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Long non-coding RNA PVT1 regulates glioma proliferation, invasion, and aerobic glycolysis *via* miR-140-5p

Y. SHAO[1], H.-T. CHEN[2], Q.-R. MA[1], Y.-W. ZHANG[1], Y.-Q. HE[1], J. LIU[1,3]

[1]Department of Anatomy, Histology and Embryology, School of Basic Medical Sciences, Ningxia Medical University, Yinchuan, Ningxia, P.R. China
[2]School of Nursing, Hunan University of Chinese Medicine, Changsha, Hunan, P.R. China
[3]Ningxia Key Laboratory of Cerebrocranial Diseases, Yinchuan, Ningxia, P.R. China

Yu Shao and *Hongtao Chen* contributed equally to this study

Abstract. – OBJECTIVE: To investigate the regulation of long non-coding RNA plasmacytoma variant translocation 1 (LncRNA PVT1) on proliferation, invasion, and aerobic glycolysis in glioma cells via miR-140-5p.

PATIENTS AND METHODS: Sixty p with glioma treated in our hospital were ed. The expression of PVT1 in tissues and ls was determined by quantitative Real Time-P merase Chain Reaction (qRT-PCR), and the fects on the prognosis were ob ed. Glion cell lines U87 and T98MG we stably o transiently transfected with r-ex sion or inhibition vectors. Cell ing kit CCK-8), transwell, glucose, and te de employed to measu ce ion, sion, and aerobic lysis ransfection. The correlation b en PVT1 iR-140-5p was determined l-Luciferas rter assay. RNA pull n RNA immun precipitation (RIP) tes ere ad to indicate the correlation b en PVT1 a -140-5p.

RESU: PVT1 was hig expressed and had s ior di nostic value in gliomas, and the expr on of PVT1 resulted in poor pro s of ents. er-expressing PVT1 increas olifera , invasion, and aerobic olysi ile in ting PVT1 yielded oppo- utcom Luciferase reporter assay co ed tha 1 could target miR-140-5p. Fu nal anal sis showed that over-expressi -5p inhibited proliferation, inva- , and a bic glycolysis in glioma cells. Res- e experiment found that the inhibitory effect iR-140-5p could be eliminated by up-regulat- in T1 expression.

CONCLUSIONS: PVT1 promotes proliferation, invasion, and aerobic glycolysis in glioma cells by regulating miR-140-5p.

Words:
cRNA PVT1, 40-5p, Glioma, Proliferation, In- Aerobic gl sis.

Introduction

Gl as, one of the most common malignant tumors in the nervous system, not only have high morbidity and mortality, but also pose a serious threat to human life and health[1]. Due to its strong invasiveness, tumor tissues cannot be completely removed by surgery, even after radiotherapy and chemotherapy, the 5-year survival rate of patients is still less than 30%[2,3]. However, the advancement of molecular biology provides new ideas for the treatment of gliomas, and effective molecular gene markers are the focus of glioma research[4].

Long non-coding RNAs (LncRNAs) are about 200 nucleotides in length and act as transcription regulators to regulate target genes at a transcription level, thus affecting cell functions[5]. Although the study of lncRNA function is still at its infancy compared with miRNAs, increasing reports[6,7] have indicated the key role of lncRNA in cell proliferation, invasion, and metabolism. Plasmacytoma variant translocation 1 (PVT1, also known as PVT1 oncogene) encodes a lncRNA and has been found to be closely related to the development and progression of various tumors. It inhibits colorectal cancer by down-regulating miR-216a-5p[8]. Besides, PVT1 has been reported to be associated with malignant behavior of glioma cells. Moreover, knocking down the expression of PVT1 reg-

Corresponding Author: Juan Liu, MD; e-mail: ljsy0518@163.com

ulates the expression of miR-424 and inhibits the progression of gliomas[9].

Aerobic glycolysis is a unique phenotype of cancer cells, which can accelerate tumor development by increasing glucose uptake and lactate production[10]. Part of lncRNAs are able to regulate aerobic glycolysis in glioma cells. Likewise, lncRNA LINC00174 promotes aerobic glycolysis and tumor progression by regulating miR-152-3p/SLC2A1 axis in gliomas[11]. However, we found for the first time that PVT1 could affect the aerobic glycolysis in glioma cells by regulating miR-140-5p.

Patients and Methods

Clinical Specimens

Sixty patients with an average age of (59.42±4.15) who underwent glioma resection in the Second Affiliated Hospital of Ningxia Medical University (the First People's Hospital of Yinchuan) from March 2016 to March 2018 were enrolled. With the consent of the patients, 60 samples of each glioma tissue and peritumoral edematous brain tissue were obtained respe- ly during surgery and stored in a liquid n tank. Inclusion criteria: patients diagnose th glioma by pathological diagnosis. Exclusion teria: patients receiving radiotherapy and c motherapy before the study; pa with oth malignant tumors, severe re tion, o serious infectious diseases ients w refused to offer samples. All pa and th agreed to participate in signed an informed sent.T dy has been approved by the ical Ethics mittee of the Second Aff spital of N a Medical Universi The People's Hospital of Yinchuan).

