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ORIGINAL RESEARCH

# CDKN3 Overcomes Bladder Cancer Cisplatin Resistance via LDHA-Dependent Glycolysis Reprogramming

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**Background:** Aerobic glycolysis plays an important role in bladder cancer (BLCA) progression and chemoresistance. Cyclin-dependent kinase inhibitor-3 (CDKN3), a dual-specificity protein tyrosine phosphatase, has aberrant upregulation in multiple cancer types and is associated with tumorigenesis. However, the role of CDKN3 in BLCA progression and glycolysis has not been elucidated.

**Purpose:** In this study, we investigated the effect and underlying mechanisms of CDKN3 on bladder cancer chemoresistance.

**Results:** This study confirmed that CDKN3 was overexpressed in BLCA tissues and promoted proliferation and migration. Additionally, our results showed a CDKN3-dependent mechanism on chemoresistance; chemoresistance cells were transformed into chemosensitivity cells by CDKN3 knockdown. Additionally, we showed that CDKN3 knockdown decreased glycolysis by inhibiting LDHA expression in BLCA chemoresistance cells. The results also proved that LDHA was an important mediator of CDKN3-regulated BLCA resistance. LDHA overexpression reversed glycolysis inhibition and chemosensitivity induced by CDKN3 downregulation.

**Conclusion:** These data collectively identified a vital role of CDKN3 in glycolysis and chemoresistance by regulating LDHA expression in BLCA cells, providing a possible therapeutic strategy for treating BLCA.

**Keywords:** bladder cancer, glycolysis, CDKN3, chemoresistance

## Introduction

Bladder cancer (BLCA), a urological malignancy, is the 10th most frequent tumour globally. The incidence rate of BC has an ascending trend. Based on GLOBOCAN data, approximately 550,000 people were diagnosed with BLCA and 200,000 deaths occurred in 2018[1] compared with that of up to 573,278 new BLCA cases and 22,536 BLCA-related deaths have occurred globally in 2020.[2] The BC is divided into two subtypes including non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) that have various molecular patterns.[3] As reported by a study of tumor epidemiology in China, BC is the seventh most frequent and tenth most lethal malignancy in China that the total incidence and mortality of BC in Chinese men are 10.9/100,000 and 4.2/100,000, respectively.[4] The onset of BLCA occurs due to the lack of cancer-specific Clinical symptoms, almost 25% incidence of patients with muscularis infiltration when diagnosed.[5,6] The surgery, chemotherapy, radiotherapy and immunotherapy are extensively applied for BLCA patients. Presently, cisplatin-based neoadjuvant chemotherapy is the major regimen for BLCA treatment, including GC (gemcitabine and cisplatin) or M-VAC (methotrexate, vinblastine, doxorubicin, and cisplatin).[7] Unfortunately, the acquired chemoresistance after long-term chemotherapy limits the cytotoxic efficacy of cisplatin-based regimens for BLCA, even though no standard of care exists after cisplatin-based neoadjuvant

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chemotherapy. Thus, a better understanding of the molecular regulatory mechanisms in developing chemoresistance is necessary for developing more efficient treatment strategies for patients with BLCA.

Previous studies reported that aerobic glycolysis, one mode of energy metabolism in cancer, plays an essential role in chemo-resistant cancer cells.[8] To facilitate sustained cell survival, excessive proliferation, metastasis and escape from the immune system, cancer cells would actively choose glycolysis as the main way to obtain energy, even in an oxygen-rich environment, a process known as the Warburg effect, which characterized by increased glycolysis and lactate production.[9] Another study by Wu et al also provided compelling evidence that Epididymal protein 3A enhanced aerobic glycolysis mediated by upregulation of HIF-1α and subsequently increased target glycolytic genes and decreased mitochondrial biogenesis, thereby promoted tumor growth and metastasis in GC cells.[10] It has been widely reported that improved glycolysis capacity is beneficial to the proliferation of cancer cells in response to chemotherapy and induced BLCA recurrence and metastasis.[11,12] As shown by Hao et al that AlkB homolog 5 RNA demethylase suppressed the progression and sensitized bladder cancer cells to cisplatin through a casein kinase 2α-mediated glycolysis pathway in an m6A-dependent manner.[13] Consequently, therapeutic regimens targeting glycolysis to conquer BLCA chemoresistance have become promising approaches.

Cyclin-dependent kinase inhibitor-3 (CDKN3), a dual-specificity protein tyrosine phosphatase, is a component of the CDC14 group, which functions to dephosphorylate CDK1/CDK2 and other proteins. CDKN3 has been proved to be overexpressed and associated with poor survival in several human cancers, such as lung adenocarcinoma, cervical cancer.[14,15] Notably, recent reports have shown that CDKN3 expression was remarkably increased in cisplatin-resistant CRC patients. The suppression of CDKN3 reduced the survival rate of colorectal cells with cisplatin resistance.[16] However, the role and the underlying mechanism of CDKN3 in BLCA chemoresistance remain largely unknown. Therefore, this study investigated whether and how CDKN3 regulates glycolysis and chemoresistance in BLCA.

# Materials and Methods

## Public Databases Analysis

Expression of CDKN3 and correlation between CDKN3 and LDHA in BLCA was analyzed by the Cancer Genome Atlas (TCGA) data database with GEPIA (http://gepia2.cancer-pku.cn/). Expression of CDKN3 based on nodules metastasis and cancer stage in BLCA tissues was analyzed with Ualcan (http://ualcan.path.uab.edu). Relationship between CDKN3 expression and overall survival in BLCA patients was analyzed with the Human Protein Atlas (https://www.proteinatlas.org/).

## Cell Culture and Treatment

The T24 and RT4 cell lines purchased from American Type Culture Collection (ATCC) were cultured in RPMI-1640 medium (Sigma-Aldrich, St.Louis, USA) with 10% fetal bovine serum. All cells were grown at 37°C with 5% $CO_2$.

The GC-resistant cancer cell derivate from T24 and RT4 with 10 μM gemcitabine and 10 μM cisplatin (Sigma-Aldrich) continuously for 6 days (a new drug added every 72 hours), then replaced normal medium for 12 days, finally repeated drug treatment for 6 days. GC-resistant cancer cell was selected by 10 μM gemcitabine and 10 μM cisplatin every 2 weeks to remain drug resistance characteristics.

