

## Original Article

# A critical role of CDKN3 in gastric cancer and regulates tumor cell proliferation

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**Abstract:** The aim of this study was to clarify the role of cyclin-dependent kinase inhibitor 3 (CDKN3) in the development of gastric cancer. CDKN3 functions as a key negative regulatory of cell cycle progression. Deregulation or mutations of CDKN3 have been implicated in various cancers. Small interfering RNA was used to silence CDKN3 expression in gastric cancer derived SGC-7901 cells. Proliferation, invasion and adhesion assays were done in engineered cells to assess the effect of CDKN3 silencing. Our data showed that CDKN3 loss dramatically decreased cell proliferation and significantly decreased cell invasive and adhesion potential. Additionally, in the limited set of genes whose expression status was determined CDKN3 loss was associated with decreased CDK1 and CDK2 expression. These results show that CDKN3 silenced in SGC-7901 cells are markedly influenced the genetic and phenotypic in gastric cancer, indicating that CDKN3 preferentially targets certain gene promoters.

**Keywords:** Gastric cancer, CDKN3, cell cycle, proliferation, invasion

## Introduction

Despite a steady drop incidence over the last decades, gastric cancer (GC) is still a major health problem worldwide due to its frequency, poor prognosis and limited treatment options [1]. GC is a multifactorial disease and risk factors including diet, *helicobacter pylori* infection, genetic factors and age [2]. Currently, surgical resection remains the most important way to prolong gastric cancer patients, but the recurrence rate frequently after surgery [3]. Compared with other more intensively investigated cancers, such as breast, colorectal cancer, the molecular mechanism of gastric cancer are largely unclear. Therefore, it is essential to identify novel targets and define molecular mechanisms to develop effective clinical therapeutic methods of GC patients.

Cyclin-dependent kinase inhibitor 3 (CDKN3, also called CDI1 or KAP) belongs to the protein phosphatases family and has dual function in regulating the cell cycle [4-6]. The gene encoding CDKN3 protein is located on chromosome mapping 14q22 [7]. CDKN3 is a cyclin-dependent kinase inhibitor that binds to CDK2 kinase, and also interacts with CDK1 [6, 8]. Fur-

thermore, CDKN3 was reported to be deleted or over-expressed in a variety of cancers [9, 10]. In the breast and prostate cancers, CDKN3 is highly expressed and can inhibit the transformation by antisense CDKN3 expression [9]. In addition, renal cell carcinoma also has elevated levels of CDKN3, and high CDKN3 expression enhances xenograft tumor growth and cell proliferation, suggesting CDKN3 is an oncogene [11]. These findings suggest that CDKN3 may potentially play an important role in cancer, but the expression pattern and biological functions of CDKN3 in human gastric carcinoma remain to be elucidated.