Reag s and Materials

C a cell s U87, U251, HS683, T98MG, and n cell HEB (American Type Culture tion, A ; Manassas, VA, USA); itativ ti polymerase chain reaction (q CR) a se transcription kit (Trans-Ge otech, Be ing, China); Roswell Park Me- -1640 (RPMI-1640) medium (In- ogen, Carlsbad, CA, USA); Lipofectamine 00 (Invitrogen, Carlsbad, CA, USA); lncRNA and miR-140-5p lentiviral vectors (BioRad, Be ey, CA, USA); methyl-thiazolyl-tetrazolium (MTT) kit (Beyotime Biotechnology Co., Ltd., C0009); TRIzol reagent (Invitrogen Co., Ltd., 10296010); Dual-Luciferase reporter gene assay kit (Solarbio, Beijing, China); Transwell kit, phosphate-buffered saline (PBS), fetal bovine serum (FBS) (Gibco, Grand Island, NY, U immunoprecipitation assay (RIPA; ng B logics Co., Ltd.); bicinchoninic a BCA) protein kit (Thermo Fisher Scientific ham, MA, USA), Annexin V-FITC/PI a optosi Meilun Biology Co., Ltd., China atrigel hai Huiying Biotechnology C Ltd.). Glucose porter type 1 (Glut1) okinas (HK2), tate dehydrogenase DH- and g ceral-dehyde-3-phosph de ase PDH) antibodies (Cel gnaling nolo o., Ltd, Danvers, M A); goat a immunoglobulin G econdary a ody (Boster Biological echno o., Ltd., Wuhan, China); enhanced chemilumi nce (ECL) developer (Th sher Scientifi altham, MA, USA); P instrument (ABI, Foster City, CA, USA). All ers were de ed and synthesized by Shangangon Bioe eering Co., Ltd., (China).

Cel d Transfection

The g cell lines U87, U251, HS683, T98MG, and normal cell line HEB were trans- to RPMI-1640 medium containing 10% BS a 100 IU/mL penicillin and 100 μg/mL streptomycin, then incubated at 37°C and 5% CO_2. When reaching 85% confluence, the cells were digested with 25% pancreatin and then cultured to complete passage. U87 and T98MG cells were transfected using Lipofectamine 2000. Suppression and over-expression plasmids were constructed with pcDNA3.1 vector, and blank vector was used as negative control (NC). The cells were seeded in a 96-well plate and transfected for 48 h for further determination.

QRT-PCR

Total RNAs were extracted from the collected cells and tissues by TRIzol. The purity, concentration, and integrity of the RNAs were measured by an ultraviolet spectrophotometer and agarose gel electrophoresis. Reverse transcription was performed with a TaqMan Reverse Transcription Reagents kit in strict accordance with instructions. SYBR_Premix ExTaq II and ABI 7500PCR were used for amplification. The amplification was carried out with a 20-μL reaction volume containing 10 μL of SYBR Premix Ex Taq II (2X), 2 μL of cDNA, 0.8 μL of each upstream and downstream primers, and made up to the final volume with sterile purified water. The amplifi-

cation condition: pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing and extension at 60°C for 30 s, for a total of 40 cycles. Each sample was tested in 3 repeated wells, and the experiment was carried out 3 times. U6 was used as a miR internal reference, GAPDH as a gene internal reference, and $2^{-\Delta\Delta ct}$ was used to analyze the data. Primer sequence: PVT1: upstream primer: 5'-ATAGATCCTGCCCTGTTTGC-3', downstream primer: 5'-CATTTCCTGCTGC-CGTTTTC-3'; miR-140-5p: upstream primer: 5'-GCTTAA CTGTAAACGCCCTTG-3', downstream primer: 5'-GGGCATCGTCGAG GGTT-3'; GAPDH: upstream primer: 5'-AGAAGGCT-GGGGCTC ATTTG-3', downstream primer: 5'-AGGGG CCTCCACAGTCTTC-3'.

Western Blot

The cells were lysed using RIPA lysis buffer to extract total proteins, and the concentration was detected with BCA method and adjusted to 4 μg/μL. The proteins were separated by 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, then blocked with 5% skimmed milk powder for 2 h. Glut1, HK2 (1:500), LDH-A (1:500), and GAPDH (1:1000) primary antibodies were added to seal the membrane overnight at 4°C. After washing to remove the primary antibodies, horseradish peroxidase (HRP)-labeled goat anti-mouse secondary antibody (1:1000) was added for 1 h incubation at 37°C. Next, the membrane was washed 3 times with PBS, for 5 min each time. The liquid was dried with filter paper. The development was performed with ECL in a darkroom.

