

# MicroRNA-221 Targets FBXW11 to Inhibit NPC Cell Proliferation by Regulating PTEN Signaling

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## Research

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# Abstract

**Background:** In the current study, we aim to demonstrate the biological function and molecular regulatory mechanisms of miR-221 in human nasopharyngeal carcinoma (NPC).

**Methods:** The quantitative real time-polymerase chain reaction was used to measure the expression of miR-221 in NPC clinical tissues and cells. And the flow cytometry assay was used to demonstrate the role of miR-221 on cell cycle, and the potential target of miR-221 was predicted and identified using luciferase reporter assay.

**Results:** Our results demonstrate that miR-221 expression was significantly decreased in NPC tissues and cell lines. We also confirmed that inhibition of miR-221 could induce G1/S cell cycle transition through upregulation of the cyclin D-CDK4/6 complex but not cyclin E-CDK2 complexes. Furthermore, luciferase reporter assay demonstrated that miR-221 could directly bind to the 3'-UTR of FBXW11. FBXW11 expression was found to increase in NPC, and was inversely correlated with miR-221 expression; thus, FBXW11 expression interfered in the biological function of miR-221. We further confirmed that miR-221 targeted FBXW11 to inhibit proliferation and promote apoptosis in NPC cell lines through regulating the PTEN signaling pathway.

**Conclusion:** our findings suggest that miR-221 plays an important role as a tumor suppressive factor in the occurrence and progression of NPC.

## Introduction

Nasopharyngeal carcinoma (NPC) is a type of squamous cell carcinoma that is caused by malignant transformation of the nasopharyngeal epithelium. NPC is most frequently found in eastern and Southeast Asia, East Africa, and North Africa, and it represents the main cause of cancer deaths worldwide [1]. Many risk factors, such as Epstein-Barr virus infection, tobacco addiction, and genetics, may increase the incidence of NPC [2]. Although the development of a novel treatment strategy has had a certain therapeutic effect on NPC, the prognosis of patients is still below the expected standard. NPC can easily invade local tissues, and distant metastasis may occur in 30–40% of patients, which leads to poor prognosis [3, 4]. Therefore, it is of great importance to establish new prognostic markers and therapeutic targets for the treatment of NPC.

MicroRNAs (miRNAs) are a class of small noncoding RNAs around 21–25 nucleotides in length. miRNAs can be directly targeted mRNA translation area (3'-UTR), inhibit the implementation regulation by mRNA degradation and/or translation function [5, 6]. A large number of literatures have confirmed that the human genome contains more than 1000 miRNAs, which can be used to regulate thousands of human protein-encoding genes to affect multiple cellular processes, including proliferation, differentiation, apoptosis, and development [7, 8]. Recently, miRNAs have been confirmed to be involved in the development and progression of NPC [9], thus providing potential targets for the design of novel therapeutic strategies and novel clinical markers for disease diagnosis.

MicroRNA-221 (miR-221) has been reported to be involved in and modulated the progression of a variety of cancers, and there is increasing evidence of association between miR-221 and cancers, including gastric cancer and hepatocellular carcinoma [10–12]. Moreover, miR-221 can also act as a tumor suppressor depending on its targets in human cancers, including NPC, although its molecular regulatory mechanism are not fully understood. Therefore, this study used the bioinformatics-based target prediction methods TargetScan, miRanda, and DIANA to identify the potential target of miR-221 as molecule, known as F-box and WD repeat domain containing 11 (FBXW11) [13]. The function of FBXW11 has been reported in many previous studies, and it mainly affects the ubiquitination of phosphorylated substrates such as  $\beta$ -catenin and ATF4, and thus regulates multiple signaling pathways, which plays an irreplaceable role in biological processes such as cell cycle, differentiation, development and metabolism [14, 15]. FBXW11 recognizes and regulates PTEN degradation by deubiquitination and ubiquitination, which as a tumor suppressor, governs a variety of biological processes [16, 17].

Therefore, this study proposed the hypothesis that miR-221 could bind FBXW11 and attenuating its expression, thus affect the progression of NPC, and further explored the molecular mechanism. Specifically, we first examined the expression level of miR-221 in NPC tissues and cell lines, and verified regulatory role of miR-221 expression in the process of NPC progression *in vitro*, including cell proliferation and apoptosis. We also utilized a dual luciferase reporter assay to determine whether the 3'-UTR of FBXW11 mRNA is the binding target of miR-221, and demonstrate the regulatory mechanism of miR-221/FBXW11 axis on NPC progression. Our findings could provide a strong complement to the regulatory mechanism for the occurrence and development of NPC, as well as identify new drug targets for NPC therapy.

## Materials And Methods

### Patients and clinical specimens

A total of 80 NPC specimens and normal nasopharyngeal epithelial tissues were collected from the patients of the Affiliated Hospital of Southwest Medical University. The tissues acquired by surgical operation were immediately frozen in liquid nitrogen for subsequent experiments. All participants signed written informed consent, and the research plan was approved by the Medical Ethics Committee of the Affiliated Hospital of Southwest Medical University. Human NPC cell lines (CNE-1, CNE-2, C666-1, 5-8F, and HONE-1) and the normal nasopharyngeal epithelial cell line NP69 were obtained from ATCC. Human NPC cell lines were cultured in RPMI 1640 (Gibco; Grand Island, NY, USA) supplemented with 10% FBS (Gibco), while NP69 was maintained in Keratinocyte-SFM medium (Gibco).

### Quantitative real-time polymerase chain reaction

qRT-PCR with TaqMan microRNA assays (Applied Biosystems) was used to measure the expression levels of miR-221 from tissues and various transfected cells, while qRT-PCR with SYBR Green assays (Takara, Japan) was used to quantify the expression levels of FBXW11. U6 or glyceraldehyde 3-

phosphate dehydrogenase (GAPDH) was used to normalize the relative expression levels of miR-221 or FBXW11 in samples, respectively. The primer sequences were as follows:

miR-221, F-5'-TCTACGTCGTAAGTCGACTGACGATGCTAAGTGCAAGC-3', R-5'-TACGCTGACGTGCGCGTAGCTGACGATGATCCCTC -3'; FBXW11, F-5'-GTCCTGCGAATGCGATGACGTGA-3', R-5'-CGAGTGAGTGACGTAGTAGCAG-3'; GAPDH, F-5'-AAAGCGTAGACTGACGTAGGAAA-3', R-5'-TGAACCCTGAGCTGACGTACA-3'; U6, F-5'-CTCAGTAGAACCTGACGATGATA-3'; R-5'-AAGCGTAGACGATGTGTC-3'.

