

RETRACTED ARTICLE: miR-96-5p Suppresses the Progression of Nasopharyngeal Carcinoma by Targeting CDK1

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Background: Nasopharyngeal carcinoma (NPC) is a malignant tumor that occurs in the nasopharyngeal mucosa. Clinically, radiotherapy is the preferred treatment for NPC, and cervical lymph node metastasis is easy to emerge in the early stage. Therefore, this study aimed to investigate the role and potential molecular mechanisms of miR-96-5p in NPC cells to develop new therapeutic horizons.

Methods: The expression of miR-96-5p and CDK1 was measured by RT-qPCR or Western blot. The target relationship between miR-96-5p and CDK1 was confirmed by luciferase reporter assay. CCK-8, sphere formation, flow cytometry and colony formation assay were employed to examine cell viability, stem-like property, apoptosis and cycle, respectively. Male BALB/c nude mice model (6–8 weeks, weigh 18–20 g) was used to evaluate the effect of miR-96-5p on tumor growth in vivo.

Results: miR-96-5p was lowly expressed and CDK1 was highly expressed in NPC tissues and cell lines. CDK1 was identified as a direct target of miR-96-5p, and its expression was negatively regulated by miR-96-5p. By targeting CDK1, miR-96-5p overexpression significantly inhibited tumor sphere formation, promoted apoptosis and cell cycle arrest in CNE-2Z cells. Importantly, CCK-8 and colony formation assay demonstrated that elevated miR-96-5p enhanced the radiotherapy and chemotherapy sensitivity of CNE-2Z cells. Animal experiments showed that the overexpression of miR-96-5p reduced tumor weight and size in tumor-bearing mice and inhibited the expression of stem-like marker proteins and apoptosis-related proteins.

Conclusion: These results, together, suggested that miR-96-5p induced cell cycle arrest and apoptosis, inhibited stem-like property, and enhanced the radiochemical sensitivity of NPC by targeting CDK1. In short, miR-96-5p may be a diagnostic and therapeutic target for NPC.

Keywords: nasopharyngeal carcinoma, miR-96-5p, CDK1, stem-like property, radiochemosensitivity

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common squamous cell carcinomas involving the nasopharyngeal epithelium.¹ In China, the incidence rate of NPS is nearly 20 times that of in Western countries.² Currently, no significant improvement in overall survival³ of NPC patients has been observed even with radiochemotherapy for NPC.⁴ Since the pathogenesis of NPC has not been fully elucidated, it is urgent to seek effective treatment strategies.

It is well known that microRNAs (miRNAs) are a small class of noncoding single stranded RNA, which act a pro-cancer or anti-cancer role in the malignant progression of different tumors, such as colorectal cancer,⁵ oral cancer,⁶ lung

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cancer⁷ and NPC.¹ MiR-96-5p, a member of the miR-183-96-182 cluster, plays an important role in tumor regulation.⁸ Importantly, miR-96-5p has been studied as an oncogene and bio-marker of oral tumorigenesis.⁹ Furthermore, miR-96-5p has carcinogenic effects in the progression of colorectal cancer.¹⁰ However, the role and mechanism of miR-96-5p in nasopharyngeal carcinoma remains unknown.

Cyclin-dependent kinase 1 (CDK1) is a cell cycle regulator that interacts with cyclin B to drive the G2-M transition and binds to other interphase cyclin (cyclin D1, E and A) to regulate G1 progression and G1-S transition.^{11,12} CDK1 may regulate apoptosis through caspase phosphorylation.^{13,14} In addition, studies have demonstrated that CDK1 plays an important role in maintaining pluripotency and genomic stability of human pluripotent stem cells.¹⁵ CDK1 is overexpressed in cancers and inhibition of CDK1 is considered a potential cancer treatment.¹⁶

In this study, we detected the miR-96-5p expression in NPC tissues and cells. Also, we investigated its effect on the cell viability, cell cycle, apoptosis, stem-like property and radio-chemosensitivity of NPC cells as well as the interaction between miR-96-5p and CDK1, to provide a basis for the study of NPC targeted therapy.

Materials and Methods

Tissue Samples

A total of 49 NPC tissues and para-carcinoma normal tissues (5 cm away from tumors) were obtained from affiliated traditional Chinese medicine hospital of southwest medical university and immediately collected in liquid nitrogen. Before obtaining these specimens of fresh tissues, all NPC patients signed a written informed consent. This study was strictly in accordance with the Declaration of Helsinki (2013 revision) and approved by the ethics committee of southwest medical university.

Cell Culture

Human immortalized nasopharyngeal epithelial cell line NP69 and NPC cell lines (CNE-2Z, HNE-2, C666-1, 5-8F and SUNE-1) were obtained from China Center for Type Culture Collection (Wuhan, China). These six cells were kept in RPMI-1640 or DMEM (High Glucose) medium (Thermo Fisher, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, CA, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL), and were

grown in a 5% CO₂ incubator at 37°C. Cisplatin (DDP)-resistant (CNE-2Z-DDP) and radiotherapy-resistant (CNE-2Z-R) cell lines established as previously described.^{17,18}

