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MircoRNA-629 promotes proliferation, invasion and migration of nasopharyngeal carcinoma through targeting PDCD4

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Abstract. – OBJECTIVE: MicroRNAs (miRNA) have been demonstrated to be involved in the development and progression of several tumors, including nasopharyngeal carcinoma (NPC). However, the expression and function of miR-629 in NPC have not been elucidated before. Here, we explored the role of miR-629 in NPC cells and investigated the possible underlying mechanism.

MATERIALS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was first utilized to detect the expression of miR-629 in NPC tissues and adjacent normal samples, as well as NPC cell lines and normal nasopharyngeal cell line NP69. MiR-629 mimics and inhibitor was transfected in NPC cells to up-regulate or down-regulate the expression of miR-629. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay and flow cytometry were used to explore the effects of miR-629 on the proliferation and cell circle of established NPC cells, respectively. Cell invasion and migration abilities were evaluated by transwell Matrigel assay and wound healing assay. Meanwhile, the underlying mechanism of miR-629 in NPC was detected using bioinformatics prediction and dual-luciferase analysis. In addition, Western blotting was employed to identify the expression of the miR-629 targeted protein.

RESULTS: MiR-629 expression in NPC tissues was significantly higher than that of adjacent normal samples. Expression of miR-629 in NPC cells was significantly higher than that NP69 cells. Over-expressing miR-629 remarkably promoted 6-10B cell proliferation, while knocking down miR-629 significantly inhibited 5-8F cell growth compared with negative control group. Cell migration and invasion abilities were remarkably increased by miR-629 mimics transfection. However, the miR-629 inhibitor transfection in cells significantly decreased cell migration and invasion. Furthermore, dual-luciferase analysis verified that PDCD4 was a direct target gene of miR-629 in NPC cells. Knockdown of PDCD4 in cells over-expressing miR-629 restored cell proliferation and metastasis.

CONCLUSIONS: In this study, the expression level of miR-629 was significantly increased in 83 NPC tissues and 4 cell lines. MiR-629 promoted NPC cell growth, migration, and invasion via repressing PDCD4 expression, which might provide a novel target for the future biotherapy for NPC.

Key Words:
MiR-629, Proliferation, Invasion, Migration, NPC, PDCD4.

Introduction

Nasopharyngeal carcinoma (NPC), whose main type is non-keratinized squamous cell carcinoma, is highly malignant due to local infiltration and early distant metastasis[1]. The causes of NPC include three aspects: environmental factors, genetic susceptibility, and EBV infection[2]. However, the specific underlying molecular mechanism of NPC has not been fully explained. Unfortunately, 30% to 40% of patients have already in the advanced stage when diagnosed. Meanwhile, these patients often have distant metastasis or local recurrence[3]. Therefore, it is urgent to understand the molecular mechanisms of NPC, to develop novel biomarkers for early diagnosis and prognosis, and to find new treatment options.

MiRNAs are a class of endogenous non-coding RNA molecules with 19-22 nucleotides in length. Recently, they have been found to play a post-transcriptional regulatory role in all eukaryotes. As early as 1993, miRNA was first discovered in the new rod nematode lin-4[4]. About

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50% of miRNAs are located on fragile chromosomal regions. During tumorigenesis and development, DNA amplification, deletion, and translocation may occur in these chromosomal regions[5]. MiRNAs play key functions in every step of nasopharyngeal tumorigenesis, including cell proliferation and metastasis[6]. For example, miR-184 suppresses NPC cell migration and invasion through regulating Notch2. MiR-342 inhibits NPC cell growth and metastasis by directly targeting ZEB1. Meanwhile, miR-495 down-regulates GRP78 expression *via* regulating epithelial-mesenchymal transition (EMT), eventually enhancing the sensitivity of NPC to radiotherapy[7-9]. Previous studies[10,11] have indicated that miR-328 can bind to the 3'-UTR of CD44. Subsequently, decreased cell migration, and EMT of NPC and exosomal miR-9 inhibit NPC angiogenesis *via* PDK/AKT axis though targeting MDK. MiR-629 has been reported to participate in the regulation of several malignancies. For example, down-regulation of miR-629 reduces tumor cell proliferation and metastasis by repressing testis-specific Y-like protein 5. Moreover, miR-629 promotes metastatic phenotypes of clear cell renal cell carcinoma *via* targeting TRIM33 through TGFβ/Smad pathway[12,13]. However, the exact function of miR-629 in NPC has not been fully elucidated.