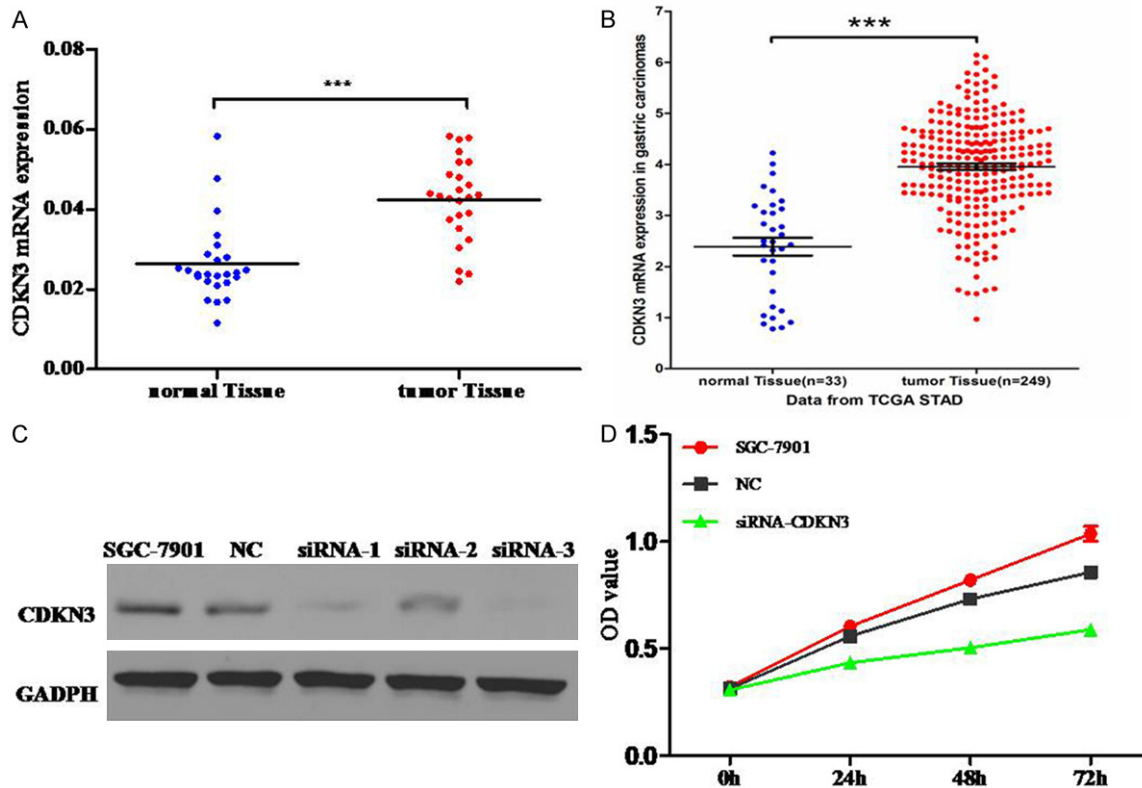
In this study, we aimed to assess the function and molecular mechanisms of CDKN3 in GC. We found that CDKN3 was over-expressed in GC, and its expression was correlated with cell cycle signaling. We also demonstrated the effect of CDKN3 siRNA on proliferation, cell cycle distribution, invasion and adhesion.

## Materials and methods

### Cell culture and transfection

Human gastric cancer SGC-7901 cells were purchased from American Type Culture Collection

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**Figure 1.** The expression of CDKN3 in gastric cancer. A. The mRNA level of CDKN3 in 25 patients' gastric cancer tissues and adjacent tissues ( $***P < 0.001$ ). B. CDKN3 mRNA expression was significantly increased in gastric tumor tissues when compared with the adjacent tissues from TCGA dataset ( $***P < 0.001$ ). C. Western blot analysis of CDKN3 protein expression after SGC-7901 cells was transfected with CDKN3-shRNA or shRNA control (NC). D. Cell proliferation was detected at 24, 48 and 72 h after transfection. Data were based on at least 3 independent experiments, and shown as mean  $\pm$  SD. NC: Negative Control.

(ATCC) and cultured in RPMI1640 containing 10% heat-inactivated fetal bovine serum (FBS), antibiotics 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin in a humidified incubator of 5% CO<sub>2</sub> at 37°C. Transfection of SGC-7901 cells was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Transfections were performed in triplicate for each treatment.

### TaqMan real-time PCR

Total RNA was extracted using TRizol reagent (Invitrogen, Carlsbad, CA, USA). The expression level of CDKN3 was quantified by TaqMan real-time assay. The following primers were used for real-time PCR: human CDKN3 forward, (5'-AG-CTGCACATCTATCATC-3') and reverse (5'-CACT-GGTGGTTTCATTTT-3'). Expression level of GADPH (forward: 5'-CACCCACTCCTCCACCTTTG-3', reverse: 5'-CCACCACCCTGTTGCTGTAG-3') was used as a control.

### Cell growth assay

After transfection with vector control or CDKN3-knockdown plasmid, the cells were seeded into 96-well plates at 2000 cell/well. The cck-8 assay was used to determine relative cell growth according to the manufacturer's instructions. Data shown are representative of three independent experiments.

