Paclitaxel inhibits the migration of CD133+ U251 malignant glioma cells by reducing the expression of glycolytic enzymes

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Abstract. Energy metabolic reprogramming (EMR) allows for the rearrangement of a series of metabolic genes and proteins when tumor cells adapt to their microenvironment. EMR is characterized by changes in the metabolic pattern and metabolic intermediates to meet the needs of tumor cells for their malignant proliferation and infiltrative growth. The present study investigated the role of low-dose paclitaxel (PTX) in changing the expression levels of key genes and proteins during glycolysis in CD133+ U251 glioma cells and explored the relevant regulatory mechanisms of action at the molecular level. CD133 immunomagnetic beads were applied to malignant CD133+ U251 glioma cells, which were then divided into a negative control and an experimental group treated with 1, 2, 4 or 8 μM PTX for 72 h. Cell Counting Kit-8 (CCK-8) was used to measure U251 cell proliferation. RNA and protein were extracted from the malignant glioma cells in all groups to observe changes in the expression levels of key glycolytic enzymes, such as glucose transporter 1 (GLUT1), pyruvate kinase M (PKM) and lactate dehydrogenase A (LDHA), using reverse transcription-quantitative PCR and western blot assays. Transwell migration assays were performed to quantify the effects of PTX solution on U251 cells. CD133+ U251 glioma cells were isolated successfully. CD1133+ cells had a higher rate of proliferation compared with CD1133- cells. In CD1133+ cells treated with PTX, a dose-dependent reduction in the expression levels of the key glycolytic enzymes GLUT1, PKM and LDHA was observed at both the mRNA and protein levels. PTX solution also inhibited cell migration. Differences between the control and experimental groups were statistically significant ($P<0.05$). Since glycolysis plays an indispensable role in the proliferation and migration of stem cell-like glioma cells, PTX may inhibit tumor cell growth by downregulating the gene and protein expression levels of glycolytic enzymes in CD133+ glioma cells.

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Introduction

Gliomas are the most common malignant primary brain tumor that contribute to >50% of malignant primary brain tumor cases worldwide (1,2). Surgical intervention, postoperative radiotherapy and chemotherapy comprise the mainstay of treatment for patients with high-grade glioblastoma (GBM); however, the median survival rate remains relatively low at 12-15 months (3,4). Thus, more antitumoral treatments are required; therefore, further research must be performed into some of the most promising medicinal substances to develop further therapies to treat various types of cancer.

Considering the multi-directional differentiation potential and other biological characteristics of glioma stem cells (GSCs), researchers have focused on the use of GSCs for the treatment of high-grade GBM (5). It was demonstrated that GSCs share a metabolic pattern similar to that of other tumor cells (6). Even when GSCs receive sufficient oxygen, they remain highly dependent on glycolysis to maintain their energy supply and thereby achieve high proliferation and malignant invasion, similar to tumor cells (7-9). At present, the development of anticancer drugs mainly focuses on malignant tumor cell survival/proliferation-related pathways, aiming to inhibit tumor tissue growth by suppressing the proliferation and migration of malignant tumor cells and inducing cell apoptosis (10,11). Paclitaxel (PTX), among many promising medicinal substances, is a natural diterpenoid product used as a novel anticancer drug for patients with breast cancer, ovarian cancer, lymphoma and non-small cell lung cancer (12). Generally, PTX regulates cell cycle signal transduction and promotes microtubule polymerization, which triggers G2/M-phase arrest (13). Currently, research into the interactions between PTX and GSCs is limited to signaling pathways of glioma genesis, whereas the medicinal effect on the GSCs metabolism is yet to be uncovered.

In the present study, CD133+ glioma cells were treated with PTX at various concentrations to investigate the effect of PTX on the expression levels of relevant glycolytic genes

in stem cell-like glioma cell lines, which hopefully provided an experimental basis for the development of novel treatments for gliomas.