Transfection

Briefly, CNE-2Z cells were plated and grown to 80–90% confluence, each well was added with 2 mL medium without antibiotics. CDK1 overexpression vector (pc-CDK1), miR-96-5p mimics or NC mimics were synthesized by GenePharma (Shanghai, China). According to the manufacturer's instructions, CNE-2Z cells were transfected using Lipofectamine 2000 (Invitrogen, CA, USA) and were harvested 24 h after transfection for the following experiments.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA was extracted with Trizol reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. RT-qPCR was performed to determine the expression of miR-96-5p or CDK1 using SYBR mix kit (Takara, Otsu, Japan) on 7500 RealTime PCR System (BioRad, California, USA). Data were normalized with the 2^{-ΔΔCt} means, and the primers were listed below:

miR-96-5p-F: 5'-ATGCTTTCTCAACTTGTGG-3',
miR-96-5p-R: 5'-TCACCG CTCTGGCCGTCACA-3';
CDK1-F: 5'-CAATGACCCCGCACGATTTC-3',
CDK1-R: 5'-CATGGAGGGCGGATTGGAA-3';
GAPDH-F: 5'-AACGGATTTGGTCGTATTGGG-3',
GAPDH-R: 5'-TCGCTCCTGGAAGATGGTGAT-3';
U6-F: 5'-AGGGGCCATCCACAGTCTTC-3',
U6-R: 5'-AACGCTTCACGAATTTGCGT-3'.

Sphere Formation Assay

CNE-2Z cells with a concentration of about 1.5 × 10³ cells/mL were seeded in 6-well plates (Corning, NY, USA) and cultured in serum-free DMEM-F12 medium (Gibco, CA, USA) supplemented with 20ng/mL EGF (Sigma-Aldrich, St. Louis, USA), 20ng/mL bFGF (Sigma-Aldrich, St. Louis, USA) and 1% B27 (Invitrogen, CA, USA). Post 14 days, the number of spheres was captured and quantified using an inverted microscope (Zeiss, Oberkochen, Germany).¹⁹

Radio-Chemosensitivity Administration

CNE-2Z, CNE-2Z-DDP and CNE-2Z-R cells were seeded into a 96-well plate (5 × 10³ cells/well) and incubated for 24 h. CNE-2Z and CNE-2Z-DDP were treated with DDP

of 0, 2.5, 5.0, 10 or 20 μM , CNE-2Z and CNE-2Z-R were irradiated with X-rays of 0, 2, 4, 6 or 8 Gy. Then, these cells were employed for subsequent clone formation and CCK-8 assay.

Clone Formation Assay

Post transfection 24 h, the 1×10^5 CNE-2Z, CNE-2Z-DDP and CNE-2Z-R cells were uniformly planted in a 6-well plate (Corning, NY, USA) and incubated in a 5% CO_2 , 37°C environment for 2 weeks. After that, the cell colonies were fixed with 10% formaldehyde (Beyotime, Haimen, China) and stained with 1% crystal violet (Beyotime, Haimen, China) for 5 min. The visible colonies were captured under an inverted microscope (Zeiss, Oberkochen, Germany) and counted by a Digital Imaging Software QCapture Pro 7 (EBSCO Industries, Inc., Birmingham, USA).

Cell Viability (CCK-8) Assays

1×10^3 transfected cells (CNE-2Z, CNE-2Z-DDP and CNE-2Z-R) were cultured in a 96-well plate for 24 h, respectively. Post radio-chemosensitivity administration, cells were supplemented with 10 μL CCK-8 solution (Sigma-Aldrich, St. Louis, USA) for another 24 h incubation, according to the manufacturer's instructions. Finally, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad, California, USA). Cell survival rate = (OD value in treated group - OD value in control group) / (OD value in the non-treated group - OD value in control group) $\times 100\%$.

Luciferase Activity Assay

The interaction relationship between has-miR-96-5p and CDK1 3'UTR was predicted by targetscan software (<http://www.targetscan.org/ver72/>). CDK1 3' UTR wild-type (wt) or mutant (mut) sequences targeting miR-96-5p were constructed by GenScript (Nanjing, China). The cells were transfected with miR-96-5p mimic and CDK1 3' UTR (wt) or CDK1 3' UTR (mut) using Lipofectamine 2000 (Invitrogen, CA, USA). Luciferase activity was evaluated at 48 h by Dual-Luciferase Reporter Assay System (Promega, WI, USA).

Western Blot (WB)

Whole cell lysates were prepared in RIPA buffer (Beyotime, Haimen, China). Proteins were separated by SDS-PAGE, followed by protein concentration measurement, and then were transferred onto PVDF membrane.

After the membranes were blocked with 5% skim milk, they were incubated with primary antibodies against CDK1 (ab133327, 1:10,000), Bmi1 (ab126783, 1:10,000), SOX2 (ab93689, 1:100), NANOG (ab109250, 1:1000), ALDH1A1 (ab215996, 1:1000), Bax (ab32503, 1:1000), Bcl-2 (ab32124, 1:1000), cleaved Caspase-3 (ab2302, 1:1000), cleaved Caspase-9 (ab32539, 1:1000), cyclinB1 (ab32053, 1:5000) and Human 14-3-3- σ antibody (AF44241, 1 $\mu\text{g}/\text{mL}$) overnight at 4°C. The next day, the samples were supplemented with the corresponding secondary antibody at room temperature for 1h. Protein bands were visualized using ECL substrates (BioRad, #170-5061) and imaged on Kodak X-COMAT 2000A (Rochester, NY, USA). Human 14-3-3- σ antibody was purchased from R&D Systems (Minneapolis, USA), and the rest from Abcam China (Shanghai, China).

