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Cyclin-Dependent Kinase Inhibitor 3 Promotes Cancer Cell Proliferation and Tumorigenesis in Nasopharyngeal Carcinoma by Targeting p27

Huimin Wang,* Hexin Chen,† Hang Zhou,* Wenfa Yu,* and Zhenmin Lu*

*Department of Otolaryngology, The First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan, P.R. China

†Otorhinolaryngology Hospital, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, Guangdong, P.R. China

Nasopharyngeal carcinoma (NPC) is a common malignancy of the head and neck that arises from the nasopharynx epithelium and is highly invasive. Cyclin-dependent kinase inhibitor 3 (CDKN3) belongs to the dual-specificity protein phosphatase family, which plays a key role in regulating cell division. Abnormal expression of CDKN3 has been found in numerous types of cancer. In the current study, we explored the possible role of CDKN3 in cell proliferation, ability to invade, and radiosensitivity in NPC cells. We reported that CDKN3 was upregulated and p27 was downregulated in NPC tissues and is associated with a worse prognosis for patients. In addition, downregulation of CDKN3 and upregulation of p27 decreased cell proliferation, induced cell cycle arrest, increased apoptosis, decreased cell invasion, and enhanced radiosensitivity. Silencing of p27 significantly inhibited the effects of the knockdown of CDKN3. Moreover, downregulation of CDKN3 and upregulation of p27 inhibited the increase in tumor volume and weight in implanted tumors, decreased the phosphorylation of Akt, and increased the expression of cleaved caspase 3 in tumors. CDKN3 expression was also inversely correlated with p27 expression in NPC patients. Knockdown of CDKN3 increased p27 expression. Silencing of p27 markedly inhibited the effects of CDKN3 on cell proliferation, cell cycle progression, apoptosis, invasion, and radiosensitivity. These results demonstrate that upregulation of p27 is involved in the knockdown of CDKN3-induced decrease in cell proliferation, increase in cell cycle arrest and apoptosis, decrease in invasion, and increase in radiosensitivity. The results demonstrate that the CDKN3/p27 axis may be a novel target in the treatment of NPC.

Key words: Nasopharyngeal carcinoma (NPC); Cyclin-dependent kinase inhibitor 3 (CDKN3); p27; Cell proliferation; Radiosensitivity

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a common head and neck malignancy that arises from the nasopharynx epithelium and is highly invasive¹. NPC occurs infrequently in most regions of the world, with a high incidence rate mainly in Southern China, Southeast Asia, and Northern Africa^{2,3}. Radiotherapy combined with chemotherapy is an effective treatment against advanced NPC⁴. Owing to the great improvement in diagnostic and therapeutic approaches, most early stage NPC patients have been successfully cured. However, in the middle-late and late stages, the survival rate is as low as 50% in most patients because of drug resistance and radioresistance⁵. Drug resistance and radioresistance are the major causes of treatment failure, leading to incomplete cure, recurrence, and metastasis⁶. As a result, the mortality rate of advanced stage NPC patients is high due to distant

metastasis and local regional relapse^{7,8}. Therefore, deep research is needed in order to find new molecular mechanisms and novel targets for the treatment of NPC.

Cyclin-dependent kinase inhibitor 3 (CDKN3), also named CDK inhibitor 3, CDI1, or KAP, belongs to the dual-specificity protein phosphatase family, which plays a key role in regulating cell division^{9–12}. The gene encoding the CDKN3 protein is located on chromosome 14q22¹³. CDKN3 can regulate cell cycle progression through binding to cyclin proteins and forming cyclin-CDK complexes^{14,15}. CDKN3 can dephosphorylate CDK1 at Thr161, resulting in a reduction in phosphorylation of CK β at Ser209 and inhibition of cell cycle progression¹⁴. CDKN3 can also dephosphorylate and inactivate CDK2, thereby inhibiting G₁/S cell cycle progression¹⁵. Abnormal expression of CDKN3 has been found in numerous types of cancer^{15–19}. For example, silencing CDKN3 inhibits the

Address correspondence to Zhenmin Lu, Department of Otolaryngology, The First Affiliated Hospital of Xinxiang Medical University, Jiankang Road No. 88, Weihui 453100, Henan, P.R. China. E-mail: luzhenmin76@163.com or Hexin Chen, Otorhinolaryngology Hospital, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, 510080 Guangdong, P.R. China. E-mail: chenhexin136@yeah.net

migration of breast cancer cell lines²⁰. Knockdown of CDKN3 inhibits proliferation and invasion in human gastric cancer cells¹⁷. CDKN3 knockdown reduces cell proliferation and invasion and promotes apoptosis in human ovarian cancer²¹. CDKN3 is overexpressed in hepatocellular carcinoma and promotes tumor cell proliferation²². CDKN3 plays a role in hepatitis/cirrhosis and hepatocellular carcinoma transformation²³. Moreover, CDKN3 is upregulated and associated with low survival in cervical cancer patients²⁴. CDKN3 is considered to be an independent prognostic factor and promotes cell proliferation in ovarian cancer^{18,19}. p27 is a negative regulator of cell cycle progression and is downregulated in various types of cancer²⁵⁻²⁷. Whether CDKN3 plays a role in the development of NPC and the possible interaction between CDKN3 and p27 is not known.

