PVT1 Knockdown Inhibits Autophagy and Improves Gemcitabine Sensitivity by Regulating the MiR-143/HIF-1α/VMP1 Axis in Pancreatic Cancer

Yun-Fei Liu, MD, Dong Luo, MD,* Xia Li, MM,† Zhi-Qiang Li, MD,* Xiao Yu, MD,* and Hong-Wei Zhu, MD**

Objectives: Elucidation of the regulatory mechanisms of gemcitabine sensitivity is needed to improve the therapeutic effects of this drug in pancreatic cancer.

Methods: PANC-1 cells were transfected with small hairpin RNA against PVT1 or microRNA (miR)-143 mimics or inhibitor. The gemcitabine sensitivity of pancreatic cancer was evaluated. Autophagosomes were analyzed with an immunofluorescence assay. Cell viability and proliferation were examined with MTT assays. Quantitative reverse transcription–polymerase chain reaction and Western blotting were used to analyze the expression of PVT1, miR-143, HIF-1α, VMP1, LC3I/II, p62, and Beclin-1. The interactions of PVT1/miR-143 and miR-143/HIF-1α were assessed by dual-luciferase reporter assays.

Results: PVT1 was upregulated while miR-143 was downregulated in pancreatic cancer. Both PVT1 knockdown and miR-143 overexpression suppressed autophagy and improved gemcitabine sensitivity in pancreatic cancer. PVT1 directly sponged miR-143 to regulate HIF-1α expression. MiR-143 inhibitor reversed the effect of PVT1 knockdown on autophagy and gemcitabine sensitivity.

Conclusions: PVT1 knockdown inhibited autophagy and improved gemcitabine sensitivity via the miR-143/HIF-1α/VMP1 axis in pancreatic cancer. Our investigation elucidated a novel regulatory mechanism of gemcitabine sensitivity and may contribute to improve the therapeutic effects of chemotherapy drugs on pancreatic cancer.

Key Words: lncRNA PVT1, miR-143/HIF-1α/VMP1 axis, pancreatic cancer, gemcitabine sensitivity

Abbreviations: BCA - bicinchoninic acid, cDNA - complementary DNA, GEM - gemcitabine, HIF-1α - hypoxia-inducible factor 1-alpha, LC-3 - microtubule-associated protein 1A/1B-light chain 3, lncRNA PVT1 - long noncoding RNA plasmacytoma variant translocation 1, MiR-143 - microRNA-143, MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, PBS - phosphate-buffered saline, PVDF - polyvinylidene difluoride, SD - standard deviation, shRNA - short hairpin RNA, UTR - untranslational region, VMP1 - vacuole membrane protein 1

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Pancreatic cancer is the fourth leading cause of cancer-related mortality and is regarded as one of the most progressive and lethal malignancies, with an 8% overall 5-year survival rate worldwide.[1] Gemcitabine is the most widely used chemotherapy agent for pancreatic cancer treatment and improves the overall survival of patients.[2,3] However, most pancreatic cancer patients treated with gemcitabine rapidly develop gemcitabine resistance, which is the primary cause of chemotherapy failure in pancreatic cancer.[4] To improve the therapeutic effect of gemcitabine, researchers must clarify the mechanisms underlying resistance. Although several factors have been found to be associated with gemcitabine resistance,[5] this process has not been fully elucidated.

MicroRNAs (miRNAs) are highly conserved short noncoding RNAs that posttranscriptionally regulate gene expression by directly binding to the 3′untranslational region (UTR) of mRNAs,[6] which are vital regulators of various physiological and pathological processes, including autophagy and pancreatic cancer.[7,8] MicroRNA-143 (miR-143) is significantly downregulated in pancreatic cancer and has been reported to suppress autophagy and the metastasis of pancreatic cancer.[9,10] Vacuole membrane protein 1 (VMP1) has been demonstrated to trigger autophagy in many cancers, including pancreatic cancer, and promote the progression of pancreatic cancer.[11] Although gemcitabine induces VMP1-mediated autophagy to facilitate its cytotoxicity, increasing data have proven that autophagy could lead to gemcitabine resistance.[12,13] In addition, HIF-1α (hypoxia-inducible factor 1-alpha)/VMP1–mediated autophagy has been demonstrated to be involved in therapy resistance in colon cancer.[14]

Long noncoding RNA plasmacytoma variant translocation 1 (lncRNA PVT1) is upregulated in pancreatic cancer and reduces gemcitabine sensitivity; thus, it could be used as a predictor of the efficacy of gemcitabine treatment.[15] As a potential downstream target of lncRNA PVT1, miR-143 suppresses the invasion and metastasis of pancreatic cancer.[9] Moreover, it has been reported that miR-143 inhibits metastasis and epithelial-mesenchymal transition by suppressing HIF-1α expression in gallbladder cancer.[16] However, the roles of the PVT1/miR-143/HIF-1α/VMP1 axis and related regulatory mechanisms in pancreatic cancer remain unknown and require further investigation.

