miR-221-3p Promotes Pancreatic Cancer Progression via Targeting P53 and PTEN

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Abstract

Background: To assess the regulatory role of microRNA-221-3p (miR-221-3p) as a modulator of pancreatic cancer (PC) cell apoptosis, invasion, migration, and proliferation through its ability to regulate P53 and PTEN expression.

Methods: PCPATU8988 cells were used as a model in which high-level or low-level models of miR-221-3p expression were established, with qPCR being used to confirm transfection efficiency. CCK-8 assays were employed to evaluate the proliferation of these cells, with migration and invasion being assessed through appropriate *in vitro* assays. Western blotting was used to assess PTEN and P53 protein levels in these experimental cells. Flow cytometry was additionally used to assess the impact of experimental manipulations on cellular apoptosis.

Results: MiR-221-3p overexpression enhanced the migratory, proliferative, and invasive activity of PCPATU8988 cells (P<0.01) and suppressed their apoptotic death (P<0.05), while miR-221-3p inhibition had the opposite impact. No differences in P53 and PTEN protein levels were detected when comparing the NC miR-221-3p mimic or inhibitor groups (P>0.05), whereas miR-221-3p mimic transfection significantly reduced the levels of these proteins (P<0.05).

Conclusion: This analysis showed that miR-221-3p can drive PC cell proliferative, migratory, and invasive activity while suppressing the apoptotic death of these cells. Functionally, this miRNA can suppress P53 and PTEN protein expression. Overall, this suggests that miR-221-3p can regulate PC development by controlling the expression of these key oncogenic proteins.

1. Introduction

Pancreatic cancer (PC) is highly malignant and often insidious in onset, resulting in a poor patient prognosis that necessitates an early diagnosis to ensure optimal patient outcomes. Over 85% of PC patients exhibit advanced -stage PC at the time of initial diagnosis, and are thus unable to undergo radical curative surgical treatment. Without treatment, these patients exhibit a median 5-8 month survival period and a < 5% five-year survival rate.[1] PC tumor cells exhibit a range of highly malignant properties including abnormal proliferative activity, a high potential for metastasis, and invasivity that make them well-suited to rapid disease progression.[2] However, further research is necessary to fully clarify the molecular mechanisms underlying PC cell malignancy.

MicroRNAs (miRNAs) are short (~22 nucleotide) post-transcriptional regulators of gene expression without coding potential.[3] Many miRNAs can shape tumorigenesis and associated processes, or can offer value in the context of cancer patient diagnosis, prognostic evaluation, and treatment.[4-6] Specific miRNAs function by regulating tumor cell differentiation, survival, proliferation, and metastasis through their ability to control the expression of specific target genes.[7]

Prior work has identified miR-221-3p as an anti-angiogenic miRNA encoded within an X chromosome gene cluster that is expressed at high levels within human atherosclerotic vascular endothelial cells of the intimal layer.[8] In several cancers, miR-221-3p has also been shown to regulate abnormal cellular proliferation and differentiation,[9-12] although only a few studies have examined it in the context of PC.[13-15] *In vitro*, there is evidence that miR-221-3p can promote PC cell proliferation,[16] whereas Sarkar et al. found that it was able to suppress PC cell proliferation by promoting the upregulation of PUMA and other downstream proteins.[17] In triple-negative breast cancer patients, low levels of miR-221-3p expression have been linked to a poor prognosis through a mechanism linked with its regulation of PARP1.[18] In most solid tumors, however, miR-221-3p upregulation is commonly observed, and the mechanistic basis for such upregulation remains unclear. There is thus a clear need for further studies of the specific target genes of this miRNA in PC and for the identification of effective treatments centered around this key genetic regulator.

The transcription facto P53 plays an essential role in maintaining genomic integrity, monitoring for abnormalities that arise during DNA replication and cell cycle progression, inducing repair gene upregulation when errors are detected, and promoting apoptotic cell death when they are not repaired, thereby eliminating cells with the potential to undergo oncogenic transformation. Owing to its potent tumor suppressor activity, P53 is frequently inactivated in tumors through deletion or inactivation, thereby enhancing the proliferative activity of cancer cells and decreasing their susceptibility to apoptotic death.[19] A range of translation and post-translational modifications control P53 expression and function, including phosphorylation, ubiquitination, and proteasomal degradation. Wild-type (WT) P53 expression is observed in roughly half of human tumors, and in these cases, the suppression or inactivation of P53 negative regulators can prove an effective therapeutic intervention.[20] For example, the accumulation of P53 and MDM2 overexpression within hepatocellular carcinoma tumors is negatively correlated with patient survival.[21] In chondrocytes, MDM2 has been established as a miR-221-3p target.[22] PTEN is another critically important tumor suppressor gene in many cancers that is encoded on chromosome 10q23.31.[23,24] PTEN regulates protein phosphatase and 3'-phosphoinositol phosphatase functionality, dephosphorylating the second messenger in the PI3K pathway, phosphatidylinositol 3,4,5-triphosphate (PIP3), thereby inhibiting the activity of AKT family serine/threonine kinases, which are important serine/threonine protein kinases. [25,26] AKT activity regulates the proliferative activity, invasive potential, and apoptotic death of many tumors.[27] In tumors exhibiting PTEN inactivation, AKT overactivation is observed, resulting in reduced apoptotic tumor cell death and enhanced proliferative activity.[28,29] In gastrointestinal cancer, miR-221-3p has been suggested to promote enhanced tumor cell apoptosis via KIT/AKT signaling pathway regulation.[30]

In light of the above evidence, this study was formulated with the goal of clarifying the role of miR-221-3p as a regulator of the survival, migration, proliferation, and invasion of PC cells. Through a series of *in vitro* analyses, P53 and PTEN were validated as miR-221-3p target genes in PC tumors, suggesting the

potential diagnostic relevance and therapeutic promise of targeting this unique tumor-regulating signaling axis in PC patients.