Here we report that CDKN3 was upregulated and p27 was downregulated in NPC tissues and associated with worse patient prognosis. In addition, downregulation of CDKN3 and upregulation of p27 decreased cell proliferation, induced cell cycle arrest, increased apoptosis, decreased cell invasion, and enhanced radiosensitivity. Silencing of p27 significantly inhibited these effects of knockdown of CDKN3. Moreover, downregulation of CDKN3 and upregulation of p27 inhibited the increase in tumor volume and weight in implanted tumors, decreased the phosphorylation of Akt, and increased the expression of cleaved caspase 3 in tumors. The results demonstrated that the CDKN3/p27 axis may be a novel target for the treatment of NPC.

MATERIALS AND METHODS

Chemicals and Materials

β -Actin was purchased from Bioworld Technology (Nanjing, P.R. China). CDKN3 and p27 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Most of the other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Patients and Ethics Statement

Samples were collected from 43 patients at The First Affiliated Hospital of Xinxiang Medical University from March 2015 to September 2016. The tissue samples were obtained from patients who did not receive chemoradiotherapy. Fresh tissues were stored at -80°C before use. All patients signed consent forms, and the study was approved by The First Affiliated Hospital of Xinxiang Medical University and complied with the Declaration of Helsinki.

Cell Culture and Lentivirus Transfection

Normal nasopharyngeal epithelial cells (NP69) and NPC cell lines (6-10B, 5-8F, CNE2, CNE1, HNE1, HONE1, and C666-1) were purchased from the American

Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher, Waltham, MA, USA), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin and incubated at 37°C and 5% CO_2 . Lentivirus (LV) vectors expressing CDKN3 shRNA, p27, and p27 shRNA were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). LVs were transfected into HNE1 according to the manufacturer's protocols.

Cell Proliferation and Viability

Cells were plated into 96-well plates, and proliferation was assayed by cell counting kit-8 (CCK-8; Beyotime Biotechnology, Jiangsu, P.R. China). Cell viability was determined by MTT {(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue)} assay.

Cell Cycle and Apoptosis Analysis

For the detection of cell cycle distribution, cells were fixed in 100% ice-cold ethanol, washed, and incubated in 1 mg/ml propidium iodide with RNase A (200 $\mu\text{g}/\text{ml}$). Cell cycle population was analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). For apoptosis measurement, cells were stained with TUNEL using the In Situ Cell Death Detection Kit, Fluorescein (Roche; Basel, Switzerland) following the manufacturer's protocol. Apoptosis was analyzed by flow cytometry (BD).

Quantitative Real-Time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 20 μl . Quantitative real-time PCR was performed to analyze the relative expression of mRNA levels of target genes using the SYBR Premix Ex Taq II (TaKaRa, P.R. China). The PCR conditions were as follows: initial denaturation at 95°C for 10 min followed by 30 cycles at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR was conducted using a CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). β -Actin was used as an internal control.

Western Blot Analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis reagent (Thermo Fisher). The protein concentration was determined using a Bradford protein assay (Sigma-Aldrich). A 50- μg sample was separated on a 10% denaturing polyacrylamide gel (with 5% polyacrylamide stacking gel) and transferred electrophoretically onto a nitrocellulose membrane. After blocking with 5% non-fat dry milk in Tris-buffered saline plus Tween (TBST),

the membranes were incubated with primary antibodies and then incubated with HRP-conjugated secondary antibody (Thermo Fisher). Bands were visualized by chemiluminescence reaction using an enhanced chemiluminescence detection system (Thermo Fisher) and captured using Bio-Rad Imaging Systems (Bio-Rad).

Transwell Assay

The ability of cells to invade was evaluated by the Transwell assay using Matrigel-coated cell culture chambers (8- μ m pore size; EMD Millipore, Billerica, MA, USA). In brief, confluent cells were trypsinized and resuspended in 200 μ l of serum-free RPMI-1640 medium and then added into the upper chamber of the insert coated with Matrigel. FBS (5%) (Gibco; Thermo Fisher Scientific) was added to the medium in the lower chamber, which functioned as a chemoattractant. After 24 h of incubation, the cells remaining on the upper membrane were carefully removed. Paraformaldehyde was used for fixing, staining, and washing. Cell population was then calculated using a light microscope.

Tumor Xenograft In Vivo

HNE1 cells (5×10^6) transfected with LV-shCDKN3 or LV-p27 were suspended in 100 ml of a 1:1 mixture of culture medium and growth factor-reduced Matrigel, and then implanted subcutaneously into the forelegs of nude mice. Three weeks after implantation, the mice were treated for 18 days with five sessions of radiation (4 Gy) that was applied every 3 days. Two days after the last treatment, all of the mice were sacrificed. Tumor volume was calculated using the equation $(L \times W^2)/2$, and tumor weights were measured and recorded in grams. All animal studies were approved by The First Affiliated Hospital of Xixiang Medical University.

