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Original Article

CDKN3 knockdown reduces cell proliferation, invasion and promotes apoptosis in human ovarian cancer

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Abstract: Cyclin-dependent kinase inhibitor 3 (CDKN3) has been reported to promote tumor genesis. The aim of this study is to investigate the possible mechanisms of silence of CDKN3 exerting the suppressive role on epithelial ovarian cancer (EOC). To study the potential function of CDKN3 enrolled in the regulation of ovarian tumor, we monitored the EOC cells SKOV3 and HO8910 behaviors including proliferation, cell cycle, apoptosis and invasion. First, we found that CDKN3 was frequently over-expressed in EOC. Functional studies showed that silence of CDKN3 inhibited cancer cell proliferation by promoting cell cycle progression in G1 phase, decreased cell invasion and promoted EOC cells apoptosis. Western blot analysis of CDKN3-silence cells revealed down-regulation of DNA-replication and cell cycle related proteins. And, a significant correlation level of CDKN3 was observed which has been demonstrated to be a novel oncogene. These findings indicated that CDKN3 might serve as a useful potential target for treatment of ovarian cancer.

Keywords: CDKN3, ovarian cancer, proliferation, invasion, apoptosis

Introduction

Ovarian cancer is complex disease composed of different types. Epithelial ovarian cancer (EOC) is the leading cause of death from gynecologic malignancies in women and accounts for 4% of all cancers [1]. Each year, despite the medical and surgical improvements, the long-term survival remains poor and has high rates of recurrence [2]. Till now, the prognosis and treatment of ovarian cancer are still unfavorable, which are associated with unsatisfactory prognosis and high mortality [3, 4]. The initiation and progression of EOC still poorly understand [5]. Therefore, there is an urgent requirement to investigate the molecular mechanisms underlying ovarian tumorgenesis and identify novel therapeutic and diagnostic strategies against this disease.

Cyclin-dependent kinase inhibitor 3 (CDKN3, also called CDI1 or KAP) belongs to the protein phosphatases family, plays a key role in regulating cell division [6-8]. Chromosomal mapping describes the sites of gene encoding CDKN3 protein is located on 14q22 [9]. CDKN3 is showed essential for mitosis and down-regulated in brain tumors, has also been suggested to function in some of other cancers [8, 10]. High expression gene CDKN3 inhibited cell cycle that associated with hepatitis/cirrhosis and hepatocellular cancer [11]. Over-expression of CDKN3 significantly enhances cell proliferation, xenograft tumor growth and resistance to apoptosis in renal cancer cells and associated with poorly differentiated [12]. In leukemic cells, CDKN3 acted as a tumor suppressor that delayed G1/S transition in Bcr-Abl-induced tumorigenesis [13]. This gene has been reported to be deleted or over-expressed in some of cancers, but the expression and biological functions of CDKN3 in human ovarian cancer remain to be elucidated. As so, more work is needed to dissect the role of the CDKN3 in ovarian cancer.

In this study, we aimed to assess the role of CDKN3 in ovarian cancer. We found that CDKN3 was over-expressed in ovarian cancer. Firstly, we found that knockdown of CDKN3 was involved in cell proliferation, apoptosis and invasion. And western blot showed that siRNA-

CDKN3 notably inhibited the cell cycle and DNA replication signal pathways related protein. These data suggest that CDKN3 is a potential targeted anticancer therapeutics of ovarian cancer.

Materials and methods

Cell culture and treatment

A2780, SKOV3, OVCAR3, HO-8910, CAOV3 and 3AO cells are human ovarian cancer cells. All cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 μ/ml penicillin and 100 μg/ml streptomycin, and incubated in a humidified atmosphere at 37°C with 5% CO_2.

siRNA tranfection

SKOV3 and HO-8910 cells were seeded in antibiotic-free medium the day before tranfection. The cells were transfected that knockdown of CDKN3 according to the instructions provided by the manufacturer. After 48 hours, the transfected cells were collected and processed for real-time PCR, western blot, proliferation, cell cycle, apoptosis and invasion assay.