### ***Cell transfection***

Firstly, cDNA of FBXW11 gene was cloned into pcDNA3.1 vectors to construct the plasmid pcDNA-FBXW11. And that, miR-221 mimics (5'-GCTCGCGTGCTGCTCTCGCGCGTATGCGCC-3'), miR-negative control (NC) mimic (5'-UUCGCGCCGAGUUCGCGAAUUGUTT-3'), miR-221 inhibitors (5'-GGCCTCGCGACGTATGAGCGAGCGAGC-3'), miR-NC inhibitor (5'-CAGCGCGUUUUCGAGACGAGCGAA-3'), Wnt1 siRNAs (siRNA1: 5'-GCGGCGCUCGAGCGCUCUUUGCGATT-3'; 5'-GCTGAGTGCGCGTATUCGCGAGGTT-3'), and siRNA control (5'-UUGAGCGAGAGACGCGUCGAGAGTT-3') were provided by Novogene (Beijing, China). Lipofectamine 2000 (Invitrogen) was used for cell transfection according to the protocol of manufacturer.

The abovementioned miRNAs (50 nM) were transfected into C666-1 and 5-8F cell lines with a density of  $10^5$ /well in 6-well plates (Corning; NY, USA) by Lipofectamine 2000 reagent (Invitrogen), and the cells were collected for analysis 48 h after transfection.

### ***MTT assay***

Cell suspensions were seeded in 96-well plates (Corning) and incubated overnight, before being transfected with 50 nM miR-221 mimic or inhibitor, miR-NC mimic or inhibitor, pcDNA-FBXW11 or pcDNA-3.1, and shFBXW11 or negative control. The cell viability was determined by MTT (Sigma; Louis, MO, USA) staining. Live cells were incubating with 20  $\mu$ l of MTT (5 g/L, Sigma). The supernatant from each well was then aspirated, and 150  $\mu$ l of dimethyl sulfoxide (DMSO, Sigma) was added to each well to dissolve the MTT methoxypyrimidine crystal to obtain the absorbance, which was measured by a microplate reader at 570 nm (Molecular Devices; Shanghai, China).

### ***Flow cytometry analysis***

Cell apoptosis was analyzed by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (BD, San Jose, CA, USA). Briefly, C666-1 and 5-8F cells were seeded into 6-well plates at a density of  $1 \times 10^5$  cells/well. After transfection, cells were collected and apoptosis was observed. The apoptotic cells were labeled with FITC-Annexin V and PI and kept dark at 25°C for 30 min. The data were collected and analyzed by a cytoFLEX LX flow cytometer from Beckman-Coulter Electronics (Jiangsu, China).

### ***Cell cycle analysis***

C666-1 and 5-8F cells were transfected with miR-221 or NC for 24 h. The cells were then collected by trypsinization and washed with PBS that had been precooled on ice. The cells were fixed in 70% methanol and incubated at 4°C for 1 h. The cells were then centrifuged and incubated with RNase at 37°C for 30 min. The cells were stained with propidium iodide for 1 h, and the cell cycle was analyzed using a FACScan flow cytometer; the data were presented using CellQuest software.

### ***Western blot assay***

The assay was performed according to previous methods, and described as below. Proteins were isolated from cultured cell using the Protein isolation kit (Tiangen), and the protein concentrations were determined using the Pierce BCA assay (Thermo Scientific; CA, USA). SDS-PAGE gel was used to separate the protein samples (20 µg), after which, the gel was electrophoretically transferred to nitrocellulose membranes (Bio-Rad; Hercules, CA, USA). The membranes were incubated with 10% skimmed milk (Sigma-Aldrich; St. Louis, MO, USA) for blocking, before the addition of the primary antibody and incubation overnight at 4°C. The following primary antibodies were used in Western blot, including FBXW11, Ki67, CDK4, Cyclin-D1, CDK2, Cyclin-E, β-actin, PTEN, phosphor-PI3K (p-PI3K), total-PI3K (t-PI3K), phospho-Akt (p-Akt), and total-Akt (t-Akt), β-catenin, c-myc and GAPDH, and all primary antibodies were purchased by Cell signaling Technology company (Beverly, MA, USA). Peroxidase-conjugated anti-IgG (1:10,000; purchased from Santa Cruz Biotechnology, Inc.) was used as the secondary antibody. The exposure detection of blots was carried out with an enhanced chemiluminescent kit (BD; San Jose, CA, USA), and the corresponding gray value was calculated by Image J software for statistical analysis.

### ***Luciferase reporter assay***

A luciferase reporter assay was performed to confirm whether FBXW11 was a direct target of miR-221. Subsequently, miR-221-overexpressing 5-8F cells were seeded in a 48-well plate, and the plasmids psiCHECK-3'UTR-WT (the wild-type 3'UTR fragment of FBXW11, including conserved binding sites for miR-221) and psiCHECK-3'UTR-MUT (the mutant 3'UTR fragment of FBXW11, in which the mutations occur in the conserved binding sites for miR-221) were transfected using Lipofectamine 2000 (Thermo Scientific). Following incubation for 48h, cells were collected and determined by a dual luciferase assay kit (Thermo Scientific).

### ***Statistical analysis***

GraphPad Prism 5 software (GraphPad Software Inc.; San Diego, CA, USA) was used for all statistical analyses. Values are presented as mean ± standard deviation (SD), and significance was determined by two-tailed Student's paired t-test or one way analysis of variance (ANOVA) followed by Tukey's post hoc test. *P*-values < 0.01 were taken to indicate statistical significance.

## **Results**

# miR-221 is decreased in NPC patients and is correlated with poor overall survival

We examined the expression levels of miR-221 in NPC tissues to determine the biological function of miR-221 in the development and progression of NPC. Our results showed that miR-221 expression was significantly reduced in NPC tissues compared to normal tissues (Fig. 1A). Kaplan-Meier analysis also demonstrated that down-regulation of miR-221 was obviously correlated with worse overall survival (Fig. 1B,  $P = 0.0036$ ). We then confirmed the expression level of miR-221 in NPC cell lines. Compared to normal nasopharyngeal epithelial cells, significantly lower expression levels of miR-221 were observed in NPC cell lines (Fig. 1C). As shown in Table 1, we also analyzed the relationships between clinical characteristics and miR-221 expression, and found that downregulated expression of miR-221 was significantly correlated with the late clinical stage and no response to chemotherapy ( $P < 0.001$ ). These findings suggest that miR-221 can be not only used as a prognostic biomarker, but may be used to determine chemotherapy tolerance in NPC.