2. Methods

2.1 Cell culture

The PATU8988 **PC cell line and** the control HPDE6-C7 normal pancreatic ductal epithelial cell line (Procell,Wuhan,China) were cultured in DMEM containing 10% FBS and penicillin/streptomycin in a 37°C 5% CO_2 incubator.

2.2 Transfection

Cells were transfected with miR-211-3p mimics or inhibitors through use of Lipofectamine 3000 (Invitrogen, Shanghai, China) based on provided directions. Cells were collected for downstream analyses at 24, 48, or 72 h post-transfection.

2.3 qPCR

Cells were cultured for 48 h in 6-well plates, after which total RNA was collected using Trizol and RNA purity was measured. ATP was diluted based on the amount of RNA used, after which the reverse transcription kit (Mei5 Biotechnology, China) was used based on provided directions to prepare a miRNA plus poly-A tail reaction system (total volume: 25 ul) in an enzyme-free reaction tube on ice. The reaction solution was mixed, centrifuged briefly at a low speed, and incubated for 15 min at 37°C. A miRNA reaction was then prepared for cDNA synthesis (20 uL volume) in enzyme-free reaction tubes on ice, with the reaction system being mixed, spun at a low speed, incubated for 50 min at 42°C, and heated for 5 min at 85°C. The resultant cDNA was stored at -20°C. For qPCR, 20 uL reactions were prepared containing: 1 uL cDNA, 10 uL ROX, 0.4 uL of each primer (10 uM), and 8.2 uL of ddH2O. miRNA expression was detected with SYBR GREEN I. Relative gene expression was assessed via the $2^{-\triangle\triangle CT}$ method as follows: $\triangle\triangle CT = (CT_{target\ gene}\text{-}CT_{reference\ gene})$ in the experimental group-$(CT_{target\ gene}\text{-}CT_{reference\ gene})$ in the control group.The primer sequence and transfection gene sequence (5’-3’) required for this experiment are shown(Table 1).

2.4 CCK-8 analysis

To assess cellular viability, cells were culture for 24, 48, or 72 h post-transfection, after which 10 uL of CCK-8 reagent was added to each well for 2 h during which the reagent was reduced by the dehydrogenase activity of mitochondria in live cells. Absorption (OD) at 450 nm in each well was then assessed using a microplate reader.

2.5 Migration assay

After transfection cells were cultured to 80-90% confluence. They were then washed thrice with PBS, harvested using 0.25% trypsin-EDTA for 1 min, and 1 mL of serum-free media was then added. A single-cell suspension was then prepared, and Trypan blue was added to the suspension for 2 min at room temperature. Next, 10 uL of these cells were counted using a hemocytometer. Cells were counted under low magnification (10x10 times), with cells being counted in the four corners of the hemocytometer field, after which cell number was measured as follows: cell number/ml=(a+b+c+d)/4x10x10^4/ml. Target cell number per counting field were between 20 and 300, with samples otherwise being diluted again and re-counted. Next, 800 uL of culture media containing 10% FBS was added to the upper chamber of a 24-well Transwell insert, after which 200 uL of prepared cell suspensions from appropriate transfection and control groups were added per well (4×10^3/well). Cells were incubated for 48 h, after which the Transwell chamber was taken out, washed with PBS, fixed with 4% formaldehyde for 20 min, and stained with 0.1% crystal violet staining solution for 30 min. Background cells were removed by washing with PBS and gently swabbing with a cotton swab. Cells that migrated to the lower layer of the Transwell chamber were counted with an inverted microscope (10×20 times). Groups of cells were selected at random and counted in 5 fields of view per sample.

2.6 Invasion assay

Matrigel was thawed at 4℃ overnight, and was then diluted 1:3 using serum-free media. The upper chamber of a 24-well Transwell insert was then coated with 40 uL of diluted Matrigel and placed in an incubator for 2 h to allow the gel to solidify. After transfection, the cells in each group were cultured to 80-90% confluence, washed thrice with PBS, digested with 0.25% trypsin containing EDTA for about 1 min, and 1 ml of serum-free medium was added to terminate the digestion. A single-cell suspension was prepared and cells were stained for 2 min with Trypan blue, after which 10 uL of cells were assessed in a counting plate and quantified via microscopy. Next, 800 uL of media containing 10% serum was added to the upper chamber of a 24-well Transwell insert, followed by the addition of 200 uL of prepared cell suspensions (2x10^4 cells/well). After a 48 h incubation, chambers were removed, rinsed with PBS, fixed for 20 min with 4% formaldehyde, and stained for 30 min with 0.1% crystal violet. Background cells were removed by washing with PBS and gently swabbing with a cotton swab. Cells that invaded to the lower layer of the Transwell chamber were counted with an inverted microscope (10×20 times). Groups of cells were selected at random and counted in 5 fields of view per sample.

2.7 Protein collection and preparation for Western blotting

At 48 h post-transfection, cells in 6-well plates were washed thrice with chilled PBS, lysed with enhanced RIPA lysis buffer (Boster Company,Wuhan,China) containing a protease inhibitor (PMSF) (Boster Company), scraped, allowed to stand for 30 min on ice, vortexing every 10 min, and spun for 15 min at 12,000 rpm at 4°C. Protein levels in supernatants were assessed via BCA assay. Protein amounts were then mixed with 4 uL of SDS-PAGE loading buffer (5X) (Boster Company), and the volume was increased to 20 uL with RIPA lysis buffer. Samples were then denatured for 5 min at 95°C, after which they were stored at -20°C.