Here, we first measured the relative expression of miR-629 in 83 pairs of NPC tissues and matched normal tissues. Results showed that miR-629 expression was significantly increased in NPC tissues. Meanwhile, the expression level of miR-629 in NPC cell lines was remarkably higher than normal cell line NP69. By transfecting miR-629 mimics or inhibitor, we up-regulated or down-regulated miR-629 expression in 6-10B or 5-8F cells, respectively. Next, MTT assay and flow cytometry analysis were used to explore the effects of miR-629 on the proliferation and cell circle of NPC cells, respectively. Cell migration and invasion were detected using wound-healing and transwell assays. PDCD4 was identified as a target gene of miR-629. All these results indicated miR-629 served as an onco-miR in NPC. Our findings might provide a novel target for NPC diagnosis and therapy.

Patients and Methods

NPC Tissues

A total of 83 NPC patients admitted to Nasopharyngeal Carcinoma Department of Inner People's Hospital from January 2013 to January 2016 were selected as research subjects. The investigation was approved by the Ethics Committee of Inner People's Hospital. The informed consent was obtained from each subject before the study. Tissues were stored in liquid nitrogen after removal.

Cell Lines

Human nasopharyngeal carcinoma cell lines (6-10B, CNE2, HNE1, and 5-8F) and human normal nasopharyngeal cell line (NP69) were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), and maintained in a 5% CO_2, 37°C incubator. After adherent growth, cells were sub-cultured and digested with trypsin. Cells in the logarithmic growth phase were selected for subsequent experiments.

Cell Transfection

MiR-629 mimics (Mimics) and negative control (NC), miR-629 inhibitor (Inhibitor), and miR-629 inhibitor negative control (INC) were synthesized by Shanghai Genepharma Technology Co., Ltd. (Shanghai, China). When the density of cells reached 40%-50%, miR-629 mimics, inhibitor, NC, INC were transfected into experimental cells according to the instructions of lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. SiRNA-PDCD4 was obtained from Genepharma (Shanghai, China), and was transfected into cells using lipofectamine 2000. Transfection efficiency was confirmed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Primer sequences used in this study were as follows: microRNA-miR-629, F: ACTTGTCCTATAGAAGCACAAC, R: AACATTTCCACAGCCCTGTGA. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'.

RNA Extraction and qRT-PCR

Total RNA in NPC tissues and cells was extracted in strict accordance with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reverse transcribed into complementary deoxyribose nucleic acid (cDNA) according to the Fast Quant RT Kit (TaKaRa, Otsu, Shiga, Japan) instructions. The QRT-PCR analysis was performed using cDNA as a template. The relative

expression level of miR-629 was normalized to U6. The expression of miRNA was calculated by the $2^{-\Delta\Delta Ct}$ method.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide) Assay

6-10B or 5-8F cells transfected with miR-629 mimics or inhibitor, and corresponding control cells were digested with trypsin and collected. The concentration of cells was adjusted to 3×10^5/mL. Then, cells were inoculated into 96-well plates at a density of 3000/well, followed by culture in a 37°C, 5% CO_2 incubator for 0, 24, 48, 72 h, respectively. 20 μL MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated in the dark for 4 h. After removing the old culture solution , a total of 150 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and shaken at room temperature for 10 min. Light absorption value of each well at the wavelength of 490 nm was measured by a microplate reader. The experiment was repeated for 3 times.

Cell Cycle Detection

Flow cytometry was used to analyze cell distribution. 6-10B or 5-8F cells after treatment were seeded into 6-well plates and cultured. Then, the cells were collected and washed twice with ice-cold phosphate-buffered saline (PBS; Beyotime, Shanghai, China). After fixing with 70% ethanol overnight at -20°C, the cells were stained with propidium iodide (PI) (Vazyme, Nanjing, China) at 4°C for 30 min in the dark. The stained cells detected by BD FACS Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The experiment was replicated for 3 times.

Wound Healing Assay

Transfected 6-10B or 5-8F cells were cultured in 6-well plates until the cells were spread over the entire 6-well plate. After washing with PBS, a 200 μL tip was used to vertically draw three spikes on cells at the bottom. Then, the cells were cultured in serum-free DMEM medium for 48 h. The healing of cell scratches was observed under a microscope and photographed. The healing rate of the cells was calculated based on three random positions.

Transwell Assay

8-μm transwell insert (Millipore, Billerica, MA, USA) was purchased for transwell assay. Matrigel gel (BD Sciences, Franklin Lakes, NJ, USA) was diluted with DMEM medium (3:1), added into the upper chamber of the insert (30 μL/well), and incubated at 37°C, 5% CO_2 for 5 h. A total of 200 μL FBS-free cell suspension (5×10^5/mL) was inoculated into the upper chamber, and 600 μL DMEM medium containing 20% FBS were added into the lower chamber. After incubation for 36 h, the insert was washed 3 times with PBS, fixed with methanol and stained with crystal violet. After wiping off the upper chamber cells using cotton swabs, the membrane of inserts was photographed using an inverted microscope. 5 fields of view were randomly selected for each sample.