Cell Proliferation

The proliferation of cells was determined using CCK-8. Cells transfected for 24 h were collected, diluted to 3*10^4 cells/mL, then inoculated in a 96-well plate (100 μL/well) and cultured at 37°C and 5% CO_2. Each well was added with 10 μL of CCK-8 solution at 0 h, 24 h, 48 h, and 72 h of adherent culture, and cultured another 2 h at 37°C and 5% CO_2. The optical density (OD) value was measured at 450 nm using a microplate reader to detect cell proliferation and plot a growth curve. The experiment was repeated 3 times.

Cell Invasion

Cell invasion of cells was measured by transwell. First, 200 μL Dulbecco's Modified Eagle's Medium (DMEM) containing 1x10^5 cells and 500 mL DMEM containing 20% FBS were added to the upper and lower chambers, respectively. After culture at 37°C for 48 h, the matrix and cells not penetrating the membrane in the upper chamber were wiped off. The remaining cells were rinsed 3 times with PBS, fixed with paraformaldehyde for 10 min, rinsed 3 times with double distilled water, then air dried and stained for 5 min with 0.1% crystal violet. Cell invasion was observed under a microscope.

Glucose Consumption and Lactate Content

The collected cells were seeded in a 6-well plate at a density of 3*10^5 cells/well and cultured at 37°C, 5% CO_2 for 48 h. Afterwards, the culture medium was used to measure glucose consumption and lactate production. The glucose and lactate levels were determined in strict accordance with the operation instructions of the determination kits.

Dual-Luciferase Reporter Assay

The bioinformatics database (Starbase v2.0) was employed to search for candidate miRNAs that can bind to PVT1. Oligonucleotide containing target sequence was amplified and cloned into pmirGLO plasmid (WT). PmirGLO-PVT1-3'UTR wild type (Wt) and mutant (Mut) were respectively established and transferred to downstream of luciferase reporter gene to sequence and identify the constructed plasmids. The luciferase reporter plasmid and miR-140-5p-Mimic or miR-NC were co-transfected into U87 cells with Lipofectamine 2000. After 48 h, luciferase activity was checked using the dual-luciferase reporter assay system.

RNA Pull-Down

A magnetic RNA-protein pull-down kit (Pierce, Rockford, IL, USA) was applied in our study. Biotin-labeled PVT1 (1 μg) was placed in an Eppendorf (EP) tube, 500 μL of buffer was added, and the mixture was bathed at 95°C for 2 min, followed by an ice bath for 3 min. Fully resuspended beads (50 μL) was incubated in the EP tube overnight at 4°C. The beads were then centrifuged at 3500 rpm for 3 min, the supernatant was discarded, and the pellet was washed 3 times with 500 μL of RNA-binding protein immunoprecipitation (RIP) wash buffer. Afterwards, 10 μL of lysis buffer was added, left at room temperature for 1 h. The cultured bead-RNA-protein mixture was centrifuged at low speed, the supernatant was collect-

ed and washed 3 times with 500 μL of RIP wash buffer. Next, 10 μL of the lyses was used as Input protein sample. After the protein concentration was determined, Western blot was performed to measure the expression. The experiment was repeated three times.

RIP Analysis

RIP analysis was performed with a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA). Cells were washed with pre-cooled PBS, and RIP lysis buffer was added. The suspension was then centrifuged to extract the supernatant. One part of the extract was removed as Input, and another part was incubated with antibodies for co-precipitation. In each co-precipitation reaction system, 50 μL of beads were washed and resuspended in 100 μL of RIP wash buffer. Five micrograms of antibody were added to each group. The magnetic bead-antibody complex was washed and resuspended in 900 μL of RIP wash buffer, and then incubated overnight at 4°C with 100 μL of cell extract. The sample was placed on a magnetic substrate to collect magnetic bead-protein complexes. Then, the Input sample was treated with prot e K to digest the protein, RNA was extrac then analyzed by Western blot. The 30-fold t-ed Argonaute2 (AGO2) antibody (1:2000, Ab MA, USA) was used for RIP determination and I (1:100, Abcam, MA, USA) was us C. The e periment was repeated three ti

Statistical Analysis

SPSS 20.0 package (IBM N1,) was used for statisti analys he collected data, and the Gr ad 7 packa building graphs. Inter-g parison wa nducted with indepen *t*-tes multi-group compar-ison with o way analys ariance (ANOVA). The pos c comparison onducted with Fisher ast sig ificant differe ce-*t*-test, the ex-pre at mu time points was analyzed with repe ement OVA, and the post hoc test wa d out Bonferroni. A value of p 5 indi a stical difference.

Results

T1 Is Up-Regulated in Glioma Tissues Cells

1 was found to be highly expressed in glioma tissues and cells, with an area under the curve (AUC) of 0.815 by the receiver operating characteristic (ROC) curve. According to the median value of PVT1, patients were divided into high and low expression groups (29/31). The 1-year survival rate in high PVT1 expression was significa than that in low expression ($p<0.05$; F e 1).