Materials and methods

Reagents. The human glioma cell line U251 was purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Other experimental materials include the human-lyophilized CD133 MicroBead kit (cat. no. 130097049; Miltenyi Biotec GmbH), Cell Counting Kit (CCK)-8 (Dojindo Molecular Technologies, Inc.), glucose transporter 1 (GLUT1; cat. no. 12939), pyruvate kinase M (PKM; cat. no. 3190), lactate dehydrogenase A (LDHA; cat. no. 3582) and GAPDH (cat. no. 8884) primary antibodies (Cell Signaling Technology, Inc.), goat anti-rabbit imunogobulin G secondary antibody (cat. no. SA00001-2; ProteinTech Group, Inc.), FBS and DMEM (Gibco; Thermo Fisher Scientific, Inc.), SYBR Green I-based real-time PCR reagent (cat. no. 03003230001; Roche Molecular Diagnostics), reverse transcription-quantitative PCR (RT-qPCR) primers (Wuhan Jin Kairui Biological Engineering Co., Ltd.) and gelatin-coated polycarbonate membrane filters (Corning Inc.).

Cell culture and sorting of CCD133$^+$ U251 glioma cells with D133 immunomagnetic beads. Following resuscitation, U251 glioma cells were added to DMEM containing 10% FBS and placed in an incubator at 37˚C and 5% CO_2, for culturing. During the exponential phase of growth, 1 ml of culture media containing ~1x10^9 cells, were collected for isolation of CD133$^+$ cells according to the instructions provided in the CD133 MicroBead kit.

CCK-8 assay for evaluating the viability of PTX-treated CD133$^+$ U251 cells. CCK8 experiments were performed according to the manufacturer's instructions. CD133$^+$ U251 cells were used as the experimental group and the CD133$^-$ cells in the residue were used as a control group. The CD133$^-$ cells were cultured in a 96-well plate, with each well containing 1x10^4 cells. After 24, 48 or 72 h, CCK-8 reagent (10 μl/well) was added to each adherent culture to observe cell growth in each well. Meanwhile, CD133$^+$ U251 cells were cultured in another 96-well plate at a density of 1x10^4 cells/well. After becoming adherent, PTX at various concentrations (1, 2, 4 and 8 μM/well) was added to the cells. The cells were then cultured for 72 h before addition of CCK-8 reagent (10 μl/well). Finally, the optical density in each well was measured at a wavelength of OD 450 nm to evaluate cell growth.

RT-qPCR analysis of glycolytic gene expression levels. Following cell culture, U251 cells were plated on a 6-well plate at a density of 1x10^6 cells/ml. Various concentrations of PTX (1, 2, 4, and 8 μM/well) were added once the cells became adherent. The cells were harvested following treatment with PTX for 72 h. TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used for RNA extraction and 3 μg RNA obtained from each culture, RT kit (cat. no. 04913850001; Roche Molecular Diagnostics) was used for reverse transcription. cDNA was synthesized after incubation using the following temperature protocol: 55˚C for 30 min, 85˚C for 5 min and 4˚C for 10 min in the presence of 0.5 μl reverse transcriptase, 1.0 μl oligDT$_{18}$, 4.0 μl buffer, 2.0 μl dNTPs, and 12 μl DEPC water. NCBI primer-designer (www.ncbi.nlm.nih.gov/tools/primer-blast) was employed for designing primers. Target genes and GAPDH were subject to RT-qPCR using the SYBR Green I-based real-time PCR reagent with 40 cycles of the following thermocycling conditions: 95˚C for 2 min, 50˚C for 1 min and 72˚C for 1 min. The primer sequences of GLUT1, PKM, LDHA and GAPDH used for the PCR are shown in Table I. Fluorescence collection and data analysis were conducted using the software attached to the RT-qPCR system. The relative expression levels of the target genes in the different concentration subgroups were calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to GAPDH (14).

Western blot analysis for GLUT1, PKM and LDHA protein expression detection. Cells were treated as aforementioned. Subsequently, 1 ml pre-cooled PBS was added to each well to wash the cells twice. Protein lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) was added (100 μl/well) to extract proteins from the cells. The BCA protein assay kit was used for protein quantification. Equal amounts of protein (30 μg/lane) were analyzed using 10% SDS-PAGE before being electro-transferred onto a PVDF membrane. Membranes were then blocked with 5% BSA (cat. no. P0252; Beyotime Institute of Biotechnology) for 2 h at room temperature. The protein bands were normalized to GAPDH (cat. no. P0063; Beyotime Institute of Biotechnology). Membranes were incubated with primary antibodies diluted to 1:2,000 and placed on a shaking table at 4˚C for overnight culture. Following primary antibody incubation, membranes were incubated with secondary antibodies diluted to 1:10,000 at room temperature for 1 h. Enhanced chemiluminescent reagent (cat. no. P0018AS; Beyotime Institute of Biotechnology) was added before exposure using an automatic exposure system. Grayscale values were measured using ImageJ (version no. V1.8.0.112; National Institutes of Health).