Real-time PCR

Total RNA was isolated from transfected cells using Trizol reagent (Invitrogen, Shanghai, China). Real-time PCR was performed using a standard SYBR Green PCR kit protocol on ABI 7300. The PCR primers for CDKN3 were 5'-AGCTGCACATCTATCATC-3' (forward) and 5'-CACTGGTGGTTTCATTTC-3'. The primers for GADPH were 5'-CACCCACTCCTCCACCTTTG-3' (forward) and 5'-CCACCACCCTGTTGCTGTAG-3' (reverse).

Western blot

Cultured or transfected cells were harvest and washed twice with PBS. Proteins was run on 10% SDS-PAGE gel and transferred electrophoretically to a membrane. The blots were blocked with 5% skim milk, followed by incubation with antibodies specific against CDKN3, GADPH, MCM2, MCM6, PCNA and CDK2. Blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China).

Cell proliferation assay

Cell viability was assessed by Cell Counting Kit (CCK) -8 (Tongren, Shanghai, China). Briefly, 4×10^3 cells were seeded in each 96-well plate, and further incubated for 24 and 48 hours, respectively. CCK-8 reagent was added to each well at 1 h before the endpoint incubation. The optical density (OD) 450 nm values in each well were determined by a microplate reader. Experiments were repeated at least three times.

Cell cycle assay

After 48 h of CDKN3 transfection, cells were harvest and cell cycle distribution was analyzed using flow cytometry (FACSCalinur, BD Biosciences).

Cell apoptosis assay

Cells were collected after CDKN3 transfection for 48 h, and stained using annexin V-fluorescein isothiocyanate and apoptosis rates were analyzed.

Cell invasion assay

For transwell assay, after tranfected for 24 h, 1×10^5 SKOV3 cells in serum-free RMPI-1640 or HO-8910 cells in DMEM were seeded into the upper chambers of each well coated with Matrigel. DMEM (RMPI-1640) containing 1% FBS was placed in the lower chambers as a chemoattractant. After 48 h of incubation, cells on the upper membrane surface were wiped off, and fixed with 100% methanol and stained with 0.5% crystal violet. The number of invasive cells was then counted under a microscope.

Statistical analysis

Experimental data were presented as mean ± SD of at least three independent replicates through analyzing with GraphPad Prism 5.0 (San Diego, CA, USA) and assessing comparisons between different groups by the student's t test. Differences were considered significant at values of $P<0.05$.

Results

Over-expression of CDKN3 is frequent in ovarian cancer

To study the biological role of CDKN3 in OC, we first detect the expression levels of CDNK3 in