Effects of PVT1 on Glioma ration, Invasion, and Aerobic Glycoly

U87 and T98MG cells sfected Si PVT1 showed significan ower PVT1 sion than those trans d with NC. Ex ining the biological ons lls in the two groups, it was fo th liferati invasion, and aerob ycolysis PVT ansfected cells wer nificantly d compared with Si-NC ed cells, an expression of Glut1, 2 and -A proteins was also significantly decreased (5). The expression of PV -NC-transfe nd Sh-PVT1-trans-f d cells was significantly up-regulated. The feration, in on, and aerobic glycolysis in VT1-transfe d cells were significantly re-d compare th Si-NC -transfected cells, and of Glut1, HK2, and LDH-A protein ignificantly up-regulated ($p<0.05$; Figure 2).

of MiR-140-5p on Glioma Proliferation, Invasion, and Aerobic Glycolysis

U87 and T98MG cells transfected with miR-140-5p-mimics showed significantly higher miR-140-5p expression than those transfected with miR-NC. Examining the biological functions of cells in the two groups, it was found that the proliferation, invasion, and aerobic glycolysis in miR-140-5p-mimics-transfected cells were significantly inhibited, and the expression of Glut1, HK2, and LDH-A proteins were also significantly down-regulated ($p<0.05$). The expression of PVT1 was significantly up-regulated in cells transfected with miR-140-5p-inhibitor. The proliferation, invasion, and aerobic glycolysis in miR-140-5p-inhibitor-transfected cells were significantly enhanced, and the expression of Glut1, HK2 and LDH-A proteins was also significantly up-regulated ($p<0.05$; Figure 3).

LncRNA PVT1 Directly Targets MiR-140-5p

Targeted binding sites were found between lncRNA PVT1 and miR-140-5p through online software starBase 3.0. Further, Dual-Luciferase reporter assay showed that luciferase activity of miR-140-5p in PVT1-Wt was significantly lower than that of

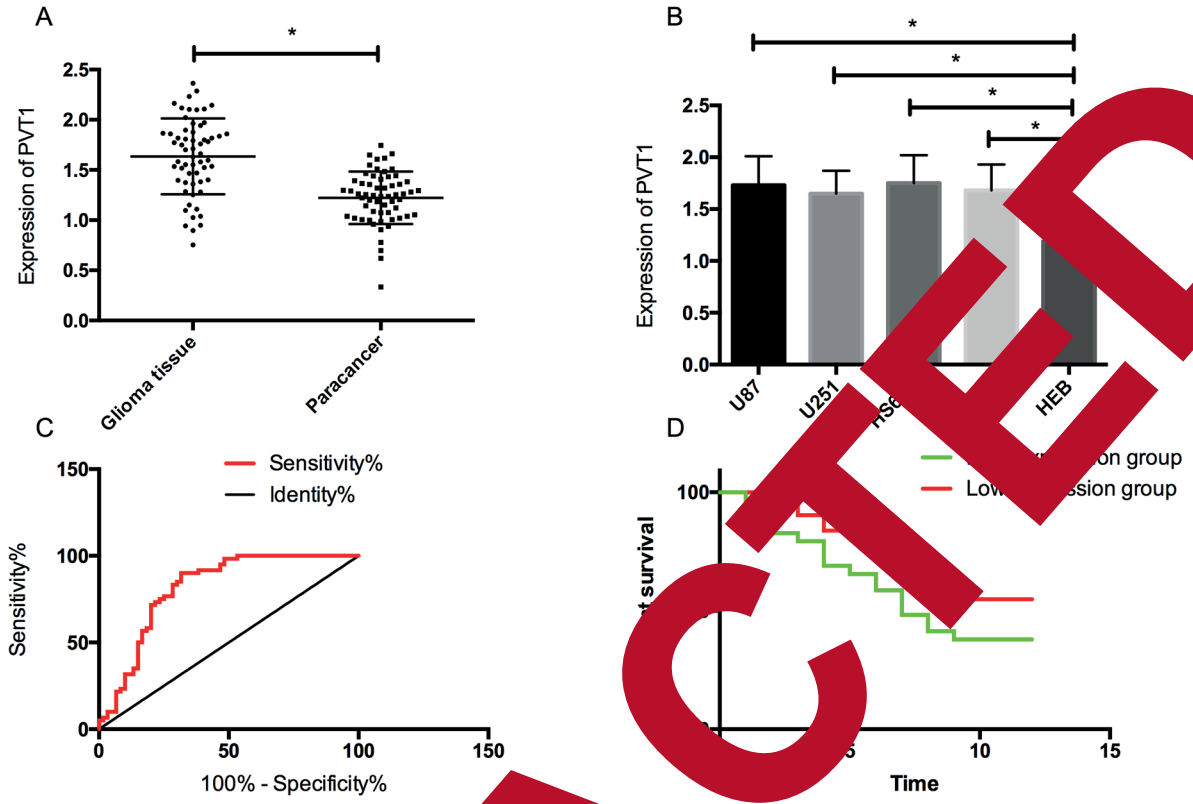

Figure 1. Expression and clinical significance of [illegible] Expression of PVT1 in glioma tissues; **B**, Expression of PVT1 in glioma cells; **C**, ROC curve analysis; **D**, [illegible] patients.

miR-NC. RNA pull-down test[illegible] the enrichment of PVT1 in AGO[illegible] compared with that in IgG, indicati[illegible] PVT1 [illegible] to AGO2. RIP test revealed tha[illegible] in miR-140-5p-Wt w[illegible] in miR-NC and miR-140-5p-M[illegible] Figure 4).