Dual-Luciferase Assay

Approximately 1×10^6 cells were uniformly seeded into 6-well plates. Four groups were established, including control group, miRNA-138 mimics group, miRNA-138 mimics co-transfected with PDCD4 3'-UTR wild-type plasmid group, and miRNA-138 mimics co-transformed with PDCD4 3'-UTR mutant plasmids group. After 48 h, the cells were collected for subsequent analysis. Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) was obtained to detect luciferase activity following the manufacturer's instructions.

Western Blot Analysis

Transfected cells were washed twice with precooled PBS on the ice, and isolated using radioimmunoprecipitation assay (RIPA) reagent (Beyotime, Shanghai, China). The concentration of extracted protein was determined by the bicinchoninic acid (BCA) Kit (Beyotime, Shanghai, China). Protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, 5% skim milk powder solution was used to block non-specific binding for 1 h. After washing with PBS solution for 3 times, the membranes were incubated with primary antibodies of PDCD4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, Abcam, Cambridge, MA, USA) at 4°C overnight. After washing with PBS for 3 times, the membranes were incubated with the corresponding secondary antibody (Abcam, Cambridge, MA, USA) at room temperature for 2 h. Immunoreactive bands were detected using a two-color infrared laser imaging system in the dark with enhanced chemiluminescence (ECL) Kit (Millipore, Billerica, MA, USA). The experiment was repeated for 3 times.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Measurement data were expressed as mean ± SD. Independent *t*-test was applied to compare the difference between the two groups. $p<0.05$ was considered statistically significant.

Results

MiR-629 Was Highly Expressed in NPC Tissues and Cells

We first employed qRT-PCR to measure the expression level of miR-629 in 83 paired NPC tissues and matched normal tissues. As shown in Figure 1A, miR-629 expression in NPC tissue samples was significantly higher than adjacent normal tissue samples. Meanwhile, we detected miR-629 expression in 4 NPC cell lines (6-10B, CNE2, HNE1, 5-8F) and 1 human normal nasopharyngeal cell line (NP69). Results found that miR-629 was highly expressed in 4 NPC cell lines when compared with NP69 cells (Figure 1B). These results indicated miR-629 was significantly up-regulated in NPC, which might act as an onco-miR. For further experiments, we elevated miR-629 expression in 6-10B cells by transfecting miR-629 mimics. Meanwhile, we down-expressed miR-629 in 5-8F cells using miR-629 inhibitor. Transfection efficiency indicated that miR-629 expression was increased in 6-10B cells, whereas decreased in 5-8F cells (Figure 1C, 1D).

MiR-629 Promoted Cell Proliferation and Cell Cycle

To describe the influence of miR-629 in cell growth and cell cycle, MTT assay and flow cytometry were performed. MTT assay results displayed that over-expression of miR-629 significantly promoted 6-10B cell proliferation comparing to NC group. However, knockdown of miR-629 significantly suppressed 5-8F cell growth comparing to INC group (Figure 2A, 2B). Next, we analyzed the effect of miR-629 on cell cycle distribution. MiR-629 mimics transfection significantly promoted 6-10B cell transition from G0/G1 phase to S phase. However, miR-629 inhibitor transfection remarkably inhibited 5-8F cell transition from G0/G1 to S phase (Figure 2C, 2D). These results suggested miR-629 could pro-