## Cell Transfection

To knockdown CDKN3, Human CDKN3 specific shRNA (shCDKN3) and a scrambled control shRNA (NC) plasmids synthesized by Genepharma (Shanghai, China) were transfected into BLCA cells using Lip3000 (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. As for LDHA overexpression cell establishment, LDHA overexpressing pcDNA3.1 plasmid (pc-LDHA; 50 nM) were purchased from Shanghai GeneChem Co., Ltd. The efficiency of transfection was detected by western blot.

## Cell Cycle Analysis

The cell cycle of BLCA cells after transfected with CDKN3 shRNA was analyzed by propidium iodide (PI) solution containing 5 μg/mL RNase A (BD Biosciences). The results were detected with a flow cytometer.

## Western Blot

The cell lysates by RIPA Lysis Buffer were quantized protein concentration with BCA protein assay kit (Beyotime Institute of Biotechnology). Then 30 μg/sample of protein were separated on 10% SDS-PAGE and then transferred to PVDF membranes (Millipore, Massachusetts, USA). After blocking with 5% skimmed dry milk, the membranes were incubated with primary antibodies (LDHA, Invitrogen; CDKN3, PKM2, GLUT1, HK2, GAPDH, Abcam) for 2 h at room temperature. Subsequently, they were hybridized with HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 2 h. The membranes were visualized using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.). Protein gray value was quantified using ImageJ software (version 1.41; National Institutes of Health). The antibody used in this study was listed in Supplementary Table 1.

## Cell Viability Assay

BLCA cells transfected with or without CDKN3-shRNA were seeded into 96-well plate ($1*10^4$ cells/well) for 12 h. Then 10 μM GC was added for 24 h. After CCK-8 incubated for 2 h, Microplate reader was used to measure the absorbance at 450 nm.

## Transwell Experiment

To measure the migration ability of BLCA cells, transwell experiment was performed. 24-well culture plate with an 8.0-μm transparent PET membrane (Corning) was used to separate the upper and lower halves of each well. BLCA cells transfected with or without CDKN3-shRNA ($1*10^4$ cells/well) were seeded into the upper halves and 600 ul 1640 containing 10% FBS was added into lower halves. After 24 h, migrated cells were fixed and stained subsequently photographed and quantified.

## Cell Proliferation Assays

To detect cell proliferation, BeyoClick™ EdU Cell Proliferation Kit with Alexa Fluor 488 (Beyotime Biotechnology, Shanghai, CN) was used. Cells were seeded into 6-well plate. After transfection with CDKN3-shRNA, cells were treated with 10 μM GC for 24 h. Then 10 μM Edu was added according to instructions provided by the manufacturer. After counterstained with DAPI, Confocal microscopy (BioRad 400) was used to detected fluorescence.

## Extracellular Acidification Rate (ECAR) Assay

To detect glycolysis-capacity, cells (5000 cells/well) were seeded into Seahorse eight-well plate. After 12 h, 10mM glucose, 2 μM oligomycin, and 2-deoxy-D-glucose were added in order, and ECAR was measured by the Seahorse XFp instrument (Agilent Technologies, Inc., Santa Clara, CA).

## Glucose Uptake and Lactate Production Assays

Cells transfected with or without CDKN3-shRNA were collected. Then Screen Quest Colorimetric glucose uptake assay kit (AAT Bioquest, USA) and D-Lactate Assay Kit (BioAssay™, USA) were used to detect glucose uptake and lactate production according to the manufacturer's instructions. Before test, all metabolic indicators were calibrated with the total number of cells.

## Statistical Analysis

All data of the results, based on three independent experiments, are exhibited as the mean ± standard deviation (SD). The differences between groups were analyzed by two-tailed unpaired Student's *t* test with GraphPad Prism 8.0 (GraphPad Software, Inc.). The value of $P<0.05$ was considered to indicate a significant difference.

# Results

## CDKN3 is Highly Expressed in BLCA Tissues and is Associated with a Poor Prognosis

CDKN3 has been reported upregulated in various cancers. As shown in Figure 1A, analysis from the TCGA database revealed that CDKN3 expression was highly upregulated in various cancer tissues, including BLCA, compared with that in normal tissues. GEPIA analysis further confirmed that CDKN3 was highly expressed in BLCA tissues (Figure 1B), suggesting that CDKN3 is associated with BLCA progression. Furthermore, survival analysis demonstrated that the overall survival rate of patients with high CDKN3 expression was significantly lower than that of patients with low CDKN3 expression ($P$ = 0.043; Figure 1C). Additionally, the analysis from the TCGA database suggested that the CDKN3 expression was markedly increased in Stage I–IV of BLCA tissues compared with normal tissues (Figure 1D). Meanwhile, CDKN3 expression was significantly upregulated in BLCA tissues with N0-3 compared with normal tissues (Figure 1E). These results suggested that CDKN3 is upregulated in BLCA and associated with poor prognosis.

## Knockdown of CDKN3 Inhibits Proliferation and Migration of BLCA Cells

To explore the role of CDKN3 in BLCA progression, CDKN3-downregulated BLCA cells were established by transfection with shRNA-targeted CDKN3, as shown in Figure 2A. Then, CCK-8 assay and cell cycle detection were performed to determine the effect of CDKN3 on the proliferation of BLCA cells. The results showed that the down-regulation of CDKN3 obviously inhibited colony number and arrest cell in M0/M1 phase of T24 and RT4 cells (Figure 2B and C). Additionally, the Transwell experimental results showed that the number of migratory cells was significantly decreased in CDKN3 downregulated BLCA cells (Figure 2D). Altogether, these data revealed that CDKN3 plays a vital role in proliferation and migration of BLCA cells.