Statistical Analysis

All data were expressed as the mean \pm SEM and analyzed by GraphPad Prism software. The statistical significance of the differences between the two groups was analyzed with two-sided unpaired Student's *t*-tests. The statistical significance of differences among more than two groups was analyzed by one-way analysis of variance (ANOVA) followed by a Dunnett's *t*-test for multiple comparisons. Survival analysis was conducted using a log-rank test. The data were considered to be statistically significant with a value of $p < 0.05$.

RESULTS

Expression of CDKN3 and p27 in NPC Tissues and Cells

The expression of CDKN3 and p27 in NPC tissues and cells was examined. The mRNA expression of CDKN3 was notably increased in NPC cell lines (6-10B, 5-8F, CNE2, CNE1, HNE1, HONE1, and C666-1) compared

with normal nasopharyngeal epithelial cells (NP69) (Fig. 1A). Moreover, the mRNA expression of CDKN3 was increased in tumor tissues compared to normal controls (Fig. 1B). Low expression levels of CDKN3 could increase the survival of patients (Fig. 1C). In contrast, mRNA expression of p27 was notably decreased in NPC cell lines and tumor tissues compared with normal controls (Fig. 1D and E). High expression levels of p27 could increase the survival of patients (Fig. 1F). We also found that CDKN3 expression was inversely correlated with p27 expression (Fig. 1G). Our results show that high expression levels of CDKN3 and low expression levels of p27 are associated with the malignancy of NPC.

Downregulation of CDKN3 Inhibits NPC Cell Proliferation, Induces Cell Cycle Arrest and Apoptosis, Reduces Migration, and Increases Radiation Sensitivity

To examine the role of CDKN3 in NPC growth, HNE1 cells were transfected with LV carrying CDKN3 shRNAs. We confirmed the knockdown efficiency of CDKN3 (Fig. 2A and C). Knockdown of CDKN3 significantly increased p27 mRNA and protein expression (Fig. 2B and C). Silencing CDKN3 significantly decreased cell proliferation in HNE1 cells (Fig. 2D). Cell population in the G_0/G_1 phase was markedly increased by silencing CDKN3, while cell population in the S phase was notably decreased, indicating significant G_0/G_1 cell cycle arrest (Fig. 2E). Knockdown of CDKN3 significantly increased the percentage of apoptotic cells (Fig. 2F). The Transwell cell number was notably decreased by silencing CDKN3 (Fig. 2G). Moreover, knockdown of CDKN3 promoted a radiation-induced decrease in cell viability (Fig. 2H). These results demonstrated that CDKN3 is a crucial regulator of cell proliferation, cell cycle progression, apoptosis, cell invasion ability, and radiosensitivity in HNE1 cells.

Upregulation of p27 Inhibits NPC Cell Proliferation, Induces Cell Cycle Arrest and Apoptosis, Reduces Migration, and Increases Radiation Sensitivity

To examine the role of p27 in the NPC growth, HNE1 cells were transfected with LV expressing p27. We confirmed the transfection efficiency of LV-p27 (Fig. 3A and B). Upregulation of p27 significantly decreased cell proliferation in HNE1 cells (Fig. 3C). Cell population in the G_0/G_1 phase was markedly increased by upregulation of p27, while cell population in the S phase was notably decreased, indicating significant G_0/G_1 cell cycle arrest (Fig. 3D). Upregulation of p27 significantly increased the percentage of apoptotic cells (Fig. 3E). The Transwell cell number was notably decreased by upregulation of p27 (Fig. 3F). Moreover, upregulation of p27 promoted a radiation-induced decrease in cell viability (Fig. 3G). These results demonstrate that p27 is a negative regulator

