EFFECT AND MECHANISM OF LNCRNA PVT1 ON PROLIFERATION, MIGRATION, INVASION AND AUTOPHAGY OF GASTRIC CANCER CELLS

Lan Pang[1], Cancan Liang[2], Jiang Wu[1], Peng Chen[1], Wenjing Ji[2,*]
[1]Department of the Endoscopic Diagnosis and Treatment, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830000, PR China - [2]Department of Digestive Internal Medicine, The Second Affiliated Hospital of Xinjiang Medical University, Urumqi 830000, PR China

ABSTRACT

Objective: *To explore the mechanisms underlying the effects of the long non-coding RNA plasmacytoma variant translocation 1 (LncRNA PVT1) on the proliferation, migration, invasion, and autophagy of gastric cancer cells.*

Methods: *The human gastric cancer cell line SGC-7901 was cultured in vitro, and gastric cancer cells were transfected with an LncRNA PVT1 overexpression vector, an LncRNA PVT1 silencing vector, or the corresponding control vectors, resulting in an LncRNA PVT1 overexpression group, an overexpression control group, an LncRNA PVT1 knockout group, and a knockout control group. Cell proliferation was detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell migration and invasion were detected by Transwell assay, autophagy-related 5 (ATG5), and large tumor suppressor kinase 2 (LATS2) protein expression was detected by Western blot assay, and LATS2 mRNA expression was detected by real-time quantitative PCR assay.*

Results: *The LncRNA PVT1 overexpression group and the overexpression control group significantly promoted the proliferation, migration, and invasion of gastric cancer cells at each time point assessed ($P<0.05$). The LncRNA PVT1 knockout group and the knockout control group displayed significant inhibitory effects against cell proliferation, migration, and invasion at each time point assessed ($P<0.05$). The expression of ATG5 protein was significantly increased in the LncRNA PVT1 overexpression group compared with that in the overexpression control group ($P<0.05$). The expression of ATG5 protein was significantly reduced in the LncRNA PVT1 knockout group compared with that in the knockout control group ($P<0.05$). The expression levels of both LATS2 mRNA and protein significantly decreased in the LncRNA PVT1 overexpression group compared with those in the overexpression control group ($P<0.05$). LATS2 mRNA and protein expression levels were significantly increased in the LncRNA PVT1 knockout group compared with the knockout control group ($P<0.05$).*

Conclusion: *LncRNA PVT1 was able to promote the proliferation, migration, and invasion of gastric cancer cells, which may be associated with the regulation of LATS2 expression. LncRNA PVT1 directly affected cellular autophagy by upregulating ATG5 protein expression.*

Keywords: *LncRNA PVT1, gastric cancer, proliferation, migration, invasion, autophagy, mechanism of action.*

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Introduction

Gastric cancer is a malignant tumor that originates from the gastric mucosal epithelium and is the second-leading cause of cancer-related deaths worldwide. In recent years, changes in the structure of diets and increasing stress levels have resulted in gastric cancer affecting younger patients. Gastric cancer has resulted in the deaths of 300,000 patients, and cancer-related deaths are often associated with a lack of early diagnosis[(1)]. Because many patients with gastric cancer have already entered the advanced stages of cancer at the time of treatment, the efficiencies of surgical and chemotherapeutic treatments remain low, and the 5-year survival rate of patients is currently less than 25%. Therefore, identifying molecular markers that can be used to diagnose early gastric cancer has extremely important clinical significance for improving the prognosis of patients[(2)]. Long non-coding RNA

(LncRNA) is a type of RNA that is longer than 200 nucleotides in length and is commonly expressed in eukaryotic cells. LncRNA regulates gene expression, which plays an important role in controlling the malignant progression of tumor cells. Compared with other tumor markers, it has the advantages of simple operation and strong repeatability[3].

The plasmacytoma variant translocation 1 (PVT1) gene is located on chromosome 8q24.21, and the dysregulation of LncRNA PVT1 has been identified in various human diseases[4]. Studies have confirmed that LncRNA PVT1 is upregulated in various malignant tumors, such as thyroid cancer, bladder cancer, and prostate cancer, and LncRNA PVT1 upregulation has been shown to promote cell proliferation, migration, and invasion and is associated with the poor prognosis of patients[5]. Therefore, this study aimed to analyze the underlying mechanisms associated with the effects of LncRNA PVT1 on the proliferation, migration, invasion, and autophagy of gastric cancer cells.