Transwell migration assay for assessing cell invasion ability. The Transwell migration assay was performed using the 24-well Transwell with gelatin-coated polycarbonate membrane filter. Firstly, cells were plated on a 6-well plate at a density of 1x10^6 cells/well and cultured until they reached 90-95% confluency. 1, 2, 4, 8 μM/ml PTX solution was then added into the each well for 48 h of incubation. Subsequently, the treated cells were detached from the plates using 0.5 ml trypsin and then seeded in the upper chambers which precoated with 500 ng/ml Matrigel solution (BD Biosciences) of the Transwell chamber (~1x10^4 cells/well), while DMEM supplemented with 10% FBS was added into the lower chambers. The cells were then incubated in the chambers for 24 h at 37˚C. Finally, the chambers were stained with 0.1% crystal violet at room temperature for 30 min, and imaged using a light microscope at x200 magnification.

Statistical analysis. Each experiment was replicated three times, with each concentration having internal triplicates. GraphPad Prism 6.0 (GraphPad Software, Inc.) was used for experimental drawing. Student's t-test was used to analyze the differences between two groups. One-way ANOVA followed

Table I. Sequences of primers used in the present study.

Gene	Primer sequences (5'-3')	Base pairs	GenBank accession number
GLUT1	F: 5'-CTATGGGGAGAGCATCCTGC-3' R: 5'-CCCAGTTTCGAGAAGCCCAT-3'	195	NM_006516.3
PKM	F: 5'-GTGGCAAGCACACTGGATTAG-3' R: 5'-GAATCAATGTCCAGGCGGCA-3'	196	NM_001206799.2
LDHA	F: 5'-CGTCGATATTCCTTTTCCACG-3' R: 5'-AGCAAGTTCATCTGCCAAGTC-3'	197	NM_001165414.1
GAPDH	F: 5'-TGGACTCCACGACGTACTCAG-3' R: 5'-ACATGTTCCAATATGATTCCA-3'	162	NM_001256799.3

F, forward; R, reverse; GLUT1, glucose transporter 1; PKM, pyruvate kinase M; LDHA, lactate dehydrogenase A.