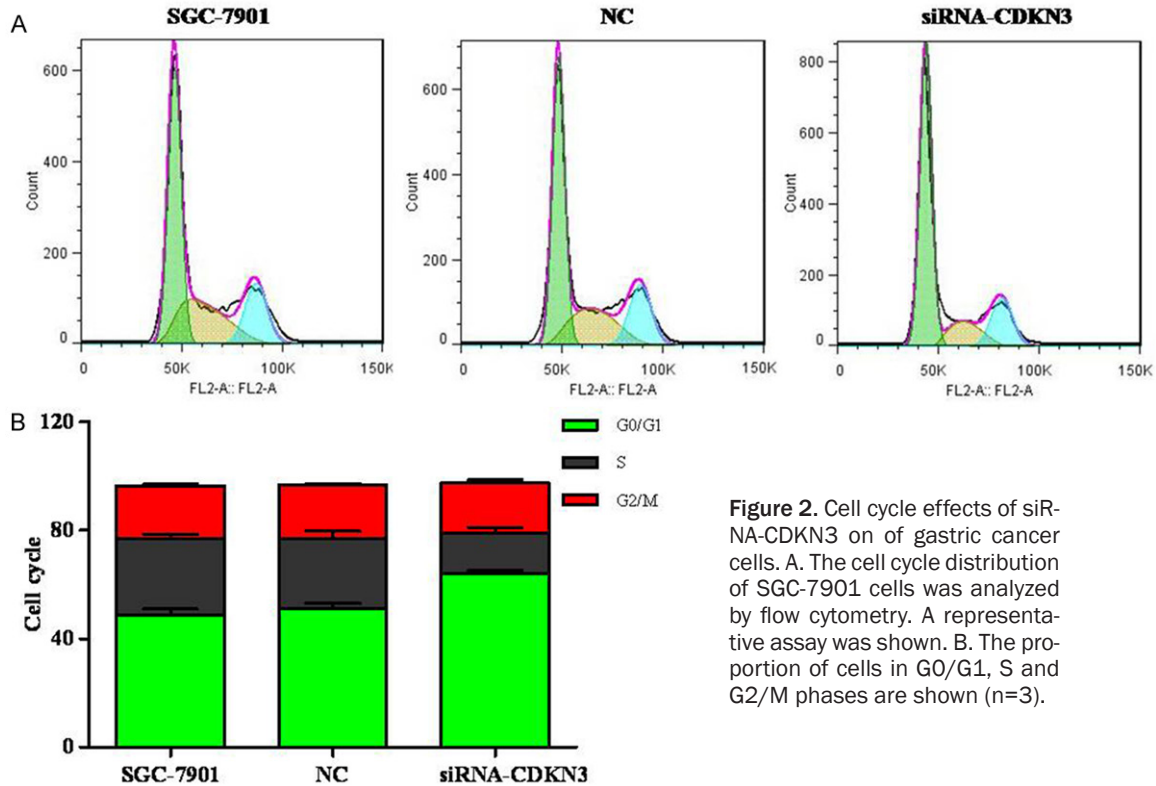
### Cell cycle assay

Briefly,  $5 \times 10^5$  of cells were harvested after transfection at the indicated time. Cells were fixed with 75% cold ethanol, washed twice with PBS, and then staining with 50  $\mu$ g/ml propidium iodide (PI) with 0.1% RNase A for 30 min. The samples were analyzed with a fluorescence-activated cell sorter (BD Bioscience).

### Transwell invasion assay

Transwell invasion assay were performed using 24-well cell culture inserts with matrigel inva-

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**Figure 2.** Cell cycle effects of siRNA-CDKN3 on gastric cancer cells. A. The cell cycle distribution of SGC-7901 cells was analyzed by flow cytometry. A representative assay was shown. B. The proportion of cells in G0/G1, S and G2/M phases are shown (n=3).

sion chambers. Briefly,  $1 \times 10^5$  cells were re-suspended and added into the inserts. RPMI-1640 with 10% FBS was added to the lower chamber. After 48 h, cells on the upper surface were washed and fixed with methanol, then stained with 0.5% crystal violet for 30 min. The number of invading cells were counted and photographed under a  $200\times$  microscope.

### Cell adhesion assay

To determine the matrix-dependent adhesion, 12-well plates was used. Cell suspension ( $1 \times 10^5$  cells/ml) was added to the well and incubated for 1 h at  $37^\circ\text{C}$ . Adherent cells were fixed with 4% methanol and stained with crystal violet for 20 min. The number of adherent cells were photographed and counted from three random selected  $200\times$  fields of microscope.