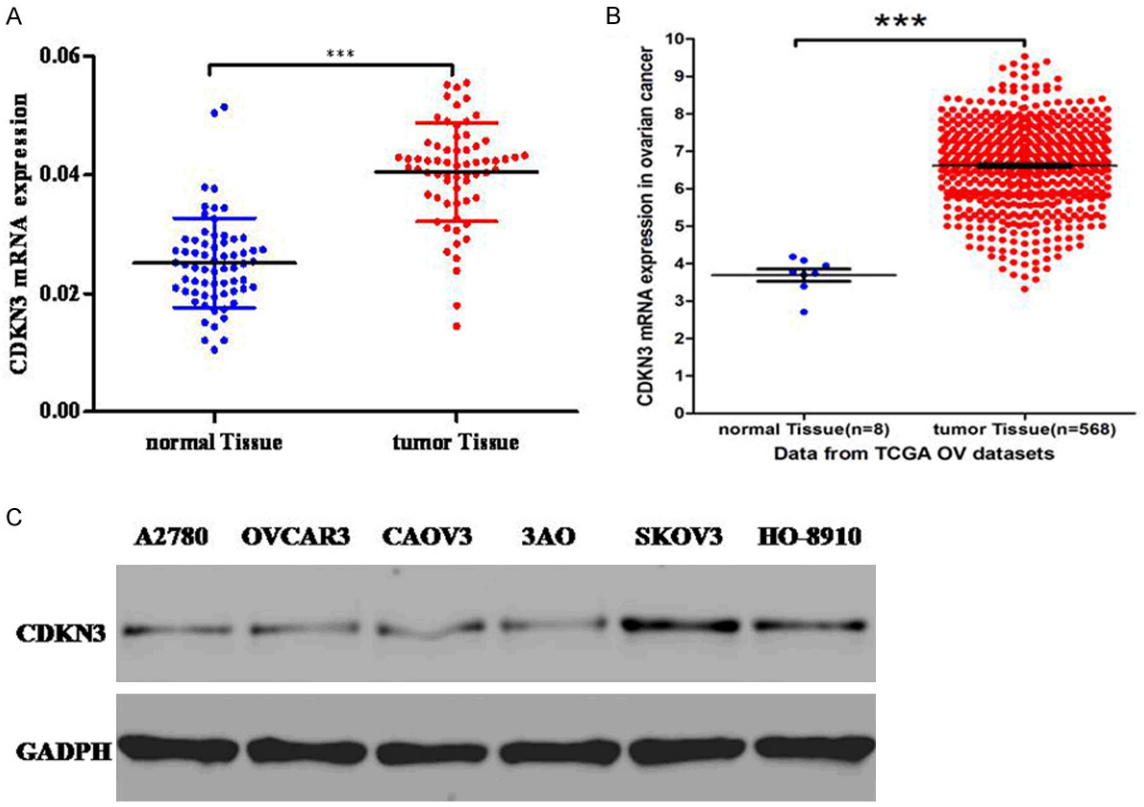

Figure 1. Over-expression of CDKN3 in ovarian cancer. A. Real-time PCR detected the CDKN3 expression level in 65 ovarian cancer tissues and their adjacent normal tissues. B. The expression level of CDKN3 investigated from TCGA dataset. C. Expression of CDKN3 in 6 ovarian cancer cell lines detected by real-time PCR.

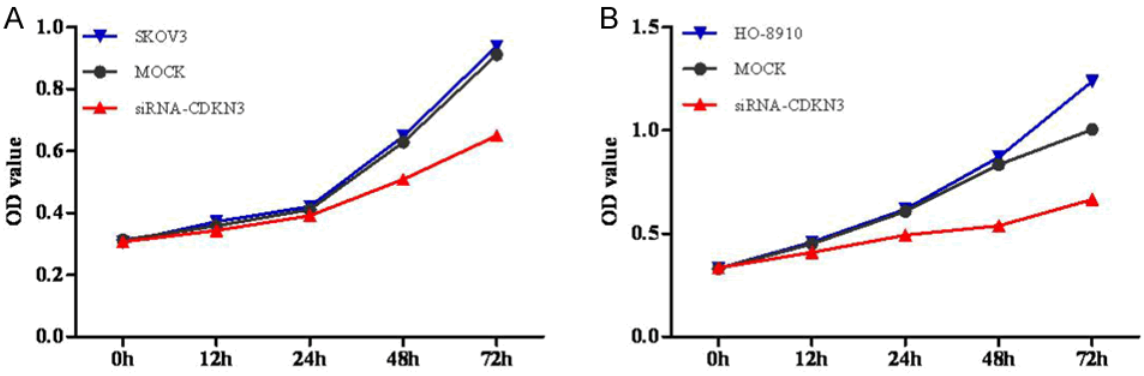

Figure 2. Knockdown of CDKN3 inhibits the proliferation of ovarian cancer cells. Cell growth was assessed using CCK-8 assay. A. The OD value was detected in SKOV3 cells after tranfection. B. The OD value was detected at 24, 48 and 72 h in HO-8910 cells.

65 ovarian cancer patients' tissues. As shown in **Figure 1A**, CDKN3 expression level was higher in OC tissues compared with adjacent normal tissue control. To validate this observation, we reanalyzed microarray data from TCGA datasets confirmed this over-expression of CDKN3 (P<0.001, **Figure 1B**).