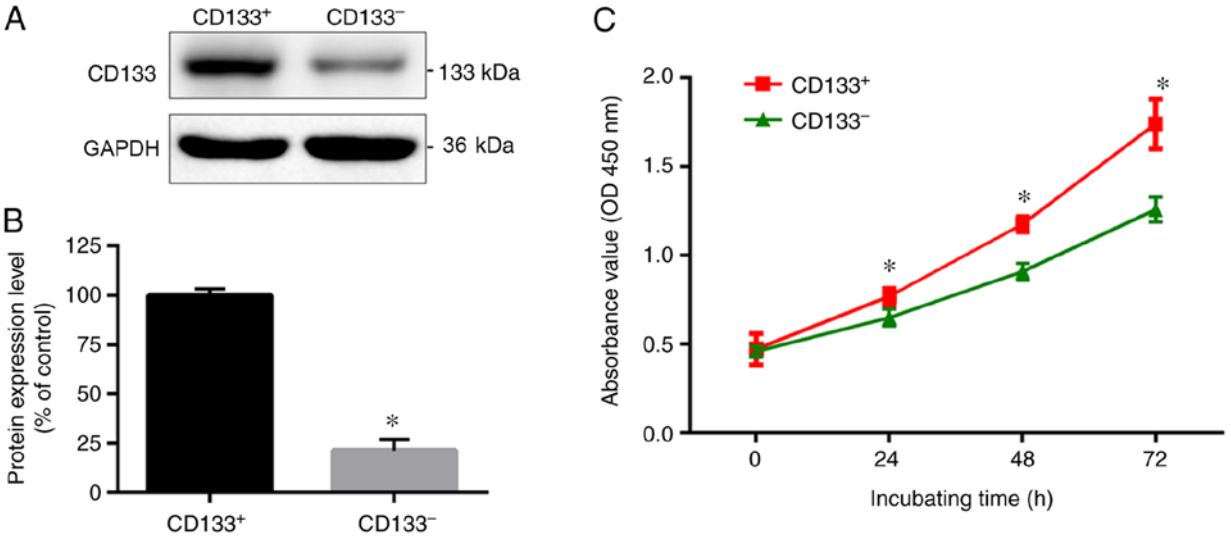

Figure 1. CD133 protein expression levels and growth curve of U251 glioma cells. (A) The CD133 protein expression levels of U251 glioma cells were detected using western blot assays. (B) Histogram of the CD133 protein expression levels in CD133$^+$ and CD133$^-$ U251 cells. (C) CD133$^+$ and CD133$^-$ U251 cell viability. Cells were cultured in DMEM medium which containing 10% FBS for 24, 48 and 72 h, then 10 μl per well cell counting kit-8 reagent was added into each well to determine the cell viability, in order to compare the proliferation rates of CD133$^+$ and CD133$^-$ glioma cells. Data are presented as the mean ± standard deviation from three independent experiments. Statistical analyses were performed using independent-samples t-tests. *P<0.05 vs. CD133$^-$ cells. OD, optical density.

by Tukey's post hoc test was used to analyze differences among multiple groups. Experimental data were counted with software SPSS 22.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

CD133$^+$ U251 glioma cells exhibit higher proliferation compared with CD133$^-$ cells. Malignant CD133$^+$ U251 glioma cells were successfully obtained using CD133 immunomagnetic beads. As shown in Fig. 1, the western blotting results indicated that CD133 protein expression levels were significantly higher in CD133$^+$ U251 cells compared with CD133$^-$ cells. From the CCK-8 growth curve, the optical densities of CD133$^+$ U251 cells at 48 and 72 h were significantly higher compared with CD133$^-$ cells (P<0.05). These data indicated that CD133$^+$ U251 glioma cells had a higher proliferation rate compared with CD133$^-$ cells.

PTX acts on CD133$^+$ U251 glioma cells as a cell growth inhibitor. Following continuous culture and sorting of CD133$^+$ U251 cells, PTX at various concentrations (1, 2, 4 and 8 μM/well) was added to each group. As shown in Fig. 2, compared with the blank control group, a higher PTX concentration resulted in greater inhibition on the cell growth rate (P<0.05). This indicated that the cell growth inhibition rate positively associated with the concentration of PTX. These results show that PTX inhibited the growth of CD133$^+$ U251 glioma cells in a dose-dependent manner.

PTX suppresses GLUT1, PKM and LDHA mRNA expression levels in CD133$^+$ U251 glioma cells. PTX at various concentrations was added to CD133$^+$ U251 glioma cells for 72 h. RT-qPCR was performed to examine GLUT1, PKM and LDHA mRNA expression levels. As shown in Fig. 3, compared with the control group, the experimental groups had significantly lower mRNA expression levels of GLUT1, PKM

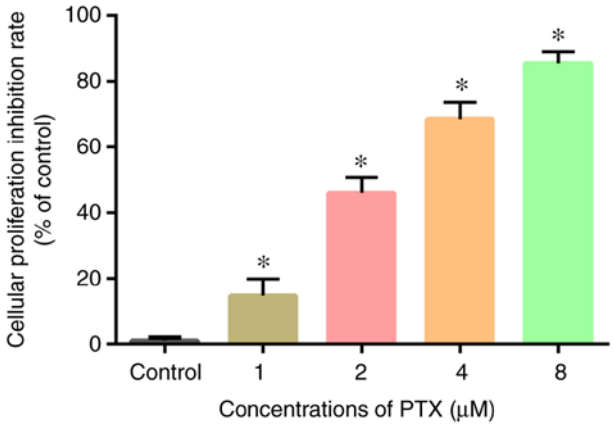

Figure 2. Inhibition of cell proliferation of PTX on CD133+ cells. Cell viability of CD133+ U251 following PTX treatment. The inhibition of the proliferation rate was measured at 72 h after administration of various concentrations of PTX. Data are presented as the mean ± standard deviation from three independent experiments. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. the control group. PTX, paclitaxel.