Animal Model in vivo

All animal experiments were conducted in accordance with NIH guidelines for the care and use of experimental animals and approved by the southwest medical university. CNE-2Z cells stably transfected with miR-96-5p mimics or NC were injected subcutaneously into the right side of male BALB/C nude mice (6–8 weeks, 18–20 g) from Beijing Vital River Laboratory Animal Technology Company (Beijing, China) to establish a xenograft tumor model, tumor weight or volume of mice were checked every 3 day after 7 days. The mice were randomly divided into two groups (n=10): the NC group and the miR-96-5p mimic group. Twenty-five days later, mice were sacrificed, and the tumor tissues were collected for RT-qPCR, WB or IHC analysis, volume = (length \times width²) / 2.

Immunohistochemistry Assay (IHC)

Post 10% formalin fixation, the tumor tissues were dewaxed and rehydrated. The sections were then blocked for 1 h in PBS with 10% normal goat serum. After that, the sections were incubated with ALDH1A1 or cleaved Caspase-3 antibody (Abcam China, Shanghai, China). Next day, the sections were rinsed and incubated with HRP-labeled secondary antibodies for 30 min at 37°C, and the visual images were captured using an optical microscopy (Leica, Wetzlar, Germany).

Statistical Analysis

The results are presented as the mean \pm the standard deviation (S.D.), and was repeated in replicate. SPSS 22.0 (IBM, Chicago, USA) was used for statistical

analysis, one-way ANOVA was employed to analyze the data differences. The difference was considered statistically significant at $P < 0.05$.

Results

miR-96-5p Was Lowly Expressed and CDK1 Was Highly Expressed in NPC Tissues and Cells

As shown in [Figure 1A and B](#), the expression of miR-96-5p in tumor tissues was significantly lower than that in adjacent normal tissues. On the contrary, the mRNA level of CDK1 in tumor tissues was significantly higher than that in adjacent normal tissues. In addition, the mRNA levels of miR-96-5p and CDK1 in NPC cell lines (CNE-2Z, HNE-2, C666-1, 5-8F and SUNE 1) and normal nasopharyngeal epithelial cell line NP69 were detected by RT-qPCR. Results in [Figure 1A and B](#) revealed that the expressions of CDK1 in NPC cell lines were up-regulated, while the expressions of miR-96-5p in NPC cell lines were down-regulated, which were consistent with those in tissues. Notably, the differential expression of miR-96-5p or CDK1 was most significant in CNE-2Z cells ([Figure 1A and B](#)). Also, Western blot assay showed that CDK1 protein expression in tumor tissues was higher than that in adjacent normal tissues ([Figure 1C](#)). Hence, we chose CNE-2Z cells for further study.

CDK1 Was a Target of miR-96-5p

TargetsCan predicted that CDK1 3'-UTR was targeted by miR-96-5p between site 585 and 591 ([Figure 2A](#)). Then, the dual luciferase reporter assay was used to assess whether CDK1 was a direct target for miR-96-5p. As shown in [Figure 2B](#), wt-CDK1-3'-UTR luciferase activity was inhibited by miR-96-5p mimic, while mut-CDK1-3'-UTR luciferase activity did not change significantly. Furthermore, we detected the mRNA levels of miR-96-5p and CDK1 by RT-qPCR. [Figure 2C](#) shows a significant increase in mRNA levels of miR-96-5p, indicating its successful transfection. The relative mRNA level of CDK1 in miR-96-5p mimic cells was significantly lower than that in control groups ([Figure 2D](#)).

miR-96-5p Inhibited the Stem-Like Properties of NPC Cells

To assess the effect of miR-96-5p and on NPC stem cells, stem-like properties were detected by spheroid

colony formation assay. As shown in [Figure 3A](#), miR-96-5p overexpression significantly reduced the pellet-forming ability of cells, while CDK1 overexpression showed the opposite effect. In addition, Western blot further demonstrated that miR-96-5p overexpression increased the expression of stem-like properties marker proteins (Bmi-1, SOX2, NANOG and ALDH1A1) in cells, while CDK1 decreased their expression, and miR-96-5p weakened the downregulation effect of CDK1 on these proteins after miR-96-5p and CDK1 co-transfection ([Figure 3B and C](#)).

miR-96-5p Promoted the Apoptosis and Cell Cycle Arrest of NPC Cells

As shown in [Figure 4A and B](#), flow cytometry showed that miR-96-5p overexpression significantly promoted CNE-2Z apoptosis while CDK1 overexpression inhibited its apoptosis compared with control. In addition, Western blot further demonstrated that miR-96-5p overexpression increased the expression of Bax, cleaved Caspase-3 and cleaved Caspase-9 in cells and decreased Bcl-2 expression. CDK1 inversely altered these proteins content ([Figure 4C and D](#)). Furthermore, miR-96-5p mimic overexpression promoted cell cycle arrest in G2/M phase ([Figure 4E](#)), and inhibited the expression of cell-cycle-associated proteins (14-3-3- σ and cyclinB1) ([Figure 4F and G](#)). After co-transfection of miR-96-5p mimic and pc-CDK1, miR-96-5p reversed the inhibitory effect of CDK1 on apoptosis and cell cycle of CNE-2Z cells.