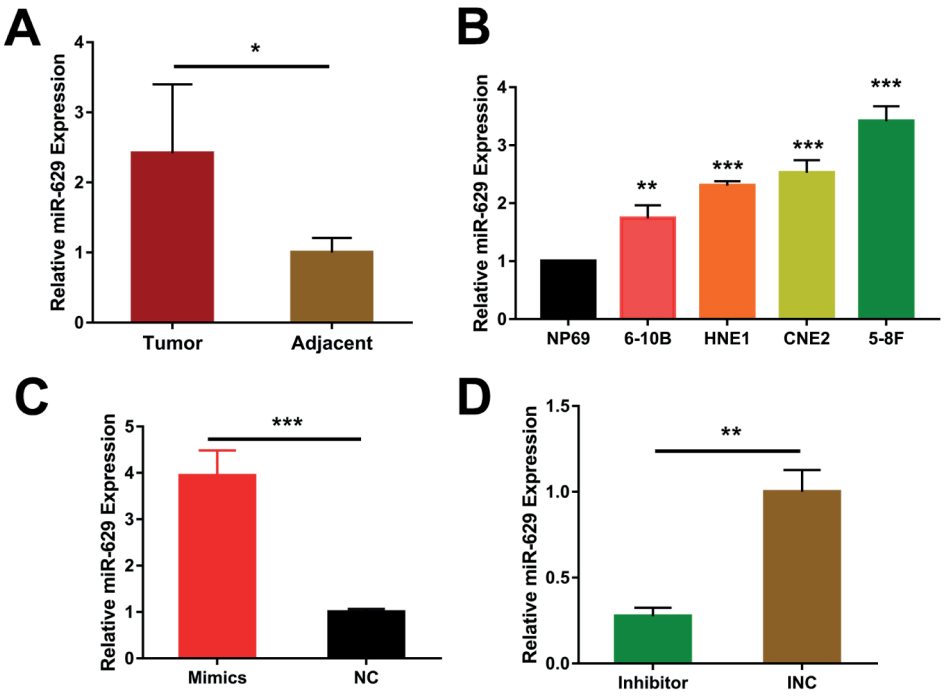

Figure 1. MiR-629 was highly expressed in NPC tissues and cell lines. ***A,*** Analysis of the expression level of miR-629 in 83 paired NPC tissue samples and adjacent normal tissues. ***B,*** Analysis of miR-629 expression level in NPC cell lines (6-10B, CNE2, HNE1, 5-8F) and human normal nasopharyngeal cell line (NP69). ***C,*** Expression of miR-629 in miR-629 mimics transfected 6-10B cells. ***D,*** Expression of miR-629 in miR-629 inhibitor transfected 5-8F cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

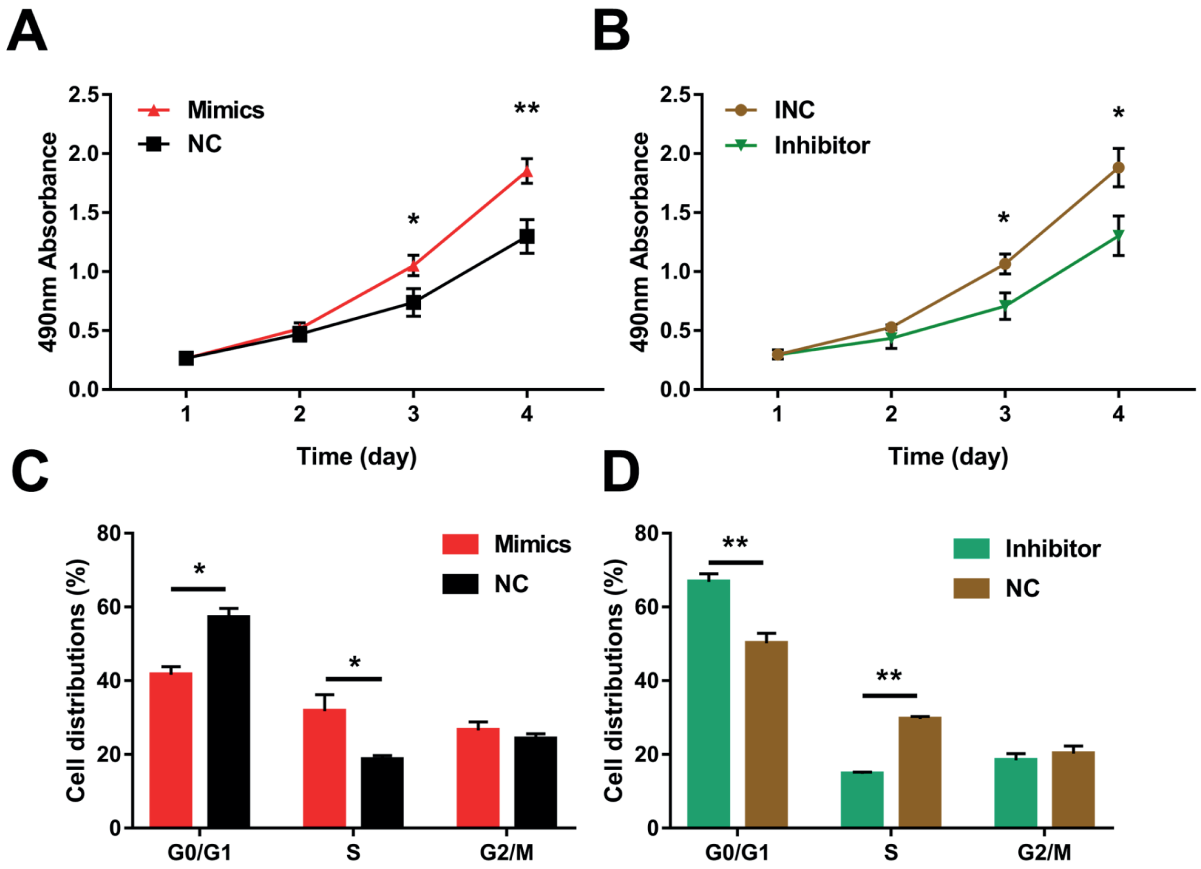

Figure 2. MiR-629 affected proliferation and cell cycle of NPC cells. ***A-B,*** MTT assay was performed to determine the proliferation of 6-10B (***A***) or 5-8F (***B***) cells transfected with miR-629 mimics or inhibitors compared to negative control cells. ***C-D,*** Flow cytometry analysis was performed to determine the cell cycle of 6-10B (***C***) or 5-8F (***D***) cells transfected with mimics or inhibitor, respectively. *p<0.05, **p<0.01, ***p<0.001.

mote cell proliferation and cell cycle transition from G0/G1 to S phase.