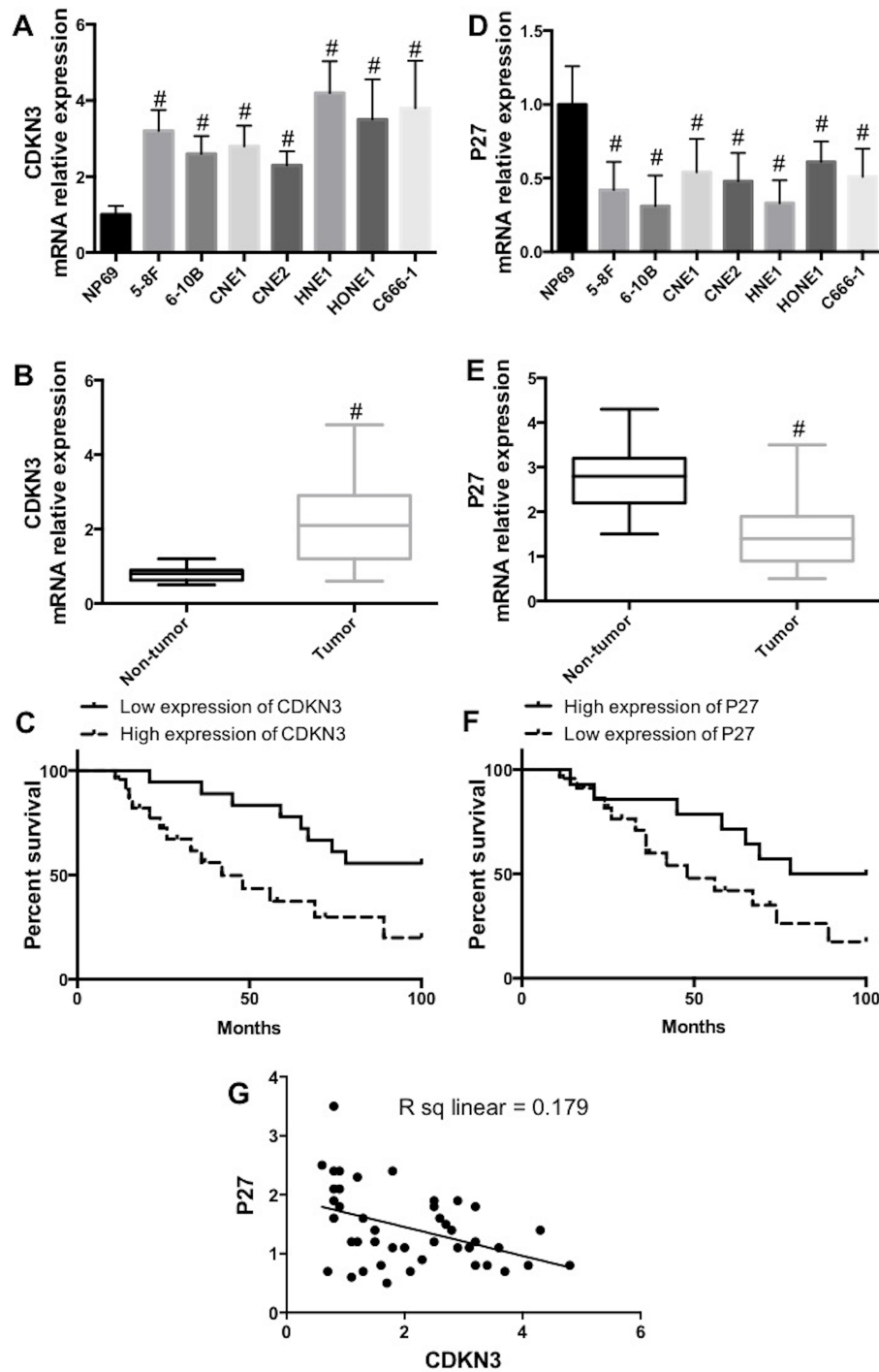


Figure 1. Upregulation of CDKN3 and downregulation of p27 expression in nasopharyngeal carcinoma (NPC) cell lines and tissues. (A) mRNA expression of CDKN3 in NP69 cells and seven NPC cell lines. (B) mRNA expression of CDKN3 in 43 NPC tissues and 16 nontumor tissues. (C) Survival analysis of overall survival duration in 43 patients according to CDKN3 gene expression levels. (D) mRNA expression of p27 in NP69 cells and seven NPC cell lines. (E) mRNA expression of p27 in 43 NPC tissues and 16 nontumor tissues. (F) Survival analysis of overall survival duration in 43 patients according to p27 gene expression levels. (G) Correlation analysis between CDKN3 and p27 in 43 clinical tissue samples. # $p < 0.05$.