## Suppression of CDKN3 Overcomes GC Resistance of BLCA Cells

A previous study has proved that CDKN3 expression induces chemoresistance of colorectal cancer.[16] To investigate the effect of CDKN3 on chemoresistance of BLCA cells, GC-resistant BLCA cells (T24/GC and RT4/GC) were established by continuous GC treatment as described in Figure 3A. And the results of the CCK-8 assay revealed that the cell ability of T24/GC and RT4/GC cells was significantly increased compared with of T24 and RT4 parental cells after treatment with 10 μM GC (Figure 3B), further proving the GC resistance of T24/GC and RT4/GC cells. Then, the CDKN3 expression in BLCA parental and resistant cells was measured. The results revealed that the protein expression of CDKN3 was significantly upregulated in T24/GC and RT4/GC cells compared with the T24 and RT4 cells (Figure 3C), suggesting that CDKN3 correlated with BLCA chemoresistance to GC. To verify this hypothesis, firstly, the CDKN3 expression was knocked down in BLCA/GC cells, as shown in Figure 3D. Then, a CCK-8 assay was performed to detect the cell viability of BLCA/GC cells with CDKN3 knockdown after GC treatment. As supposed, CDKN3 knockdown significantly decreased the viability of BLCA/GC cells treated with GC (Figure 3E). Together, these results showed that CDKN3 knockdown abrogates the GC resistance of BLCA cells.

## Inhibition of CDKN3 Suppresses Glycolysis in BLCA/GC Cells

As reported in recent studies, glycolysis is a significant factor in inducing cancer chemoresistance. Thus, the glycolysis capacity in BLCA and BLCA/GC cells was explored. As shown in Figure 4A, the extracellular acidification rate (ECAR) in BLCA/GC cells was strikingly increased compared with BLCA cells, suggesting that glycolysis capacity was enhanced in GC-resistant BLCA cells. To further explore whether CDKN3 is associated with glycolysis reprogramming in BLCA/GC cells, the ECAR in BLCA/GC cells transfected with sh-CDKN3 was measured. The results revealed that CDKN3 knockdown significantly decreased the rates of extracellular acidification in BLCA/GC cells (Figure 4B). Consistently, CDKN3 reduction considerably reduced glucose uptake and lactate production in both BLCA/GC cells (Figure 4C and D). Collectively, these results demonstrated that the inhibition of CDKN3 suppressed glycolysis in BLCA/GC cells.

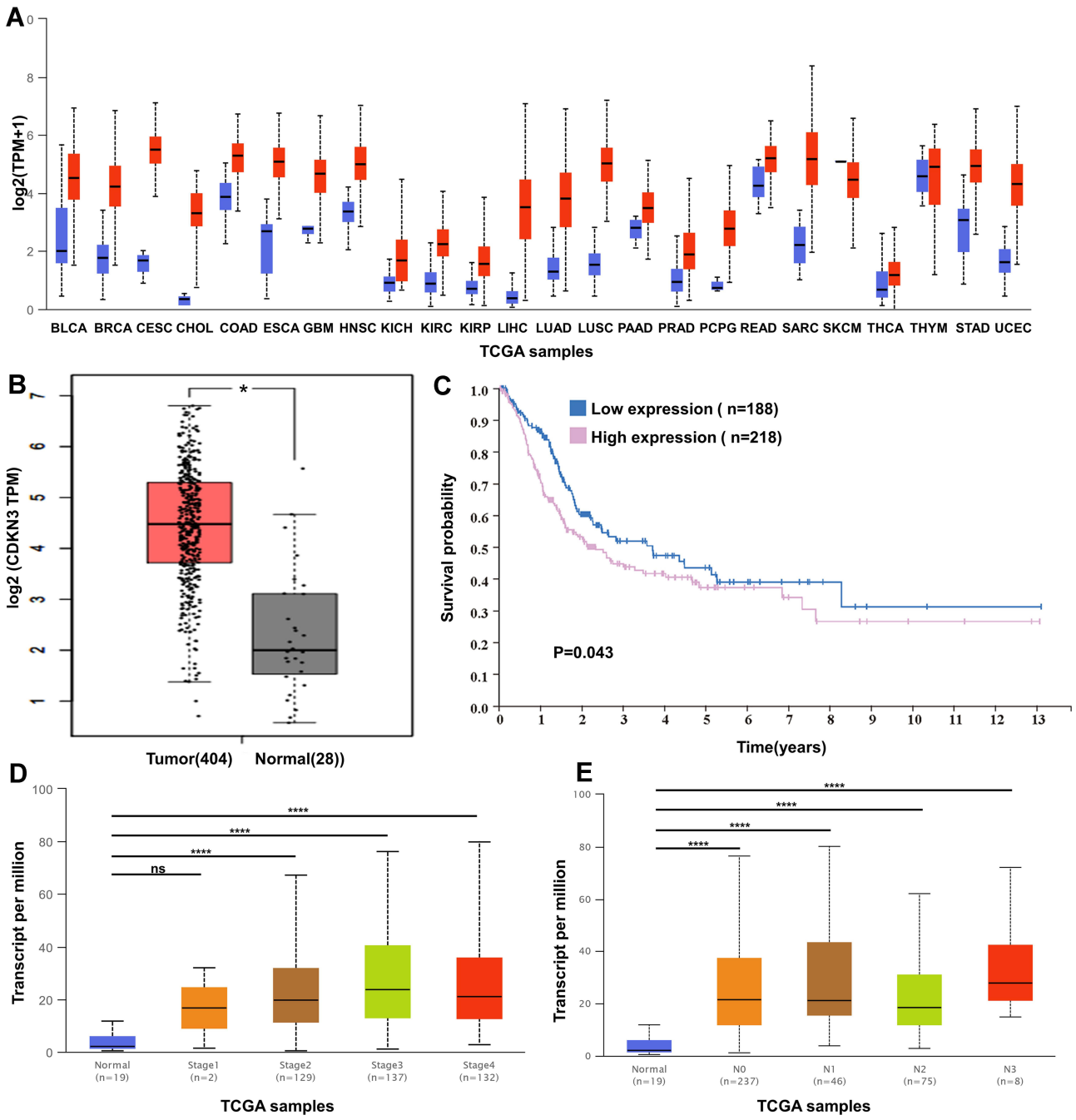


**Figure 1** CDKN3 is highly expressed in BLCA tissues and associated with poor prognosis. (**A**) Expression of CDKN3 in cancer tissues analyzed by TCGA database. (**B**) Expression of CDKN3 in BLCA and normal tissues. (**C**) Kaplan Meier overall survival curve of BLCA patients based on CDKN3 expression. (**D**) Expression of CDKN3 in BLCA based on the individual cancer stage. (**E**) Expression of CDKN3 in BLCA based on nodal metastasis status. Asterisks represent the degree of significance: P- values: ns presented $P \geq 0.05$, *$P < 0.05$ and ****$P < 0.0001$.