Table 1  
Association between expression and clinicopathologic characteristics in NPC patients.

Characteristic	MiR-221 expression <sup>#</sup>		<i>p</i> value
	Number	Low 57	
<b>Age</b>			<b>0.876</b>
< 50	46	34	12
≥ 50	34	23	11
<b>Gender</b>			<b>0.668</b>
Male	48	40	8
Female	32	17	15
<b>Chemotherapy response</b>			<b>0.027*</b>
No	60	51	9
Yes	20	6	14
<b>T stage</b>			<b>0.234</b>
T1-2	34	22	12
T3-4	46	35	11
<b>N stage</b>			<b>0.116</b>
N0-1	32	18	14
N2-3	48	39	9
<b>M status</b>			<b>0.436</b>
M0	45	32	13
M1	35	25	10
<b>Clinical stage</b>			<b>0.013*</b>
I + II	28	13	15
III + IV	52	44	8
* <i>p</i> < 0.05 was considered statistically significant.			
<sup>#</sup> Low/high expression was determined by the sample mean. Pearson chi-square test was utilized to analyze the clinical data.			

# MiR-221 suppresses proliferation and increases apoptosis of NPC cells

To explore the role of miR-221 in NPC progression, we transfected the miR-221 mimic or the miR-221 inhibitor into NPC cell lines C666-1 and 5-8F. Compared to the negative control, transfection of the miR-221 mimic remarkably enhanced the expression level of miR-221 in NPC cells. In contrast, transfection of the miR-221 inhibitor obviously reduced miR-221 expression (Figs. 2A and B). Furthermore, the MTT and ELISA-BrdU assays demonstrated that miR-221 decreased cell viability and proliferation compared with the NC (Fig. 2C–F), while miR-221 promoted the apoptosis of NPC cells (Fig. 2G and H). Taken together, these data suggest that miR-221 suppresses NPC cell proliferation and promotes apoptosis.

## MiR-221 blocks the cell cycle via regulating CDK4/Cyclin-D1 expression

Because the miR-221 mimic effectively decreased NPC cell proliferation, we then evaluated the effect of miR-221 on the cell cycle of NPC cells using flow cytometry. The results showed that compared to both C666-1 and 5-8F cells transfected with the miR-221 inhibitor, the miR-221 mimic significantly enhanced the proportion of cells in the G1/G0 peak, and reduced the proportion of cells in the S peak (Fig. 3A). Therefore, we speculate that miR-221 may slow down the transition of G1/S cell cycle, and prevent cell proliferation. We further investigated the mechanisms by which miR-221 regulates the cell cycle. As we know, G1/S cell cycle transition is governed by cyclin D-CDK4/6 and cyclin E-CDK2 complexes [18, 19]. Therefore, we examined the effects of the miR-221 mimic on cyclin D-CDK4/6 and cyclin E-CDK2 expression. As shown in Fig. 3B and C, the protein expression of CDK4 and Cyclin-D1 were upregulated in ovarian cancer cells transfected with the miR-221 inhibitor compared to those of the cancer cells transfected with the miR-221 mimic. Besides, the protein expression of CDK2 and Cyclin-E did not change significantly in miR-221-transfected cells compared to those in the NC. These data reveal that miR-221 can inhibit the cell cycle by regulating the expression of the cyclin D-CDK4 complex.

## MiR-221 inhibits cell proliferation in NPC cells through the PTEN/PI3K/AKT signaling pathway

To further investigate the molecular mechanisms of miR-221 in the progression of NPC, the miR-221 mimic, miR-221 inhibitor, or corresponding control were transfected into the 5-8F NPC cell line. We determined the expression levels of PTEN/PI3K/Akt pathway-related proteins, including PTEN, p-PI3K, t-PI3K, p-Akt, and t-Akt. The data demonstrated that the miR-221 mimic increased PTEN protein expression while suppressing PI3K and Akt phosphorylation (Fig. 4A). The inhibition of PTEN as a tumor suppressor has been reported to activate PI3K and Akt expression, thereby increasing the survival of various tumors [20, 21]. We also determined the effects of PTEN/PI3K/Akt pathway inhibition on cell proliferation, apoptosis, and cell cycle. Our results showed that the downregulation of cell proliferation and cell cycle, and the increase of apoptosis induced by the miR-301a mimic was significantly blocked by pretreatment

with the Akt inhibitor LY294002 (Fig. 4B-D). Collectively, these data confirm that miR-221 can inhibit NPC progression and increase apoptosis through regulating the PTEN/PI3K/Akt signaling pathway.

## **FBXW11 was identified as the target for miR-221**

We performed a bioinformatics strategy including TargetScan, miRanda, and DIANA to predict the potential target of miR-221, and the results showed that FBXW11 had the potential to bind miR-221 (Fig. 5A). Further, the expression levels of FBXW11 in a variety of NPC tumor tissues, noncancerous counterparts, NPC cell lines, and the normal control cell line were measured, and significantly increased expression of FBXW11 was observed in NPC tissues and cell lines compared with control groups (Fig. 5B and C). We also used Pearson's correlation coefficient analysis to analyze the correlation between miR-221 and FBXW11 expression, which indicated that the levels of miR-221 in NPC tissue samples were negatively correlated with those of FBXW11 (Fig. 5D). In addition, in 5-8F cells, the significantly decreased luciferase activity induced by FBXW11 3'-UTR in the miR-221 mimic group was observed compared with that in the control group, suggesting that FBXW11 may direct target of miR-221 (Fig. 5E). In order to further prove the regulatory function of miR-221 on FBXW11, we detected the levels of FBXW11 after the miR-221 mimic/inhibitor transfection via western blot analysis, and the data showed that miR-221 attenuating could increase the expression level of FBXW11, which indicated that miR-221 negatively regulates FBXW11 (Fig. 5F). And that, we further investigated the role of FBXW11 in the process of miR-221 regulating proliferation and apoptosis. As shown in Fig. 5G, H, and I, in the pcDNA-FBXW11 transfected cells, a significantly higher cell proliferation rate and an obvious decrease in apoptosis were observed in the pcDNA-FBXW11 group compared to that observed in the control group (Fig. 5G, H, and I). Collectively, these data demonstrate that miR-221 can directly target FBXW11, and thereby regulate tumor progression in NPC.