Ectopic Expression of miR-629 Influenced Invasion and Migration of NPC Cells

Next, we validated the effect of miR-629 on metastasis by wound-healing and transwell assays. The wound-healing rate of 6-10B cells overexpressing miR-629 was markedly higher than that of NC treated group (Figure 3A). On the contrast, 5-8F cells treated with miR-629 inhibitor showed significantly reduced wound-healing ability than INC group (Figure 3B). Meanwhile, cell invasion was detected by transwell assay. The number of invading 6-10B cells in miR-629 mimics transfection group was much more NC group. However, 5-8F cells down-expressing miR-629 presented less invaded cells than INC cells (Figure 3C, 3D). These data indicated that miR-629 promoted NPC cell migration and invasion.

PDCD4 Was a Direct Target of MiR-629 in NPC

Previous studies have demonstrated that miRNA can bind to the 3'-UTR of its target gene. Furthermore, we explored the potential underlying mechanism of miR-629 in NPC. After cross-searching several databases including miRBase, TargetScan, miRWalk, and PiTar, PDCD4 was predicted as a target for miR-629 (Figure 4A). The Dual-luciferase analysis was constructed to verify our assumption. Luciferase activity of wild-type 3'-UTR of PDCD4 group co-transfected with miR-629 mimics was significantly decreased. However, no significant difference was found in mutant group (Figure 4B). This indicated that miR-629 could directly target to the 3'-UTR