Materials and methods

Experimental reagents and instruments

The human gastric cancer cell line SGC-7901 was purchased from American Type Culture Collection (ATCC). RPMI1640 medium was purchased from Gibco Corporation; Every Green fetal bovine serum was purchased from the Beijing Meribo company. Trizol extraction reagents were purchased from American Life Technologies; the bicinchoninic acid (BCA) protein content kit and sodium dodecyl sulfate (SDS) gel preparation kit were purchased from Nanjing Keygen Biotech Co., Ltd. The chemiluminescent liquid was purchased from Thermo Fisher Scientific. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was purchased from Dojindo Laboratories. The 3'-diaminobenzidine (DAB) color development kit was purchased from the US DAKO company. Anti-autophagy-related 5 (ATG5) and large tumor suppressor kinase 2 (LATS2) antibodies were purchased from Zhongshan Golden Bridge Biotechnology Co., Ltd. Polyvinylidene difluoride (PVDF) membrane was purchased from Beijing Solarbio Company.

The ultra-clean workbench was purchased from Suzhou Antai Airtech Co., Ltd. The cell incubator was purchased from Thermo Fisher Scientific, USA. The inverted microscope was purchased from LEICA, Germany. High-speed centrifuge and micro sampler were purchased from Eppendorf, Germany. The electrophoresis instrument, electrophoresis tank, and film transfer tank were purchased from Tanon Science & Technology Co., Ltd. The vortex mixer was purchased from Haimen Kylin-Bell Lab Instruments Co., Ltd. Transwell migration and invasion chambers were purchased from Corning, USA.

Cell culture, transfection, and grouping

After cell resuscitation, the cells cultured in RPMI-DMEM, containing 10% fetal bovine serum, 100U/mL penicillin, and 100g/mL streptomycin, in a constant-temperature incubator at 37°C, in a 5% CO_2 atmosphere. Cell growth was observed. When confluency reached 90%, the cells were passaged by trypsin digestion, which was terminated when the cells retracted into spheres. The cells were evenly distributed among the wells of a six-well plate. When the cell density reached 80%-90%, transfection was performed using the Lipofectamine 2000 reagent. Both an overexpression vector and a silencing vector for LncRNA PVT1 were designed and transfected into gastric cancer cells, separately. A corresponding control group was transfected with miR-shNC transfection to observe the cell transfection status.

Detection methods

The MTT method was used to detect cell proliferation. Each group of gastric cancer cells was seeded onto 96-well plates at an initial density of 2×104 cells/well. Then, 150 μL of dimethyl sulfoxide was added to each well, 24, 48, and 72 h after the addition of MTT. Plates were shaken for 10 minutes to dissolve the crystals fully, and the absorbance at 490 nm was measured to calculate the proliferation ability of gastric cancer cells.

Transwell migration and invasion experiment: To assess migration, transfected gastric cancer cell suspensions (5×10^4 cells) were placed in the upper chamber of a Matrigel-coated Transwell migration chamber 36 h after transfection. A medium containing 10% FBS was added as a chemical attractant to the lower chamber, and the chambers were incubated at 37°C for 24 h. The remaining cells in the upper chamber were removed, and the cells that migrated to the surface of the lower chamber were fixed with 100% methanol. The numbers of migrating and invading cells were counted under an inverted microscope, with 0.5% crystal violet staining.

Western blot protein expression assay: Cells were collected from 6-well plates after intervention treatments, and proteins were extracted using a BCA

kit to determine the protein contents. A 10 μL volume of the extracted protein solution was subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transfer to a PVDF membrane. The membrane was blocked with Tris saline containing 5% skim milk for 1 h. Antibodies, diluted at 1:1000, against ATG5 and LATS2 were incubated with the membrane at 4°C overnight. Membranes were washed three times with tris-buffered saline containing Tween 20 (TBST), and the corresponding secondary antibodies were incubated for 2 h at room temperature. Electrochemiluminescence was used to visualize proteins. Real-time quantitative PCR method to detect LATS2 mRNA expression: Cells were grown to 80%-90% confluency, and 1 mL Trizol was added to extract the total RNA. RNA content and integrity were determined. Using 1.0 μg total RNA, a reverse transcription reaction was performed according to the instructions provided by the reverse transcription kit. The fluorescent dye, SYBR-Green mix, was used to detect the expression of LATS2 in gastric cancer cells using a real-time fluorescence quantitative PCR amplifier and cDNA as a template. β-actin mRNA expression levels were used to normalize and quantify the expression of PVT1 in each sample. The PCR protocol was as follows: pre-denature at 95°C for 10 min, followed by 40 cycles of extension at 95°C for 10 seconds and 60°C for 20 seconds. The $2^{-\triangle\triangle CT}$ formula was used for data analysis.