Silence of CDKN3 represses OC cell proliferation

Having documented significantly up-regulation of CDKN3 in ovarian cancer tissues, we wonder how CDKN3 affects ovarian cancer cell biological behavior. We analyzed CDKN3 expression in

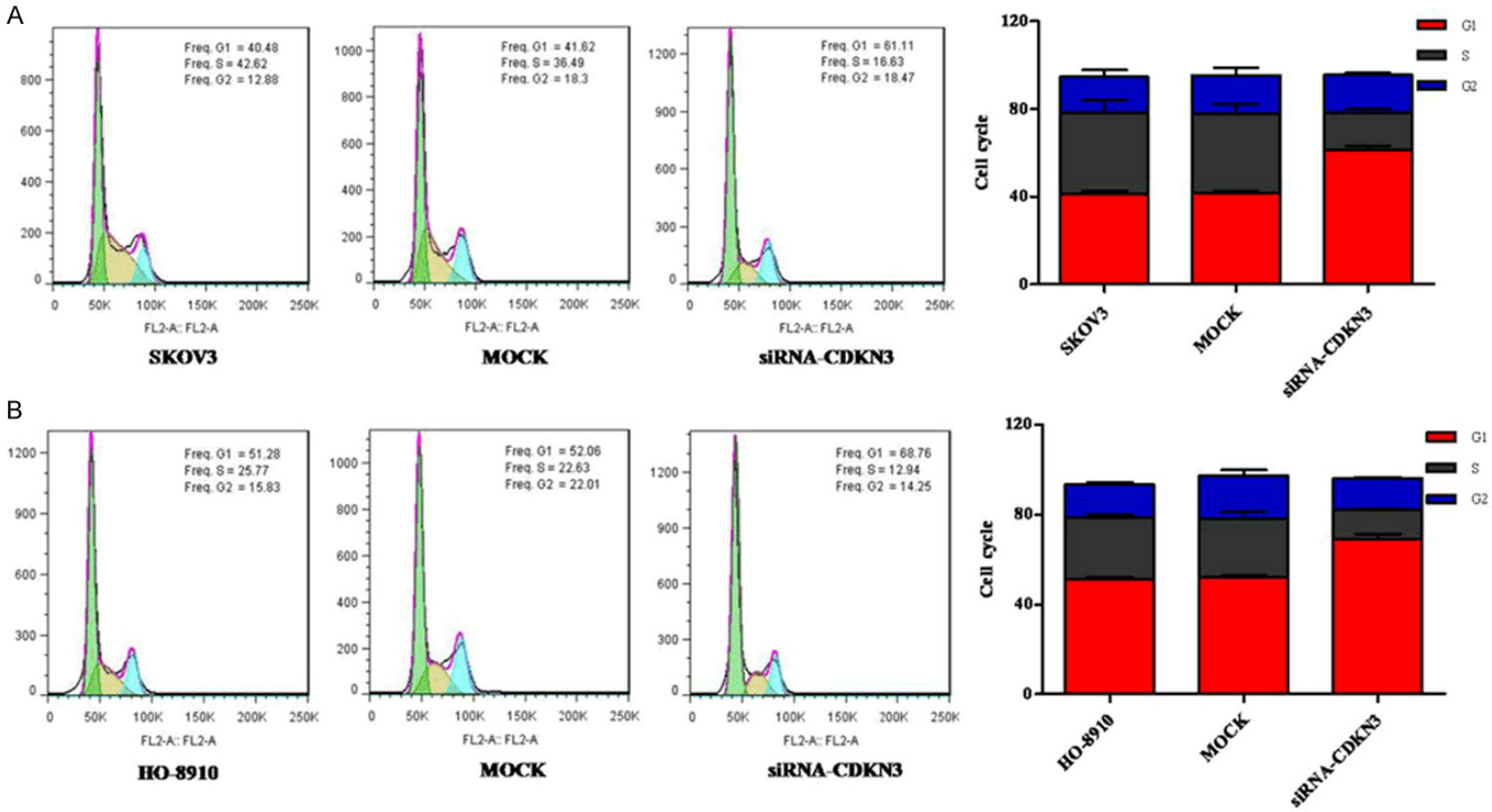

Figure 3. Flow cytometry showing that knockdown of CDKN3 could promote G1 phase transition. A. The cell cycle transition in SKOV3 cells. B. Cell cycle transition in SKOV3 cells.