2.8 Western blotting

For protein separation, 10% SDS-PAGE gels were prepared with a 5% stacking gel region. Proteins were electrophoretically separated at 80 V until proteins entered the separation gel, at which time the voltage was increased to 120 V. Proteins were then transferred onto PVDF membranes (Millipore, USA), which were blocked using 5% non-fat milk for 1 h with constant shaking (60 rpm) at room temperature, after which appropriate primary antibodies (1:1000, ABclonal, China) were added in primary antibody diluent (Boster Company, Wuhan, China), with polyclonal anti-GAPDH (Bioworld Company, USA) serving as a loading control. Blots were probed overnight at 4℃, rinsed thrice with 1×TBST (100 rpm; 10 min/wash), and probed with HRP-labeled secondary antibodies (1:5000, Boster Company, Wuhan, China) for 1 h at room temperature with constant shaking (60 rpm). Membranes were then developed using an ECL reagent and an imaging instrument, with the Image Lab software being used to detect protein bands.

2.9 Analysis of cellular apoptosis

At 48 h post-transfection, apoptotic cell death was assessed by dual staining cells with Annexin V-FITC and propidium iodide (PI) with an apoptosis detection kit (Meilun, Dalian, China) based on provided directions. A flow cytometer (Thermo Company, USA) was then used to assess apoptotic cell death, analyzing live, dead, early apoptotic, and late apoptotic cells.

2.10 Statistical analysis

SPSS 22.0 was used for all statistical analyses in this study. Data were averaged from three independent experiments. Data were compared between groups using one-way ANOVAs, and were compared within groups via LSD t-tests. $P < 0.05$ was the threshold of statistical significance. GraphPad Prism 7.0 and Adobe Photoshop CC2019 were used to prepare Figures.

3. Results

While miR-221-3p has been reported to play an oncogenic role in many cancers, its specific functions in PC are poorly understood. As such, we began by exploring miR-221-3p expression levels in PC cell lines and the relationship between this miRNA and PC cell behavior *in vitro.* We then confirmed the ability of this miRNA to regulate PTEN and P53 in PC cells to influence tumor progression.

3.1 Analyses of miR-221-3p knockdown and overexpression

The $2^{-\triangle\triangle CT}$ method was used to assess relative gene expression, yielding the results shown in Table 1 and Figure 1.

3.2 Analysis of PC cell proliferation

Following miR-221-3p inhibition or overexpression in PATU8988 cells, a CCK-8 assay was performed to assess cell viability as discussed in the Methods section above (Figure 2).

3.3 Analysis of PC cell migration

At 24 h post-transfection, cells were added to a Transwell insert at an appropriate density for 48 h. They were then fixed, stained, and counted via microscopy (Figure 3).

3.4 Analysis of PC cell invasion

At 24 h post-transfection, cells were added to the Transwell chamber at an appropriate density and cultured for an additional 48 h. Cells that had invaded through the Matrigel layer were then counted (Figure 4).

3.5 Western blotting analysis

Photoshop was used to process protein band images, with ImageJ being used for densitometric analyses (Figure 5).

3.6 Analysis of PC cell apoptosis

To evaluate the apoptotic death of PC cells, they were next subjected to Annexin V-FITC/PI staining and assessed via flow cytometry. The frequency of apoptotic PC cells rose significantly following miR-221-3p inhibition, and decreased upon miR-221-3p overexpression (Figure 6), suggesting that miR-221-3p can suppress the apoptotic death of PC cells.

4. Discussion

PC is among the deadliest cancers in the world,[31] ranking 9th and 10th in terms of tumor incidence in the USA among men and women, respectively, in addition to being the 4th leading cause of tumor-related death as of 2021.[32] Similarly, the National Cancer Center of China statistics from 2017 indicated that PC was 7th and 11th in terms of tumor incidence in men and women, respectively, and was the 6th leading cause of tumor-related death.[33] PC patients still face a poor prognosis despite improvements in associated surgical and chemotherapeutic treatment options.[34] One of the primary drivers of poor PC patient survival is that tumor invasion and distant metastasis often occur very early during the process of disease progression.[35] As such, it is critical that PC be diagnosed at an early stage to improve patient treatment outcomes, emphasizing the need for novel approaches to diagnosing and treating this cancer type.

Through their ability to regulate diverse oncogenic processes, miRNAs can act as tumor suppressors or oncogenes.[36,37] Virtually all cancer types have been found to exhibit miRNA dysregulation,[38] and these miRNAs can control cellular proliferation, apoptosis, and growth.[39] Specific miRNAs have been identified as novel biomarkers with relevance in the context of tumor diagnosis, prognostic evaluation, and treatment.[40] MiR-221-3p is an oncogenic miRNA in liver,[41] colorectal,[42] breast,[43] lung,[44] prostate,[45] and gastrointestinal stromal cancers,[46] whereas it is a tumor suppressor in cholangiocarcinoma.[47] There is

strong evidence that miR-221-3p can regulate tumor cell proliferative, invasive, metastatic, chemoresistant, and apoptotic activity.[48-50] Therefore, understanding the role of miR-221-3p in PC is of great value.

Herein, PATU8988 cells were used to establish models of miR-221-3p upregulation and downregulation as validated via qPCR. CCK-8 assays indicated that miR-221-3p overexpression enhanced proliferation, while Transwell-based assays further indicated that such overexpression enhanced PC cell migratory and invasive activity. Moreover, flow cytometry indicated that miR-221-3p overexpression reduced rates of PC cell apoptosis. The inhibition of this miRNA had the opposite effect.