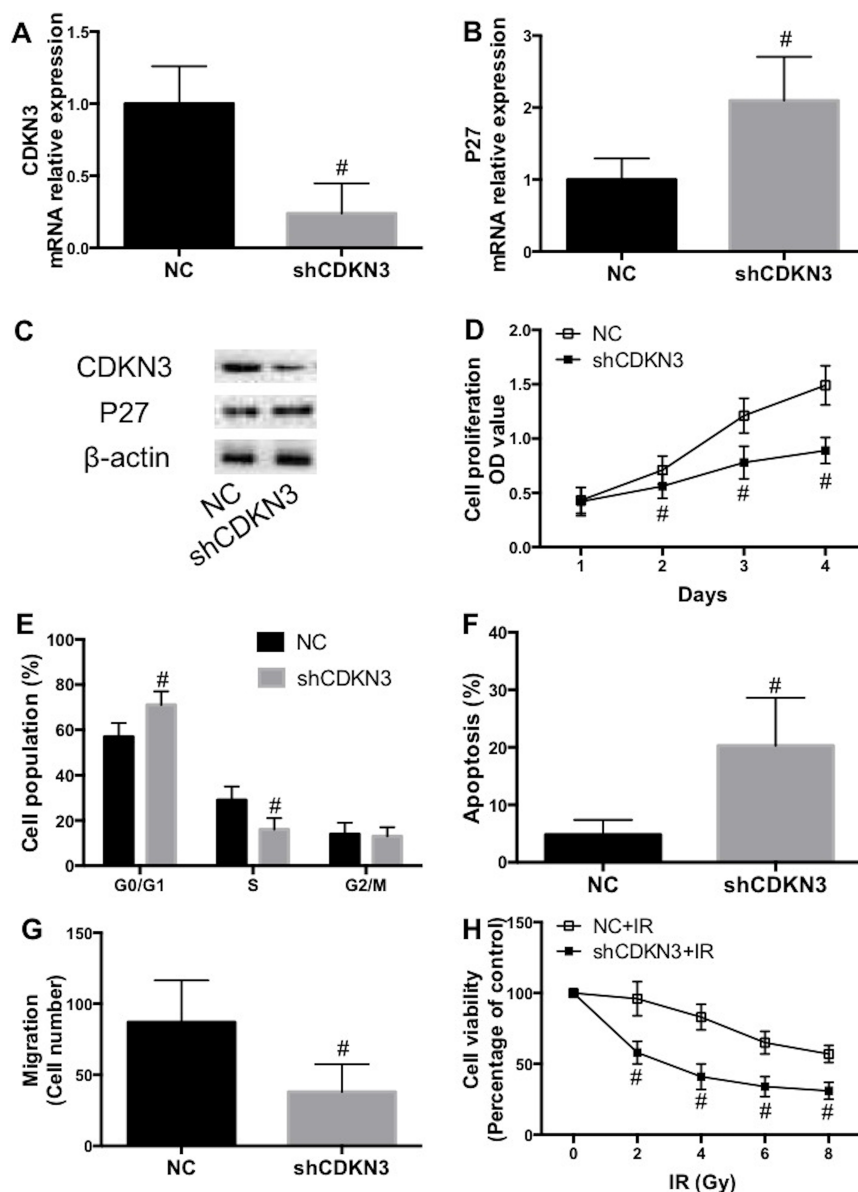


Figure 2. Downregulation of CDKN3 inhibits NPC cell proliferation, induces cell cycle arrest and apoptosis, reduces migration, and increases radiation sensitivity. HNE1 cells were transfected with CDKN3 LV-shRNAs. (A, B) mRNA expression of CDKN3 and p27. (C) Protein expression of CDKN3 and p27. (D) The proliferation of cells was measured at the indicated times using cell counting kit-8 (CCK-8). (E) Cell cycle distribution of cells analyzed by flow cytometry. (F) Apoptotic detection of cells by TUNEL staining. (G) Migration detection by counting Transwell cell numbers. (H) Cell viability after exposure to a different dose of radiation. # $p < 0.05$.

for HNE1 cell cycle progression, cell proliferation, and invasion ability, and a positive regulator of apoptosis and radiosensitivity.

Upregulation of p27 is Involved in the Downregulation of CDKN3-Induced Inhibition of NPC Cell Proliferation, Induction of Cell Cycle Arrest and Apoptosis, Reduction of Migration, and Increase in Radiation Sensitivity

Since p27 expression was increased by the downregulation of CDKN3, we further examined whether

upregulation of p27 was involved in the effect of CDKN3 on NPC. We showed that silencing p27 markedly inhibited the effect of the knockdown of CDKN3 on cell proliferation (Fig. 4A), cell cycle population (Fig. 4B), apoptosis (Fig. 4C), cell invasion (Fig. 4D), and radiosensitivity (Fig. 4E). These results demonstrate that upregulation of p27 is involved in the downregulation of CDKN3-induced inhibition of NPC cell proliferation, induction of cell cycle arrest and apoptosis, reduction of migration, and increase in radiation sensitivity.