### Western blot

After transfection, SGC-7901 cells were collected and proteins were harvested. Proteins were resolved on an SDS-PAGE and then transferred onto a nitrocellulose membrane. Antibody to CDKN3, CDK1, CDK2 (Abcam, Cambridge, USA) and antibody to GADPH (CST, Beverly, USA) were incubated with the blot overnight at  $4^\circ\text{C}$ . Membranes were washed and incubated with

respective secondary antibodies and were visualized by ECL. Shown are representative data from individual experiments that were repeated at least twice.

### Statistical analysis

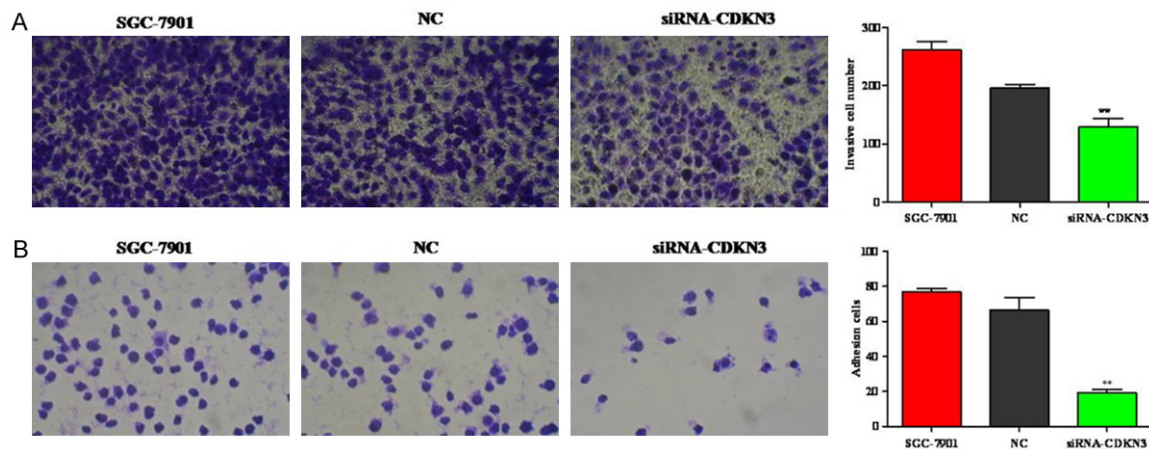
Data are represented as means  $\pm$  SD of three independent experiments, each performed in triplicate. Student's t-test was used to analyze the differences between two groups. Statistical significance was performed using GraphPad Prism 5.0 (San Diego, CA, USA). A level of  $P < 0.05$  was regarded as significant.

## Results

### High expression of CDKN3 is frequent in GC

To study the biological role of CDKN3 in GC, qRT-PCR was performed to detect the expression levels of CDKN3 in GC patients' tissues. We collected 25 gastric cancer tissues and their adjacent normal tissues. As shown in **Figure 1A**, CDKN3 expression level was higher in GC tissues compared with adjacent normal tissue control. To validate this observation, we used data from The Cancer Genome Atlas (TCGA) GC cohort to analysis its mRNA expression. A notable increase CDKN3 mRNA expres-

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**Figure 3.** Knockdown of CDKN3 inhibits invasion and adhesion of gastric cancer cells. A. Matrigel invasion chambers were used to assess the invasiveness of SGC-7901 cells. The number of invading cells was counted and averaged over three randomly selected fields. B. Cell-matrix adhesion assay was used to assess the adhesion ability of SGC-7901 cells.

sion in gastric cancer tissue compared with normal tissue (**Figure 1B**).

### *CDKN3 knockdown inhibits SGC-7901 cell growth in vitro*

CDKN3 mRNA was interfered in SGC-7901 cell line. Western blot was employed to identify the interference efficient and showed that protein level declined notably in CDKN3 siRNA group in comparison with mock group (**Figure 1C**). To investigate the functional significance of siRNA-CDKN3 in SGC-7901, we performed CCK-8 assay in 24, 48 and 72 h. As demonstrated in **Figure 1D**, the cell viability at 24, 48 and 72 h after transfection was weakened, compared to the vector control groups.