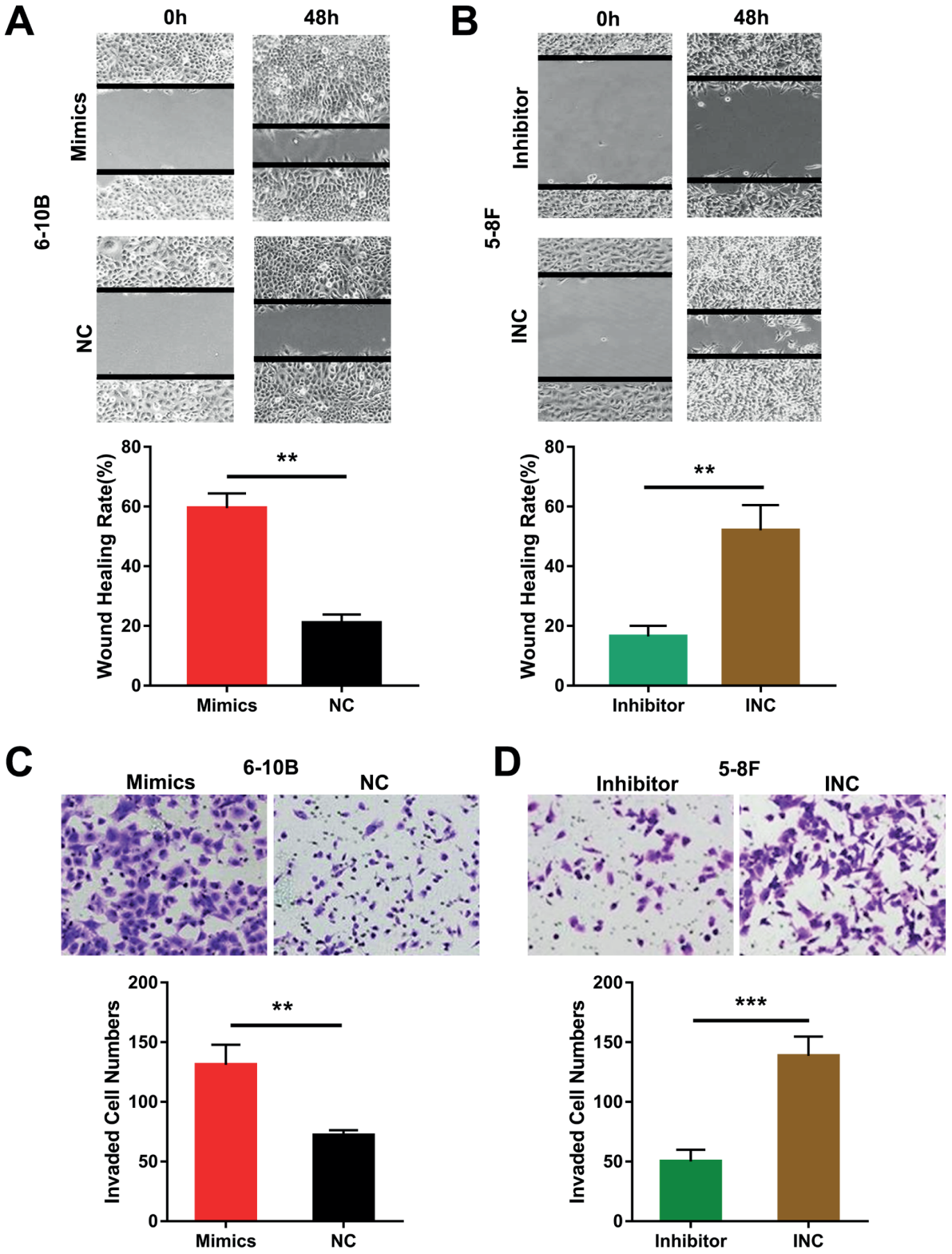

Figure 3. MiR-629 affected invasion and migration of NPC cells. ***A-B,*** Wound-healing assay was used to detect the invasion ability of miR-629 mimics transfected 6-10B cells or miR-629 inhibitors transfected 5-8F cells (Magnification: 10×). ***C-D,*** Transwell migration assay was used to detect the invasion ability of miR-629 mimics transfected 6-10B cells or miR-629 inhibitor transfected 5-8F cells (Magnification: 10×). Data were presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

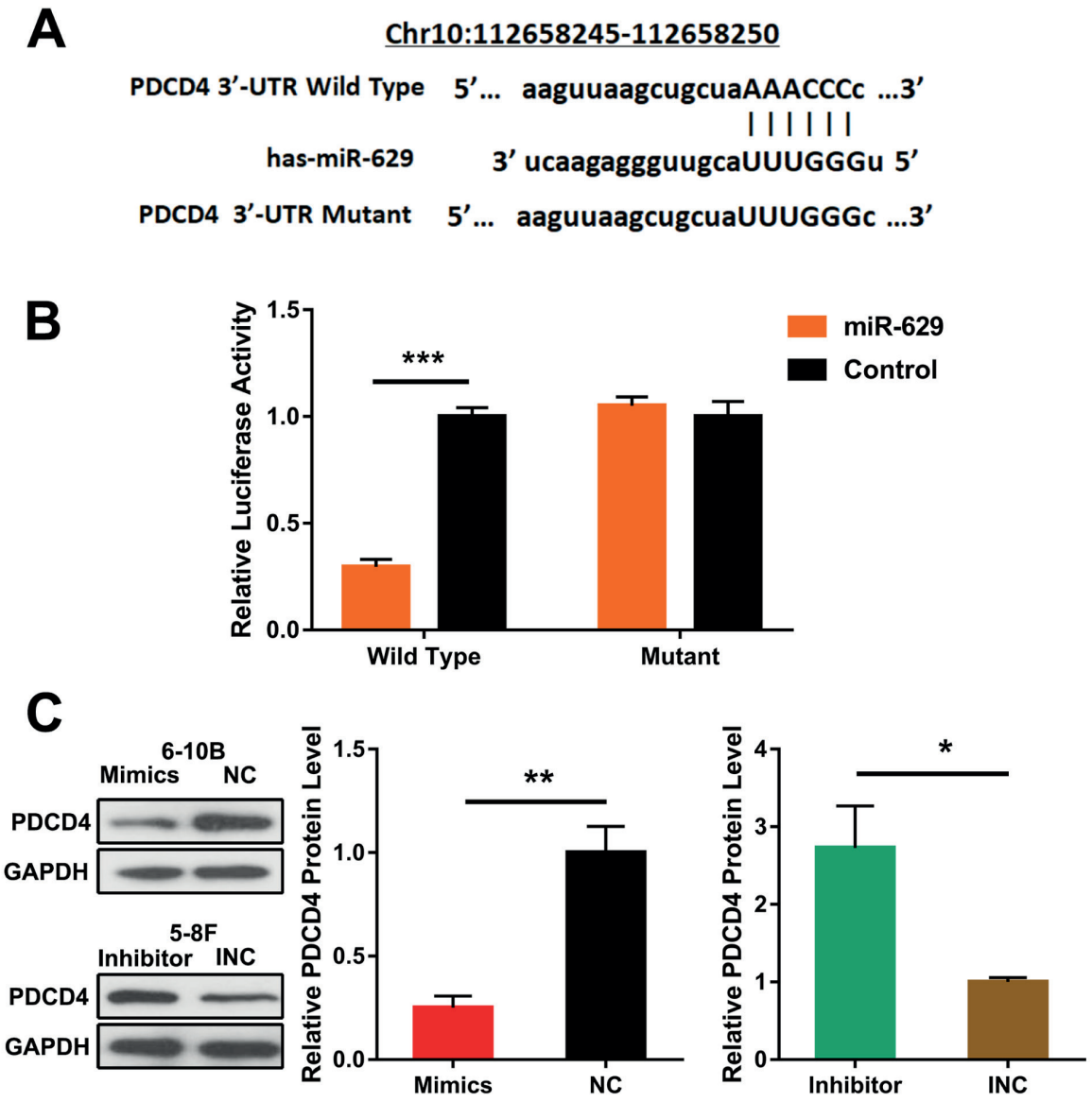

Figure 4. PDCD4 was a direct target of miR-629. ***A,*** The predicted binding sites of miR-629 in the 3'-UTR of PDCD4. ***B,*** Dual-luciferase reporter assay was used to determine the binding site. ***C,*** Levels of PDCD4 and GAPDH protein measured by western blotting in miR-629 over-expressed 6-10B cells and miR-629 knockdown 5-8F cells. The relative protein level of PDCD4 was normalized to GAPHD. Data were presented as mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