miR-96-5p Increased the Chemoradiotherapy Sensitivity of CNE-2Z Cells

To investigate the role of miR-96-5p in chemoradiotherapy resistance in NPC, we overexpressed miR-96-5p in CNE-2Z-R or CNE-2Z-DDP cells and treated with different doses of radiation or DDP, respectively. As shown in [Figure 5A](#), under 0, 2, 4, 6 or 8 Gy X-ray irradiation, cell colonies formed by CNE-2Z-R decreased with dose gradient irradiation, and the colony number of miR-96-5p mimic was higher than that in NC group. The cell survival curve showed that the cell survival fraction (SF) decreased with the ascended of the ascended irradiation. Compared with the NC, the survival curve in mimic group shifted to the right. Notably, the reduction of cell SF in CDK1 group was

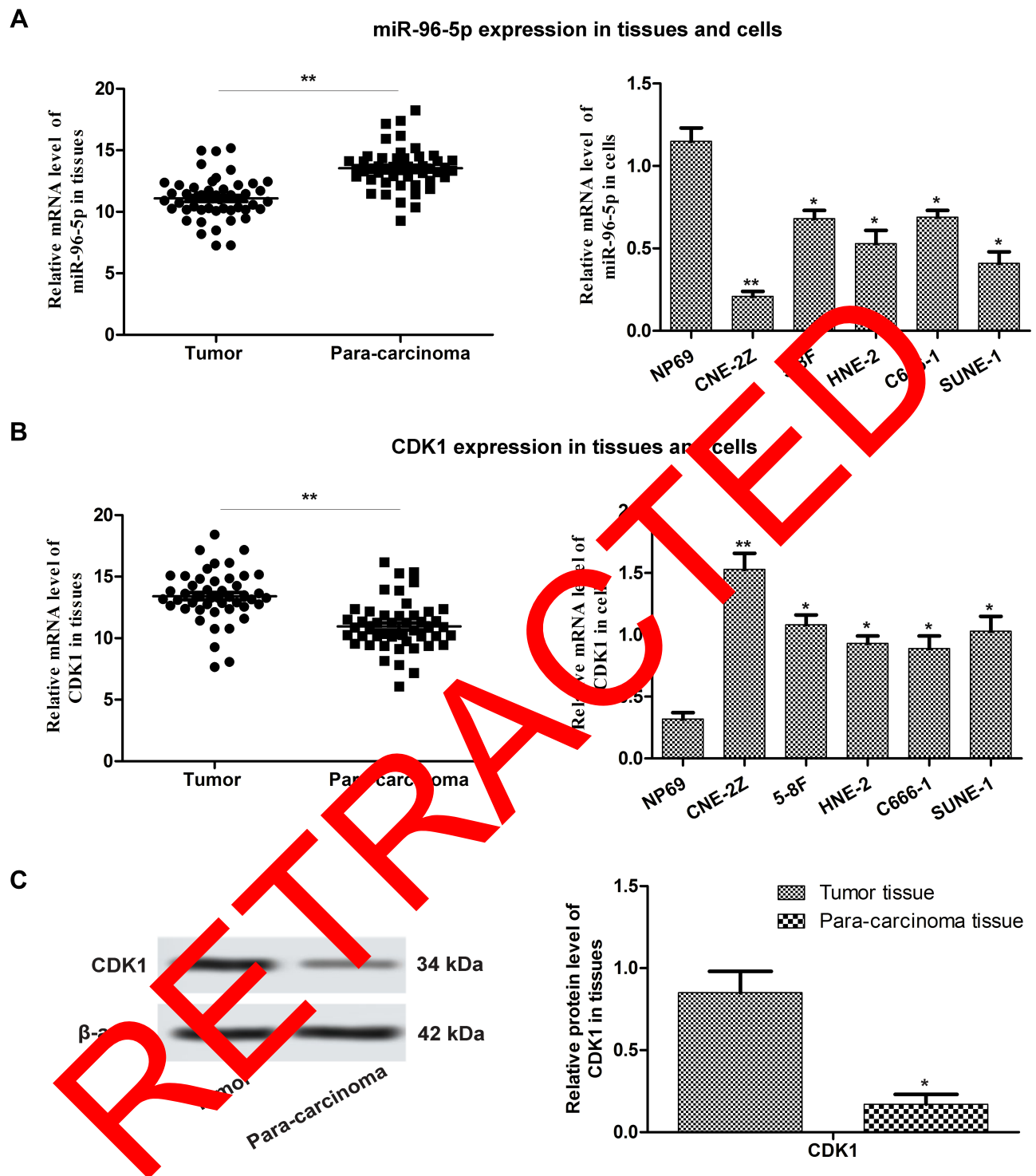


Figure 1 miR-96-5p low-expression and CDK1 high-expression in NPC tissues and cells. **(A)** The mRNA levels of miR-96-5p or CDK1 in NPC tissues and para-carcinoma normal tissues were examined by RT-qPCR. **(B)** The mRNA levels of miR-96-5p or CDK1 in NP69 cells and NPC cells (CNE-2Z, HNE-2, C666-1, 5-8F and SUNE-1) were examined by RT-qPCR. **(C)** The protein level of CDK1 was detected by Western blot. Data are presented as mean \pm SD. *P < 0.05, **P < 0.01. GAPDH or U6 as the internal control. All operations were performed in triplicate.

more obvious. After co-transfection mimic and pc-CDK1, miR-96-5p inhibited the decrease of cell SF, compared with CDK1 group (Figure 5B). In

addition, CNE-2Z-DDP cells were exposed to different doses of DDP (0, 5, 10, 15 or 20 μ M). The results of Figure 5D demonstrated that miR-96-5p