Accordingly, in this study, we aimed to explore the roles of the PVT1/miR-143/HIF-1α/VMP1 axis in pancreatic cancer and determine whether PVT1 knockdown can suppress autophagy to improve the gemcitabine sensitivity of pancreatic cancer by targeting the miR-143/HIF-1α/VMP1 pathway; the results may contribute to

From the Departments of *Hepatobiliary and Pancreatic Surgery II, and †Endocrinology, Third Xiangya Hospital, Central South University, Changsha, China.
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Address correspondence to: Xiao Yu, MD or Hong-Wei Zhu, MD, Department of Hepatobiliary and Pancreatic Surgery II, Third Xiangya Hospital, Central South University, No. 138, Tongzipo Rd, Changsha 410006, Hunan Province, China (e-mail: yuxiaoyx4@126.com or zhw_0509@csu.edu.cn).
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the development of more effective therapeutic strategies with enhanced gemcitabine sensitivity for pancreatic cancer patients.

MATERIALS AND METHODS

Human Pancreatic Cancer Tissue Collection

The clinical research protocol was approved by the Ethical Committee of Third Xiangya Hospital, Central South University. Tissue specimens were collected from 36 patients diagnosed with pancreatic cancer who had undergone pancreatic resections at Third Xiangya Hospital, Central South University. Tissues were harvested freshly after sample dissection, snap-frozen in liquid nitrogen, and finally preserved at −196°C in a liquid nitrogen tank.

Cell Culture

The human pancreatic cancer cell lines SW1990, BxPC-3, PANC-1, and AsPC-1 and the human normal pancreatic ductal epithelial cell lines HPDE6-C7 and HEK293T were purchased from the American Type Culture Collection (Manassas, Va). All these cells were seeded and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 4 mM of L-glutamine, 100 μg/mL of streptomycin, and 100 U/mL of penicillin at 37°C in a humidified atmosphere containing 5% CO_2. All of the above reagents used for cell culture were ordered from Thermo Fisher Scientific (Waltham, Mass). For gemcitabine treatment, PANC-1 cells were treated with gemcitabine (Sigma-Aldrich, St Louis, Mo) at 10 μM for 24, 48, or 72 hours. Cells were then harvested for subsequent assays.

RNA Extraction and Quantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from SW1990, BxPC-3, PANC-1, AsPC-1, and HPDE6-C7 cells and cancer and paracancerous tissues from 36 pancreatic cancer patients. For cell samples, cells were treated, and total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific) following the instructions. For cancer and paracancerous tissues, total RNA was extracted as previously described.[17] Briefly, specimens were collected and snap-frozen in liquid nitrogen. Then, the samples were transferred into 1 mL of TRIzol reagent, ground to homogenates, and used to extract total RNA. For lncRNA PVT1 quantification, RNA was reverse transcribed into complementary DNA (cDNA) with a high-capacity RNA to cDNA kit (Thermo Fisher Scientific) following the manual. MicroRNA was isolated using a miRNA Isolation Kit (Geneaid, Shanghai, China) and reverse transcribed into cDNA with a TaqMan microRNA reverse transcription kit (Thermo Fisher Scientific). The relative expression levels of lncRNA PVT1 and miR-143 were analyzed using a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, Calif) with SYBR Green QPCR Master Mix (Toyobo, Osaka, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 snRNA was used as the endogenous control. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer for miR-143 was purchased from RiboBio (Guangzhou, China). Other primers are listed in Table 1.

Western Blotting

Western blotting was conducted as previously described.[18] In brief, treated PANC-1 cells were lysed on ice for 30 minutes with cell lysis buffer containing protease inhibitors. The cell lysates were centrifuged for 10 minutes at 12,000 rpm at 4°C. The protein concentration was determined with a BCA Protein Assay Kit (Thermo Fisher Scientific). Fifty micrograms of protein was loaded onto a 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis gel, electrophoresed and transferred to polyvinylidene difluoride membranes (GE Healthcare, Pittsburgh, Pa). Then, the blots were blocked with 5% skim milk in Tris-buffered saline with Tween 20 buffer for 1 hour and incubated with primary antibodies against human HIF-1α (1:500), VMP1 (1:800), LC-3 (microtubule-associated protein 1A/1B-light chain 3) (1:1000), p62 (1:500), Beclin-1 (1:1000), and GAPDH (1:5000) overnight at 4°C on a shaker. All primary antibodies were ordered from Cell Signaling Technology (Boston, Mass). The membranes were washed with Tris-buffered saline with Tween 20 buffer on a shaker and probed with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. Enhanced chemiluminescence substrates (Thermo Fisher Scientific) were used to visualize the bands. The bands were quantified with ImageJ software (NIH, Bethesda, Md) bundled with 64-bit Java 1.8.0_172.