**Abbreviations**: BLCA, bladder cancer; BRCA, breast cancer; CESC, cervical and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; THCA, thyroid carcinoma; THYM, thymoma; STAD, stomach adenocarcinoma; UCEC, uterine corpus endometrial carcinoma.

## CDKN3 Regulates Glycolysis Through LDHA

To investigate the underlying mechanism of CDKN3 on glycolysis regulation, the protein expression of key enzymes in glycolysis progression was detected. The result revealed that CDKN3 shRNA treatment decreased the LDHA expression

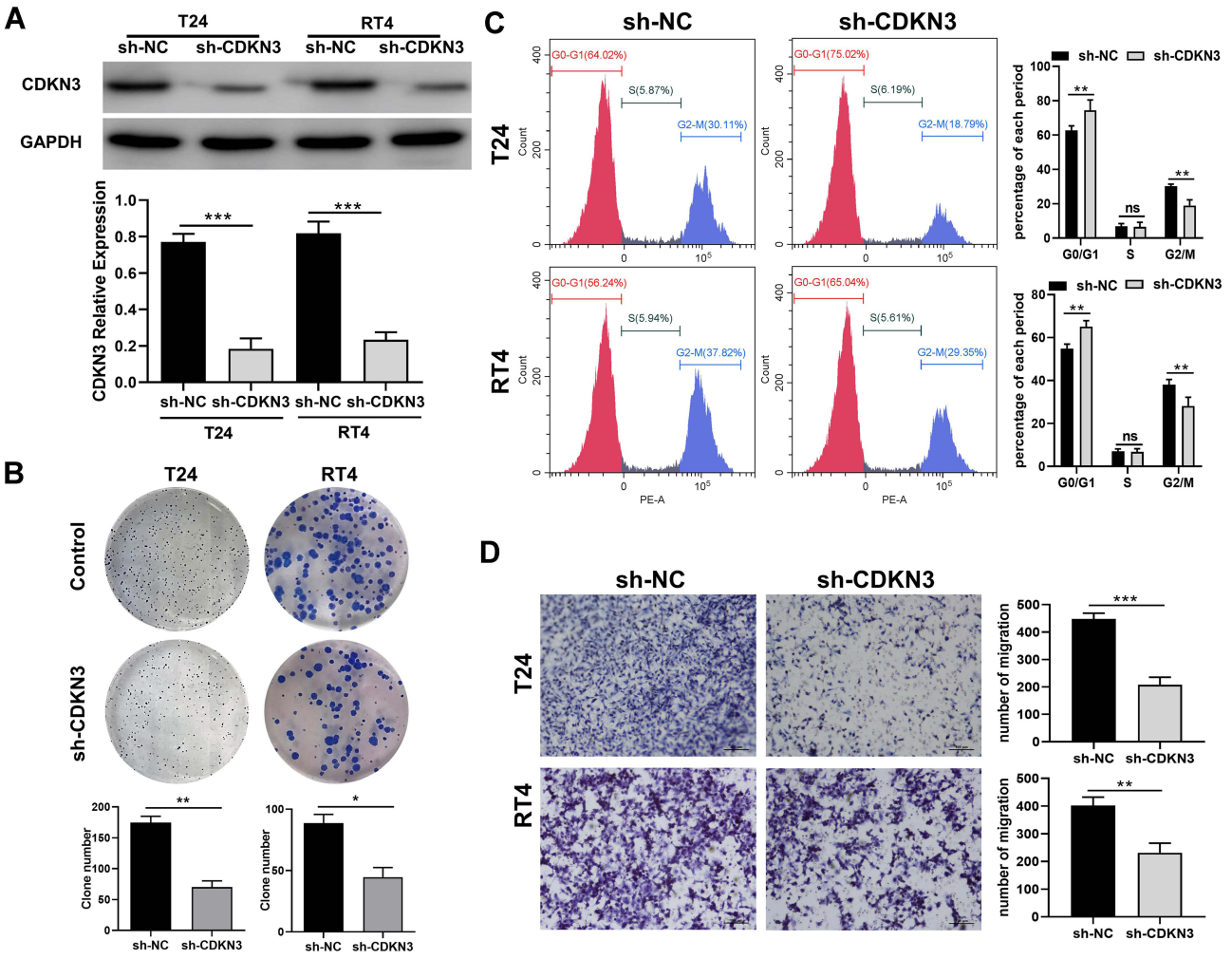


**Figure 2** Knockdown of CDKN3 inhibits proliferation and migration of BLCA cells. (**A**) Expression of CDKN3 protein in T24 and RT4 cells after transfection with CDKN3 shRNA. (**B**) Colony formation experiment was performed to detect the colony forming ability after CDKN3 knockdown in BLCA cells. (**C**) Cell cycle analysis by flow cytometry in BLCA cells transfected with CDKN3 knockdown vectors. (**D**) Transwell experiment was performed to measure the migratory ability of BLCA cells after CDKN3 downregulation (magnification: 200×). Asterisks represent the degree of significance: P- values: ns presented $P \geq 0.05$, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

but had no significant changes on the expression of GLUT1, HK2 and PKM2 (Figure 5A) in BLCA/GC cells. To further verify the regulation of CDKN3 on LDHA, a CO-IP experiment was performed. As shown in Figure 5B, CDKN3 binds to LDHA in both BLCA/GC cell lines. Furthermore, the correlation analysis of the TCGA database showed that CDKN3 mRNA expression was positively correlated with LDHA in BLCA tissues (R = 0.46, $P = 0$; Figure 5C). Next, LDHA was overexpressed in CDKN3 downregulated BLCA/GC cells. Experimentally, when LDHA was upregulated, CDKN3 knockdown induced a decrease in glucose consumption, and lactate production was markedly improved (Figure 5D and E). Together, these findings demonstrated that CDKN3 regulated glycolysis of BLCA/GC cells by binding LDHA.