To assess how miR-221-3p influenced downstream oncogenic pathways in PC, we herein performed a series of predictive bioinformatics analyses and related functional *in vitro* experiments. P53 is among the most studied tumor suppressors in mammalian cells, with the mutation or inactivation of P53 being a hallmark of a large percentage of human cancers. Mutated P53 can both lose its normal WT tumor suppressor activity and can even contribute to malignant disease progression in some cases.[51] Suzuki et al. found that miRNA maturation is dependent on P53 regulation.[52] The MDM2 gene is upregulated by P53 and binds to its inactivation domain, disrupting the recruitment of other transcriptional regulators necessary for gene expression.[53] MDM2 can also serve as an E3 ubiquitin ligase to promote P53 degradation. [54] Through this mechanism, P53 and MDM2 regulate one another in a feedback loop such that P53 promotes MDM2 upregulation which in turn promotes P53 downregulation.[55] Cyclin G1 can additionally serve as a negative feedback regulator that decreases P53 stability and promotes protein degradation.[56] PTEN is a central tumor suppressor gene with documented roles as a regulator of cellular apoptosis, proliferation, and growth.[57] PTEN is encoded on chromosome 10q23.3, and suppresses oncogenesis via its dual lipid and protein phosphatase activity.[58-60] Many malignant tumors exhibit PTEN inactivation and consequent overactivation of AKT, in turn driving enhanced invasion and radioresistance together with a decrease in apoptotic susceptibility.[61-63] PTEN regulates tumor cell survival, proliferation, and metastatic progression.[64,65] Several miRNAs including miR-21 and miR-214 are known PTEN regulators.[66-70] Yang et al.[71] posited that miR-221-3p was able to promote Capan2 PC cell proliferation via the PTEN/AKT axis. Consistently, we herein found that P53 and PTEN protein levels were reduced in PC cells following miR-221-3p overexpression and increases when this miRNA was inhibited, consistent with the ability of this miRNA to target these two genes. However, further work will be necessary to clarify the related signaling mechanisms whereby miR-221-3p regulates these two tumor suppressor genes. However, this study only evaluated this miRNA at the cellular level, and as such, our future research will focus on examining the diagnostic and prognostic relevance of miR-221-3p in clinical settings. In conclusion, miR-221-3p overexpression within PC cells can enhance tumor progression and malignancy.Further work is needed to explore the mechanisms whereby this miRNA can shape the pathogenesis of PC in order to highlight novel approaches to diagnosing and treating PC.

5. Conclusion

The upregulation of miR-221-3p in PATU8988 cells enhanced their proliferative, migratory, and invasive activity while suppressing PC Cell apoptosis. In contrast, the downregulation of this miRNA had the opposite effect. Mechanistically, miR-221-3p was able to target P53 and PTEN as a means of regulating PC progression. Together, these data may highlight a novel approach to exploring the pathogenesis of PC in order to provide patients with novel therapeutic modalities capable of improving survival rates and overall quality of life.

Declarations

Acknowledgment

Not applicable.

Author Contributions

JJW and ZHS conceived and designed the study and drafted the manuscript. JJW,MG,ZHY,FGR and SCB collected, analyzed, and interpreted the experimental data.JJW and ZHS revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study is approved by the ethics committee of the Second Hospital of Shanxi Medicine University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Primer sequence and transfection gene sequence (5’-3’) required for this experiment.

Gene and primer name	Primer sequence (5’-3’)
miR-221-3p mimics NC	5'-UUCUCCGAACGUGUCACGUTT-3’
miR-221-3p mimics	5'-AGCUACAUUGUCUGCUGGGUUUC-3’
miR-221-3p inhibitor NC	5'-UUGUACUACACAAAAGUACUG-3’
miR-221-3p inhibitor	5'-GAAACCCAGCAGACAAUGUAGCU-3’
miR-221-3p Forward Primer	5'-AGCTACATTGTCTGCTGGGTTTC-3’
miR-221-3p Reverse Primer	5'-GCAGGGTCCGAGGTATTC-3’
U6 Forward Primer	5'-GCTTCGGCAGCACATATACT-3’
U6 Reverse Primer	5'-GCAGGGTCCGAGGTATTC-3’