TABLE 1. Primers Used in This Study

miR-143	5′-AGTCAGTGAGATGAAGCACTG-3′
	5′-GTGCAGGGTCCGAGGT-3′
LncRNA PVT1	5′-CCGACTCTTCCTGGTGAAGC-3′
	5′-GTATGGTCAGCTCAAGCCCA-3′
GAPDH	5′-GGATTTGGTCGTATTGGG-3′
	5′-GGAAGATGGTGATGGGATT-3′
U6 snRNA	5′-CTCGCTTCGGCAGCACA-3′
	5′-AACGCTTCACGAATTTGCGT-3′

Cell Transfection

PANC-1 cells were seeded and cultured to 70% to 80% confluency in 6-well plates (Corning Incorporated, Corning, NY) and transfected with the pLKO.1 puro vector (Addgene, Watertown, Mass) containing short hairpin RNA oligonucleotides against lncRNA PVT1 (sh-PVT1), short hairpin RNA control, miR-143 mimics, or mimics control (mimics NC) using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) following the manufacturer's recommendation. For cotransfection, PANC-1 cells were cotransfected with sh-PVT1 and miR-143 inhibitor or inhibitor control (inhibitor NC). After 48 hours, the cells were collected for subsequent assays. Human short hairpin (sh)-PV1, sh-NCmimics NC, miR-143 mimics, inhibitor NC and miR-143 inhibitor were obtained from GenePharma (Shanghai, China).

MTT Cell Viability Assay

Cell viability was analyzed using an MTT Assay Kit (Abcam, Cambridge, United Kingdom) following the manufacturer's recommendation. PANC-1 cells (1×10^5) were seeded in 96-well plates per well, transfected and treated with gemcitabine as indicated. Then, the medium was replaced with fresh serum/phenol red-free medium (50 μL/well) and 50 μL of MTT reagent. Cells were incubated at 37°C for 3 hours. After incubation, 150 μL of MTT solvent was added to each well and incubated on a shaker for 15 minutes. The absorbance at 490 nm was recorded using a microplate reader (BioTek Instruments, Winooski, Vt). The relative cell viability was quantified by the mean absorbance of the treatment group/mean absorbance of the control group × 100%.

Immunofluorescence

Immunofluorescence assays were performed as previously described.[19] PANC-1 cells were seeded in Nunc Lab-Tek II Chamber Slides (Thermo Fisher Scientific), transfected and treated with gemcitabine as indicated. Then the cells were washed with

phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.15% Triton X-100 in PBS for 20 minutes at room temperature, and blocked for 1 hour with 5% bovine serum albumin in PBS. The cells were incubated with anti-LC3 antibody (dilution 1:200) overnight at 4°C. The cells were then washed, probed with Alexa Fluor 488 fluorescent antimouse secondary antibody (1:1000, Thermo Fisher Scientific) for 1 hour at room temperature, mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific), and analyzed with confocal microscopy (Olympus, Tokyo, Japan).

Dual-Luciferase Reporter Assay

For construction of dual-luciferase reporter plasmids, the predicted binding sequences of miR-143 in lncRNA PVT1 (PVT1-WT) or the 3′UTR of HIF-1α (HIF-1α-WT) and the corresponding mutated sequences (PVT1-MUT and HIF-1α-MUT) were cloned into the pmirGLO vector (Promega, Madison, Wis). HEK293T cells were seeded, cultured to 70% to 80% confluency, and cotransfected with the indicated construct and miR-143 mimics or mimics NC using Lipofectamine 3000 following the manual. After 48 hours, the firefly and Renilla luciferase activity was examined with the Dual-Glo Luciferase Assay System (Promega).

Statistical Analysis

Data were analyzed using GraphPad Prism software (San Diego, Calif). All data are presented as the mean (standard deviation [SD]) from at least 3 independent experiments. Statistical analysis was conducted with Student *t* test for 2 independent groups or 1-way analysis of variance for multiple independent groups. $P < 0.05$ was considered significant statistically.

RESULTS

Dysregulated Expression of LncRNA PVT1 and MiR-143 in Pancreatic Cancer

To investigate the association of lncRNA PVT1 and miR-143 with pancreatic cancer, we collected cancerous and paracancerous tissues from 36 pancreatic cancer patients and analyzed the expression of lncRNA PVT1 and miR-143. Compared with the paracarcinoma tissues, the carcinoma tissues showed significantly higher expression of PVT1 (Fig. 1A) and reduced expression of miR-143 (Fig. 1B). Based on these expression data, we discovered an obvious negative correlation between PVT1 and miR-143 expression in pancreatic cancer (Fig. 1C). In addition to cancer samples from patients, we also used the pancreatic cancer cell lines SW1990, BxPC-3, PANC-1, and AsPC-1 and the normal pancreatic ductal epithelial cell line HPDE6-C7 to examine the expression of PVT1 and miR-143. Consistent with the expression pattern in carcinoma tissues, PVT1 was highly upregulated in all 4 pancreatic cancer cell lines but not in the HPDE6-C7 cell line (Fig. 1D). In contrast, miR-143 was significantly downregulated in SW1990, BxPC-3, PANC-1, and AsPC-1 cells (Fig. 1E). The high expression of PVT1 and the negative correlation between PVT1 and miR-143 expression indicated that PVT1 might play key roles by regulating the expression of miR-143 in pancreatic cancer.