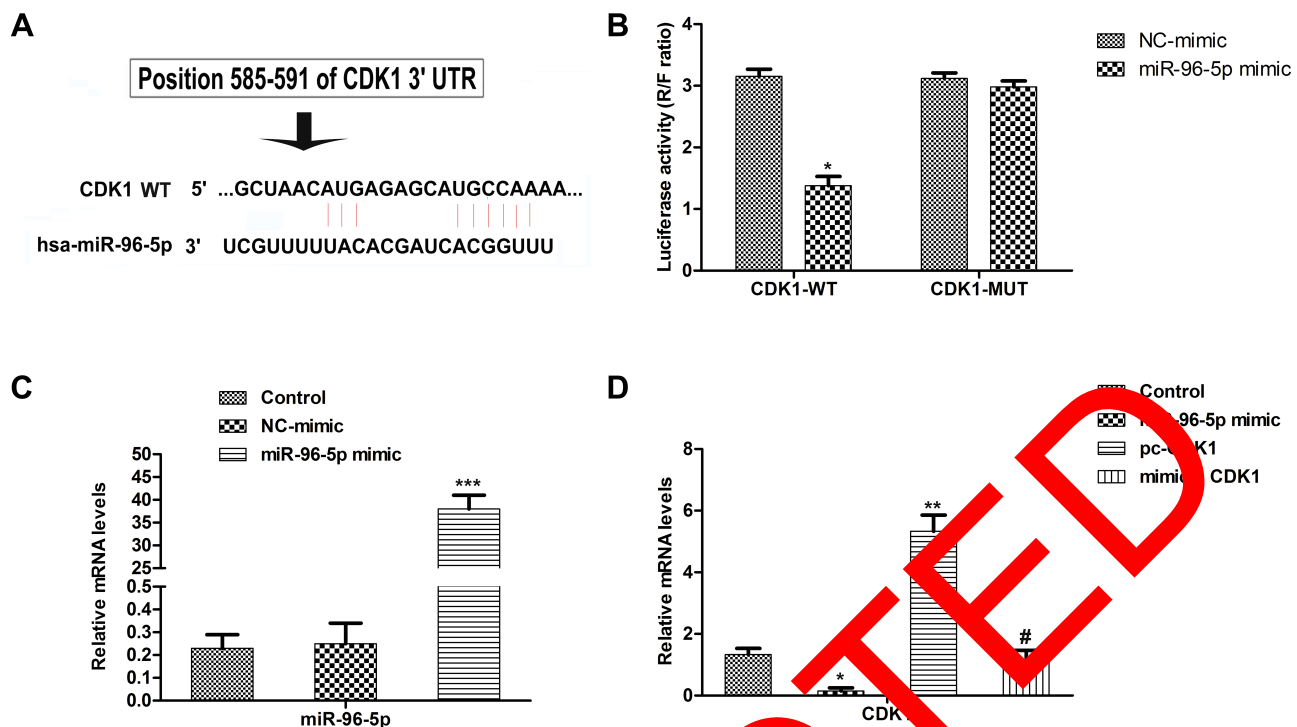


Figure 2 miR-96-5p directly targets CDK1. (A) The predicted binding sites between miR-96-5p and CDK1 3' UTR employing TargetScan software. (B) Luciferase activity of WT or MUT-CDK1 was detected using luciferase reporter. (C) CNE-2Z cells were transfected with miR-96-5p mimic or NC, the mRNA level of miR-96-5p was detected by RT-qPCR assay. (D) CNE-2Z cells were transfected with miR-96-5p mimic/NC, pc-CDK1 or co-transfection, the mRNA level of CDK1 was detected by RT-qPCR assay. Data are presented as mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control; # $P < 0.05$ vs pc-CDK1; U6 as the internal control. All operations were performed in triplicate.