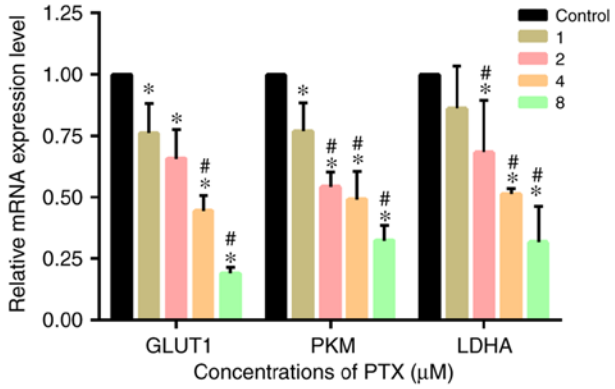

Figure 3. Effect of PTX on mRNA expression levels of glycolytic enzymes in glioma cells. GLUT1, PKM and LDHA gene expression levels in CD133+ U251 cells in the presence of various concentrations of PTX were measured using reverse transcription-quantitative PCR. All gene expression levels were normalized to GAPDH gene expression levels. Data are presented as the mean ± standard deviation. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. the control group; #P<0.05 vs. the 1 μM concentration group. GLUT1, glucose transporter 1; LDHA, lactate dehydrogenase A; PKM, pyruvate kinase M; PTX, paclitaxel.

and LDHA (P<0.05). mRNA expression levels decreased as the PTX concentration increased, indicating that PTX effectively inhibited the transcription of the key glycolytic enzymes in CD133+ glioma cells.

PTX suppresses GLUT1, PKM and LDHA protein expression levels in CD133+ U251 glioma cells. Western blot assays were performed to measure the expression levels of the three glycolytic enzymes. As shown in Fig. 4, GLUT1, PKM and LDHA protein expression levels in the experimental groups were lower compared with the control group (P<0.05), and the protein expression levels reduced in a negative association with the PTX concentration. This indicated that in terms of protein translation, PTX inhibited the expression levels of the glycolytic enzymes in CD133+ glioma cells.

PTX treatment inhibits the migration of CD133+ U251 cells. Transwell assays were used to evaluate in vitro cell migration. Transwell assay results revealed that PTX treatment significantly attenuated the migratory ability of the cells compared with controls (Fig. 5A). The differences in the colony count and the migratory ability was found to be statistically significant (P<0.05; Fig. 5B).

Discussion

Cancer stem cells (CSCs) comprise a special cell population characterized by self-renewal, cell immortalization and a multi-directional differentiation potential (15). CSCs are present in most tumor tissue and cancer cell populations, such as brain cancer, lymphoma, breast cancer and lung cancer (16,17). As a brain CSC marker, CD133 is closely associated with the malignant proliferation and infiltration of cancer cells (18). Compared with normal cells, CD133+ CSCs are more dependent on glycolysis and rely on anaerobic glucose metabolism via glycolysis for energy production (2). In the present study, immunomagnetic beads were used to sort U251 glioma cells, which expressed high levels of the CD133 protein. Based on the CCK-8 assay, it was found that the CD133+ cells had a higher proliferative ability compared with CD133- cells. Following PTX treatment of CD133+ cells, the present study found that the expression levels of the genes and proteins in the key glycolytic enzymes were significantly suppressed, which suggested that PTX acted as a cancer cell growth inhibitor by suppressing these genes.

The present study showed that GLUT1 expression is negatively affected by PTX to a degree, where a low PTX dose is less effective. According to previous reports, cancer cells require GLUTs to transport glucose into their cytoplasm, thereby strengthening glycolytic function and providing energy for cancer cell growth and proliferation (19,20). Elevated expression levels of GLUT1/3 leads to enhanced glucose uptake in GSCs, making glycolysis the primary metabolic source of energy for GSCs (21). In particular, GLUT1 serves as an important regulator for the development and progression of a range of tumors, including gliomas and other malignant tumors (22). It was reported that GLUT1 expression is abnormally high in many types of cancer, including gastric, colon, bladder, liver, colorectal and lung cancers (23). These results suggest that GLUT1, a primary metabolic gene, is possibly one of many critical contributors to the cancer suppression induced by PTX.