Statistical methods

All the measurement data in this study were expressed as the mean ± standard deviation ($\bar{x}\pm s$). An independent samples t-test was used to compare the means between two groups, and analysis of variance was used to compare the means among multiple groups. P<0.05 was considered significant. The data were analyzed using SPSS, version 20.0, software package.

Results

The effects of LncRNA PVT1 on the proliferation of gastric cancer cells

The MTT assay results showed that compared with the overexpression control group, the LncRNA PVT1 overexpression group significantly promoted cell proliferation abilities at each time point (P<0.05). Compared with the knockout control group, the LncRNA PVT1 knockout group significantly inhibited cell proliferation abilities at various time points (P<0.05, Figure 1).

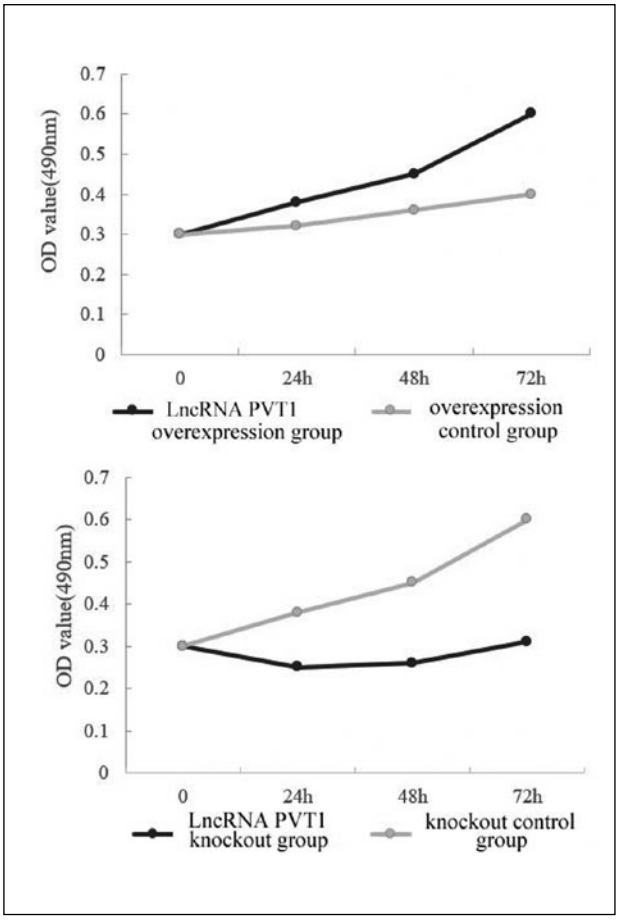

Figure 1: Effects of LncRNA PVT1 on the proliferation of gastric cancer cells.

The effects of LncRNA PVT1 on the migration and invasion abilities of gastric cancer cells

The Transwell migration and invasion test results showed that compared with the overexpression control group, the LncRNA PVT1 overexpression group significantly promoted cell migration and invasion abilities (P<0.05).

In contrast, the LncRNA PVT1 knockout group had a significant inhibitory effect on cell migration and invasion compared with the knockout control group (P<0.05, Figure 2, Table 1)

The effects of LncRNA PVT1 on the gastric cancer cell expression of the autophagy protein ATG5

Western blot analysis results showed that compared with that in the overexpression control group, the expression level of ATG5 protein in the LncRNA PVT1 overexpression group increased significantly (P<0.05); The expression level of ATG5 protein was significantly reduced in the LncRNA PVT1 knockout group compared with that in the knockout control group (P<0.05, Figure 3).