Figures

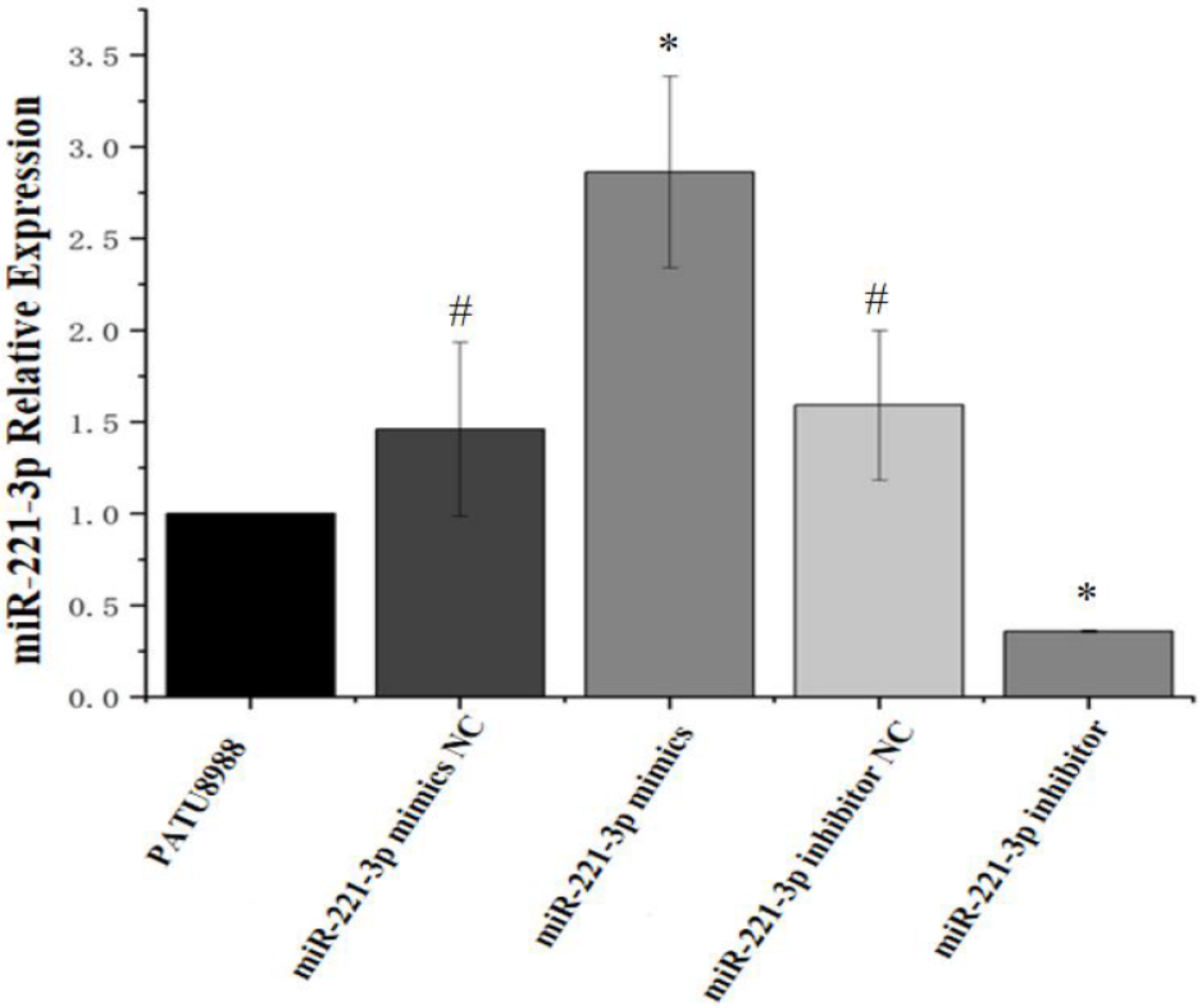

Figure 1

Figure 1

Relative to the control group, at 24 h post-transfection, PATU8988 cells transfected with miR-221-3p mimics NC or miR-221-3p inhibitor NC did not exhibit any alterations in miR-221-3p expression (#P>0.05), while miR-221-3p mimic transfection increased the expression of this miRNA (*P<0.05), after miR-221-3p inhibitor transfection downregulated this miRNA (*P<0.05).

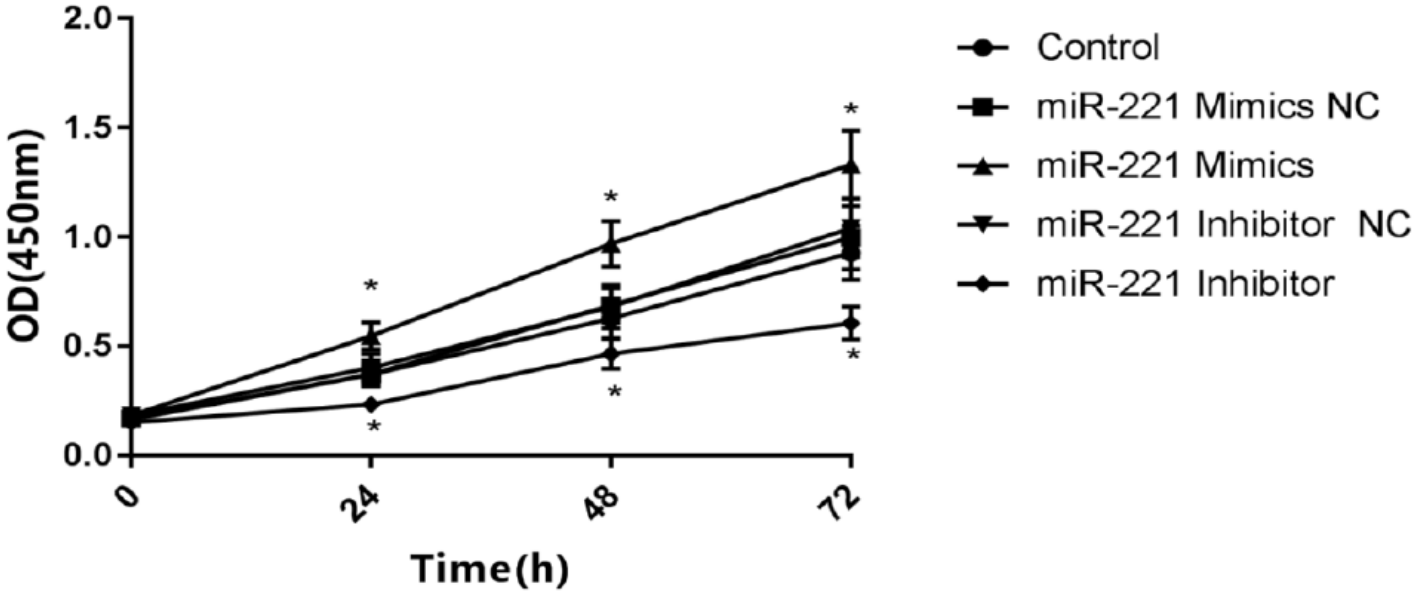

Figure 2

Figure 2

The absorbance (OD) value at 450 nm was assessed in the indicated treatment groups at 24, 48, and 72 h post-transfection. The OD value for the miR-221-3p mimic group was significantly higher than that in the control and miR-221-3p mimic groups ($P<0.01$), while the OD value in the miR-221-3p inhibitor group was significantly lower than that in the control and miR-221-3p inhibitor NC groups ($P<0.01$). There were no significant differences among the three control groups with respect to the OD450 value ($P>0.05$).