Secondly, the present study found that PTX effectively inhibited PKM mRNA and protein expression in GSCs. A previous study showed that PKM plays a role in the final step of glycolysis, namely converting the midway product into pyruvate acid, that eventually yields ATP (24). Although PKM is necessary for cancer cell growth, proliferation and metastasis, it has not investigated in relation to glioma metabolism (25,26). The effect of PTX at a lower dosage on PKM expression is much more sensitive compared to that of GLUT1 expression. These findings not only suggest that PTX has an antitumoral effect through interfering with the glycolysis process, but also suggest that PTX, possibly through a negative feedback loop from the accumulation of intermediate products, has a more substantial effect on the late steps in the glycolysis as opposed

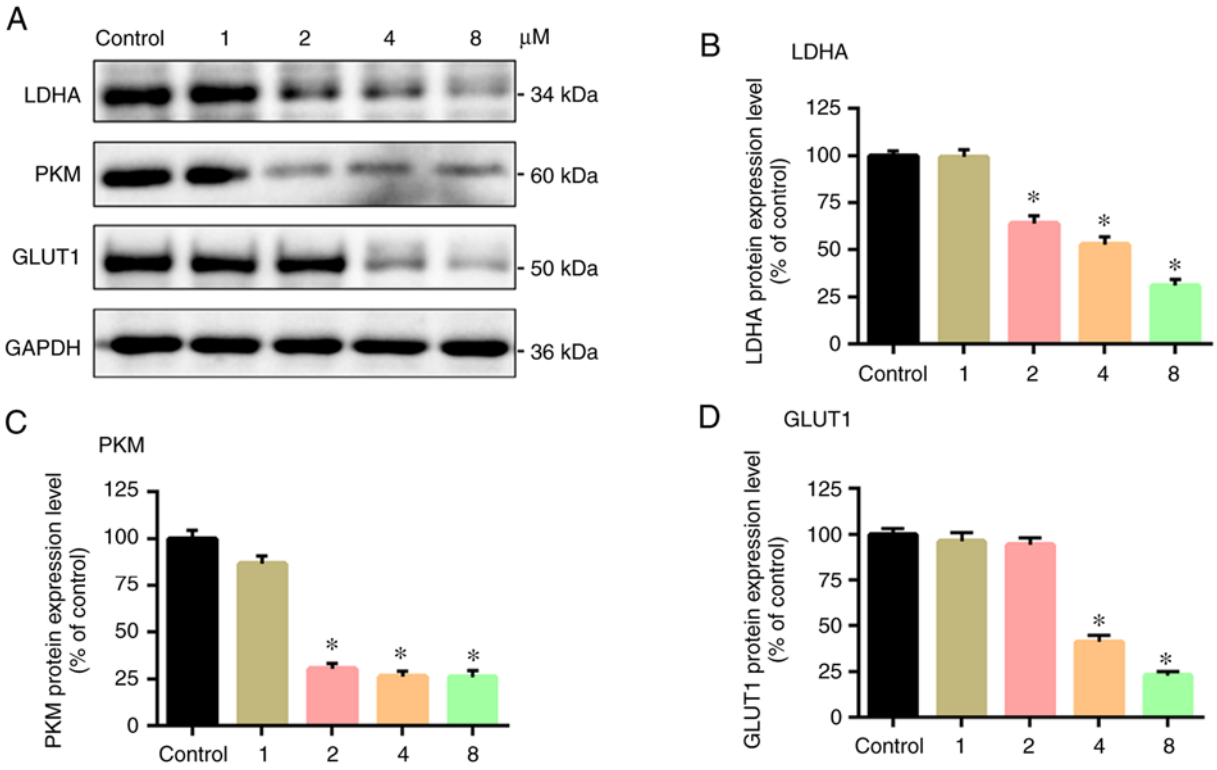

Figure 4. Effect of PTX on protein expression levels of glycolytic enzymes in glioma cells. (A) Target protein expression levels were detected using western blotting. (B) GLUT1, (C) PKM and (D) LDHA protein expression levels in CD133+ U251 cells were measured using western blotting in the presence of different doses of PTX. Representative western blot densities of the proteins normalized to GAPDH. Data are presented as the mean ± standard deviation. Differences in the mean expression levels were analyzed using one-way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. the control group. GLUT1, glucose transporter 1; LDHA, lactate dehydrogenase A; PKM, pyruvate kinase M; PTX, paclitaxel.