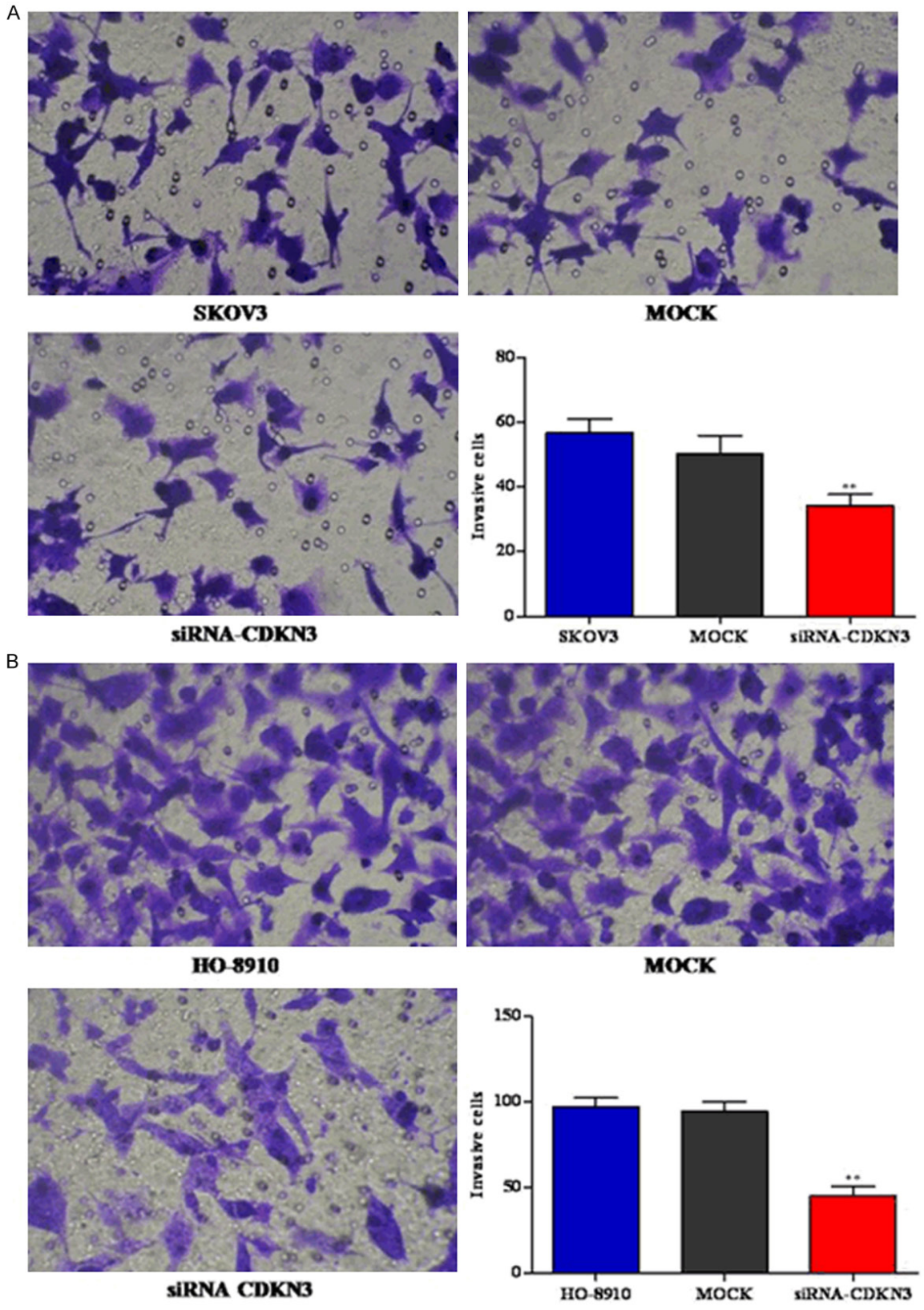

Figure 4. Knockdown of CDKN3 decreased cell invasion on ovarian cancer. A. Knockdown of CDKN3 in SKOV3 notably inhibited cell invasion. B. Silencing CDKN3 of HO-8910 cells significantly inhibited cell invasion. Data were based at least 3 independent experiments, $^{**}P<0.01$.