Knockdown of LncRNA PVT1 Inhibits Autophagy and Improves the Gemcitabine Sensitivity of Pancreatic Cancer

Because PANC-1 cells showed the highest expression of PVT1 and lowest expression of miR-143, they were selected for subsequent assays. To further study the roles of PVT1 in pancreatic cancer, we knocked down PVT1 in PANC-1 cells. Compared with the sh-NC control, sh-PVT1 markedly reduced the expression of PVT1 in PANC-1 cells, which proved that PVT1 was successfully knocked down (Fig. 2A). PANC-1 cell viability was significantly decreased upon sh-PVT1 transfection at 24, 48, and 72 hours, indicating that PVT1 might inhibit the proliferation of pancreatic cancer cells (Fig. 2B). Moreover, knockdown of PVT1 notably reduced the number of autophagosomes (Fig. 2C) and decreased the LC-3 II/I ratio and the expression Beclin-1 while increased p62 level (Figs. 2D, E), which suggested that PVT1 could suppress autophagy in pancreatic cancer. Because VMP1-mediated autophagy has been proven to be involved in many cancers[11,20,21] and VMP1 is a novel direct target of HIF-1α,[14] we found that the expression of VMP1 and HIF-1α

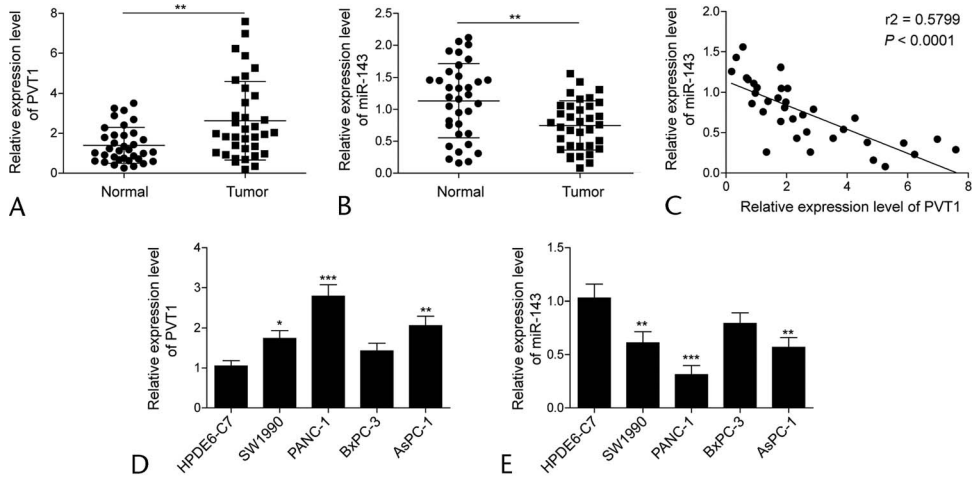

FIGURE 1. The dysregulated expression of PVT1 and miR-143 in pancreatic cancer. The relative expression of PVT1 (A, n = 36) and miR-143 (B, n = 36) in carcinoma and paracarcinoma tissues from 36 pancreatic cancer patients. C, Correlation analysis of the expression of PVT1 and miR-143. The relative expression of PVT1 (D, n = 3) and miR-143 (E, n = 3) in HPDE6-C7, SW1990, BxPC-3, PANC-1, and AsPC-1 cells. GAPDH and U6 snRNA were used as normalization controls in quantitative polymerase chain reaction analysis. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus the control. $P < 0.05$ was considered statistically significant. All data were from at least 3 independent experiments and are shown as the mean (SD).