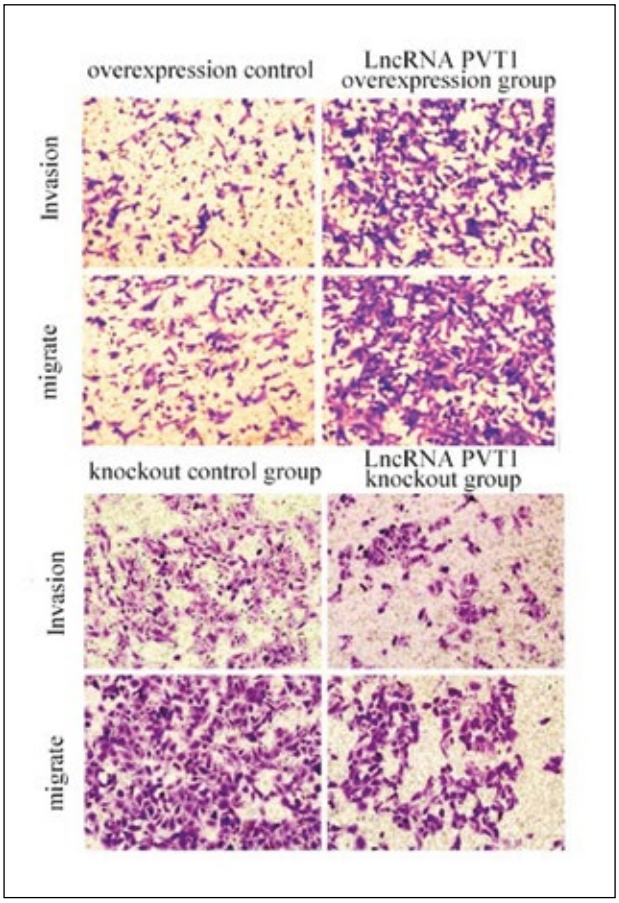

Figure 2: Effects of LncRNA PVT1 on the migration and invasion abilities of gastric cancer cells. LncRNA PVT1: long non-coding RNA plasmacytoma variant translocation 1.

Group	Number of samples	Number of invasions	Number of migrations
Overexpression control group	18	1.00±0.05	15.46±2.68
LncRNA PVT1 overexpression group	18	3.58±1.26*	40.37±5.41*
Knockout control group	18	1.00±0.02	42.10±8.23
LncRNA PVT1 knockout group	18	0.25±0.06#	15.63±2.30#

Table 1: Comparison of the migration and invasion characteristics of gastric cancer cells in each group.
*Note: Compared with the overexpression control group *P<0.05; Compared with the knockout control group #P<0.05.*

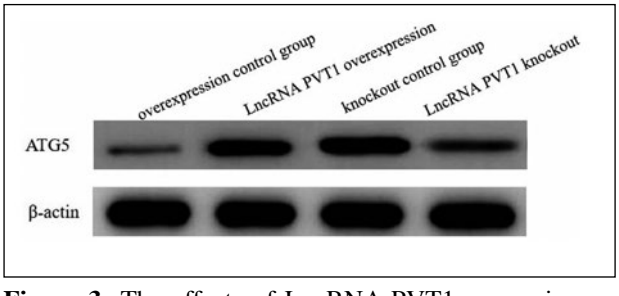

Figure 3: The effects of LncRNA PVT1 expression on the on gastric cancer cell expression of the autophagy protein ATG5.

The effects of LncRNA PVT1 expression on the LATS2 mRNA and protein expression levels in gastric cancer cells

The LATS2 mRNA and protein expression levels in the LncRNA PVT1 overexpression group were significantly lower than those in the overexpression control group (P<0.05). The expression levels of LATS2 mRNA and protein in the LncRNA PVT1 knockout group were significantly increased compared with those in the knockout control group (P<0.05, Figure 4, Table 2).

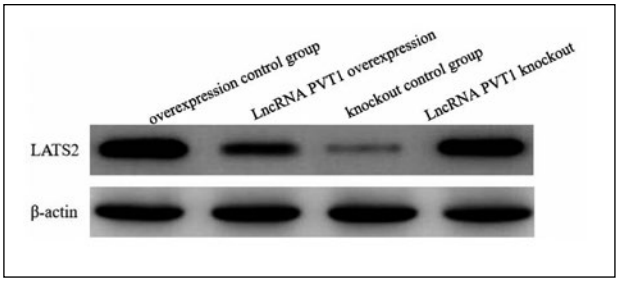

Figure 4: The effects of LncRNA PVT1 expression on LATS2 protein expression levels in gastric cancer cell.

Group	Number of samples	LATS2 mRNA	LATS2 protein
Overexpression control group	18	7.58±1.89	9.03±1.74
LncRNA PVT1 overexpression group	18	2.36±0.74*	2.36±1.05*
Knockout control group	18	1.00±0.06	1.03±0.06
LncRNA PVT1 knockout group	18	7.26±2.33#	8.26±1.42#

Table 2: Comparison of LATS2 mRNA and protein content of gastric cancer cells in each group.
*Note: Compared with the overexpression control group *P<0.05; Compared with the knockout control group #P<0.05.*

Discussion

Early diagnosis is particularly important for the treatment and prognosis of gastric cancer patients. Previously, clinical research focused primarily on the study of molecular chemoresistance and cellular mechanisms of action. This is one of the main reasons for the failure of advanced cancer treatment. Therefore, identifying effective molecular markers is the key to achieving the early diagnosis of gastric cancer. The discovery of new molecular features can be used as a potential therapeutic target for the qualitative study of tumors. LncRNAs have been found to be dysregulated in many human cancers. In recent years, the mechanisms used to regulate abnormal gene and protein expression at the epigenetic level have been associated with various diseases, which has been confirmed by an increasing

number of studies. The participation of epigenetic mechanisms in tumor metastasis primarily occurs through interactions with micro RNAs (miRNAs), which can affect the level of transcription through direct interactions with transcription factors or direct effects on the functions of target proteins[6, 7]. To confirm that LncRNAs may represent potential molecular biomarkers for the treatment of malignant tumors, this study analyzed the regulation of tumor and cell growth behaviors in response to changes in the expression of LncRNA PVT1.