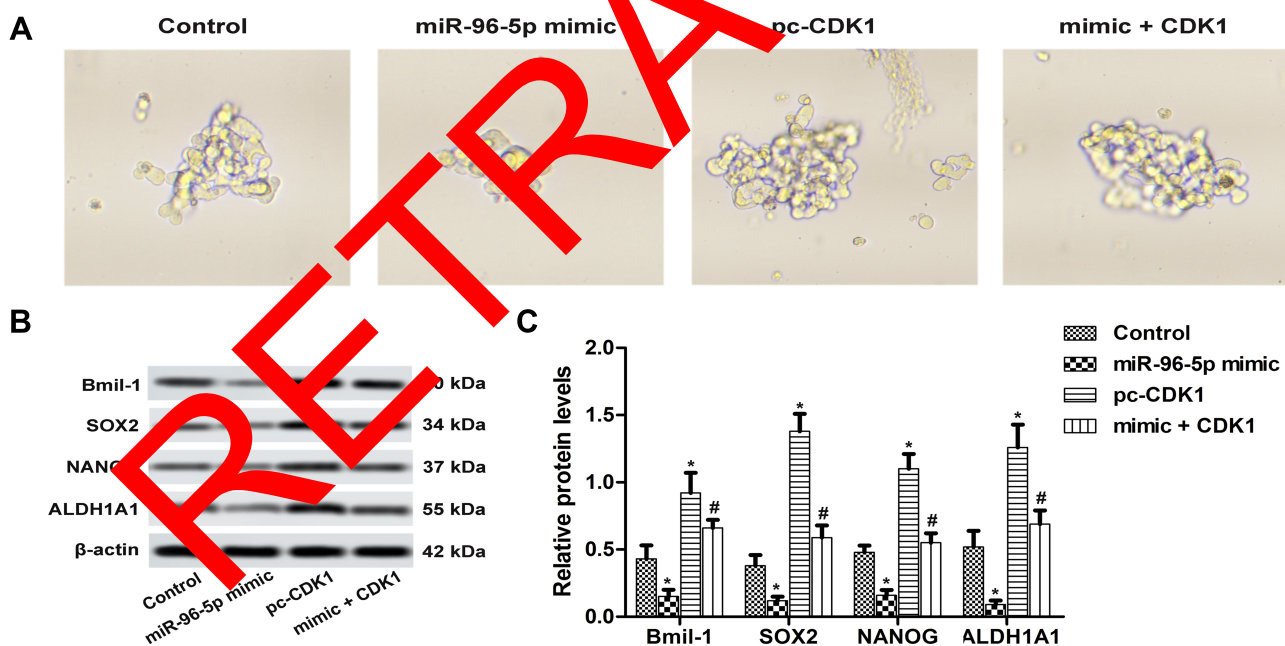


Figure 3 Effect of miR-96-5p on cell stem-like property. (A) sphere formation of CNE-2Z cells in different groups (200 \times). (B, C) The protein levels of Bmi-1, SOX2, NANOG and ALDH1A1 were detected by Western blot. Data are presented as mean \pm SD. * $P < 0.05$ vs control; # $P < 0.05$ vs pc-CDK1. β -actin as the internal control. All operations were performed in triplicate.

overexpression promoted the clonal formation ability of CNE-2Z-DDP cells, compared with the NC. Also, CCK-8 showed that CDK1 overexpression restrained

CNE-2Z-DDP cells' viability with gradient doses of DDP, but this effect was reversed by miR-96-5p mimic (Figure 5C).

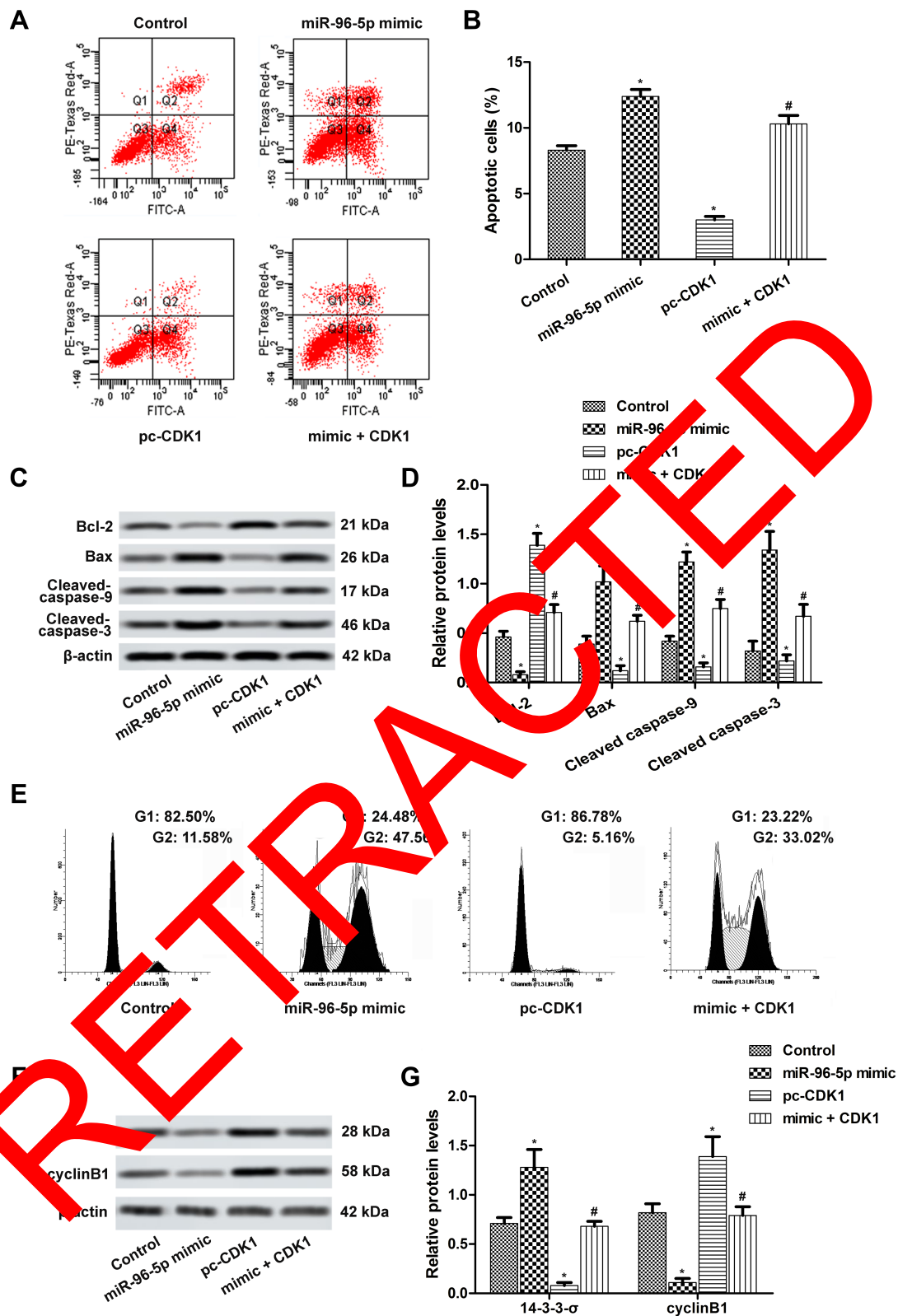
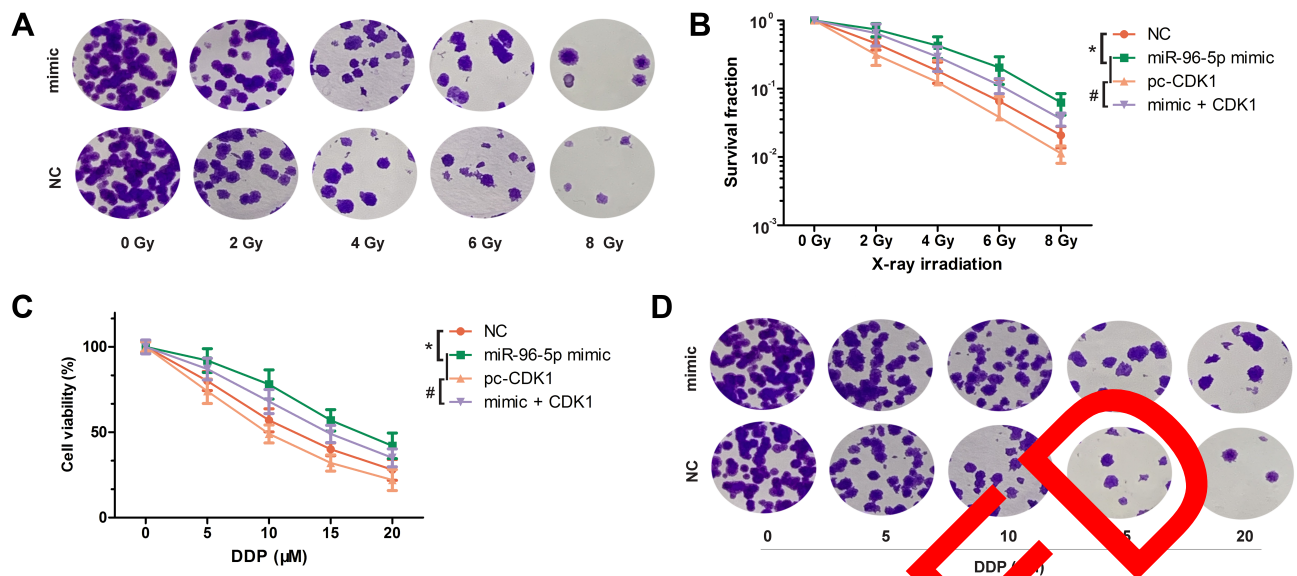