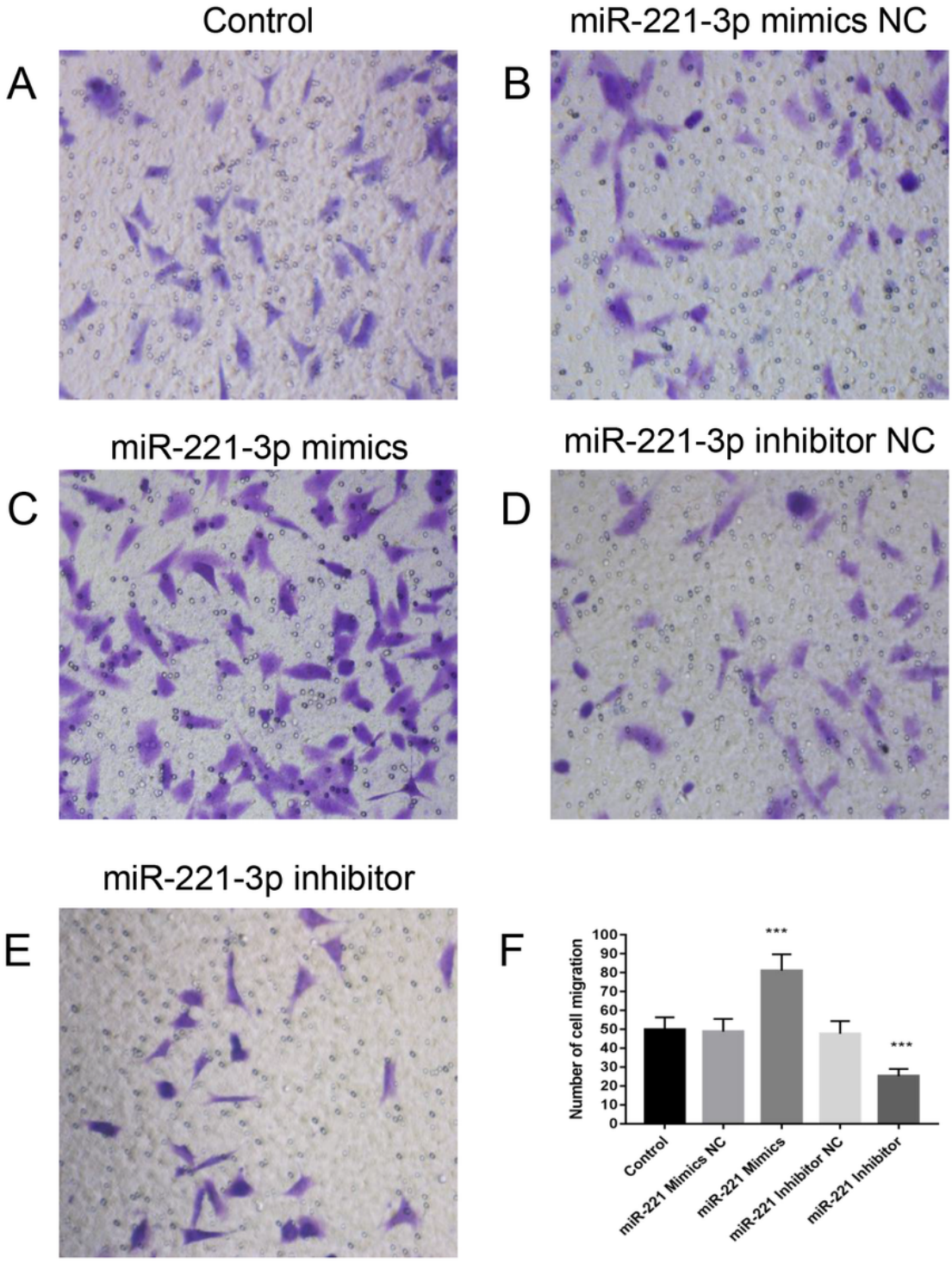

Figure 3

(A-E) Representative images of cells in the indicated treatment groups. (F) The number of migratory cells in the miR-221-3p mimic group was significantly higher than that in the control and miR-221-3p mimics NC group ($P<0.01$), while the number of migratory cells in the miR-221-3p inhibitor group was significantly lower than that in the control and miR-221-3p inhibitor NC groups ($P<0.01$). There were no differences in cellular invasion among the three control groups ($P>0.05$).