## Suppression of CDKN3 Overcomes BLCA Cells Chemoresistance to GC via LDHA-Dependent Glycolysis

It has been proven that LDHA downregulation can sensitize gastric cancer cells to cisplatin.[17] Therefore, further exploration into whether LDHA was critical for CDKN3-regulated cancer chemoresistance was performed. As described in cell viability analysis, knockdown of CDKN3-decreased GC resistance of BLCA/GC cells were reversed by LDHA overexpression (Figure 6A). Consistently, the results of the Edu experiment showed that upregulated LDHA significantly improved the proliferation of BLCA/GC cell transfection with CDKN3-shRNA after GC treatment (Figure 6B). Accordingly, LDHA overexpression reversed the increase in apoptosis-related proteins induced by CDKN3

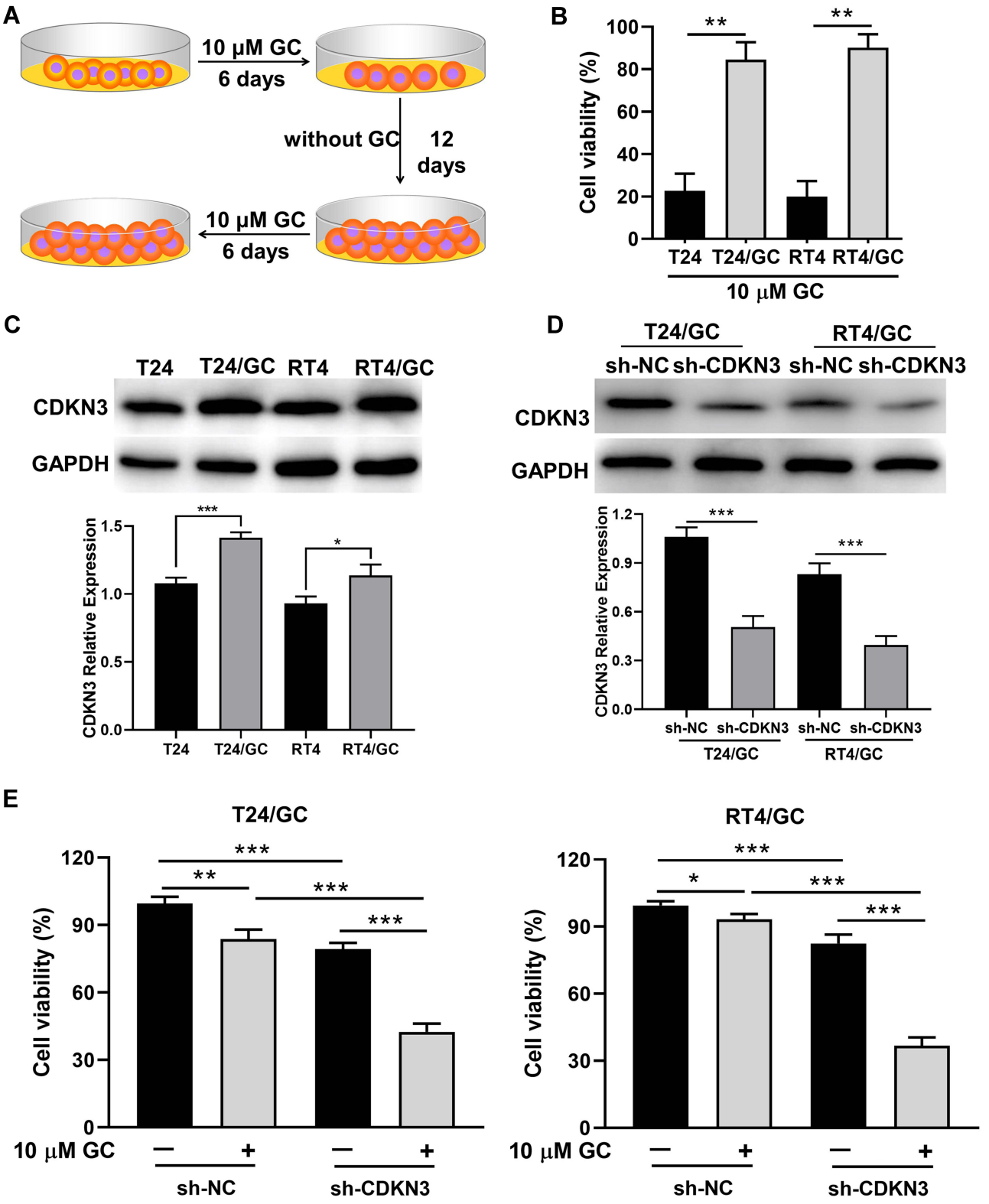


**Figure 3** Suppression of CDKN3 overcomes GC resistance of BLCA cells. (**A**) Schematic depicting the experimental approach of BLCA/GC cells established. (**B**) Cell viability of BLCA cells and BLCA/GC cells treatment with 10 μM GC detected using CCK-8 assay. (**C**) Expression of CDKN3 protein in BLCA and BLCA/GC cells was detected by western blot. (**D**) Western blot was used to analyze the expression of CDKN3 in BLCA/GC cells after CDKN3 knockdown. (**E**) Cell viability of T24/GC and RT4/GC cells transfected with CDKN3-shRNA after treatment with GC was measured by CKK-8 assay. Asterisks represent the degree of significance: P- values: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