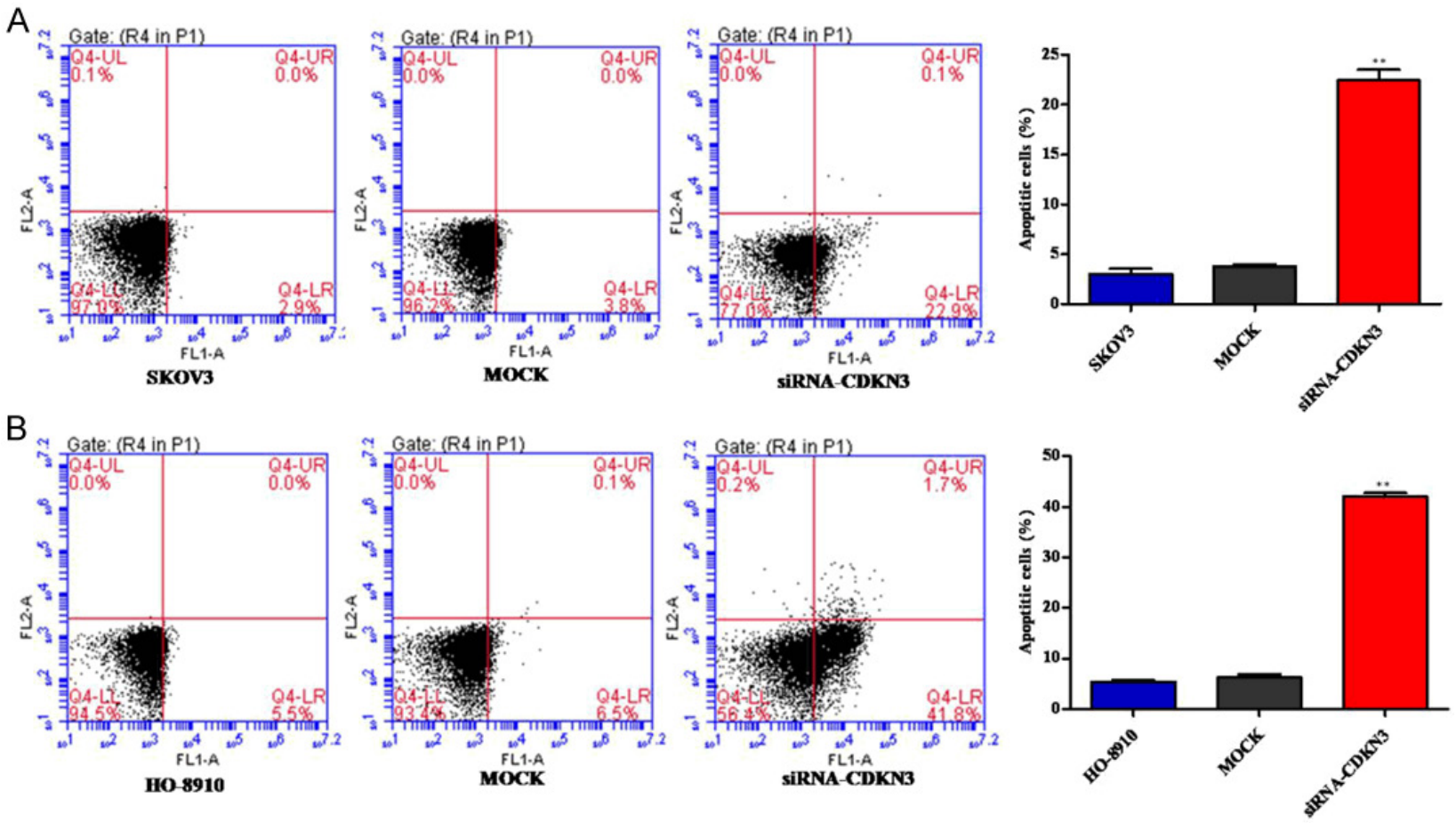

Figure 5. siRNA-CDKN3 induces cell apoptosis in OC cells. SKOV3 and HO-8910 cells were transfected and collected. A. Apoptosis rates were analyzed in SKOV3 cells using flow cytometry. B. Apoptosis rates were analyzed in HO-8910 cells.