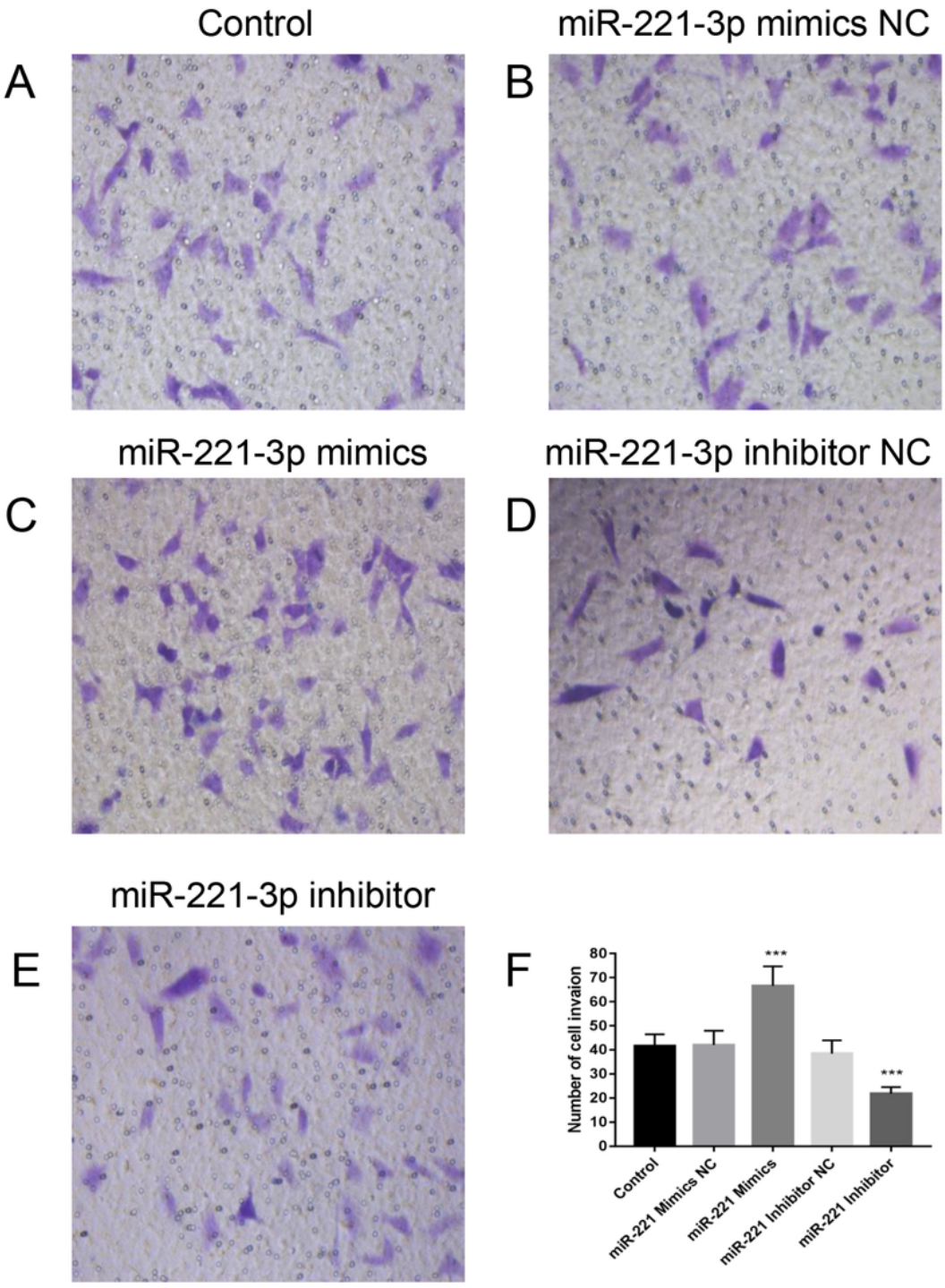

Figure 4

(A-E) Representative images of cells in the indicated treatment groups. (F) The number of invasive cells in the miR-221-3p mimic group was significantly higher than that in the control and miR-221-3p mimics NC group ($P<0.01$), while the number of invasive cells in the miR-221-3p inhibitor group was significantly reduced relative to the control and miR-221-3p inhibitor NC groups ($P<0.01$). There were no differences in cellular invasion among the three control groups ($P>0.05$).