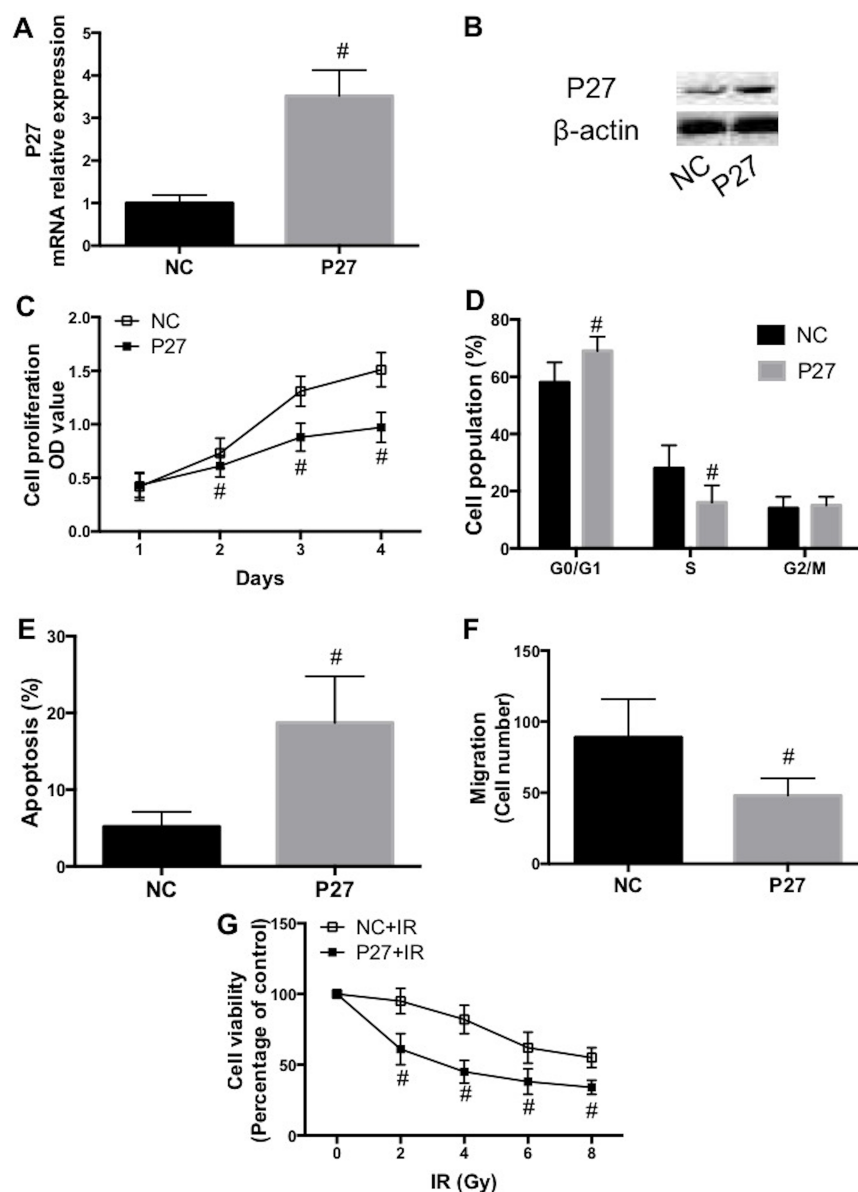


Figure 3. Upregulation of p27 inhibits NPC cell proliferation, induces cell cycle arrest and apoptosis, reduces migration, and increases radiation sensitivity. HNE1 cells were transfected with LV-p27. (A) mRNA expression of p27. (B) Protein expression of p27. (C) The proliferation of cells was measured at the indicated times using a CCK-8 kit. (D) Cell cycle distribution of cells analyzed by flow cytometry. (E) Apoptotic detection of cells by TUNEL staining. (F) Migration detection by counting Transwell cell numbers. (G) Cell viability after exposure to different dose of radiation. # $p < 0.05$.

Downregulation of CDKN3 and Upregulation of p27 Sensitize NPC Cells to Radiation in Nude Mice Xenografts

To determine whether CDKN3 and p27 regulate tumor growth and radiation sensitivity *in vivo*, we subcutaneously xenografted HNE1 cells into nude mice. Three weeks after implantation, the mice were treated with five sessions of radiation (4 Gy) every 3 days. We showed that downregulation of CDKN3 and upregulation of p27

markedly reduced tumor volume (Fig. 5A and B). In addition, downregulation of CDKN3 and upregulation of p27 notably promoted radiation-induced reduction of tumor volume (Fig. 5A and B). Moreover, downregulation of CDKN3 and upregulation of p27 resulted in and promoted radiation-resulted reduction of tumor weight (Fig. 5C and D). These results demonstrate that downregulation of CDKN3 and upregulation of p27 inhibit tumor growth and promote radiation sensitivity *in vivo*.