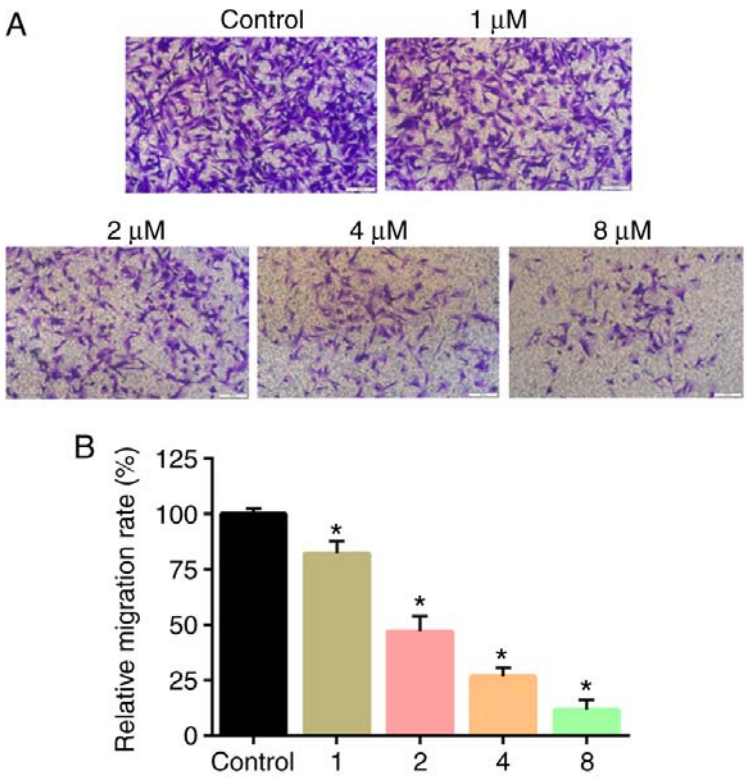

Figure 5. Inhibitory effect of PTX on invasion of CD133+ U251 glioma cells. (A) Transwell assays of CD133+ U251 glioma cells following treatment with PTX. The intensity of the crystal violet dye showed that cell groups treated with PTX showed reduced cell invasion compared with the control group, in which the PTX groups presented the lowest levels of cell invasion. (B) The relative migration rates of the observed groups. Data are presented as the mean ± standard deviation of three independent repeat experiments and analyzed using one-way ANOVA followed by Tukey's post hoc test. *P<0.05 vs. the control group. PTX, paclitaxel.

to directly affecting the GLUT1 expression levels. The PTX downregulated LDHA supports the idea also.

The present study also found, in the PTX-treated GSCs, LDHA expression was significantly inhibited. LDHA catalyzes the conversion of pyruvic acid to lactic acid and is highly expressed in most cancer cells (27). Studies have shown that lactic acid produced from glycolysis is transferred out of the cells and acidifies the local microenvironment, which not only protects cancer cells from being killed by the host immune cells but also impairs the effect of anticancer drugs on the cancer cells (28). Additionally, an acidified microenvironment is likely to induce cell matrix degradation, microvessel formation and the Warburg effect (29). Moreover, silencing LDHA inhibited proliferation, induced apoptosis and increased the chemosensitivity of glioma cells to temozolomide (30). This indicates that PTX may effectively inhibit cancer cell growth and achieve its therapeutic effect by mediating the expression of glycolysis-related apoenzymes in GSCs.

In conclusion, this present study designed a PTX-treated GSC model to preliminarily explore the effect of PTX on glycolysis in glioma cells that had high expression levels of CD133. These results provide a theoretical basis for *in vivo* experiments exploring the use of PTX for the treatment of cancers by altering the expression of other signaling proteins leading to EMR of glycolysis in gliomas.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

JL and NL designed the study. SP and JJ performed the experiments. NL analyzed the data and drafted the manuscript. LC and MD supervised the study and drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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