Rescue Exp[illegible]

The pro[illegible] and aerobic glycolysis i[illegible] cells transfected [illegible] Si-PVT1+miR-140-5[illegible] were not different from those tra[illegible] with miR-NC, and transfecting with Si-[illegible] the effects of miR-140-5p-inhibitor [illegible] cell proliferation, invasion, ae[illegible] glycolysis and up-regulation of Glut1, HK2, [illegible] LDH-A proteins. The proliferation, invasion, and aerobic glycolysis in cells [illegible] Sh-PVT1+miR-140-5p-mimics were not different from those transfected with miR-NC cells, and transfecting with Sh-PVT1 reversed the effects of miR-140-5p-mimics on inhibition of cell proliferation, invasion, and aerobic glycolysis and the down-regulation of Glut1, HK2, and LDH-A proteins (Figure 5).

Discussion

Although gliomas are the most common malignant tumors in the central system, the pathogenesis has not yet been elaborated[12]. However, lncRNA, a long non-coding RNA, has received increasing attention in recent years for its role in tumor[13]. Increasing studies[14,15] show that it can function as an oncogene or tumor suppressor. PVT1, a classical lncRNA, is found to be highly expressed in gastric cancer, colon cancer, and other tumors and has a significant impact on the biological function of cells[16]. After analyzing 60 glioma samples in our study, we found that the expression of PVT1 in glioma tissues was significantly higher than that in normal brain tissues, and was related to the survival and prognosis of patients. The 1-year survival of patients with high PVT1 expression was significantly lower than that of patients with low expression. The high expression of PVT1 was reported to be related to the poor prognosis of various tumors[17].

There have been studies showing that lncRNAs could regulate the function of glioma cells. Like-

