimental basis and new targets for the development of drugs for treatment of invasive pituitary adenoma.

Conflict of interest

None.

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Figure 4. Knockdown of PVT1 negatively modulates Wnt/β-catenin signaling in pituitary adenoma tissues and cells. (A, B) β-catenin, cyclin D1, and c-Myc expressions were evaluated using Western blot analysis in pituitary adenoma tissues or cells and normal tissues or cells. (C) The protein levels of β-catenin, c-Myc, and cyclin D1 were examined in PVT1-downregulated pituitary adenoma cell lines. Means ±SDs of results obtained from at least 3 experiments are shown. ** p<0.05 vs. Normal control (NC) group, Normal tissues, or NHPG cells.
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