of PDCD4 in 5-8F cells. Further, we detected the protein expression of PDCD4 in experimental cells. Results showed that, after miR-629 mimics transfection, the protein expression of PDCD4 in 6-10B cells was significantly decreased. However, after miR-629 inhibitor transfection, the protein expression of PDCD4 in 5-8F cells was significantly increased (Figure 4C). These results demonstrated that PDCD4 was a direct target of miR-629 in NPC.

MiR-629 Promoted NPC Cell Proliferation and Metastasis Via PDCD4

Since we have verified PDCD4 as a target gene for miR-629, we next designed rescue experiments to re-confirm our assumption. Knockdown of PDCD4 in 5-8F cells transfected with miR-629 inhibitor could decrease the protein expression of PDCD4 induced by miR-629 inhibition (Figure 5A). Next, we measured the proliferation and invasion of cells in control, miR-629 inhibitor,

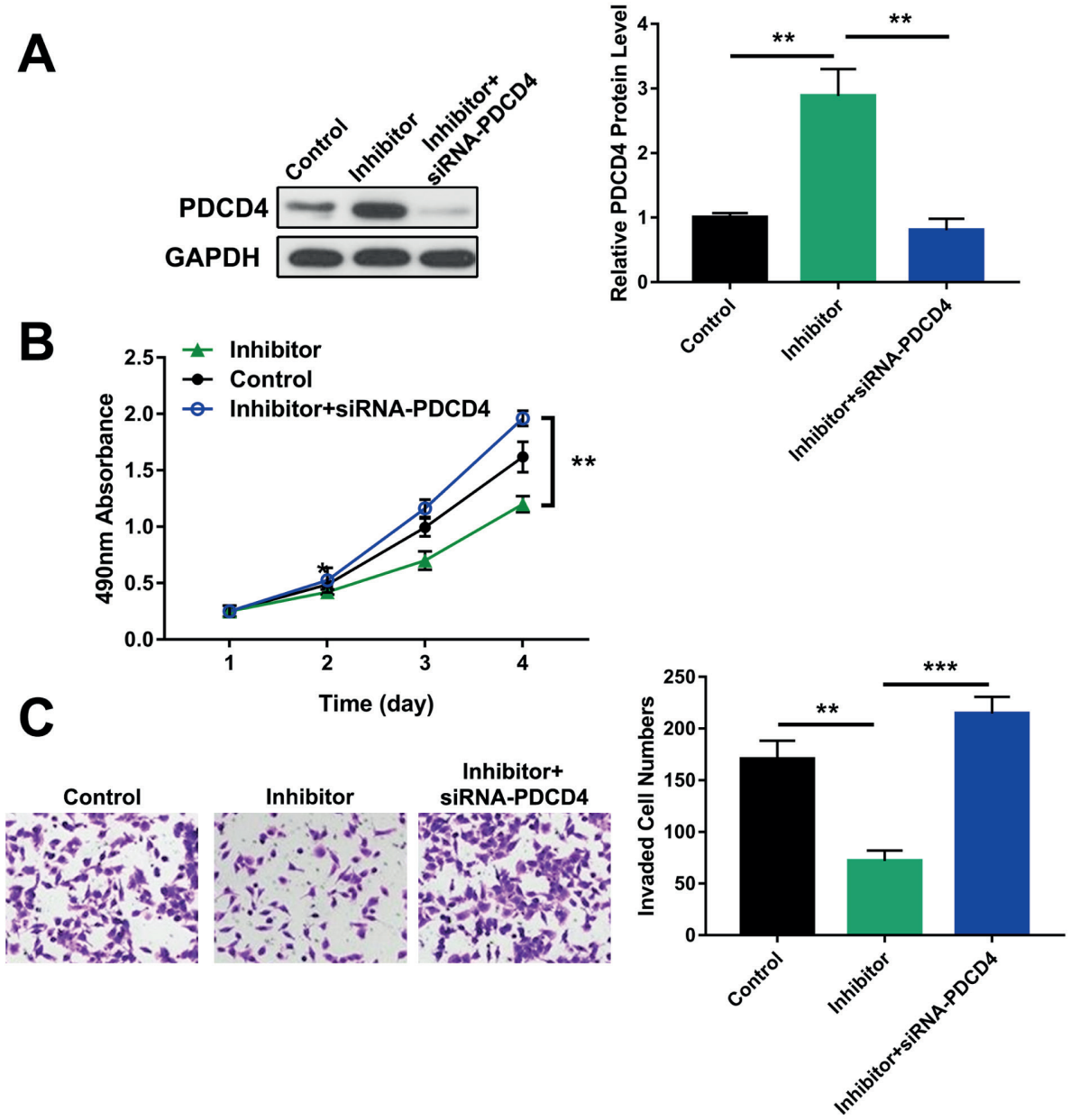

Figure 5. PDCD4 rescued the effects of miR-629 inhibition in 5-8F cells. ***A,*** Western blotting analyses of PDCD4. GAPDH was used as an internal control. ***B,*** Analysis of cell proliferation ability by MTT assay in control, inhibitor, or inhibitor + siRNA-PDCD4 treated 5-8F cells; ***C,*** Cell invasion ability was measured by transwell assay (Magnification: 10×); Data were represented as mean ± SD of three replicates. *p<0.05, **p<0.01, ***p<0.001.

and miR-629 inhibitor + siRNA-PDCD4 groups, respectively. Interference of PDCD4 significantly restored the proliferation ability of 5-8F cells reduced by miR-629 down-regulation (Figure 5B). Meanwhile, cell invasion was also rescued by siRNA-PDCD4 (Figure 5C). These results showed that miR-629 promoted NPC cell proliferation, cell cycle transition, migration, and invasion *via* repressing PDCD4 protein level.

Discussion

NPC originates from nasopharyngeal mucosa epithelium. The symptoms of NPC are often concealed, meanwhile, lymph node metastasis occurs in early stage[14]. At present, radiotherapy is the main treatment for NPC. However, the prognosis is poor due to recurrence and early metastasis[15]. Therefore, the discovery of specific molecular

markers, gene therapy targets and related mechanisms is of great significance to reduce mortality and improve prognosis of patients with NPC.