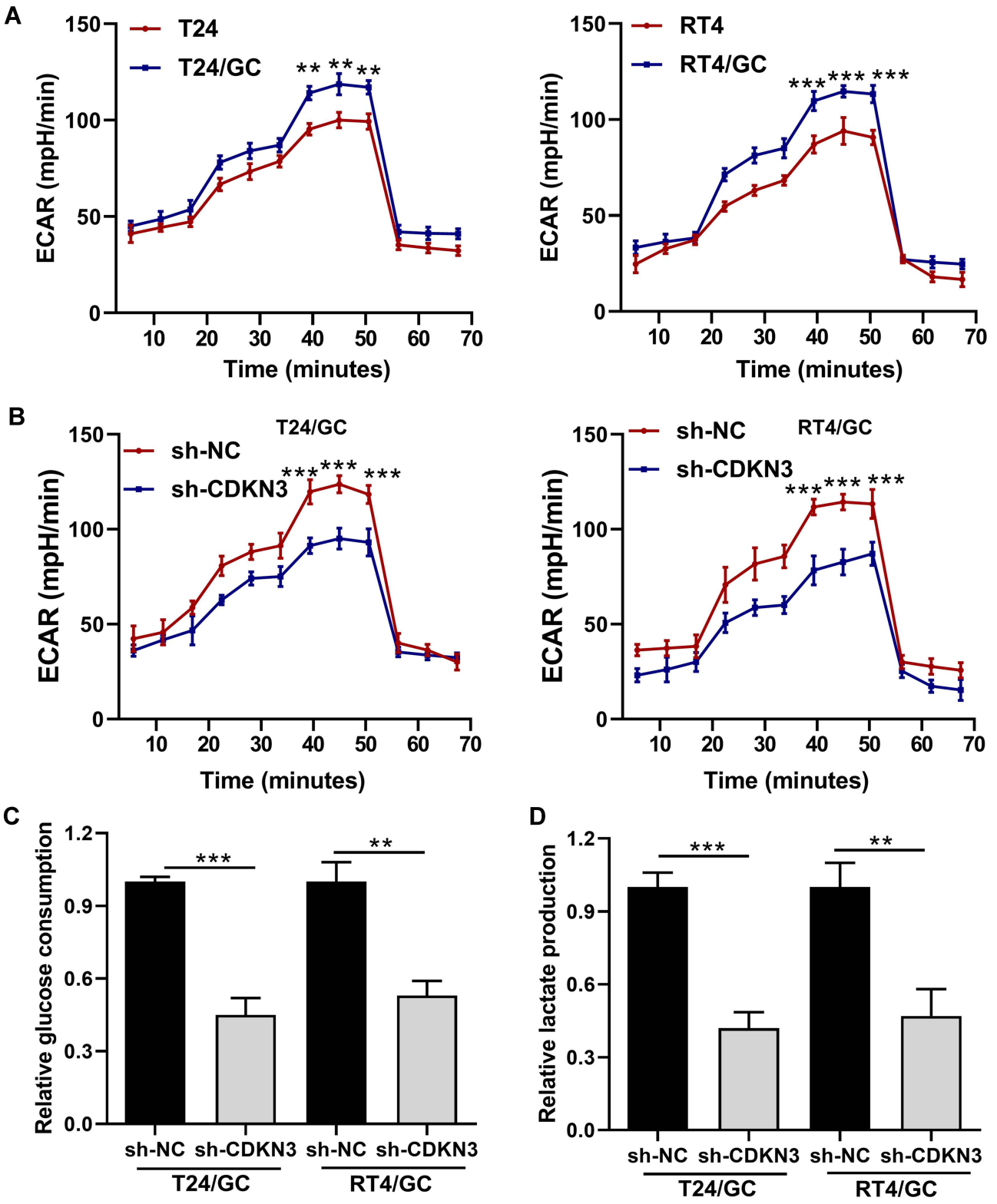


**Figure 4** Inhibition of CDKN3 suppresses glycolysis in BLCA/GC cells. (**A**) ECAR in BLCA and BLCA/GC cells was measured by Seahorse XFp instrument. BLCA/GC cells were transfected with CDKN3 knockdown vector for 24 h. (**B**) ECAR, (**C**) glucose uptake, and (**D**) lactate production were analyzed. Asterisks represent the degree of significance: P- values: $^{**}P < 0.01$ and $^{***}P < 0.001$.