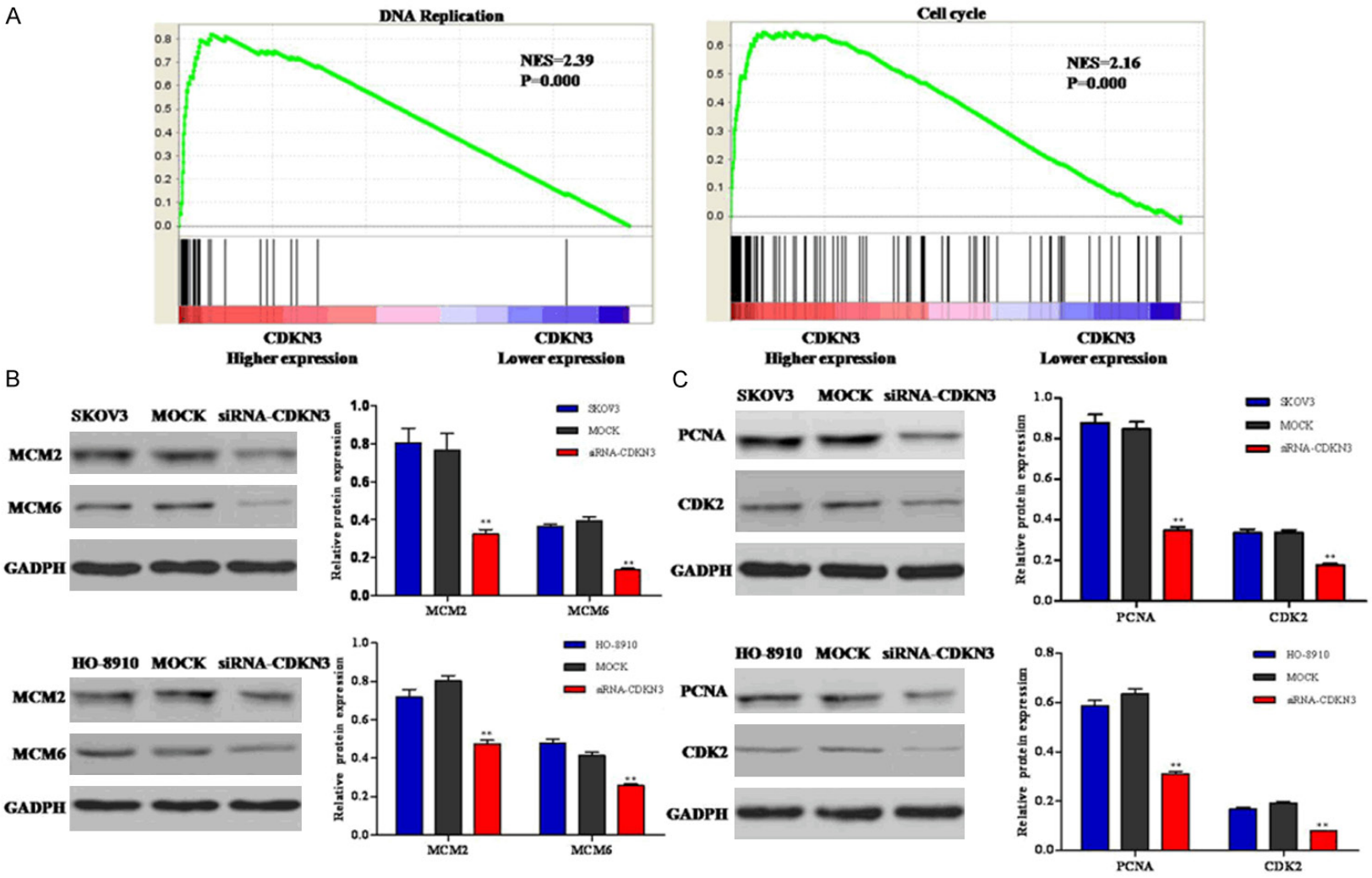

Figure 6. CDKN3 regulated the expression of protein in SKOV3 and HO-8910 cells. A. Gene Set Enrichment Analysis (GSEA) identified cell cycle and DNA replication signaling pathway associated with CDKN3 in TCGA ovarian cancer dataset. B. Western blot showed the expression levels of MCM2 and MCM6 in SKOV3 and HO-8910 cell lines. C. Cells were transfected, followed by western blot analysis for PCNA and CDK2.