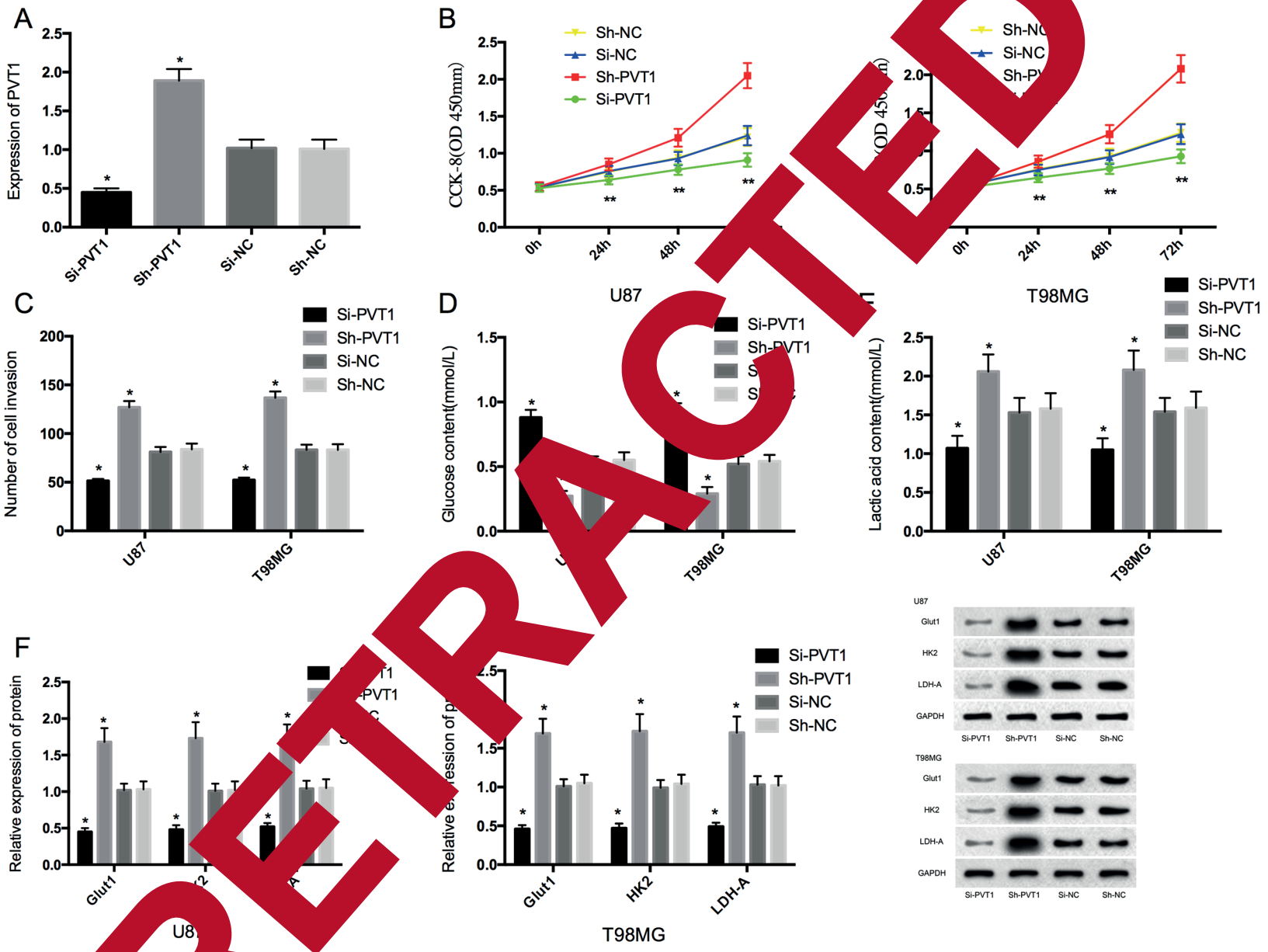

Figure 2. Effects of PVT1 on proliferation, invasion, and aerobic glycolysis. **A**, Expression of PVT1 in glioma cells after transfection; **B**, Effects of PVT1 on proliferation of glioma cells; **C**, Effects of PVT1 on invasion of glioma cells; **D**, Effects of PVT1 on glucose consumption in glioma cells; **E**, Effects of PVT1 on lactate production in glioma cells; **F**, Effects of PVT1 on aerobic glycolysis-related proteins in glioma cells. *indicated $p<0.05$.

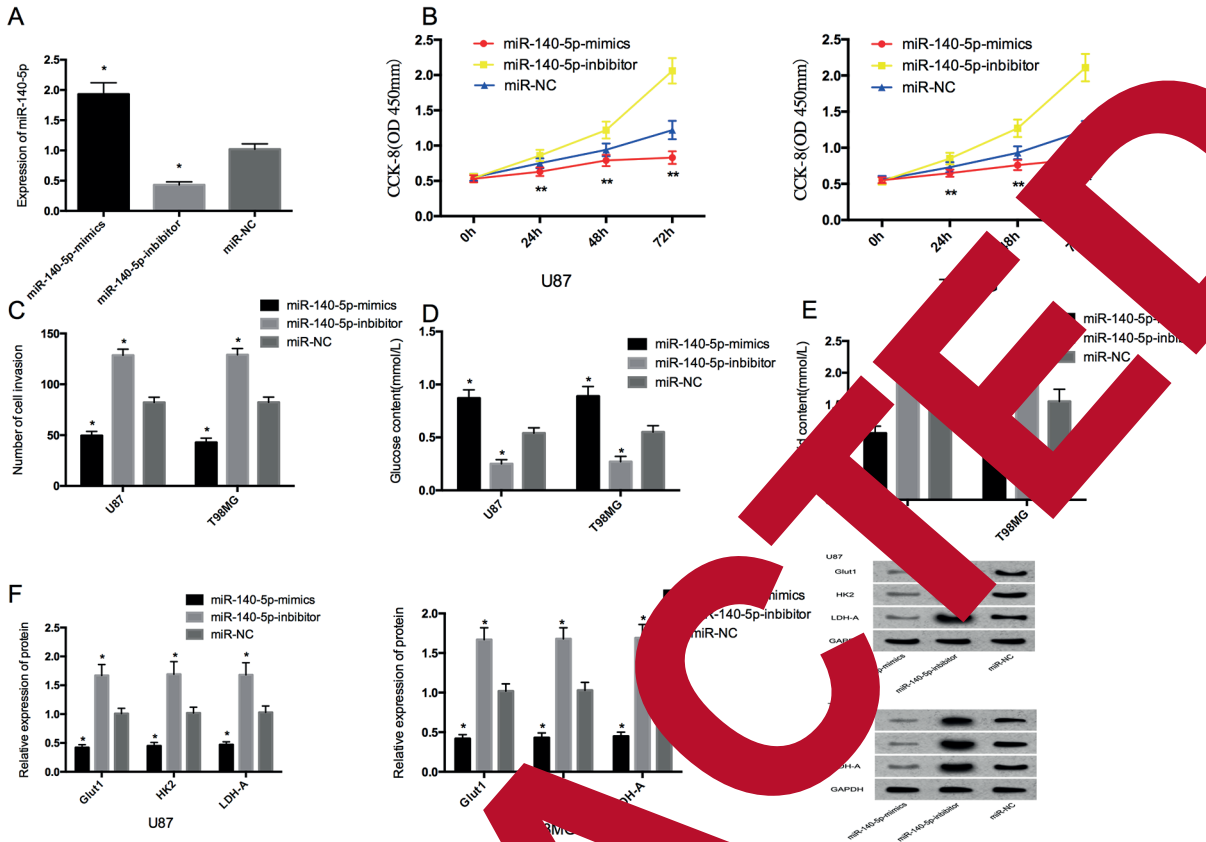

Figure 3. Effects of miR-140-5p on glioma prolifera[illegible]inv[illegible], a[illegible]obic glycolysis. **A**, Expression of miR-140-5p in glioma cells after transfection; **B**, Effects of miR-140-[illegible]iferation of glioma cells; **C**, Effects of miR-140-5p on invasion of glioma cells; **D**, Effects of miR-140-5p on glucos[illegible]umption in glioma cells; **E**, Effects of miR-140-5p on lactate production in glioma cells; **F**, Effects[illegible]-5p on ae[illegible]glycolysis-related proteins in glioma cells. *indicated $p<0.05$.