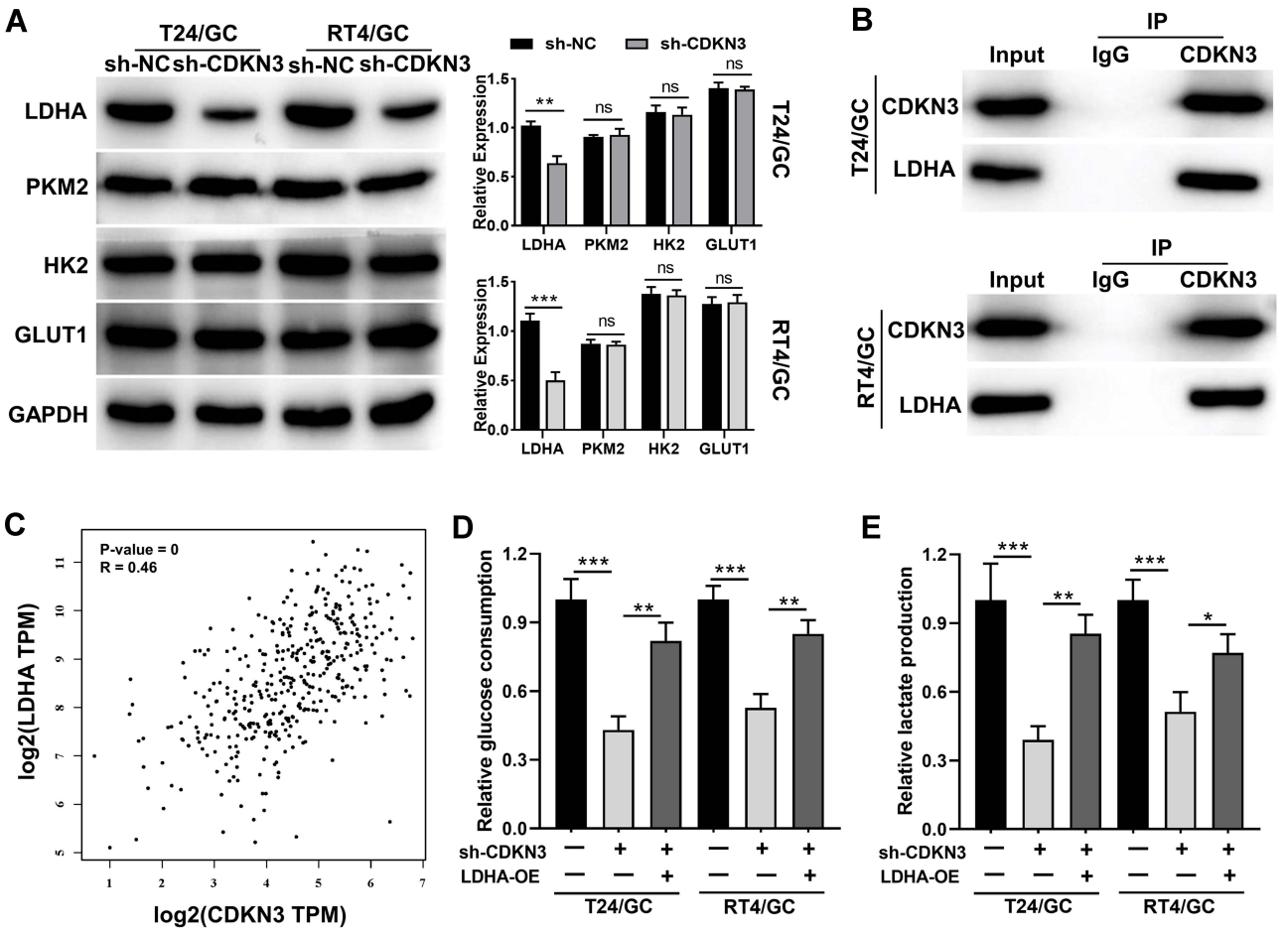


**Figure 5** CDKN3 regulates glycolysis through LDHA. (**A**) Western blot was used to detect the protein expression of LDHA, GLUT, HK2, and PKM2 in BLCA/GC cells transfected with or without CDKN3 shRNA. (**B**) CO-IP-western blot analysis of T24/GC and RT4/GC cells. The lysates were incubated with anti-CDKN3 antibodies, and the fractionated immunoprecipitates were probed on western blots using antibodies against CDKN3 and LDHA. (**C**) Correlation analysis of TCGA database with GEPIA between CDKN3 and LDHA in BLCA tissues. Knockdown of CDKN3 and overexpression of LDHA in BLCA/GC cells. The level of (**D**) glucose uptake and (**E**) lactate production was measured. Asterisks represent the degree of significance: P- values: ns presented $P \geq 0.05$, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

downregulation in GC treated-BLCA/GC cells (Figure 6C). Collectively, these results suggested that CDKN3 regulates chemoresistance of BLCA cells via LDHA-dependent glycolysis.

## Discussion

BLCA is a common malignancy of the urological system. The incidence and mortality of BLCA in men (accounting for respective 9.5 and 3.3 per 100,000 among men) are approximately 4 times those in women worldwide.[2,18] Despite advancements in treatment strategies, such as neoadjuvant chemotherapy, patients with advanced or metastatic BLCA frequently have a poor prognosis due to its chemoresistance.[19–21] Thus, it benefits BLCA therapy development to identify the underlying mechanisms of chemoresistance of BLCA cells.

The study of Wang et al reported that CDKN3 were identified the top 1 seed gene of top cluster. The level of CDKN3 mRNA and protein was significantly increased in esophageal squamous cell cancer (ESCC) patients compared to controls, and was significantly correlated with lymph node metastasis and clinical staging of ESCC patients.[22] More explicitly, CDKN3 was identified as a promoting gene in esophageal squamous cell carcinoma leading to improve the ability of proliferation, invasion and migration by activating AKT pathway.[23] Furthermore, targeted CDKN3 and suppressed expression repress the progression of non-small-cell lung cancer in vivo and in vitro.[24] These are consistent with the results in our study, which showed that CDKN3 is overexpressed in BLCA tissues and associated with a lower