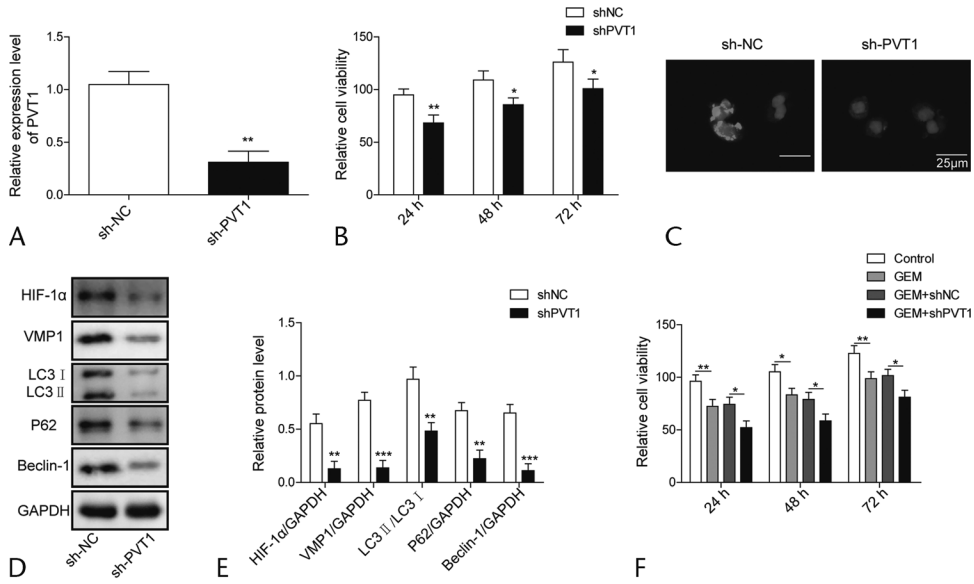

FIGURE 2. PVT1 knockdown inhibited autophagy and improved the gemcitabine (GEM) sensitivity of pancreatic cancer. PANC-1 cells were transfected with sh-PVT1 or sh-NC and used for (A) quantitative polymerase chain reaction analysis of the relative expression of PVT1 (n = 3), (B) relative cell viability analysis at 24, 48, and 72 hours (n = 3), (C) immunofluorescence analysis of autophagosomes (n = 3), and (D) Western blotting analysis of the protein levels of HIF-1α, VMP1, LC-3 I/II, p62, and Beclin-1 (n = 3). E, Intensity analysis of bands in Western blotting (n = 3). F, Analysis of the relative cell viability of PANC-1 cells transfected with sh-PVT1 or sh-NC upon gemcitabine administration at 10 μM for 24, 48, or 72 hours (n = 3). GAPDH was used as a normalization control in quantitative polymerase chain reaction and Western blotting analysis. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus control. $P < 0.05$ was considered statistically significant. All data were from 3 independent experiments and are shown as the mean (SD).

was decreased upon PVT1 knockdown (Figs. 2D, E). To determine whether PVT1 was associated with the gemcitabine sensitivity of pancreatic cancer, we used gemcitabine to treat cells with PVT1 knockdown. Sh-PVT1 transfection dramatically enhanced the cytotoxic effect of gemcitabine on pancreatic cancer cells (Fig. 2F). These observations indicated that PVT1 promoted autophagy by modulating the expression of HIF-1α and VMP1 and abolished the gemcitabine sensitivity of pancreatic cancer.

LncRNA PVT1 Directly Sponges MiR-143 to Negatively Regulate Its Expression

Because lncRNAs generally serve as competitive endogenous RNAs to sponge miRNAs,[22,23] we examined whether PVT1 exerts its function by regulating miR-143 in pancreatic cancer. We found that miR-143 was remarkably upregulated in PVT1 knockdown PANC-1 cells (Fig. 3A), indicating a possible negative regulatory relationship between PVT1 and miR-143. To further study whether PVT1 could directly target miR-143, we predicted that PVT1 harbored a binding site for miR-143 by bioinformatics analysis (Fig. 3B). The relative luciferase activity was notably suppressed upon cotransfection with the PVT1 wild-type construct (PVT1-WT) and miR-143 mimics (Fig. 3C); however, it was not affected in cells transfected with the mutated PVT1 construct (PVT1-MUT, Fig. 3C). These data suggested that PVT1 might play an important role in pancreatic cancer by directly sponging miR-143 and negatively regulating its expression.

Overexpression of MiR-143 Suppresses Autophagy and Improves the Gemcitabine Sensitivity of Pancreatic Cancer

To investigate the association of miR-143 in the regulation of autophagy and gemcitabine sensitivity in pancreatic cancer, we successfully overexpressed miR-143 in PANC-1 cells (Fig. 4A). PANC-1 cell proliferation was significantly inhibited by miR-143 overexpression (Fig. 4B). In addition, the number of autophagosomes (Fig. 4C), the LC-3 II/I ratio, and the expression of Beclin-1 (Figs. 4D, E)

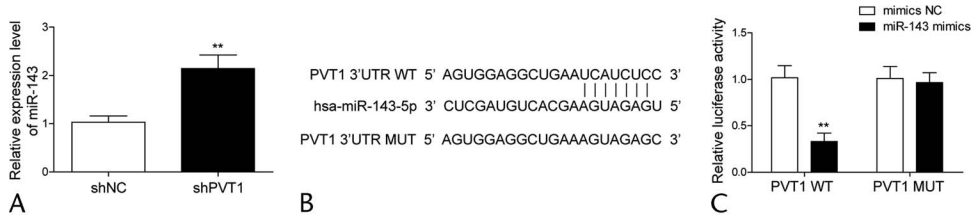

FIGURE 3. LncRNA PVT1 directly sponged miR-143 to regulate its expression. A, The relative expression of miR-143 in PANC-1 cells transfected with sh-PVT1 or sh-NC (n = 3). B, Predicted binding site of miR-143 in PVT1. C, The relative luciferase activity analysis for the interaction of miR-143 and PVT1 (n = 3). U6 snRNA was used as a normalization control in quantitative polymerase chain reaction analysis. $**P < 0.01$ vs control. $P < 0.05$ was considered statistically significant. All data were from 3 independent experiments and are shown as the mean (SD).