To further validate the cell proliferation inhibition of anti-CDKN3, cell cycle was analyzed in SGC-7901 cells (**Figure 2**). Cell cycle analysis showed that silencing CDKN3 notably increased the rate of G0/G1 phase cells and reduced S phase cell population. These results indicated that silencing CDKN3 in gastric cancer cells may inhibited cell proliferation by arresting cell cycle progression in G0/G1 phase.

### *CDKN3 siRNA inhibited cell invasion and adhesion of SGC-7901 cells*

We wonder whether CDKN3 regulated metastasis of gastric cancer cells. To investigate the invasion-inhibiting function of CDKN3 knockdown in gastric cells, the invasion capacity of

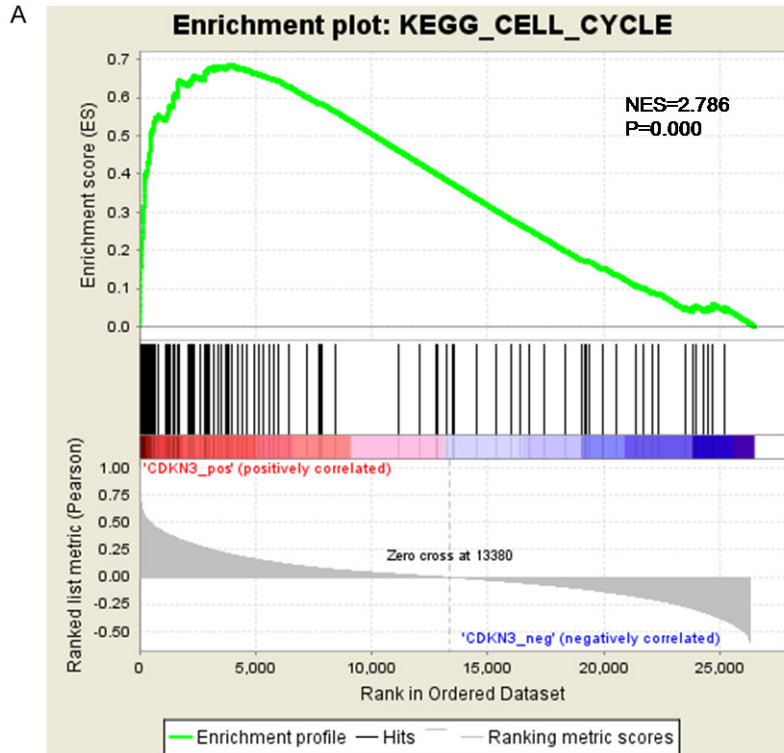
SGC-7901 cells were evaluated by transwell assay. As shown in **Figure 3A**, siRNA-CDKN3 significantly reduced the cell invasion ability compared with NC. CDKN3 knockdown also reduced abilities of cell adhesion to the extracellular matrix by adhesion assay (**Figure 3B**). These results indicate that CDKN3 knockdown inhibits motility of gastric cancer cells.

### *Identification of genes and signaling associated pathways*

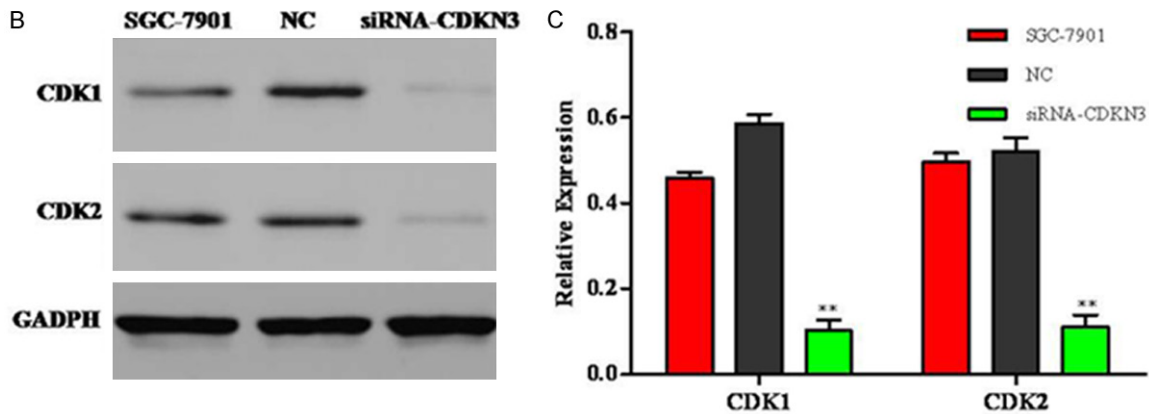
To probe the CDKN3-associated pathways on an unbiased basis, we performed GSEA using data from the Gene Expression Omnibus database. Among all the 'KEGG pathways' gene sets, the cell cycle pathway was identified with the significant association with CDKN3 expression (**Figure 4A**). Then we investigated the cell cycle regulations proteins CDK1, CDK2 by western blot, which show that the proteins were down-regulated in CDKN3 siRNA cells compared to NC (**Figure 4B, 4C**).