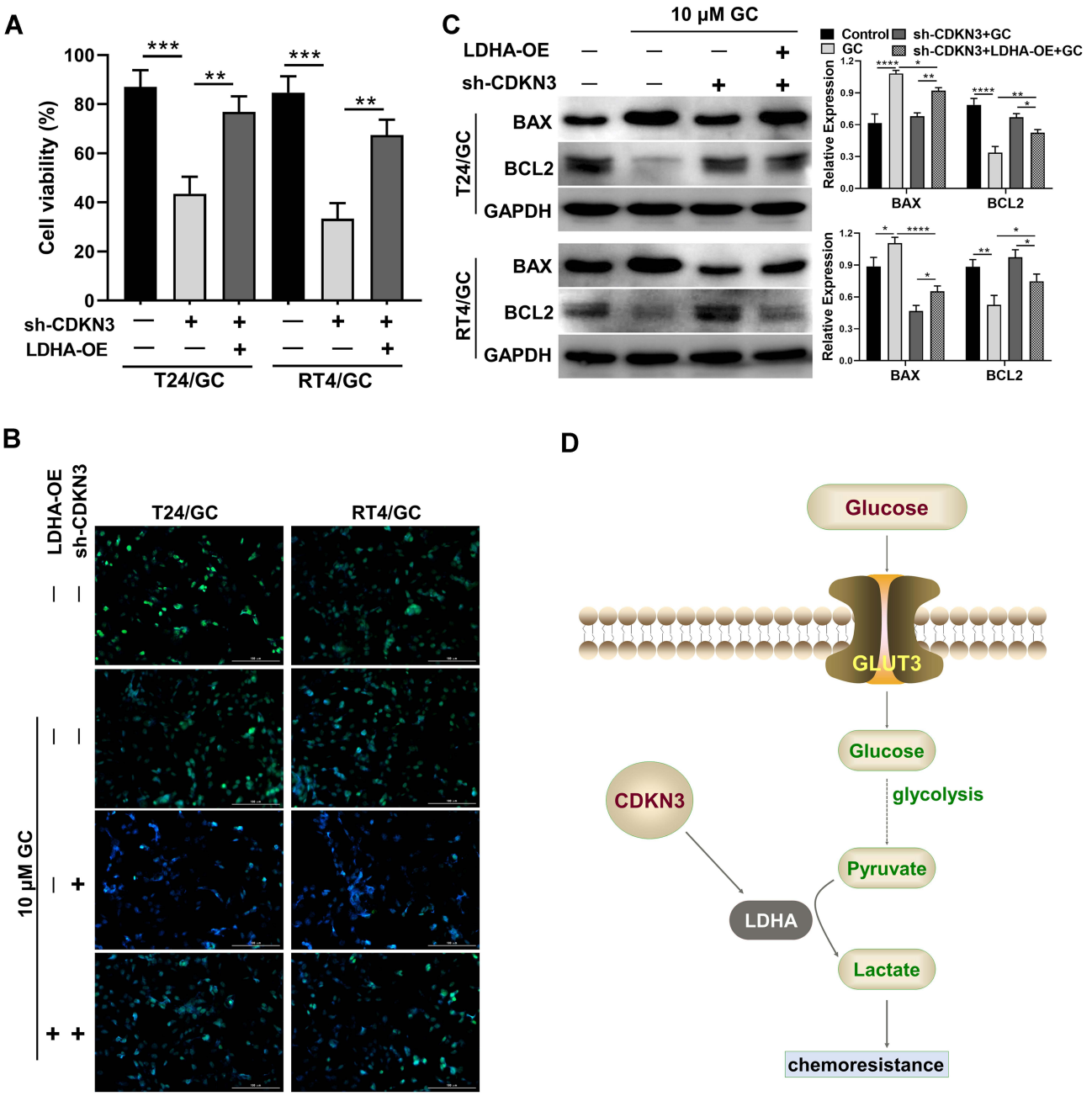


**Figure 6** Suppression of CDKN3 overcomes BLCA cells chemoresistance to GC via LDHA-dependent glycolysis. Knockdown of CDKN3 and overexpression of LDHA in BLCA/GC cells then treated with 10 μM GC for 24 h. (**A**) Cell viability was detected by CCK-8 assay. (**B**) Proliferation in BLCA/GC cells detected using the EdU experiment (bar=100 μm). (**C**) Expression of bcl-2 and BAX was detected by western blot. (**D**) The proposed molecular mechanism by which CDKN3 regulates BLCA chemoresistance through modulating glycolysis. Asterisks represent the degree of significance: P- values: **P* < 0.05, ***P* < 0.01, ****P* < 0.001 and *****P* < 0.0001.

overall survival rate. Additionally, our observation also proved the promotion of CDKN3 on the proliferation, cell cycle arrest and migration of BLCA cells. Altogether, it is suggested that CDKN3 acts as an oncogene in BLCA.

A previous study reported that most patients with bladder cancer develop disease progression within 8 months of receiving cisplatin-based regimens, and the median survival was only 13–15 months.[25] It has been reported that expression of CDNK3 is positively correlated with the chemoresistance of esophageal cancer (ESCA) patients and suppression of CDKN3 recover cisplatin sensitivity of cisplatin-resistant ESCA cells.[26] Consistently, the study of Li et al revealed that CDKN was upregulated in colorectal cancer patients with cisplatin resistance, and the knockdown of CDKN3 reduced the viability of cisplatin-resistant colorectal cancer cell lines.[16] Thus, we further explored the role of

CDKN3 in BLCA chemoresistant using the GC-resistant cells. As expected, these results demonstrated that the level of CDKN3 expression was remarkedly elevated in GC-resistant cells, and inhibition of CDKN3 obviously suppressed the cell viability of GC-resistant BLCA cells treated with GC. These results suggested that CDKN3 enhances the chemoresistance of BLCA cells, which is consistent with the finding of Li et al.[16]

Increasing evidence shows that aerobic glycolysis is an apparent characteristic of energy metabolism in cancer cells, and an increase in glycolysis is beneficial for tumour progression and recurrence, especially chemoresistance of cancer cells.[9,27,28] Jia et al reported that activation of glycolysis improves tumor malignancy and chemoresistance of hepatocellular carcinoma.[29] And the study of Lin et al found that POU2F1 directly bound to the ALDOA promoter and then decreased the sensitivity to oxaliplatin in colon cancer cells by enhancing glycolysis.[30] Furthermore, targeting metabolism reprogramming was an efficient method to overcome the chemoresistance of pancreatic ductal adenocarcinoma.[31] Consequently, targeting glycolysis has been indicated to be an effective means for overcoming chemoresistance, thereby improving cancer therapy. To the best of our knowledge, there is no study related to the effect of CDKN3 on glycolysis. This study found that knockdown CDKN3 decreased ECAR, glucose uptake and lactate production in GC-resistant BLCA cells. We, therefore, conclude that CDKN3 plays a critical role in the promotion of glycolysis in GC-resistant BLCA cells.

LDHA, a key enzyme for regulating glycolysis progression, has been reported to be overexpressed in a range of human cancers and associated with a worse prognosis.[17,32,33] Activation of LDHA could eventually elevate lactate level and glycolysis, eventually, promote the tumorigenesis of lung cancer.[34] Consistent with this, data of this study found that CDKN3 could regulate the protein expression of LDHA by binding to LDHA directly, with the TCGA database exhibiting a close correlation between CDKN3 and LDHA mRNA expression in BLCA tissues. Additionally, LDHA overexpression could reverse the inhibition of CDKN3 knockdown on glycolysis in BLCA/GC cells. Our results thus offer novel insight into a mechanism whereby CDKN3 can promote glycolysis via LDHA regulation.

Cisplatin-based treatment strategy is still the most commonly used in adjuvant treatment for bladder cancer. However, cisplatin resistance remains a serious problem for bladder cancer patients and is one of the burning issues of our times. Currently, combined treatment modalities that target glycolytic pathways hold promise for the treatment of chemoresistant cancer cell. Increasing evidence shows that downregulation of glycolysis enhances inhibition of proliferation by chemotherapy drug in ovarian cancer, lung cancer, and leukemia.[35] Because LDHA is a key regulator in glycolysis, targeting LDHA may play an important role in CDKN3-regulated chemoresistance. Indeed, our results showed that LDHA upregulation enhanced cell viability and inhibited apoptosis of CDKN3-knockdown BLCA/GC cells after treatment with GC, suggesting that LDHA-dependent glycolysis is essential in CDKN3-regulated chemoresistance. Overall, therapeutic interventions that suppress CDKN3 expression have the potential effect on surmounting chemoresistance in BLCA.

In conclusion, our data in this study demonstrated that CDKN3 was an oncogene in BLCA. Furthermore, knockdown of CDKN3 overcomes chemoresistance to GC of BLCA cells via LDHA-dependent glycolysis (Figure 6D). Thus, our study suggested CDKN3 as a novel therapeutic target for the treatment of BLCA targeting chemoresistance. However, in vivo experiments should be performed to further confirm these observations and the mechanism of CDKN3 regulation of LDHA has not been investigated. Addressing such limitations can serve as a basis for conducting future studies.

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## Disclosure

The authors declare no conflicts of interest in this work.

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