6 ovarian cancer cell lines, A2780, SKOV3, OVCAR3, HO-8910, CAVO3 and 3AO cells by western blot (**Figure 1C**). CDKN3 was expressed in higher level in SKOV3 and HO-8910 cells compared with other cell lines. Then, CDKN3 knockdown were treated into SKOV3 and HO-8910 cells respectively. Cell proliferation was analyzed using CCK-8 assay. And shown in **Figure 2A** and **2B**, CDKN3 knockdown induced great inhibition on cell growth compared with MOCK in both cells.

To further validate the cell proliferation inhibition of CDKN3, cell cycle was analyzed in SKOV3 and HO-8910 cells (**Figure 3**). Cell cycle analysis revealed that silencing CDKN3 notably increased the rate of G1 phase cells and reduced S phase cell population in both cell lines. The result indicated that silencing CDKN3 in ovarian cancer cells may inhibited cell proliferation by arresting cell cycle progression in G1 phase.

Knockdown of CDKN3 on OC decreased cells invasion

To determine whether CDKN3 is involved in cell invasion, transwell assay was then performed. The result showed the inhibited invasive ability in siRNA-CDKN3 cells (**Figure 4**).

Inhibition of CDKN3 induces cell apoptosis in OC cells

Till now, most of studies about CDKN3 mainly focus on its function of cell proliferation, and other functions of this gene are largely unknown. Then, we evaluated the apoptotic function of CDKN3 in SKOV3 and HO-8910 cells by Annexin V-FITC/PI staining assay. As shown in **Figure 5**, flow cytometry analysis revealed that inhibition of CDKN3 in cells significantly induced cell apoptosis compared to corresponding mock shRNA in both SKOV3 and HO-8910 cells.

Western blot

Cells were harvested, and total proteins were extracted. Proteins were run on 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The blots were blocked with 5% skim milk, followed by incubation with antibodies against CDKN3, MCM2, MCM6, PCNA, CDK2 and GADPH. Blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody and visualized using enhanced chemiluminescence (ECL, Millipore) (**Figure 6**).

Discussion

Ovarian cancer generally presents in advanced stages with a high case ratio. The majority of women with ovarian cancer will develop recurrent disease and need surgical [14]. At present, the treatment methods include chemotherapy, surgery and radiation [15]. CDKN3 was identified with high levels in breast and prostate cancers by using a phosphatase PCR strategy [10]. In heaptocellular cancer, functional studies showed that CDKN3 is also over-expressed and can promote cell proliferation [16]. However, the functional implication and prognostic value of CDKN3 in ovarian cancer have been poorly defined.

Here, we found that CDKN3 was over-expressed in ovarian cancer. Among several ovarian cell lines, CDKN3 was found expressing notably in SKOV3 and HO-8910 cells. Therefore, this two cell lines were determined for further investigation. CDKN3 siRNA could effectively inhibited proliferation and invasion, induced apoptotic in ovarian cancer cells. To further elucidate the molecular mechanism of CDKN3 involved in, we performed GSEA to identify associated with signaling pathways. Cell cycle and DNA replication pathways play crucial role in the process of ovarian cancer cells.

The role of CDKN3 on cell proliferation has been well studied [17]. MCM2 and MCM6 is member of MCM family. MCM (minichromosome maintenance proteins) is composed of at least six subunits, including MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7, which play an important role in DNA replication and extension [18]. And ensure DNA replication occurs only once in the cell cycle [19]. In cervical cancer cells, the research indicated that MCM2 and MCM6 were over-expressed [20]. Then we detected the DNA replication related proteins MCM2 and MCM6 expression. Moreover, PCNA and CDK2 two cell cycle related proteins were also detected. The results showed that the proteins expression were significantly decreased when CDKN3 knockdown in ovarian cells.

In summary, our results have shown that CDKN3 is frequently up-regulated in ovarian

cancer. The functional data strongly suggest that CDKN3 behaves an oncogene in ovarian cancer. Furthermore, CDKN3 is related to cell cycle and DNA replication signaling pathways. CDKN3 can act as a potential prognostic marker and therapeutic target in ovarian cancer.

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Disclosure of conflict of interest

None.

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