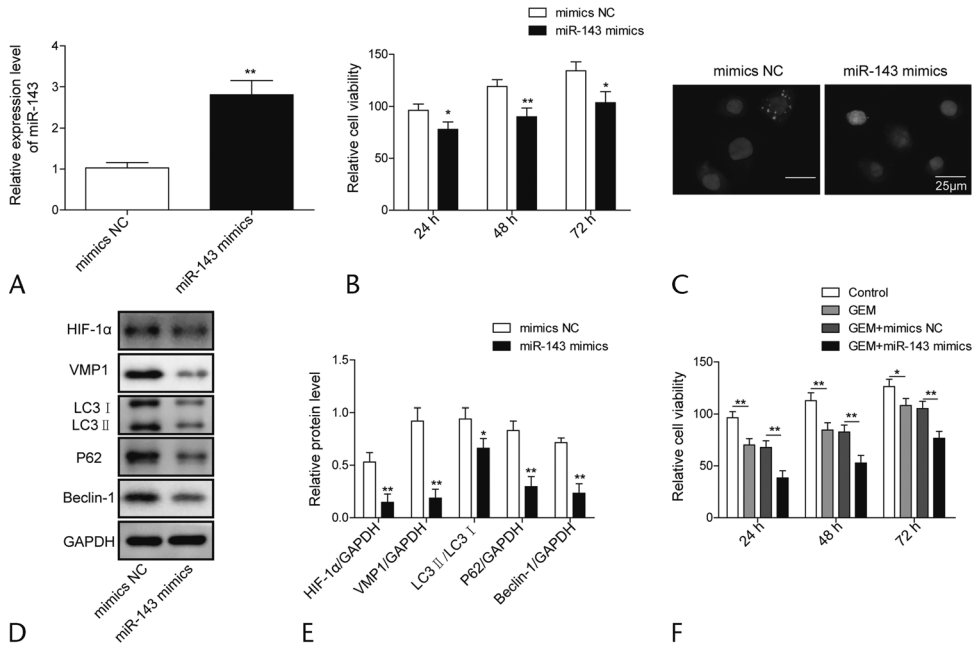

FIGURE 4. MiR-143 overexpression suppressed autophagy and improved the gemcitabine sensitivity of pancreatic cancer. PANC-1 cells were transfected with miR-143 mimics or mimics NC and used for (A) quantitative polymerase chain reaction analysis of the relative expression of miR-143 (n = 3), (B) relative cell viability analysis at 24, 48, and 72 hours (n = 3), (C) immunofluorescence analysis of autophagosomes (n = 3), and (D) Western blotting analysis of the protein levels of HIF-1α, VMP1, LC-3 I/II, p62, and Beclin-1 (n = 3). E, Intensity analysis of bands in Western blotting (n = 3). F, The relative cell viability analysis of PANC-1 cells transfected with miR-143 or miR-NC upon gemcitabine (GEM) administration at 10 μM for 24, 48, or 72 hours (n = 3). U6 snRNA or GAPDH was used as a normalization control in quantitative polymerase chain reaction and Western blotting analysis. $*P < 0.05$ and $**P < 0.01$ versus control. $P < 0.05$ was considered statistically significant. All data were from 3 independent experiments and are shown as the mean (SD).

were all markedly decreased in the PANC-1 cells overexpressing miR-143, suggesting that autophagy was inhibited by miR-143 overexpression. The expression of VMP1 and HIF-1α was also downregulated in the cells with miR-143 overexpression (Figs. 4D, E), suggesting that miR-143 might regulate autophagy by targeting the HIF-1α/VMP1 axis in pancreatic cancer. The cytotoxic effect of gemcitabine on pancreatic cancer cells was prominently enhanced upon miR-143 overexpression (Fig. 4F), implying that miR-143 could improve gemcitabine sensitivity. These results demonstrated that overexpression of miR-143 might suppress autophagy and improve the gemcitabine sensitivity of pancreatic cancer via the novel HIF-1α/VMP1 axis. Because the effects of miR-143 overexpression and PVT1 knockdown were strongly consistent and PVT1 directly targeted miR-143, we believe that miR-143 acts as a mediator of PVT1 to modify autophagy and gemcitabine sensitivity.

MiR-143 Directly Targets HIF-1α to Negatively Modulate Its Expression

HIF-1α has been reported to be a novel autophagy-related protein,[14] and its expression was significantly decreased in the miR-143–overexpressing PANC-1 cells (Figs. 4D, E and Fig. 5A). To examine whether miR-143 directly targeted HIF-1α, we predicted the potential binding site for miR-143 in the 3′UTR of HIF-1α by bioinformatics analysis (Fig. 5B). The relative luciferase activity was inhibited upon cotransfection with the HIF-1α wild-type construct (HIF-1α-WT) and miR-143 mimics, but it was unaffected in cells transfected with the mutated HIF-1α construct (HIF-1α-MUT, Fig. 5C), indicating that HIF-1α was the functional direct target of miR-143. These results suggested that miR-143 might directly target HIF-1α to modulate its expression and then regulate autophagy in pancreatic cancer.

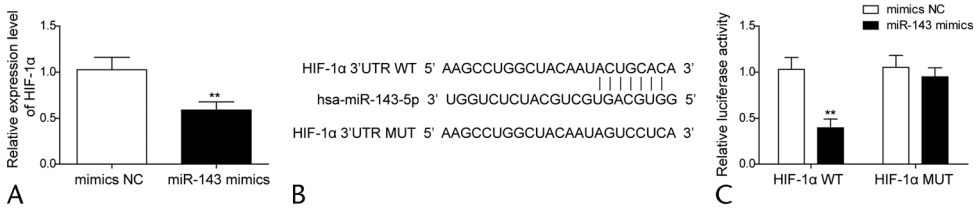

FIGURE 5. MiR-143 directly targeted HIF-1α to negatively modulate its expression. A, The relative expression of HIF-1α in PANC-1 cells transfected with miR-143 or miR-NC (n = 3). B, Predicted binding site of miR-143 in the 3′UTR of HIF-1α. C, The relative luciferase activity analysis for the interaction of miR-143 and HIF-1α (n = 3). GAPDH was used as a normalization control in quantitative polymerase chain reaction analysis. $**P < 0.01$ versus control. $P < 0.05$ was considered statistically significant. All data were from 3 independent experiments and are shown as the mean (SD).