MiRNA is a class of evolutionarily highly conserved non-coding small-molecule single-stranded RNA. It only accounts for 1% to 3% of the genomic sequence. MiRNA can guide RISC to degrade target mRNA, thereby regulating protein expression of target genes[16]. Some studies have found that most miRNAs are located on the tumor-associated region on chromosomes. Therefore, miRNAs are involved in all stages of malignant tumor development and progression, serving as onco-miR or tumor suppressor miRNAs in malignancies[17,18]. MiR-629 has been identified as an onco-miR in several malignant tumors. In pancreatic cancer, miR-629 is over-expressed, which acts as a marker for prognosis and diagnosis[19]. In colorectal cancer, miR-629 accelerate cell growth by suppressing CXXC finger protein 4 expression[20]. In hepatocellular carcinoma, miR-629 is a downstream molecule of circSMAD2, which inhibits EMT[21]. Here, we found that miR-629 was significantly up-regulated in NPC tissues and cells. This was familiar to the results that miR-629 was over-expressed in many tumors. Further, our results indicated that miR-629 could promote NPC cell proliferation and cell cycle transition from G0/G1 to S phase. Moreover, it markedly accelerated cell migration and invasion as well. All these findings demonstrated miR-629 promoted NPC development and progression.

In this work, luciferase analysis and Western blotting verified that PDCD4 was a direct target of miR-629 in NPC. PDCD4 is a novel tumor suppressor gene that inhibits cell proliferation and metastasis and promotes cell apoptosis[22]. Currently, the exact role of PDCD4 remains unclear. However, increasing evidence has shown that PDCD4 not only affects protein translation, but also plays an important regulatory role in programmed cell death and signal pathways, eventually affecting the occurrence and development of tumors[23]. PDCD4 is decreased in colorectal cancer, which may have clinical significance[24]. Meanwhile, PDCD4 degradation mediated by S6K1 and β-TRCP promotes cell growth and protein translation[25]. One study has revealed that PDCD4 functions as a RSK substrate, which is negatively regulated by 14-3-3[26]. PDCD4 can also be a target gene for miR-503 or miR-21 in colorectal cancer cells, thereby regulating cell migration and invasion[27,28]. In NPC, PDCD4 blocked cell growth and survival *via* modulating miR-184-mediated direct repression of BCL2 and C-MYC[29]. Moreover, it was part of the TGFβ/PDCD4/AP-1 signaling axis to affect NPC prognosis[30]. We elucidated that PDCD4 could be regulated by miR-629 in NPC, which functioned as a regulator for NPC development and progression.

Conclusions

We demonstrated that miR-629 served as an oncogene in NPC. Meanwhile, it promoted proliferation and metastasis of NPC *via* repressing PDCD4 expression. This investigation might provide an insight into the biological diagnosis and therapy of NPC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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