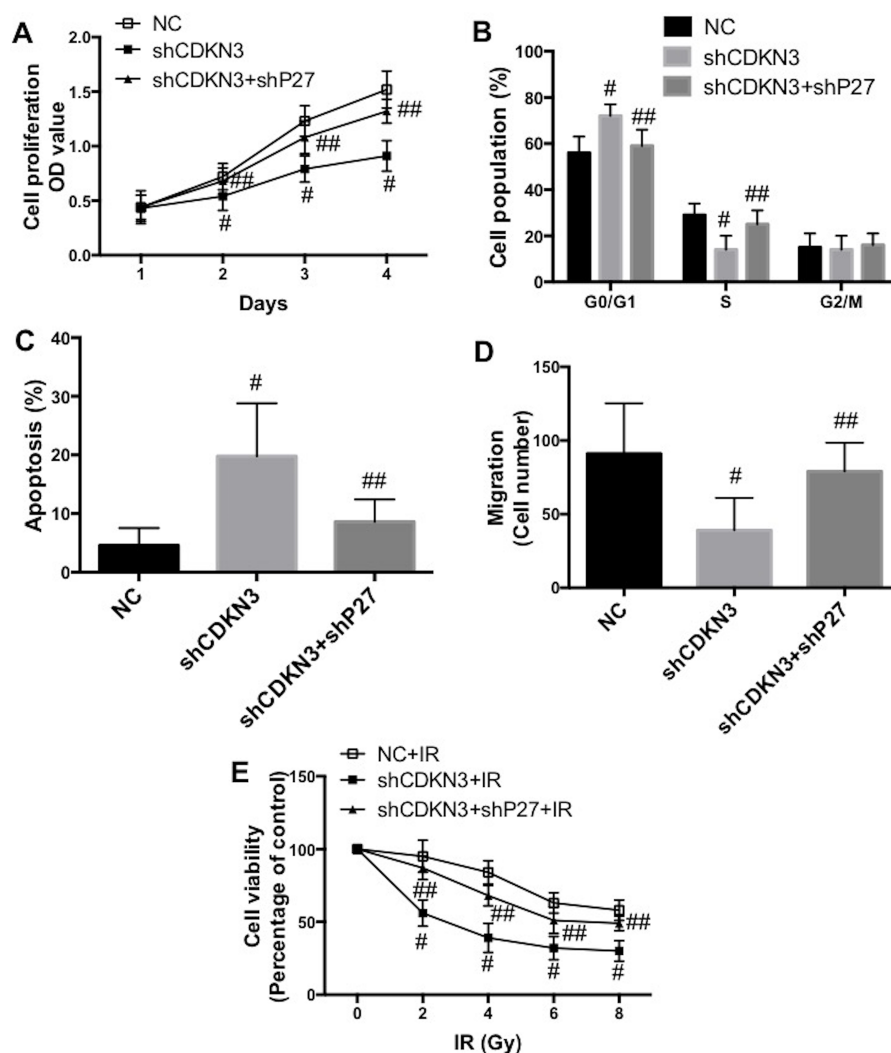


Figure 4. Upregulation of p27 is involved in the downregulation of CDKN3-induced inhibition of NPC cell proliferation, induction of cell cycle arrest and apoptosis, reduction of migration, and increase in radiation sensitivity. HNE1 cells were cotransfected with CDKN3 and p27 LV-shRNAs. (A) The proliferation of cells was measured at the indicated times using a CCK-8 kit. (B) Cell cycle distribution of cells analyzed by flow cytometry. (C) Apoptotic detection of cells by TUNEL staining. (D) Migration detection by counting Transwell cell numbers. (E) Cell viability after exposure to a different dose of radiation. $^{\#}p < 0.05$ versus control. $^{\#\#}p < 0.05$ versus shCDKN3.

DISCUSSION

A large number of studies have reported that abnormal expression of CDKN3 is associated with the development of numerous types of cancer¹⁵⁻¹⁹. However, whether CDKN3 plays a role in the regulation of NPC growth is not known. In the current study, we tested the possible role of CDKN3 in NPC cell proliferation, cell cycle progression, apoptosis, cell invasion ability, and radiation sensitivity.

It was reported that knockdown of CDKN3 inhibited proliferation in human gastric cancer cells¹⁷, ovarian cancer^{19,21}, and cervical cancer²⁴, and CDKN3 is

overexpressed in hepatocellular carcinoma and promotes tumor cell proliferation²². In the current study, we found that CDKN3 expression was increased in NPC tumor tissues and cells and that a low expression level of CDKN3 can increase the survival of patients. Using LV-shRNA, we knocked down the expression of CDKN3 in HNE1 cells and found that downregulation of CDKN3 can significantly reduce cell proliferation.

Tumor cell growth is characterized by uncontrolled cell cycle progression^{28,29}. CDKN3 can regulate cell cycle progression through binding to cyclin proteins and forming cyclin-CDK complexes^{14,15}. CDKN3 can