LncRNA PVT1 Knockdown Suppresses Autophagy and Improves the Gemcitabine Sensitivity of Pancreatic Cancer Via the MiR-143/HIF-1α/VMP1 Axis

Although a close association between PVT1 and miR-143 in the regulation of autophagy and gemcitabine sensitivity was proposed, we still need more direct evidence to prove this hypothesis. Therefore, we cotransfected PANC-1 cells with sh-PVT1 and miR-143 inhibitor and found that the number of autophagosomes was completely restored by miR-143 inhibitor transfection (Fig. 6A). Moreover, the miR-143 inhibitor restored the LC-3 II/I ratio and the expression of p62 and Beclin-1 (Figs. 6B, C), which showed that inhibiting miR-143 expression could fully reverse PVT1 knockdown-mediated suppression of autophagy in pancreatic cancer. The expression of VMP1 and HIF-1α was restored in the cells transfected with the miR-143 inhibitor (Figs. 6B, C). In addition, the miR-143 inhibitor completely impaired the PVT1 knockdown-mediated improvement in gemcitabine sensitivity in pancreatic cancer (Fig. 6D). Taken together, our results demonstrated that PVT1 regulated autophagy and gemcitabine sensitivity by modulating the miR-143/HIF-1α/VMP1 axis in pancreatic cancer.

DISCUSSION

Pancreatic cancer is currently the fourth leading cause of cancer-related death globally and is expected to be second by 2030.[24] With a very low average survival time, pancreatic cancer has long been regarded as one of the deadliest malignancies worldwide[25]; this disease is extremely difficult to treat and lacks effective therapeutic methods. Surgical resection is the best option for treatment, although the 5-year survival rate is still only approximately 20% after surgery.[26] However, only 10% of pancreatic cancer patients have resectable tumors at their diagnosis.[27] Gemcitabine is the general option and is widely used as a chemotherapy drug for pancreatic cancer; it has modest effects in improving the overall survival of patients.[28] However, most patients rapidly lose gemcitabine sensitivity, which is the primary factor limiting gemcitabine use.[4,5] Although an increasing number of studies have helped elucidate the regulatory mechanisms of gemcitabine resistance,[29–31] these mechanisms remain largely unknown and urgently need to be clarified to improve the therapeutic effects of gemcitabine.

Chemotherapy resistance, an extremely thorny problem in cancer treatment, largely compromises the therapeutic efficacy of