LncRNA PVT1 is an important cancer-associated LncRNA. PVT1 is an intergenic LncRNA, encoded by the human PVT1 gene, and is longer than 300 kb. Although PVT1 cannot produce any protein products, it has gained the attention of researchers due to its unique localization pattern[8]. Previous studies have confirmed that LncRNA PVT1 is an oncogene in non-small cell lung cancer and gastric cancer and can be used as a relatively independent regulatory factor, together with MYC, affecting many malignant biological behaviors of tumors[9]. At present, the analysis found that PVT1 primarily has the following effects: LncRNA PVT1 produces a variety of LncRNA spliceosomes and plays biological functions through 6 chimeric miRNAs; LncRNA PVT1 combines with specific DNA, RNA, or recruited transcription factors to regulate tumor-related genes; LncRNA PVT1 interacts with the oncogene MYC, which enhances the stability of MYC proteins[10]. In addition, in multiple myeloma and acute myeloid leukemia, LncRNA PVT1 can cause DNA rearrangement through fusion with exon 1, intron 1, or specific genes to promote tumor development[11]. Some studies have reported that PVT1 expression levels are significantly increased in tissues from gastric cancer patients compared with tissues from healthy controls. Its upregulation is closely related to clinicopathological features, such as the depth of gastric cancer invasion, lymph node metastasis, and TNM (tumor, node, metastasis) staging, which suggested that it may represent a molecular marker for early gastric cancer diagnosis[12]. Recent studies found that LncRNA PVT1 expression is negatively correlated with the expression of miR-152 in gastric cancer tissues, which can inhibit the expression of miR-152 through three binding sites, to promote the overexpression of oncogenes and enhance gastric cancer cell proliferation and invasion[13]. Studies have shown that miR-186 is the target of PVT1 in gastric cancer cells, and the two can interact to inhibit the expression of hypoxia-inducible factor 1α (HIF-1α) protein[14]. These studies showed that LncRNA PVT1 plays a key role in the pathogenesis of gastric cancer and is expected to become a new target for gastric cancer treatment.

In this study, we found that LncRNA PVT1 is involved in the regulation of gastric cancer cell proliferation, migration, invasion, and autophagy. These results showed that the LncRNA PVT1 overexpression group significantly promoted the effects of cell proliferation, migration, and invasion ability at each time point examined compared with the overexpression control group ($P<0.05$). Compared with the knockout control group, the LncRNA PVT1 knockout group presented a significant inhibitory effect on cell proliferation, migration, and invasion, at each time point examined ($P<0.05$). These findings suggested that LncRNA PVT1 acts as an oncogene, which participates in the regulation of gastric cancer. LATS2 is an important tumor suppressor gene and plays an important role in the maintenance of cell homeostasis. Studies have confirmed that LATS mutation or low expression levels may be related to colon cancer and gastric cancer cell proliferation. The overexpression of LATS can significantly inhibit cancer cell proliferation[15].

The results of this study showed that the expression levels of both LATS2 mRNA and protein were significantly reduced in the LncRNA PVT1 overexpression group compared with the overexpression control group ($P<0.05$). The expression levels of LATS2 mRNA and protein were significantly increased in the LncRNA PVT1 knockout group compared with those in the knockout control group ($P<0.05$). LncRNA PVT1 is thought to negatively regulate LATS and inhibit the proliferation and invasion of gastric cancer cells. In addition, this study also found that the expression level of ATG5 protein was significantly increased in the LncRNA PVT1 overexpression group compared with the overexpression control group ($P<0.05$), which indicated that LncRNA PVT1 could promote the autophagy of gastric cancer cells by binding ATG5 and inducing ATG5 expression.

In summary, LncRNA PVT1 expression significantly promoted the proliferation, migration, and invasion of gastric cancer cells, and the mechanism may be related to the regulation of LATS2 expression. LncRNA PVT1 directly affected cellular autophagy by upregulating the expression of ATG5 protein.

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Corresponding Author:
Wenjing Ji
Email: v1446p@163.com
(China)