Figure 4 Effect of miR-96-5p on cell apoptosis and cell cycle. **(A, B)** Apoptosis of CNE-2Z cells was detected by flow cytometry. **(C, D)** The protein levels of Bcl-2, Bax, cleaved-Caspase-3 and cleaved-Caspase-9 were detected by Western blot. **(E)** Cells cycle of CNE-2Z cells was analyzed by flow cytometry. **(F, G)** The protein levels of 14-3-3- σ and cyclinB1 were detected by Western blot. Data are presented as mean \pm SD. *P < 0.05 vs control; #P < 0.05 vs pc-CDK1. β -actin as the internal control. All operations were performed in triplicate.



miR-96-5p Overexpression Suppressed Tumor Formation in vivo

To examine the anti-tumor effect of miR-96-5p in vivo, CNE-2Z cells transfected with miR-96-5p mimics were used to establish a xenograft tumor model. As shown in Figure 6A–C, we observed that miR-96-5p mimic decreased the tumor weight and size, compared with NC.

In addition, the mRNA level of miR-96-5p was elevated while CDK1 was reduced in tumor tissues (Figure 6D). Compared with NC, Western blot analysis showed that elevated miR-96-5p significantly inhibited the expression of NANOG, ALDH1A1 and cyclinB1 proteins in tumor tissues, but increased the expression of p53 and cleaved caspase-3 (Figure 6E). Furthermore, the data from