## **FBXW11 affects the PTEN/PI3K/AKT signaling pathway to reverse the function of miR-221 on NPC cells**

To demonstrate the regulatory mechanisms of miR-221 binding to FBXW11 in NPC, pcDNA-FBXW11, shFBXW11, and negative controls were transfected into NPC cells. The results demonstrated that the decrease in FBXW11 expression promoted the expression of PTEN and consequently regulated the levels of PI3K and AKT (Fig. 6A). Thus, FBXW11 was shown to have a clear biological function in regulating NPC cell proliferation. Moreover, we determined the cell proliferation and apoptosis of NPC cells under the condition of inhibition of the PTEN/PI3K/AKT signaling pathway by pretreatment with the AKT inhibitor LY294002. Our data showed that the FBXW11-mediated downregulation of cell proliferation and increase in apoptosis were attenuated following PTEN/PI3K/AKT signaling pathway inhibition (Fig. 6B and C). Collectively, these data confirmed that miR-221 binds to FBXW11, inhibits NPC cell proliferation, and increases apoptosis by regulating the PTEN/PI3K/AKT signaling pathway.

## **Discussion**

Abnormal expression of miRNAs has been shown in a variety of cancers, and the role of miRNAs in regulating the occurrence and development of tumors has become the focus of attention. Because miRNAs are critical factors involved in regulating basic functions of cells, their functions in tumorigenesis are very complex. Indeed, the expression of single miRNA may be increased in one cancer, but decreased in another. Emerging evidence has confirmed low expression of miR-125b in human breast cancer clinical biopsy samples and cell lines [22], whereas miR-125b has been shown to be highly expressed in prostate cancer clinical tissue samples [23]. Although previous studies have reported that miR-221 can act as an oncogene and is involved in the regulation of various cellular processes, including cell differentiation, apoptosis, proliferation, survival, and metastasis in different types of cancers [24, 25], in this study, miR-221 may act as a tumor suppressor in NPC.

By transfecting an miR-221 mimic into NPC cells, we determined that miRNA-221 inhibited cell proliferation. In addition, the correlation between miR-221 and FBXW11 in NPC cells prompted us to explore whether miR-221 could directly or indirectly regulate the expression of FBXW11 in NPC cells. Our results demonstrated that the miR-221 mimic increased FBXW11 expression (Fig. 5). Typically, miRNAs are characterized by the inhibition of target protein expression rather than enhancement; thus, we would not expect the direct target of miR-221 to be the FBXW11 protein. As FBXW11 expression induced cell proliferation, we hypothesized that miR-221 may directly target mRNAs that are repressed by FBXW11 expression. FBXW11 (also known as  $\beta$ -TrCP2) is related to a variety of human tumors, and its targets include various oncogenes (Mcl-1, MYC, and cyclin D), further highlighting its vital role in human cancers [26]. Moreover, FBXW11 has been reported to be negatively regulated by miR-106b-25, which increases the migration of NSCLC cells [27]. Consistent with the above studies, our data revealed that FBXW11 is downregulated by miR-221 in NPC cells, inducing increased cell proliferation and reduced apoptosis, and verifying its tumor-suppressing role in NPC.

In addition, miRNAs have been reported to act as mediators in the regulation of cell proliferation and cell cycle. Most of the previous studies are related to miRNA-induced cell cycle arrest, and these miRNAs have been shown to target key proteins regulating the cell cycle, such as cyclin D1, cyclin E, CDK4, CDK2, and E2F3 [28–30]. However, the molecular mechanisms that underlie miRNAs promoting the cell cycle and eliciting G1/S transition have not been reported, and the biological relevance of such a miRNA-mediated regulation of the cell cycle machinery *in vivo* remains unclear. CDK4 and CDK6 are activated by D-type cyclin complex formation that acts as a cell cycle growth sensor, whereas CDK2 binds to cyclin E or cyclin A, and also regulates the G1-S phase [31]. In this study, our results showed that inhibition of miR-221 could induce G1/S cell cycle transition through upregulation of the cyclin D-CDK4/6 complex but not cyclin E-CDK2 complexes. Notably, most previous studies have observed that CDK4/6 and CDK2 are upregulated and downregulated respectively and simultaneously in the cell cycle [32, 33]. However, cell cycle upregulation by an miR-221 inhibitor only upregulated CDK4 expression, whereas CDK2 expression was unchanged. This may indicate that miR-221 inhibition promotes cell proliferation via targeting CDK4, but the detailed mechanisms need to be further elucidated.

Furthermore, we demonstrated that miR-221 inhibits cell proliferation via the PTEN/PI3K/Akt signaling pathway. The PTEN gene has been found to be involved in many physiological and pathological processes, and can inhibit the occurrence and development of tumors, but it is prone to mutation [34]. PTEN, as a tumor suppressor, plays a critical biological function in the regulation of cell growth, proliferation, migration, invasion, and apoptosis [35, 36]. PTEN attenuates the survival and growth of tumor cells through inhibition of the PI3K/Akt signaling pathway, and vice versa. Downregulation of PTEN expression can activate Akt, thereby promoting cell proliferation and angiogenesis [37]. In addition, previous studies have shown that miRNA can regulate the progression of esophageal cancer through the PTEN signaling pathway [38]. Therefore, our data reveal that the PTEN/PI3K/Akt signaling pathway plays a critical role in the progression of NPC. Collectively, our results validated that miR-221 can inhibit cell growth and proliferation by upregulating PTEN levels, which indicates that miR-221 is a vital negative regulator for the PTEN/PI3K/Akt signaling pathway. Our research expands the mechanisms of the PTEN/PI3K/Akt signaling pathway in the development of NPC, and clarifies the correlation between miR-221 and the PTEN/PI3K/Akt signaling pathway, which goes some way to elucidate the regulatory mechanisms of NPC progression.

## Conclusion

In conclusion, our study confirmed a novel mechanism of miR-221 in the progression of NPC. Moreover, we highlight low miR-221 expression levels in NPC, and demonstrate that miR-221 inhibits cell proliferation but promotes apoptosis in NPC by targeting FBXW11 through regulating the PTEN/PI3K/Akt signaling pathway. Overall, our data reveal that miR-221 may be a potential targets for the treatment of NPC in the future.

## Declarations

### Compliance with Ethical Standards

#### Funding:

None

#### Conflict of interest:

The authors have completed the ICMJE uniform disclosure form, and confirmed that there is no conflict of interests.