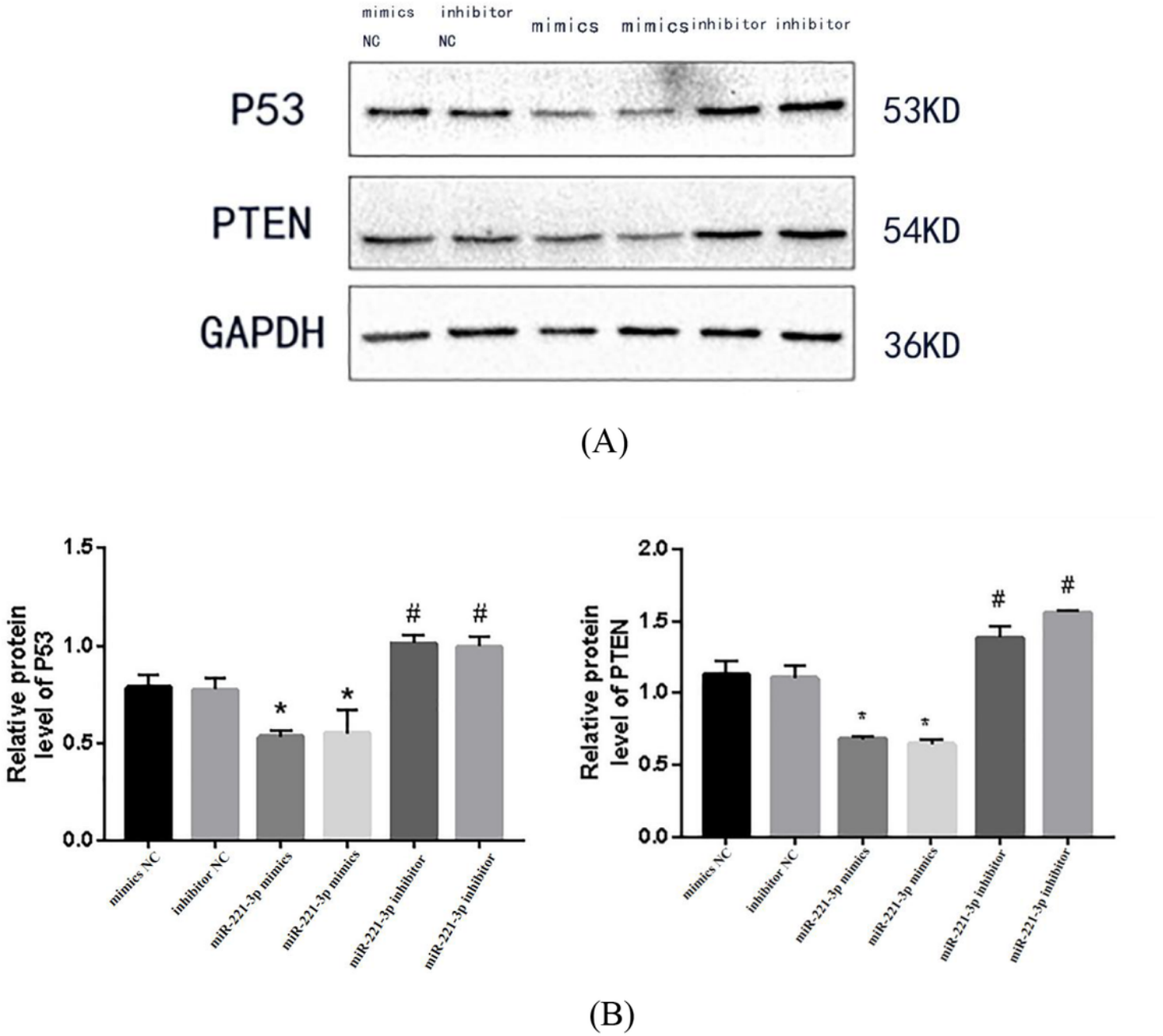

Figure 5

Figure 5

(A) P53, PTEN, and GAPDH protein levels were measured in the indicated groups. (B) At 24 h post-transfection, P53 and PTEN protein levels were measured by Western blotting, revealing no differences in these levels when comparing the NC and miR-221-3p inhibitor NC groups (P>0.05). P53 and PTEN levels were significantly reduced in the miR-221-3p mimic group as compared to the mimic NC group (*P<0.05).

Relative to the inhibitor NC group, P53 and PTEN protein levels were significantly increased in the miR-221-3p inhibitor group (#P<0.05).

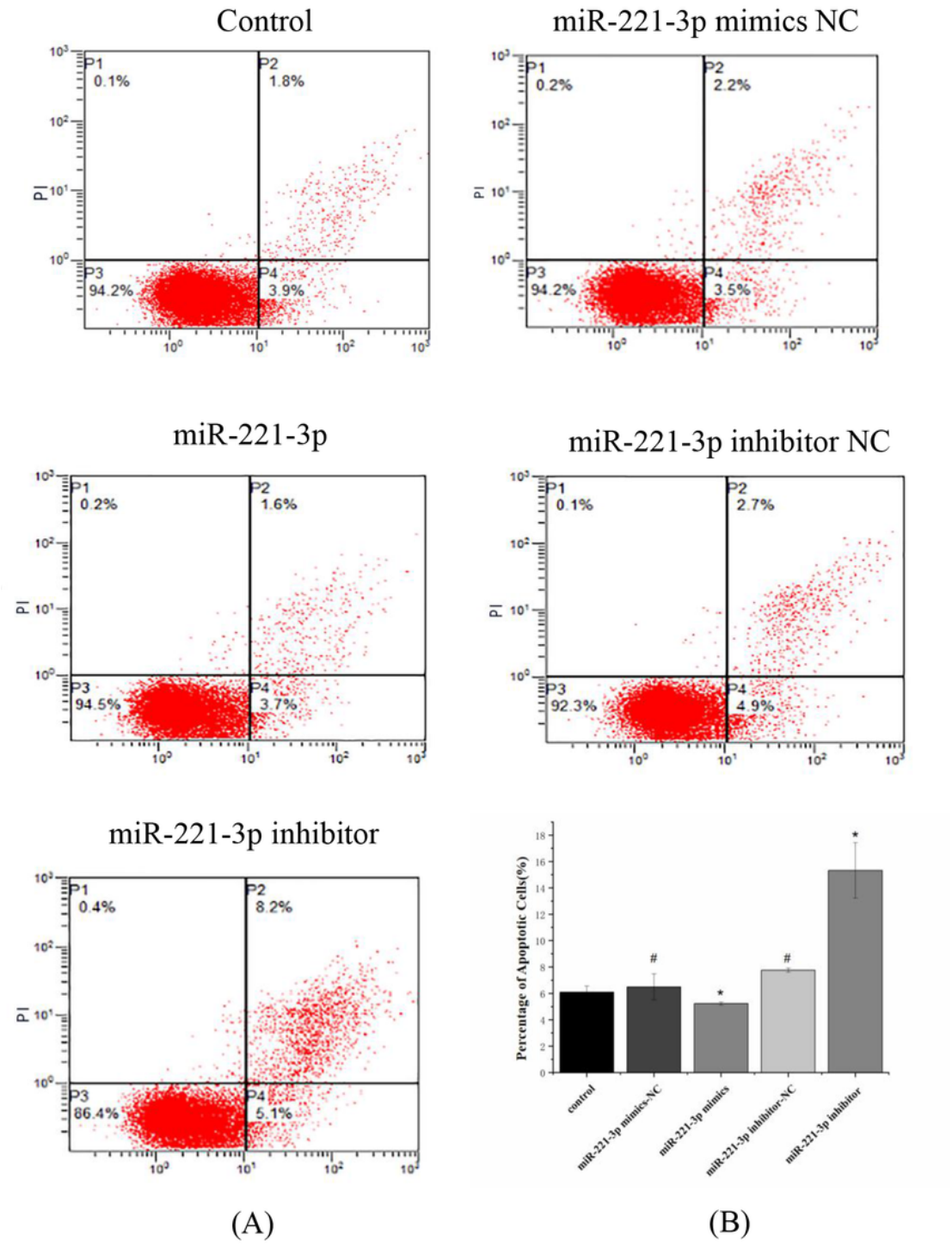

Figure 6

Figure 6

(A) Apoptotic induction in the indicated treatment groups. (B) Rates of apoptosis were significantly increased in the miR-221-3p inhibitor group relative to the control and the miR-221-3p inhibitor NC groups,

while miR-221-3p mimic transfection significantly reduced the rate of apoptotic induction relative to control treatment. #P>0.05,*P<0.05.