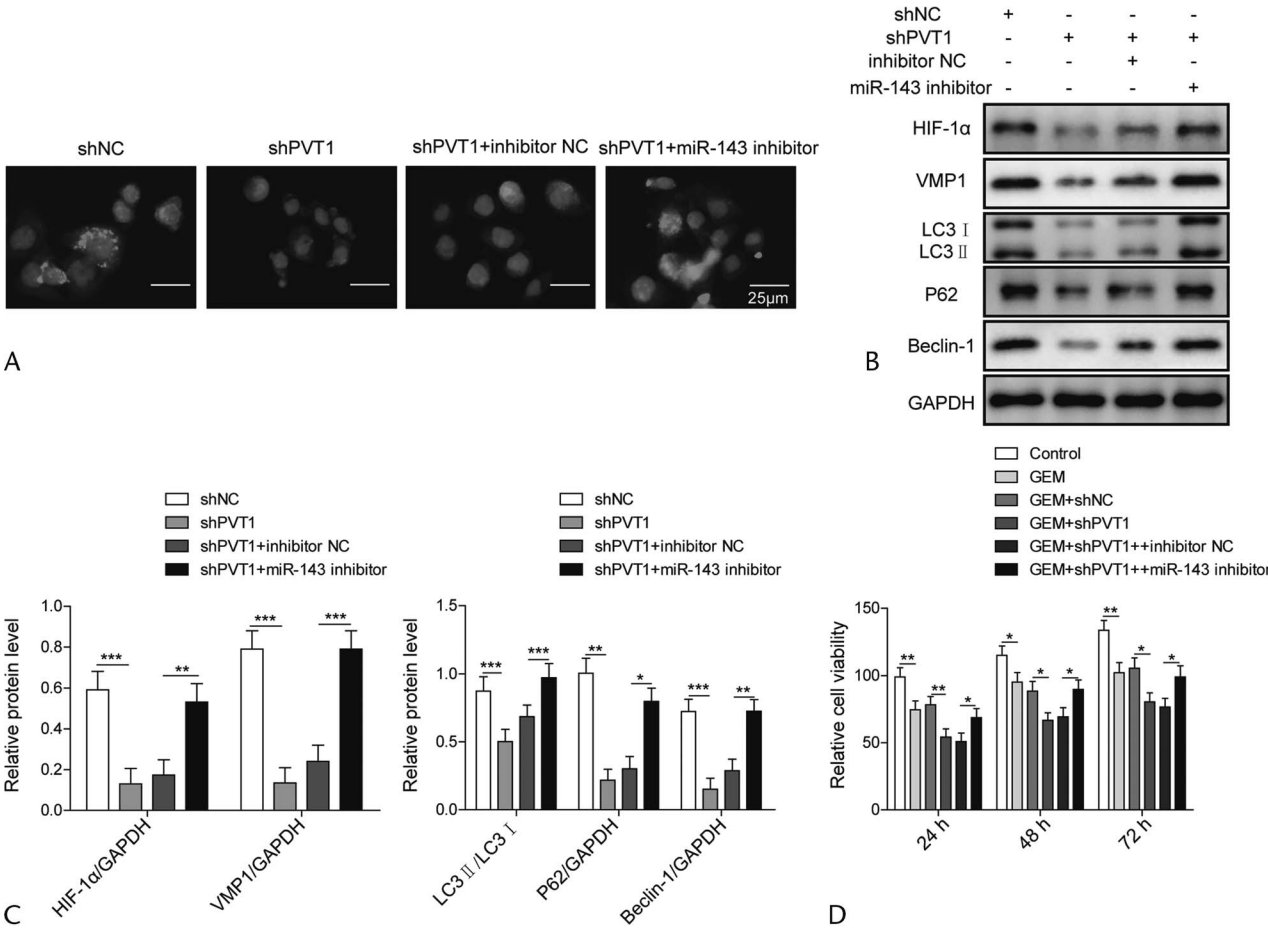

FIGURE 6. LncRNA PVT1 regulated autophagy and gemcitabine sensitivity by modulating the miR-143/HIF-1α/VMP1 axis in pancreatic cancer. PANC-1 cells were transfected with sh-NC, sh-PVT1, sh-PVT1/inhibitor-NC, or sh-PVT1/miR-143 inhibitor and used for (A) immunofluorescence analysis of autophagosomes (n = 3) and (B) Western blotting analysis of the protein levels of HIF-1α, VMP1, LC-3 I/II, p62, and Beclin-1 (n = 3). C, Intensity analysis of bands in Western blotting (n = 3). D, The relative cell viability analysis of PANC-1 cells with the indicated transfection upon gemcitabine (GEM) administration at 10 μM for 24, 48, or 72 hours (n = 3). GAPDH was used as a normalization control in Western blotting analysis. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control. $P < 0.05$ was considered statistically significant. All data were from 3 independent experiments and are shown as the mean (SD).

anticancer agents. Autophagy, a well-recognized process of self-digestion, has been shown to facilitate cancer resistance to chemotherapeutic agents.[32,33] Novel HIF-1α/VMP1–mediated autophagy has been proven to induce colon cancer cell resistance to photodynamic therapy.[14] Suppression of autophagy could sensitize cancer cells to anticancer drugs.[34] MicroRNAs have been demonstrated to regulate autophagy and are closely associated with chemotherapy resistance in cancers.[34] For example, the decreased expression of miR-199a enhanced cisplatin resistance by promoting autophagy in hepatocellular carcinoma.[35] MiR-30a sensitized cancer cells to cisplatinum by inhibiting autophagy.[36] In this study, increased PVT1 expression and decreased miR-143 expression were observed, indicating their involvement in pancreatic cancer. Both PVT1 knockdown and miR-143 overexpression suppressed cell proliferation and autophagy and improved gemcitabine sensitivity in pancreatic cancer. Combined with the direct interaction of PVT1 and miR-143 and the HIF-1α/VMP1 expression pattern, we presumed that PVT1 might directly target miR-143 to activate HIF-1α/VMP1–mediated autophagy, which might induce pancreatic cancer cell resistance to gemcitabine.

MicroRNA-143 inhibitor transfection completely reversed the effects of PVT1 knockdown on the number of autophagosomes, autophagy-associated protein expression, and gemcitabine sensitivity in pancreatic cancer, providing direct evidence showing that PVT1 exerts its function by sponging miR-143. Our results demonstrated that PVT1 directly sponged miR-143 to increase the expression of HIF-1α and VMP1 and regulated autophagy and gemcitabine sensitivity in pancreatic cancer. However, the detailed regulatory mechanisms of autophagy and the relationship between autophagy and pancreatic cancer resistance to gemcitabine still need more investigation in future studies.

Taken together, in our study, we demonstrated for the first time that knockdown of PVT1 could suppress autophagy and sensitize pancreatic cancer cells to gemcitabine by regulating the miR-143/HIF-1α/VMP1 axis. Although many advances have been made, we only have a limited understanding of chemotherapy resistance. Our study helps elucidate the regulatory mechanism of gemcitabine sensitivity in pancreatic cancer. More importantly, our investigation provides novel potential targets for developing therapeutic strategies to improve gemcitabine sensitivity in pancreatic cancer. Combination therapy with drugs against PVT1 should be a promising option to enhance the therapeutic effects of gemcitabine for pancreatic cancer patients.

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