#### Ethics approval:

This study was approved by the Institutional Research Ethics Committee of the Affiliated Hospital of Southwest Medical University (IRB approval number: 20180408)

#### Informed consent:

All participants provided written informed consent.

**Data availability statement:**

The data are available on request and provided by authors.

**Author contributions:**

**(I) Conception and design:** Jun Liu

**(II) Administrative support:** Chengyi Song, Zhuoping Liang, Xiang Long, Min Guo and Jun Liu

**(III) Provision of study materials or patients:** Chengyi Song and Zhuoping Liang

**(IV) Collection and assembly of data:** Min Guo and Jun Liu

**(V) Data analysis and interpretation:** Xiang Long and Jun Liu

**(VI) Manuscript writing:** All authors

**(VII) Final approval of manuscript:** All authors

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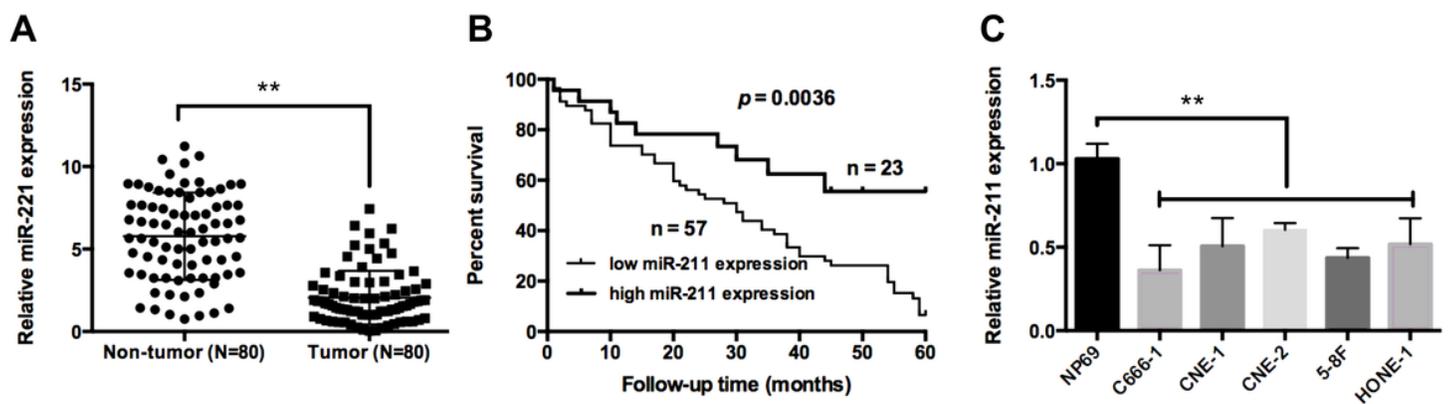
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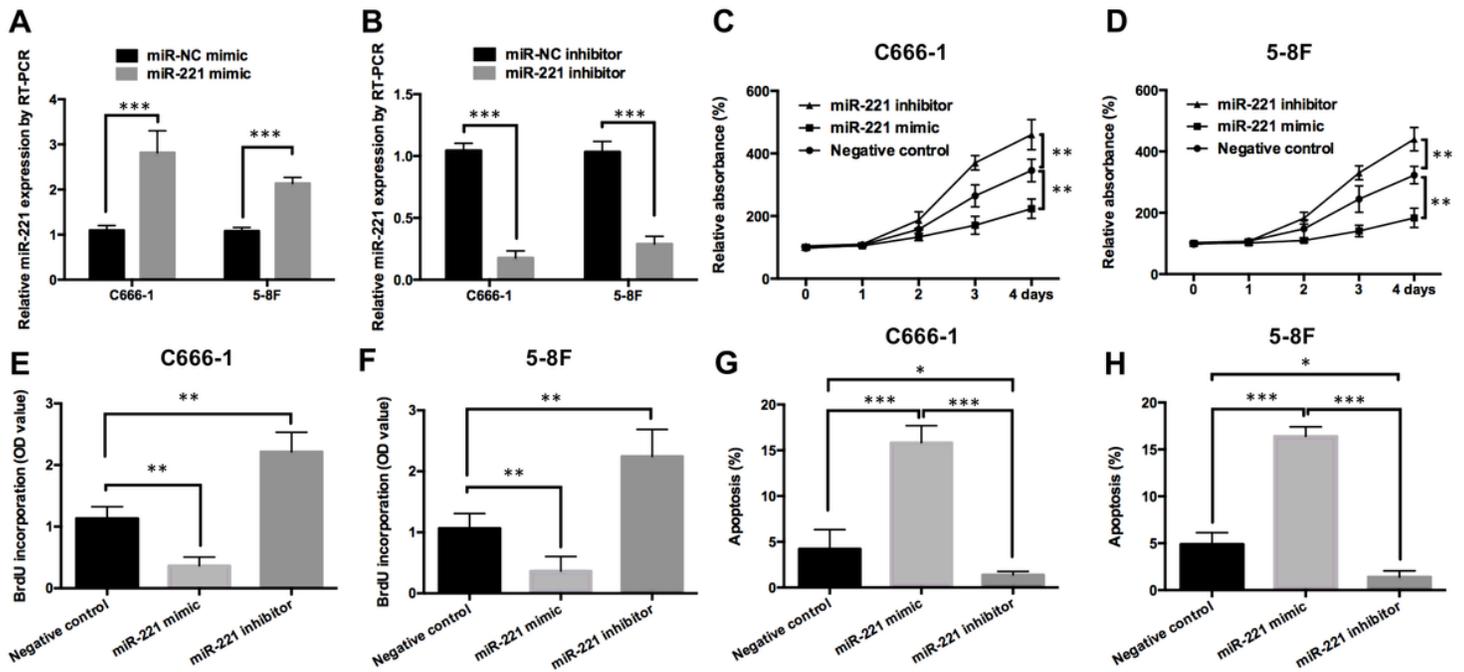
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## Figures