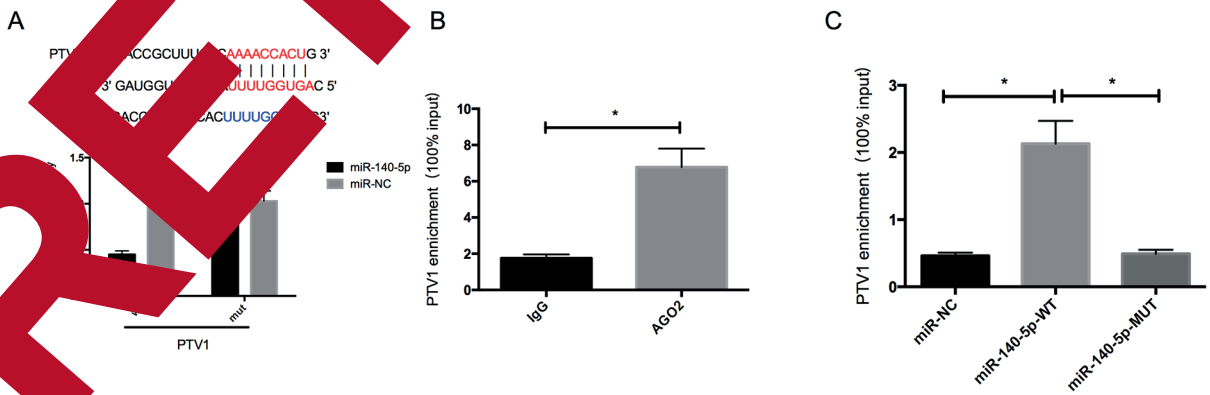

Figure 4. Correlation between PVT1 and miR-140-5p. **A**, Dual-Luciferase reporter assay confirmed that there were targeted binding sites between PVT1 and miR-140-5p. **B**, Assessment of PVT1 and AGO2 enrichment by RIP assay. **C**, Assessment of PVT1 and miR-140-5p enrichment by RNA pull-down. *indicated $p<0.05$.