## Discussion

In the present study, we investigated the biological function of CDKN3 in gastric cancer. The clinical data shows that CDKN3 was high-expressed in gastric cancer patients that also supported by the data from TCGA STAD. In vitro experiments, the results showed that knockdown of CDKN3 in gastric cancer cells inhibited cell growth and metastasis. Cell cycle pathway was also inhibited in CDKN3 siRNA condition.



**Figure 4.** Levels of cell cycle related proteins were detected by western blot. GADPH was used as the loading control. A. GSEA was performed using KEGG dataset. Cell cycle pathway was identified with the strongest association with CDKN3-higher expression. B, C. Expression of two cell cycle related proteins CDK1 and CDK2 were evaluated by western blot. Data were based on at least 3 independent experiments, and shown as mean  $\pm$  SD. NC: Negative Control.



Thus, CDKN3 may serve as a useful prognosis marker and potential target for treatment of gastric cancer. CDKN3 was reported to be over-expression in prostate, breast, hepatocellular and some other cancers [9, 12, 13]. Here, we found that CDKN3 was frequently up-regulated in GC tissues and cell lines, suggesting that increased expression of CDKN3 is associated with GC. Then we examined the effect of CDKN3 on proliferation, cell cycle, invasion and adhesion in SGC-7901 gastric cancer cells.

Cell proliferation is a key process in the growth of carcinoma. We found that knockdown of CDKN3 significantly reduced cell growth ra-

te. Moreover, cell cycle analysis showed that CDKN3 siRNA inhibited cell proliferation by promoting G1/S cell cycle transition in gastric cancer cells. CDKN3 has been investigated to be correlated with cell proliferation in some cancers [14, 15]. Abnormal changes of cyclin-dependent kinases (CDKs) will lead to tumorigenesis [16]. CDK1 and CDK2 are key proteins through running G1/S and G2/M phase restriction point in cell cycle [17-19]. We used GSEA dataset identified that CDKN3 significant association with cell cycle signaling pathway, then the protein levels of CDK1 and CDK2 were detected. The over-expression of CDK1 and CDK2 is closely related to tumor progression

and poor prognosis [20]. The results suggested CDKN3 siRNA inhibited cell proliferation might by decreasing both CDK1 and CDK2 cell cycle related protein expression levels.

Metastasis is a key event in tumor progression. The role of CDKN3 on cell invasion and adhesion is rarely reported and prompted us to investigate the relationship with CDKN3. In renal cancer cells, over-expression of CDKN3 has been reported to have greater ability of cell invasion [11]. The functional study demonstrated that there were significantly decreased changes of cell invasion and adhesion after CDKN3 interfered, indicating CDKN3 could affect the metastasis potential of GC cells. To our knowledge, this is the first report of CDKN3 on tumor adhesion.

In summary, our study provides for the first time that CDKN3 played a key role in proliferation, invasion and adhesion of gastric cancer cells, and CDKN3 might regulate the biological progress through cell cycle related signal pathways. CDKN3 provide a therapeutic strategy in GC treatment, and it needs further study in vitro and vivo to validate its therapeutic function in the future.

### Disclosure of conflict of interest

None.

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