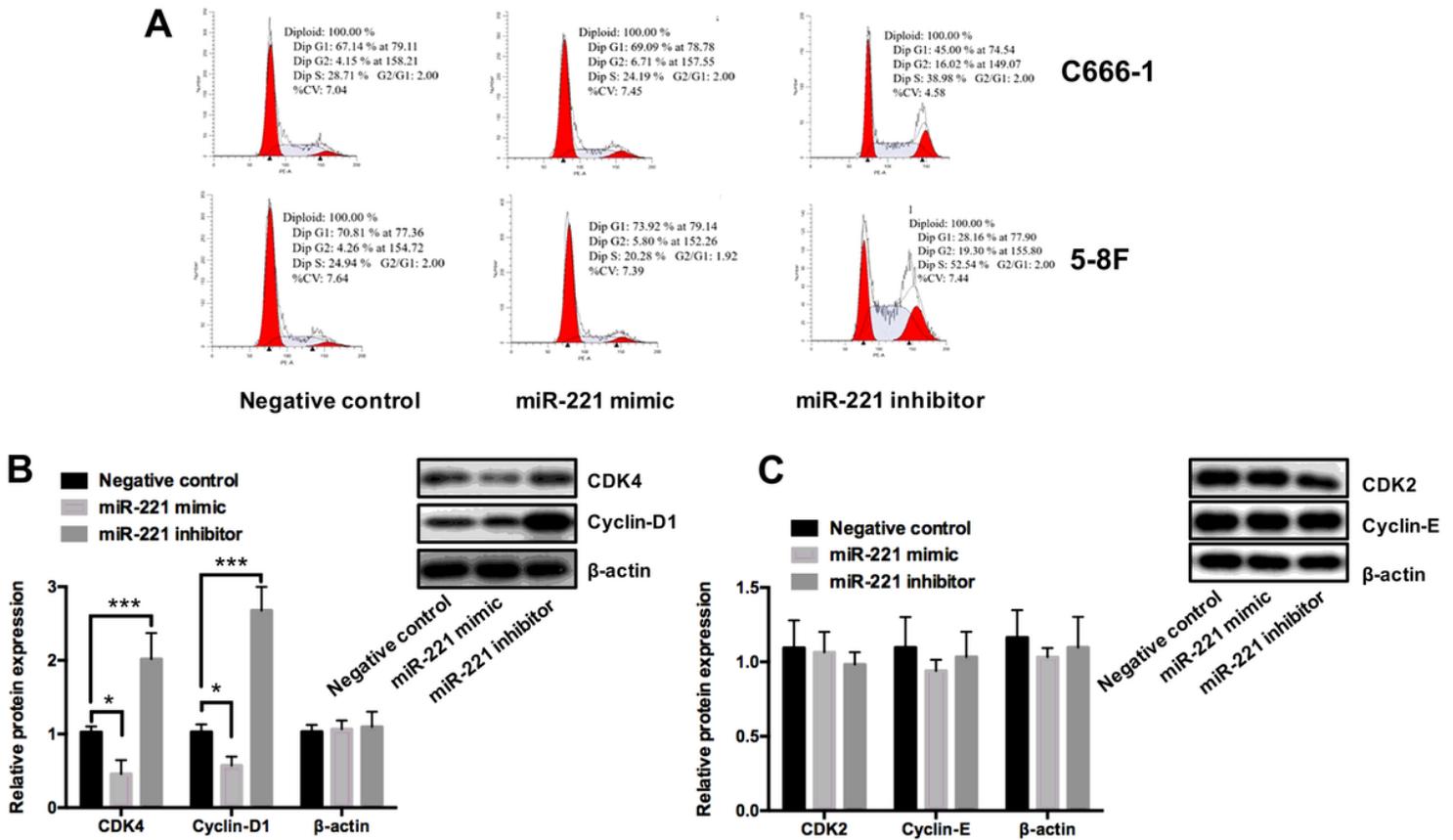
**Figure 1**

miR-221 is frequently down-regulated and associated with poor overall survival of NPC patients. (A) Relative expression of miR-221 in 90 NPC tissues and 13 non-tumor nasopharyngeal epithelial tissues. (B) Kaplan-Meier overall survival curves for patients with NPC stratified by miR-221 expression. (C) Relative expression of miR-221 in NPC cell lines and NP69 cells. \*P < 0.05; \*\*P < 0.01.



**Figure 2**

MiR-221 inhibited proliferation and increased apoptosis in NPC cells. (A) Transfection with the miR-221 mimic promoted miR-221 expression in C666-1 and 5-8F cells. (B) Transfection with the miR-221 inhibitor reduced miR-221 expression in C666-1 and 5-8F cells. MiR-221 inhibitor was transfected into C666-1 (C) and 5-8F (D) cells and induced a higher proliferation rate than the miR-221-mimic-transfected group and miR-NC-transfected group as controls. The significantly increased cell proliferation levels in C666-1 (E) and 5-8F (F) cells transfected with miR-221 inhibitors and the obviously downregulated cell proliferation of the miR-221-transfected group were observed and compared with those of the controls by ELISA-BrdU assay. MiR-221 inhibitor or miR-221 mimic was transfected into C666-1 (G) and 5-8F (H) cells, and the miR-221 mimic triggered a significantly high apoptotic rate compared with controls. \*\*P < 0.01 and \*\*\*P < 0.001.



**Figure 3**

The effects of miR-211 transfection on cell cycle and cell cycle-related proteins in C666-1 and 5-8F cells. Cells were transfected with the miR-221 mimic and the corresponding inhibitor or negative control. (A) Cell cycle was detected using flow cytometry. (B) and (C) The cyclin D-CDK4/6 and cyclin E-CDK2 complex protein expressions were determined by Western blotting using  $\beta$ -actin as an internal control. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