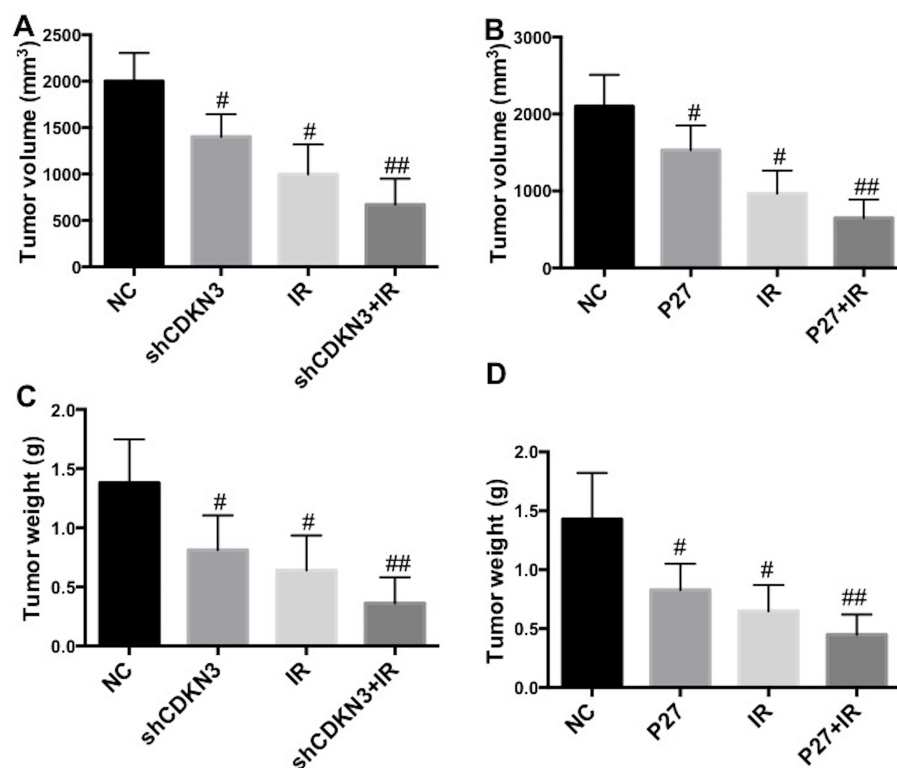


Figure 5. Downregulation of CDKN3 and upregulation of p27 sensitize NPC cells to radiation in nude mice xenografts. Three weeks after implantation of cells transfected with LV-shCDKN3 or LV-p27, the mice were randomly divided into groups: those receiving radiation and those that are not. (A, B) Tumor volumes were measured. (C, D) Xenograft weights were measured. # $p < 0.05$ versus control. ## $p < 0.05$ versus IR.

dephosphorylate CDK1 at Thr161, resulting in a reduction in phosphorylation of CK β at Ser209 and inhibition of cell cycle progression¹⁴. CDKN3 can also dephosphorylate and inactivate CDK2, thereby inhibiting G₁/S cell cycle progression¹⁵. Silencing CDKN3 in human gastric cancer cells can induce G₀/G₁ cell cycle arrest¹⁷. In the current study, we also found that knockdown of CDKN3 could result in G₀/G₁ cell cycle arrest in NPC cells. Apoptosis, a programmed cell death, is considered to play an important role against tumor growth³⁰⁻³². Knockdown of CDKN3 was shown to induce apoptosis in human gastric cancer cells¹⁷, ovarian cancer^{19,21}, and cervical cancer²⁴. We also found that knockdown of CDKN3 in NPC cells induced a significant increase in apoptosis.

Invasion ability represents the potential for tumor metastasis. Knockdown of CDKN3 inhibited invasion in human gastric cancer cells¹⁷, ovarian cancer^{19,21}, cervical cancer²⁴, and glioblastoma³³. In our study, we showed that knockdown of CDKN3 in NPC cells decreased cell invasion ability. Radioresistance significantly limited the efficiency of radiotherapy⁵. We also tested the effect of knockdown of CDKN3 on radiosensitivity and showed that downregulation of CDKN3 notably increased the sensitivity to radiotherapy. Moreover, we showed that

knockdown of CDKN3 inhibited the increase in tumor volume and weight in implanted tumors in mice. These results demonstrate that downregulation of CDKN3 inhibits NPC cell proliferation, induces cell cycle progression and apoptosis, decreases cell invasion ability, and increases radiation sensitivity. However, there are studies reporting the opposite results. For instance, it has been found that CDKN3 has a remarkable effect in suppressing colorectal cancer cell proliferation and migration and in inducing cell cycle arrest and apoptosis in a colorectal cancer cell line, SW480³⁴. It is suggested that the role of CDKN3 in the development of tumorigenesis is cell type specific.

p27 (CDKN1B) is an important regulator of cell cycle and apoptosis^{35,36}. p27 is an enzyme inhibitor that is encoded by the CDKN1B gene. Reduced expression of p27 is associated with multiple tumor types, including lung cancer³⁷, hepatocellular carcinoma³⁸, gastric cancer³⁸, and colorectal cancer³⁹. In particular, decreased p27 protein expression is correlated with the progression and poor prognosis of NPC⁴⁰. Nuclear p27 expression confers a favorable outcome for NPC patients⁴¹. In the current study, we found that p27 expression was decreased in NPC tumor tissues and cells and that high expression

levels of p27 could increase the survival of patients. Upregulation of p27 significantly inhibited NPC cell proliferation, induced cell cycle progression and apoptosis, decreased cell invasion ability, and increased radiation sensitivity. Moreover, CDKN3 expression was inversely correlated with p27 expression in NPC patients. Knockdown of CDKN3 increased p27 expression. Silencing p27 markedly inhibited the effects of CDKN3 on cell proliferation, cell cycle progression, apoptosis, invasion, and radiosensitivity. These results demonstrated that upregulation of p27 was involved in the knockdown of a CDKN3-induced decrease in cell proliferation, increase in cell cycle arrest and apoptosis, decrease in invasion, and increase in radiosensitivity.

In conclusion, we found that CDKN3 may be an important regulator of NPC growth and metastasis through the regulation of p27. An aberrant CDKN3/p27 signal may be a novel therapeutic target for the treatment of GC.

ACKNOWLEDGMENT: The authors declare no conflicts of interest.

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