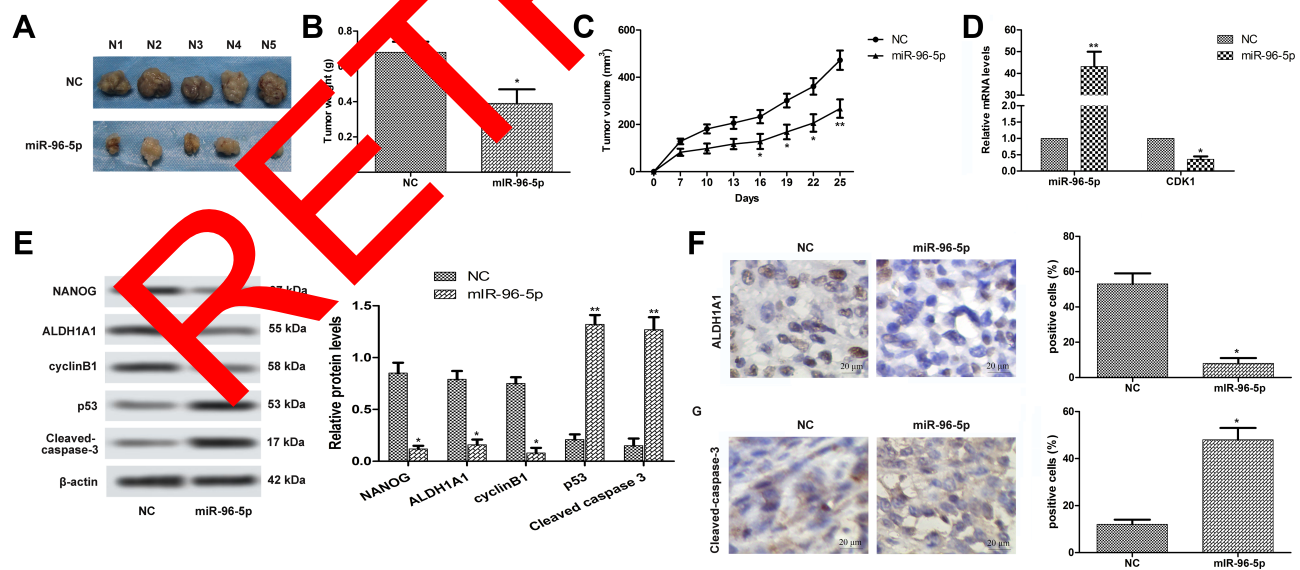


Figure 6F and G showed that miR-96-5p overexpression significantly increased the positive cell number of cleaved Caspase-3 in tumor tissues and decreased the number of ALDH1A1 cells, compared with NC.

Discussion

Nasopharyngeal carcinoma is a malignant tumor originating from the nasopharyngeal epithelium, which is prone to local recurrence and distant metastasis. Although radiotherapy or induction chemotherapy is the basic method of clinical treatment of NPC,⁴ it is necessary to find new tumor markers and targets for the treatment of advanced NPC.²⁰ In this study, we found that miR-96-5p was lowly expressed in NPC. Moreover, the abnormal expression of miR-96-5p was related to the stem-like properties, apoptosis, cell cycle arrest and radiochemotherapy sensitivity of NPC cells. Animal experiments showed that miR-96-5p overexpression inhibited tumor formation in tumor-bearing mice. Notably, we also found that CDK1 was the miR-96-5p target, and miR-96-5p regulated the progression of NPC by targeting CDK1.

Previous studies have confirmed that abnormal expression of miRNAs is closely related to tumorigenesis and development, such as apoptosis, cell cycle, cell proliferation, metastasis and radiochemotherapy resistance.^{6, 9, 11} It was observed that miR-96-5p expression was dysregulated in some cancers.^{8–10} Here, we detected that the relative mRNA level of miR-96-5p was remarkably down-regulated in NPC tissues and cells. miR-96-5p overexpression could significantly promote the apoptosis and cell cycle arrest of NPC cells. Additionally, luciferase activity was decreased in the CDK1 3' UTR (wt) by miR-96-5p mimic. CDK1 is highly conserved in eukaryotic cell cycle regulation.²² The G₂M transition is mainly regulated by the CDK1/Cyclin B1 complex, which is blocked by the depletion of CDK1.^{19, 23} Notably, we found that the positive effects of miR-96-5p-induced-apoptosis and cell cycle arrest of NPC cells could be reversed by CDK1 overexpression. Thus, miR-96-5p modulated NPC cell cycle arrest and apoptosis through suppressing the expression of CDK1.

Cancer Stem Cells (CSCs) are a group of tumor-initiating cells that can self-renew and mediate tumorigenesis and metastasis.²³ Initially, CSCs were found to have the ability to self-renew and form new tumors in acute myeloid leukemia.^{24–26} Later, the study of CSCs extended to solid tumors, such as brain cancer,²⁷ lung cancer²⁸ and colon cancer.²⁹ Unlike tumor cells, CSCs differentiate slowly on their own but are resistant

to radiotherapy and chemotherapy. Conventional chemotherapy drugs only kill ordinary tumor cells, not CSCs. Therefore, CSCs are considered to be the root cause of NPC recurrence. Su et al identified CD44⁺-like cells in human NPC cell lines.^{30,31} In our study, the microsphere formation culture obtained the stem-like characteristics of NPC cells. Sox2 is a critical member of the Sox family involved in regulating the transformation of pluripotent stem cells.³² NANOG is another critical stem cell transcription factor, together with Sox2, maintains the pluripotent differentiation potential of human embryonic stem cells as well as cell differentiation in early embryonic development. Our results found that overexpression of miR-96-5p significantly reduced the pellet size of stem-like cells and inhibited the expression of stem-like marker proteins (Bmi-1, SOX2, NANOG and ALDH1A1), suggesting that miR-96-5p overexpression inhibited NPC stem cells to form large tumor spheres.

Generally, miRNAs can modify cellular responses to irradiated or precise drugs through mechanisms such as interference DNA repair and targets.^{34,35} Interestingly, studies reported that up-regulated miRNA-21 promoted resistance of NPC cells to cisplatin-induced apoptosis.²⁰ CDK1 mediated cell cycle arrest in response to DNA damage in response to radiation.³⁶ In this study, we found that up-regulation of miR-96-5p increased the radiochemical sensitivities of NPC cells by targeting CDK1, overexpression CDK1 might be correlated with radiochemical sensitivities.³⁷

Conclusion

Collectively, the present study demonstrated that miR-96-5p was down-regulated and CDK1 up-regulated in NPC. CDK1 was identified as a direct target of miR-96-5p. Our data highlighted the tumor inhibition of miR-96-5p in NPC by targeting CDK1, it provided new biochemical markers for clinical examination or treatment of NPC.

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Disclosure

The authors report no conflicts of interest in this work.

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