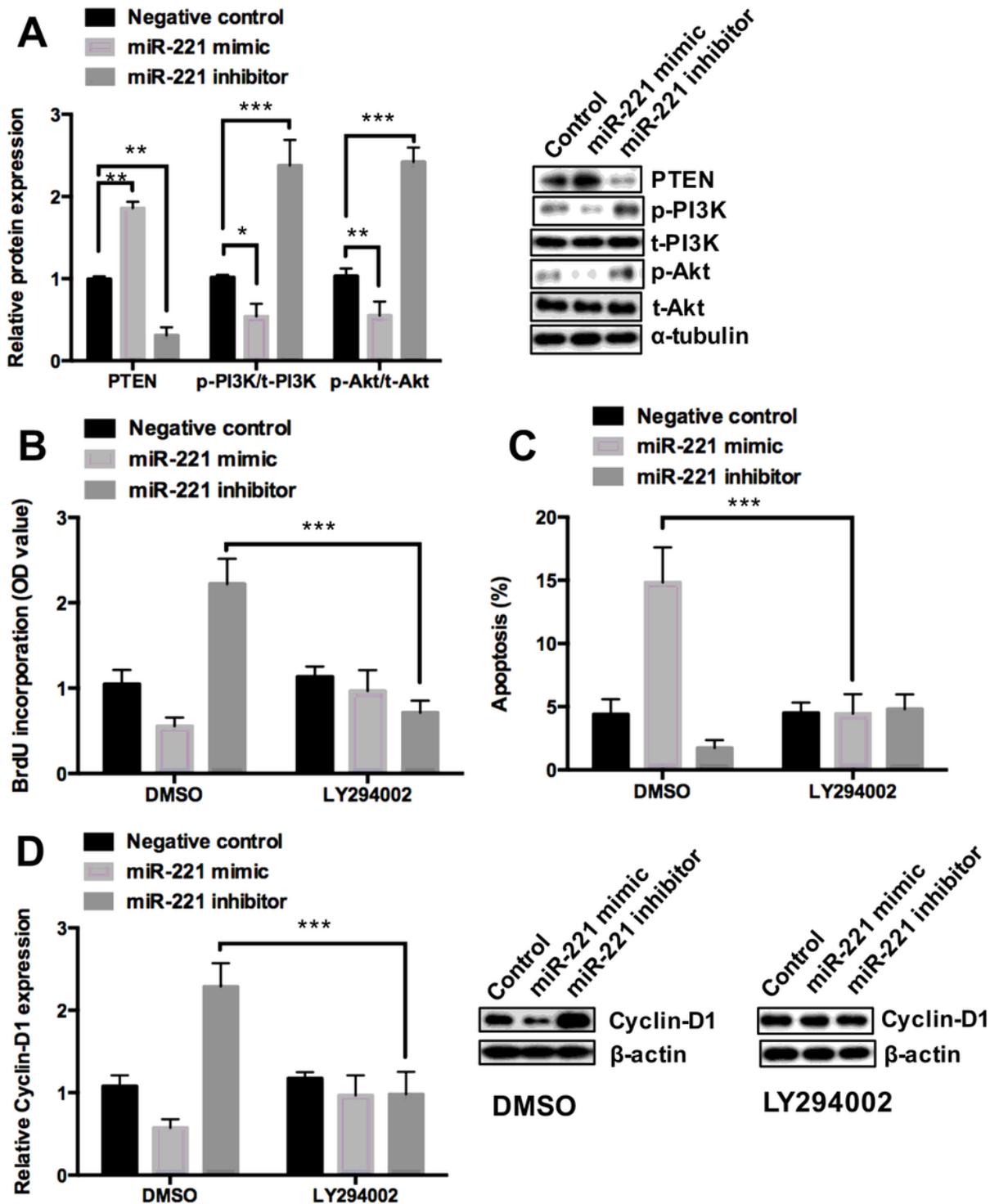


Figure 4

MiR-221 inhibited NPC progression and increased apoptosis via regulating the PTEN/PI3K/AKT signaling pathway. (A) Western blot analysis was performed for determining the PTEN, p-PI3K and t-PI3K, and p-Akt and t-Akt expression levels in 5-8F cells transfected with the miR-221 mimic, miR-221 inhibitor, and corresponding control, respectively. Furthermore, 5-8F cells were pretreated with DMSO or LY294002 for 6 h and then transfected with the miR-221 mimic, miR-221 inhibitor, and corresponding control for 24 h. (B)

ELISA-BrdU assay was performed to analyze the cell proliferation. (C) Flow cytometry assay was performed to evaluate the cell apoptotic rate. (D) Cyclin-D1 expression level was used to evaluate cell cycle progression. \*\*P < 0.01 and \*\*\*P < 0.001.