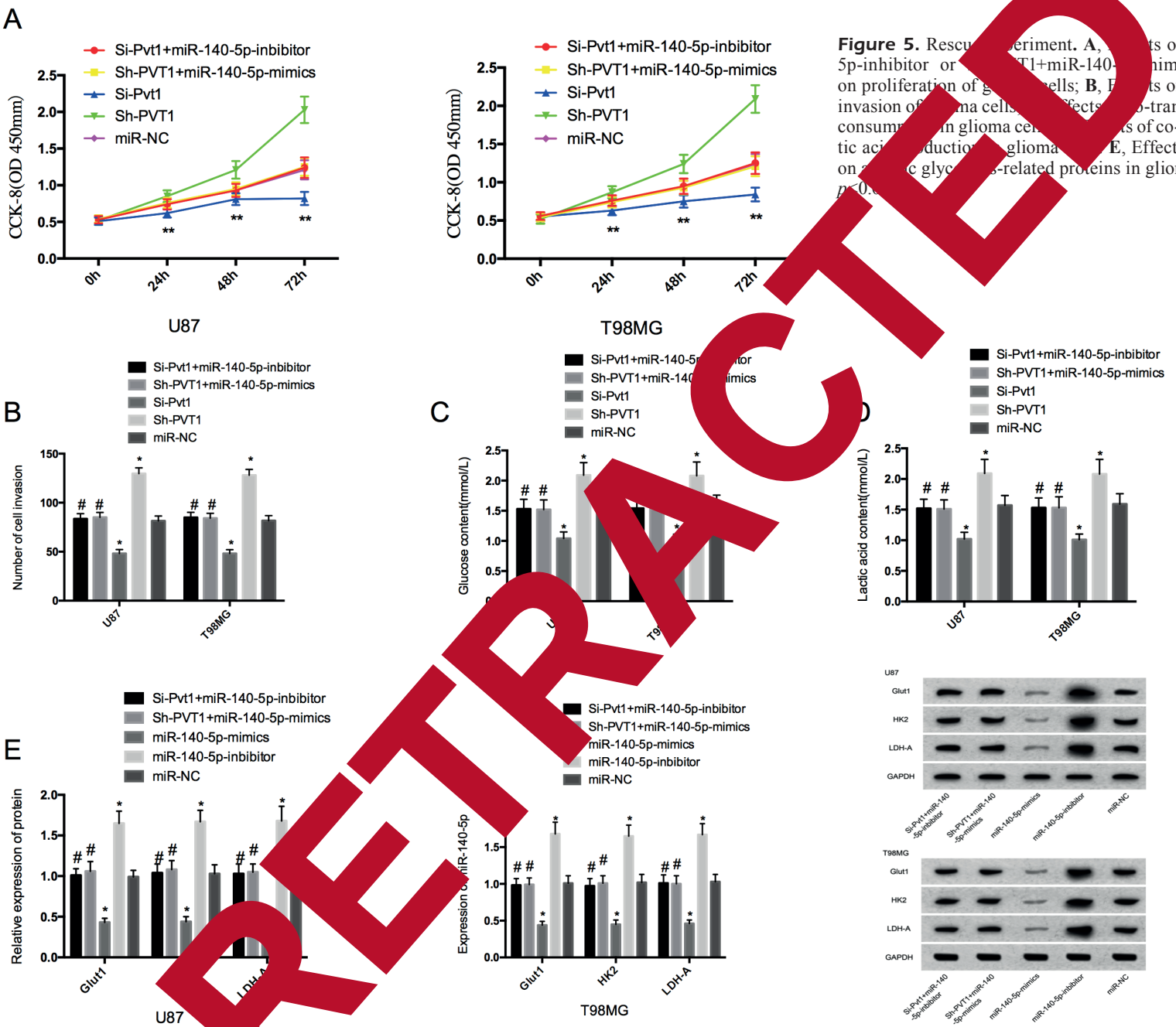

Figure 5. Rescue experiment. **A**, Effects of Si-Pvt1+miR-140-5p-inhibitor or Sh-PVT1+miR-140-5p-mimics co-transfection on proliferation of glioma cells; **B**, Effects of co-transfection on invasion of glioma cells; **C**, Effects of co-transfection on glucose consumption in glioma cells; **D**, Effects of co-transfection on lactic acid production in glioma cells; **E**, Effects of co-transfection on glycolysis-related proteins in glioma cells. *indicated $p<0.$

wise, Xie et al[18] pointed out that lncRNA CASC15 accelerated the proliferation and metastasis of glioma cells by targeting miR-130b-3p. In our study, we found that up-regulating the expression of PVT1 facilitated the proliferation and invasion of glioma cells, but inhibiting the expression yielded opposite outcome. As one of the main features of tumor, metabolic reprogramming plays a critical role in its development and progression, with aerobic glycolysis as its main phenotype[19]. Aerobic glycolysis supplies energy to tumor cells by consuming glucose, which leads to the extracellular secretion of lactate to promote cell invasion and metastasis[20]. Therefore, it is of great significance to the upstream molecular mechanism in tumors. It was also found for the first time in our study that PVT1 could be used as an oncogene to promote aerobic glycolysis in glioma cells, and that over-expression of PVT1 could up-regulate the expression of Glut1, HK2, and LDH-A. Those proteins were key molecules in the aerobic glycolysis process that affected the glucose uptake and lactate production of tumor cells[21]. All the above conclusions suggested that PVT1 was served as an oncogene in gliomas.

Subsequently, biological analysis demo that miR-140-5p could bind to PVT1, whic as confirmed by dual-luciferase reporter assay, test, and RNA pull-down. MiR-140-5p has b reported to play the role of tu pressor gliomas and is regulated by va JAs. Fo example, research found th cRNA DXA11-AS acted as miRNA sp to pro tumorigenesis by tar sides, miR-140-5p i ted th feration and invasion of glioma s by regula scular endothelial growt VEGFA)/ma metallo-protein 2 (M) sig pathway[23]. All these studies ind ted the imp e of miR-140-5p in gliomas our research, m -5p expression in gli cells w regulated, and the up-regulation bited t roliferation and invasion of glioma wh as dow gulation reversed. It is also fo regul the expression of miR- p als ete robic glycolysis in glioma cel o dete whether PVT1 promoted aerobi ycolysis in glioma cells by regulating miR-14 experiment was conducted. The lts showed that Si-PVT1 abolished the effects miR-140-5p inhibition on promotion of prolif- invasion, and aerobic glycolysis in glioma cel s well as on Glut1, HK2, and LDH-A proteins. Similarly, Sh-PVT1 reversed the effects of miR-140-5p over-expression on inhibition of cell proliferation, invasion, and aerobic glycolysis, as well as on Glut1, HK2, and LDH-A proteins. The above results suggested that PVT1 affected glioma proliferation, invasion, and aerobic by inhibiting the expression of miR- p.

Conclusions

PVT1 is highly expres n gliomas, wh dicates a poor prognosi the pat . Moreo , it can promote the pr tion asion, d aerobic glycolysis in mi regula miR-140-5p. Howev here ar some utations in this study example, th periments have not b med to obs e the effects of PVT1 umor th. Secondly, the possible mechanisms of miR- in gliomas have not bee ed in depth. efore, we will carry o ore basic and clinical trials to supplement experimenta ta.

Co sts

The Auth are that they have no conflict of interests.

Acknowledgements

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