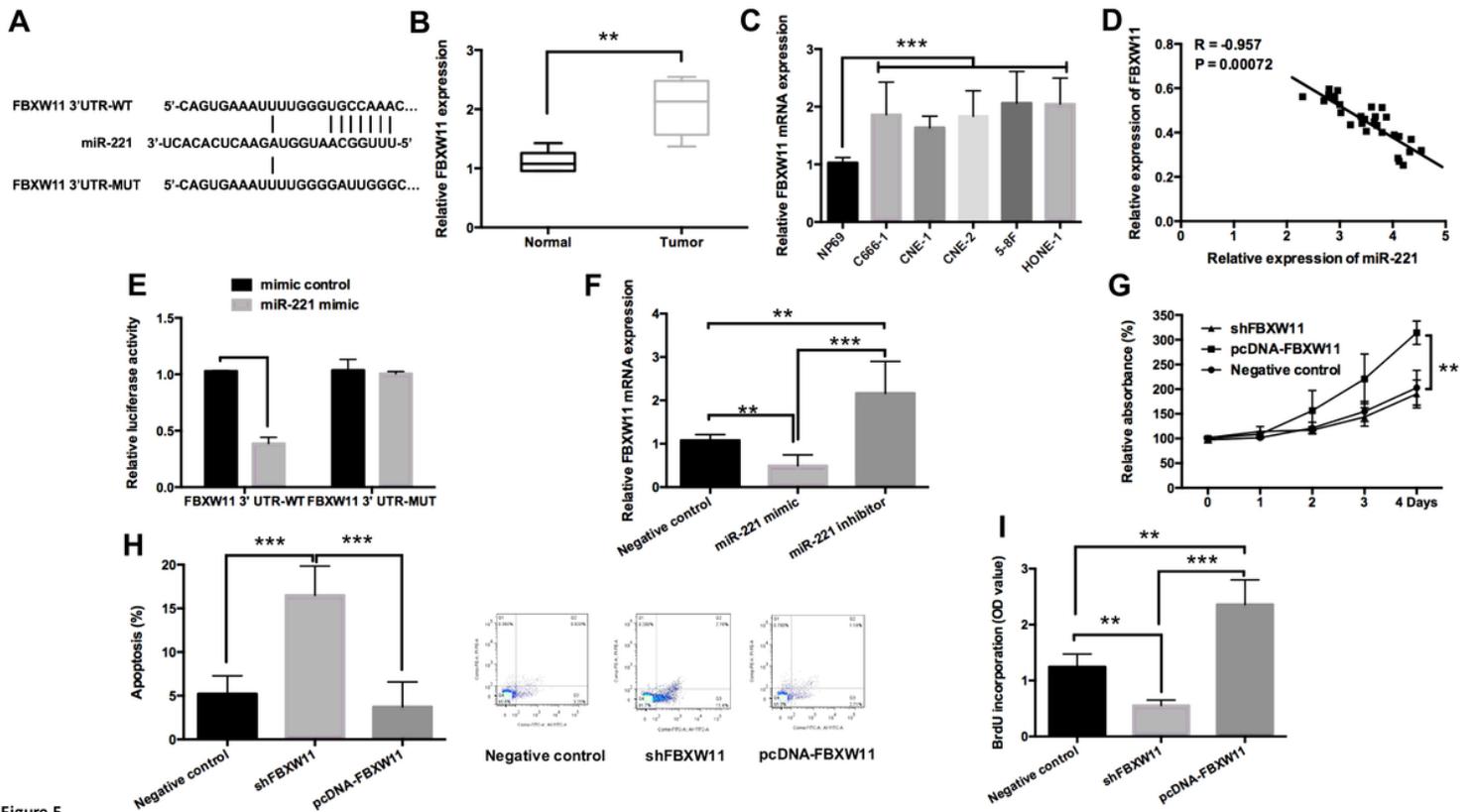


Figure 5

## Figure 5

FBXW11 can directly target miR-221. (A) A potential target of miR-221, known as FBXW11, has been predicted by a bioinformatics-based target prediction analysis and the putative binding site is on the 3'-UTR of FBXW11. (B) RT-PCR analysis of FBXW11 expression in NPC tissues and their noncancerous counterparts. (C) A significantly downregulated expression of FBXW11 mRNA is observed in NPC cell lines compared with that of normal cells. (D) A negative Spearman's correlation between miR-221 and FBXW11 mRNA levels is evident in 80 NPC tumor tissues. (E) Significantly decreased luciferase activity, driven by the 3'-UTR of FBXW11, is observed in the miR-221 mimic group and the 3'-UTR-MUT group compared with the negative control. (F) The downregulation of miR-221 induces a dramatic overexpression of the FBXW11 protein. (G) A significantly higher cell proliferation rate is observed in the pcDNA-FBXW11 group than in the control group. (H) Significantly lower apoptosis is observed in the pcDNA-FBXW11 group than in the control group. (I) Significantly increased cell proliferation is observed in the pcDNA-FBXW11 group compared with the control group. \*\*P < 0.01 and \*\*\*P < 0.001.

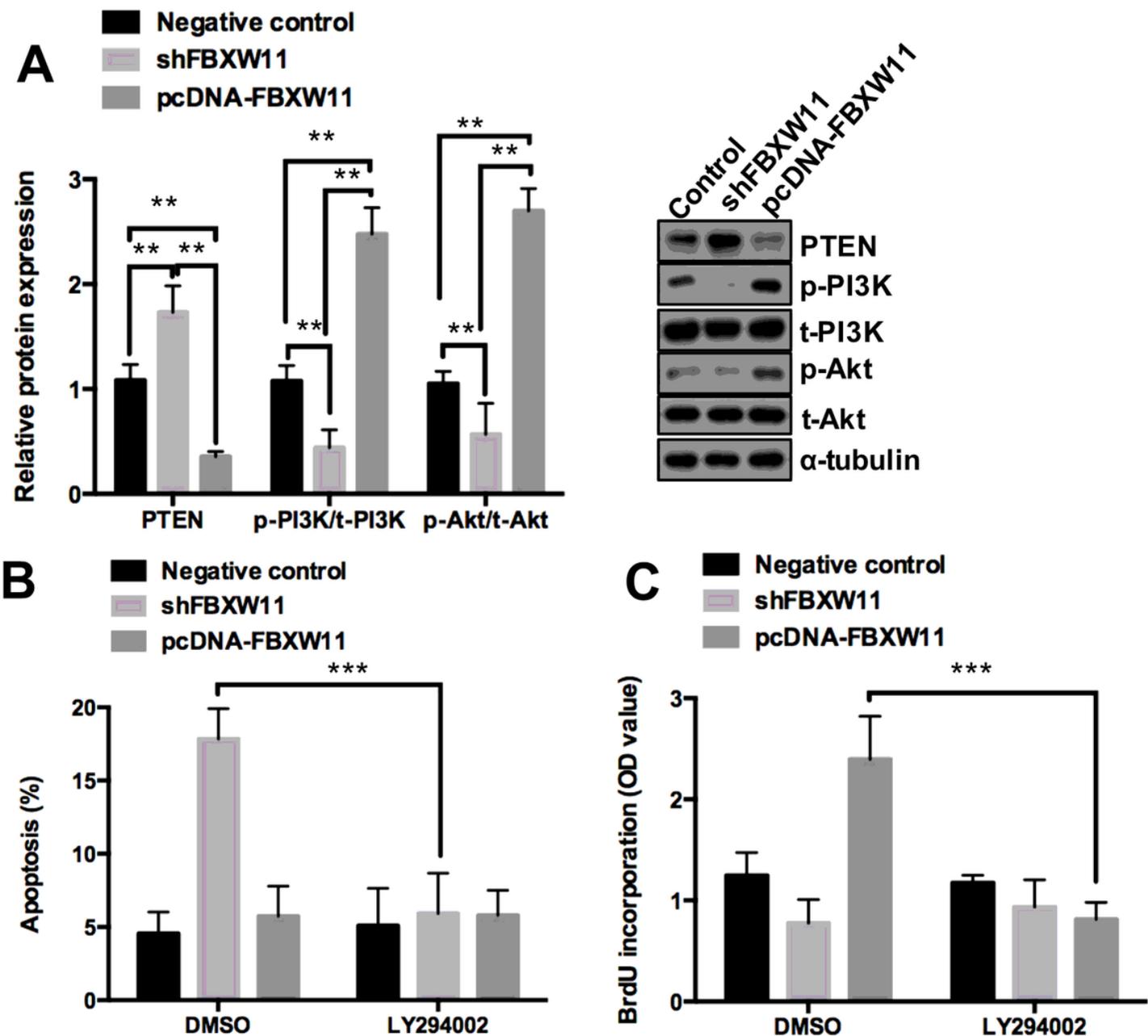


Figure 6

FBXW11 reverses the effects of miR-221 on NPC cells via the PTEN/PI3K/AKT signaling pathway. (A) Western blot analysis was performed for determining the expression level of PTEN, p-PI3K and t-PI3K, p-Akt, t-Akt in NPC cells transfected with pcDNA-DDX3, shDDX3 and corresponding control, respectively. OVCAR3 cells were pretreated with DMSO or LY294002 for 6 h and then transfected with pcDNA-DDX3, shDDX3 and corresponding control for 24 h. (B) mean that ELISA-BrdU assay was performed to analyze the cell proliferation. (C) mean that flow cytometry assay was used to evaluate the cell apoptosis rate. \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .