

# Overexpression of miR-140-3p Could Reverse Radioresistance of Nasopharyngeal Carcinoma by Targeting XIAP

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## Research

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# Abstract

## Background

Malignant behavior and radioresistance, which severely limits the efficacy of radiotherapy (RT) in nasopharyngeal carcinoma (NPC), lead to tumor progression and poor prognosis. The aim of this study was to investigate the underlying molecular mechanism of miR-140-3p on NPC development and radioresistance.

## Methods

The expression of genes and proteins was detected by Western blotting, and quantitative reverse transcription PCR (qRT-PCR). Proliferation, apoptosis, and radioresistance of NPC were detected. Luciferase reporter assays were used to detect the interactions of miR-140-3p with its downstream targets.

## Results

The expression of miR-140-3p was associated with the occurrence and radiation resistance of NPC. XIAP as the direct target of miR-140-3p was verified. We found alleviated radioresistance of NPC cells upon miR-140-3p overexpression or XIAP knockdown.

## Conclusion

miR-140-3p is downregulated in radioresistant NPC. miR-140-3p could hamper radioresistance of NPC via directly target XIAP, suggesting miR-140-3p/XIAP axis is vital in the regulation of irradiation sensitivity.

# Introduction

Nasopharyngeal carcinoma (NPC) is a common malignant tumor in China, with an incidence of 30/100,000 [1]. Many NPC patients already acquire advanced-stage NPC at first diagnosis; moreover, the prognosis of NPC is generally poor [2, 3]. Distant metastasis, which is common among NPC patients, remains a primary reason for failure of the treatment [4]. Radiation therapy (RT) is currently the chief treatment for NPC [5]. Although radiochemotherapy indeed improve the treatment outcome, 19% to 29% of patients still suffer local recurrence due to radioresistance, which eventually lead to treatment failure [6, 7]. Therefore, it is urgent to explore molecular mechanisms of NPC radioresistance, and thereby to identify new targets which contribute to developing current treatment for NPCs.

MicroRNAs (miRNAs) are a class of small, noncoding single-stranded RNA molecules (approximately 22-nucleotide (nt)) that can play vital roles in almost many physio-pathological processes via negatively regulating gene expression [8, 9]. Numerous studies have been reported that miRNAs participant in many cancer progressions, including apoptosis, proliferation, invasion, and migration [10-12]. Many previous studies also have presented a lot of miRNAs, including miR-23a, miR-101, miR-34a, and miR-203a-3p,

have been associated with NPC progression, absolutely including radioresistance [12-14]. Recently, a microRNA expression profiling analysis [15] has been suggested that miR-140-3p were down-regulated in NPC tissue; therefore, there might be a potential association between miR-140-3p and NPC, especially NPC radioresistance. Moreover, little relevant study on the role of the miR-140-3p in NPC has been ever reported.

This study aimed to investigate the involvement of miR-140-3p in NPC and explore its potential molecular mechanism of NPC radioresistance.

## Materials And Methods

This study was approved by the ethics committee of the The First Hospital of Hunan University of Chinese Medicine. Written informed consent was obtained from each enrolled participant in the study.

### Patients and tissue samples

Samples were collected from 103 NPC patients who underwent received radiation therapy (RT) alone at the The First Hospital of Hunan University of Chinese Medicine from January 2013 to December 2018 and analyzed for miR-140-3p expression. Clinical characteristics, including gender, age, tumor-node-metastasis (TNM) stage and pathologic type, were collected. All the patients were newly diagnosed by histopathology and had no prior RT or chemotherapy before the collection of tissue.

### Cell Lines

CNE2-IR (radioresistant NPC cell line) and CNE2 (radiosensitive NPC cell line) obtained from SHUNRAN (Shanghai, China) were employed in the study. Both types of cells were cultivated in the mixture composed of RPMI-1640 (BI, Jerusalem, Israel) medium plus 10% fetal bovine serum (BI, Jerusalem, Israel) at a condition of 37°C, 5% CO<sub>2</sub>, and 95% humidity.

### RNA Isolation and qPCR analysis

The total RNA was isolated from the normal and the NPC cell lines using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The cDNA was reverse-transcribed synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA), and Platinum SYBR Green qRT-PCR SuperMix-UDG reagents (Invitrogen Carlsbad, CA, USA) by the CFX96 sequence detection system (Bio-Rad, Hercules, CA, USA) was used to amplification. All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia) was applied to perform poly (A) addition of mature miRNAs, miRNA reverse transcription and qPCR assay. U6 was used as the internal control to measure the expression levels of miR-140-3p. The expression was estimated by  $2^{-\Delta\Delta C_t}$  method. The Primer sequences for miR-140-3p and XIAP were: miR-140-3p, 5'- GCGCGTACCACAGGGTAGAA-3' (forward) and 5'- AGTGCAGGGTCCGAGGTATT-3'(reverse);FGF2, 5'- TGGCATTGCTGAACGCATTT-3' (forward) and 5'- TGCAGCCAGGTCTAATTGTTTT-3' (reverse).

## Cell Transfection

Lentivirus particles for expression of miR-140-3p, miR-140-3p inhibitor, XIAP, shXIAP, miR-NC, as well as NC inhibitor were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Lipofectamine 2000 (Invitrogen, USA) was used to transfect cells according to the manufacturer's instructions. All the following experiments were carried out using cells harvested at 24h post-transfection.

## Cell viability and colony formation assay

The cell counting kit-8 (Beyotime, China; CCK-8) assay was adopted to detect cell viability. Briefly, cells were inoculated three times into a 96-well plate with a density of  $1 \times 10^4$  cells per well. One day later, the cells were irradiated at a dose of 4Gy. Then, the CCK-8 reagent was added into the cell medium every day for the following four days. One hour after CCK-8 addition, the absorbance of each well at 450 nm was recorded using a spectrophotometer (BioTek, WI, USA).

For colony formation assay, the cells were seeded in six-well plates with a density of  $1 \times 10^3$  cells per well and received 4Gy irradiation 24h later. Crystal violet staining was used to count the colonies. ImageJ was used to calculate the colonies, which were considered as the number of cells of >50.

## Flow cytometric analysis of cell apoptosis

Stably transfected cells were plated in six-well plates with a density of  $5 \times 10^5$  cells per well exposed to 4Gy irradiation. The cells were cultured for 48h, collected, and washed twice using ice-cold phosphate buffer solution (PBS). Flow cytometer using Annexin V-FITC/PI staining was employed to detect the apoptotic cells.

## Western Blot

Cells were lysed by RIPA buffer to obtain the total protein. The protein concentration was measured by bicinchoninic acid (BCA) protein assay kit (Beyotime). About 20 mg per protein sample was applied for Western blot analysis. The following antibodies were used: monoclonal anti-XIAP antibody (1:1000; Abcam, HK, ab2051) and anti- $\beta$ -actin antibody (1:2000; CST, USA, #3700). The protein amounts were visualized by Electrochemiluminescence detection reagent (Millipore).

## Dual Luciferase Reporter Assay

The wild-type (WT) and mutant XIAP-3'UTR (MUT) plasmids were constructed by GenScript (Nanjing, China). Cells were inoculated into 24-well plates and grown for 24h before transfection. Transfection of miR-140-3p mimics or the miR-NC and wild-type or mutant XIAP-3'UTR plasmids into cells was using Lipofectamine 2000 (Invitrogen, USA). Transfected cells were harvested after forty-eight hours and then detected using a Dual-Luciferase Reporter Assay System (Promega, USA). The relative luciferase activity was presented by the ratio of firefly luciferase to Renilla luciferase activity.

## Statistical Analysis

Statistical analyses were conducted by Graphpad Prism 8.0 software. Measurement data were presented as mean  $\pm$  standard deviation. Paired *t*-test or chisquare test was performed for comparison between the two groups. The Kaplan– Meier method was using to draw the survival curve, and the statistical comparison was conducted by using the Log rank test.  $P < 0.05$  indicates that the difference was statistically significant.

## Results

### miR-140-3p is downregulated and correlates to the Prognosis in NPC

The expressions of miR-140-3p in CNE2 and CNE2-IR cells were determined using qPCR. As Figure 1A shown, the miR-140-3p level is significantly decreased in CNE2-IR cells. Subsequently, we further tested the expression of miR-140-3p in NPC and adjacent normal tissue (ANT) samples and checked the relationships between miR-140-3p expression and clinicopathological factors of NPC. Accordingly, the expression level of miR-140-3p in NPC was obviously reduced, compared with that in ANT (Figure 1B). Furthermore, the level of miR-140-3p in radiosensitive NPC tissues was significantly upregulated, compared with that in radioresistant NPC tissues (Figure 1C, Table 1). Similarly, Table 1 listed that low expression of miR-140-3p was positively associated with primary T stage, lymph node metastasis, and advanced TNM stage (Table 1), suggesting that miR-140-3p may correlate with prognosis of NPC. Indeed, after performing Kaplan-Meier survival analysis, the results have been indicated the lower expression of miR-140-3p lead to the poorer overall survival of NPC patients (Figure 1D). Therefore, we revealed that miR-140-3p is downregulated in NPC, especially for radioresistant NPC, which is correlated with the poorer prognosis in NPC.

### **Table 1** Correlation Between miR-140-3p Level and Clinicopathological Characteristics in NPC

Variables	N	miR-140-3p level		P-value
		Low (65)	High (38)	
Gender				
Male	64	38	26	0.237
Female	41	29	12	
Age				
≤ 45	44	25	19	0.205
> 45	61	42	19	
Primary T stage				
T1-2	40	18	22	<b>0.001*</b>
T3-4	65	49	16	
Lymph node (N) metastasis				
N0	47	14	33	<b>0.006*</b>
N1-3	58	53	5	
TNM stage				
I-II	42	20	22	<b>0.004*</b>
III-IV	63	47	16	
Radiation response				
Sensitivity	50	26	24	<b>0.016*</b>
Resistance	55	41	14	
Smoking history				
Yes	51	30	21	0.301
No	54	37	17	

\* P value was considered significantly different.

### miR-140-3p hampered radioresistance of NPC cells

Since miR-140-3p is downregulated in radioresistant CNE2-IR cells, we subsequently investigated the effects of miR-140-3p expression on the radioresistance of NPC cells. Firstly, we established the stable cell lines, including CNE2-IR-miR-140-3p-inhibitor, and CNE2-miR-140, as well as the control cells, using

lentivirus particles transfection. Subsequently, the radiation sensitivity of NPC cells was detected by CCK-8, colony formation assay, and apoptosis assays under irradiation treatment of 4Gy. Ectopic expression of miR-140-3p significantly sensitized CNE2IR cells to irradiation due to decreased cell viability (Figure 2A, left panel), fewer survival clones (Figure 2B, left panel), and higher apoptotic rate (Figure 2C, left panel); whereas, miR-140-3p-inhibitor remarkably enhances the tolerance of CNE2 cells to irradiation indicating by improved cell viability (Figure 2A, right panel), more survival clones (Figure 2B, right panel), and reduced apoptotic rate (Figure 2C, right panel). Thus, all these results demonstrated that miR-140-3p can destroy radioresistance of NPC cells.

### **miR-140-3p can directly target XIAP in NPC**

The functions of miRNAs are dependent on its regulated mRNAs. Therefore, we explored the target of miR-140-3p in NPC using Starbase 3.0 online. Then, XIAP was identified as a potential target of miR-140-3p. Therefore, we measured the mRNA and protein level of XIAP in CNE2 and CNE2-IR cells transfected with miR-140-3p and miR-140-3p-inhibitor, respectively. As the qPCR and Western blot indicated (Figure 3A and B), overexpression of miR-140-3p significantly reduced XIAP expression, while miR-140-3p-inhibitor upregulation remarkably increased the mRNA and protein level of XIAP in CNE2 and CNE2-IR cells, respectively. These results manifested that miR-140-3p could regulate XIAP in NPC cells; however, whether miR-140-3p could directly target XIAP in NPC cells remains unclear. Therefore, dual luciferase reporter assay was used to verify the underlying mechanism. As shown in Figure 3C, miR-140-3p mimics could remarkably impair luciferase signaling in CNE2 cells transfected with wild 3'UTR XIAP plasmids, but not in cells transfected with mutant 3'UTR XIAP plasmids, proving that XIAP was a direct target of miR-140-3p in NPC cells.

### **miR-140-3p release radioresistance of NPC Cells via Suppressing XIAP in vitro**

Considering XIAP was the target of miR-140-3p in NPC cells, we further analyzed the role of XIAP in miR-140-3p associated NPC radioresistance. The influences of ectopic XIAP expression or XIAP silence on the radiation sensitivity of NPC cells were detected. Accordingly, CNE2-miR-140-3p and CNE2-IR-miR-140-3p-inhibitor cells was transfected lentivirus particles with XIAP expression or knockdown. Ectopic expression of XIAP could significantly rescue the level of XIAP (Figure 4A, left panel) and re-enhanced the tolerance of the NPC cells to irradiation, shown by improved cell viability (Figure 4B, left panel), more survival clones (Figure 4C, upper panel), and lower apoptosis rate (Figure 4D, left panel), in CNE2-miR-140-3p cells. Whereas, silence of XIAP successfully decreased the level of XIAP (Figure 4A, right panel) and re-sensitize NPC cells to irradiation, indicated by reduced cell viability (Figure 4B, right panel), fewer survival clones (Figure 4C, lower panel), and higher apoptosis rate (Figure 4D, right panel), in CNE2-IR-miR-140-4p-inhibitor cells. Therefore, these findings in vitro indicated that miR-140-3p could alleviate radioresistance of NPC cells by suppressing XIAP.

## **Discussion**

The incidence rate of NPC is high in Asia [16]. RT for various malignant tumors is an effective, standard therapeutic intervention that is commonly used. IR achieves its therapeutic effects via cause DNA damage and destroy cancer cells [17]. Although RT is the main treatment for NPC, the existence of radioresistant cells in NPC tissue could cause NPC recurrence and metastasis after radiation [18]. Many previous studies have been reported the roles of miRs in the development of NPC [19]. However, the molecular mechanisms underlying the development of NPC have not yet been fully elucidated. In this study, we found that NPC cells with lower expression of miR-140-3p presented less sensitivity to irradiation, which contributed to the radioresistance of NPC in clinic.

MiR-140 was initially reported to be involved in the development of cartilage in vivo [20]. Zhang et al. has reported that overexpression of miR-140 inhibited the proliferation and invasion of colorectal cancer by targeting VEGFA [21]. MiR-140-3p belongs to the miR-140 cluster and has been found to function in tumorigenesis [22]. MiR-140-3p expression was shown to be decreased in breast cancer patients [23]. MiR-140-3p was also reported to inhibit cell proliferation of lung cancer by downregulating ATP8A1 [24]. Furthermore, miR-140-3p was shown to promote the sensitivity of hepatocellular carcinoma cells to sorafenib [25]. However, the expression of miR-140-3p was reversely associated with the prognosis of spinal chordoma [26]. Therefore, the role of miR-140-3p in carcinogenesis seems to be unclear and complicated. To date, the expression and function of miR-140-3p in the development of NPC remains unknown. In this study, we found that miR-140-3p expression was decreased in NPC tissues, especially in radioresistant NPC. In addition, upregulation of miR-140-3p significantly suppressed proliferation and induced NPC cell apoptosis in vitro. These results demonstrated that miR-140-3p plays a vital role in the tumorigenesis and the regulation of radioresistance of NPC.

The function of miRs is dependent on its target genes. In this study, we found XIAP was the direct target of miR-140-3p. The X-linked inhibitor of apoptosis (XIAP) is a member of IAP protein family that is characterized as a well-defined inhibitor of the caspase/apoptosis pathway [27, 28]. Overexpression of XIAP is particularly related to the progression and aggression of malignant tumors [29]. The previous study has indicated four functional domains in XIAP [30]: three repeats of the baculovirus IAP repeat (BIR) domain at its NH<sub>2</sub> terminus exerting its anti-apoptotic function by inhibiting caspase-3, -7 and -9; a RING finger domain near its COOH terminus displaying E3 ubiquitin ligase activity [31]. XIAP also promotes cell proliferation of bladder cancer through the BIR domain-mediated c-Jun/miR-200a/EGFR axis [32]. The RING domain of XIAP could regulate human colon cancer cell migration by interacting with RhoGDI $\alpha$  protein to inhibit RhoGDI $\alpha$  SUMOylation at Lys-138 [33]. In addition, decreased expression of the tumor suppressor p63 $\alpha$  protein by the RING domain of XIAP could promote malignant transformation of bladder epithelial cells [33]. In this study, XIAP could be negatively regulated by miR-140-3p and affect radiation sensitivity in NPC treatment.

## Conclusion

In summary, we demonstrated that miR-140-3p is downregulated in radioresistant NPC. miR-140-3p could hamper radioresistance of NPC via directly target XIAP, suggesting miR-140-3p/XIAP axis is vital in the

regulation of irradiation sensitivity and may serve as a potential target to improve radiation sensitivity in NPC treatment.

## **Declarations**

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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.

### **Authors' contributions**

QW and ZHZ conducted the experiments. ZHZ participated in the sequence alignment. QW participated in the design of the study and performed the statistical analysis. ZHZ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

### **Ethics approval and consent to participate**

The present study was approved by the Institutional Animal Care and Use Committee of The First Hospital of Hunan University of Chinese Medicine.

### **Consent for publication**

Not applicable.

### **Conflict of interest**

Qi Wu, Zhenhua Zhu declare that they have no conflict of interest..

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## Figures

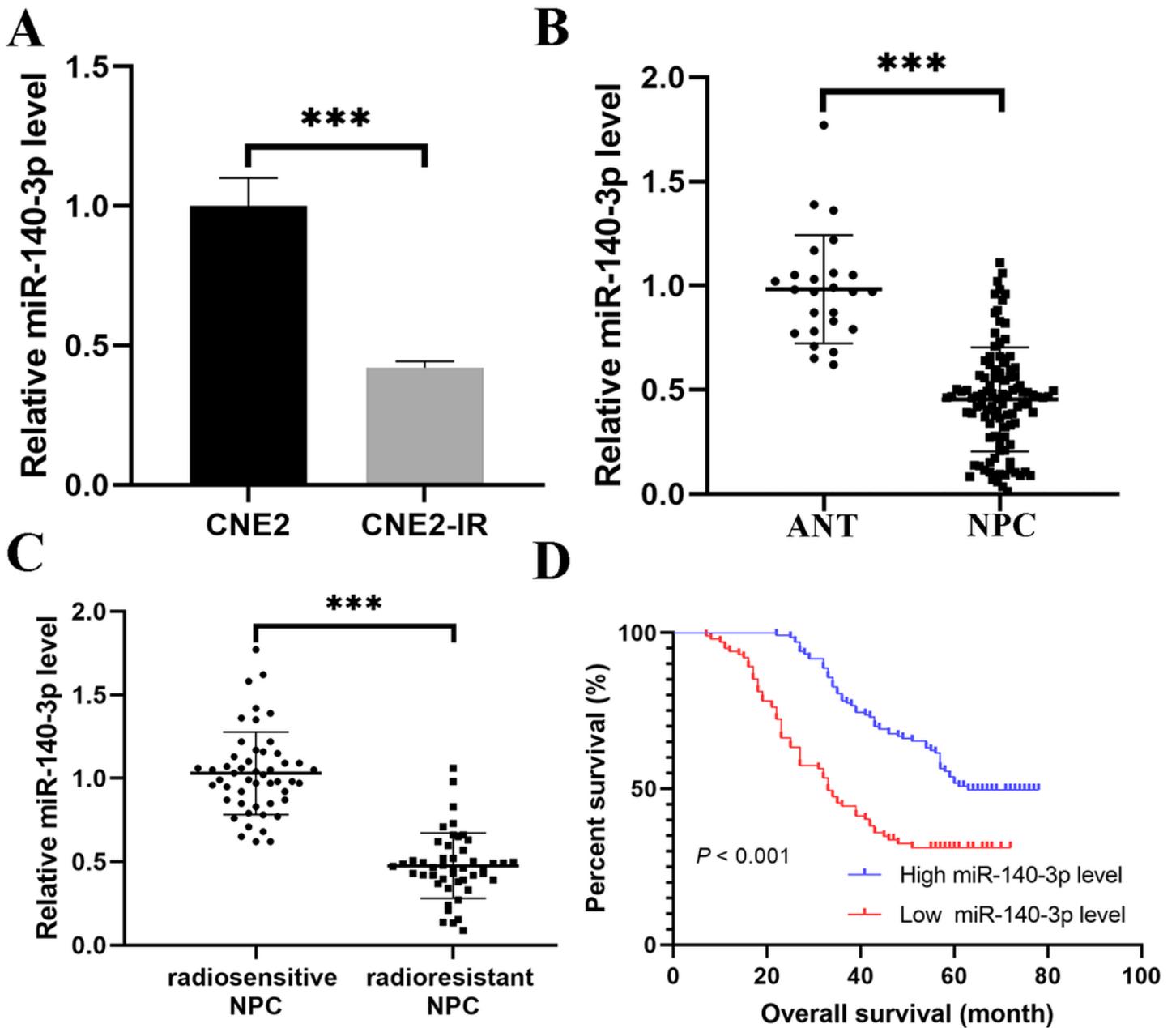
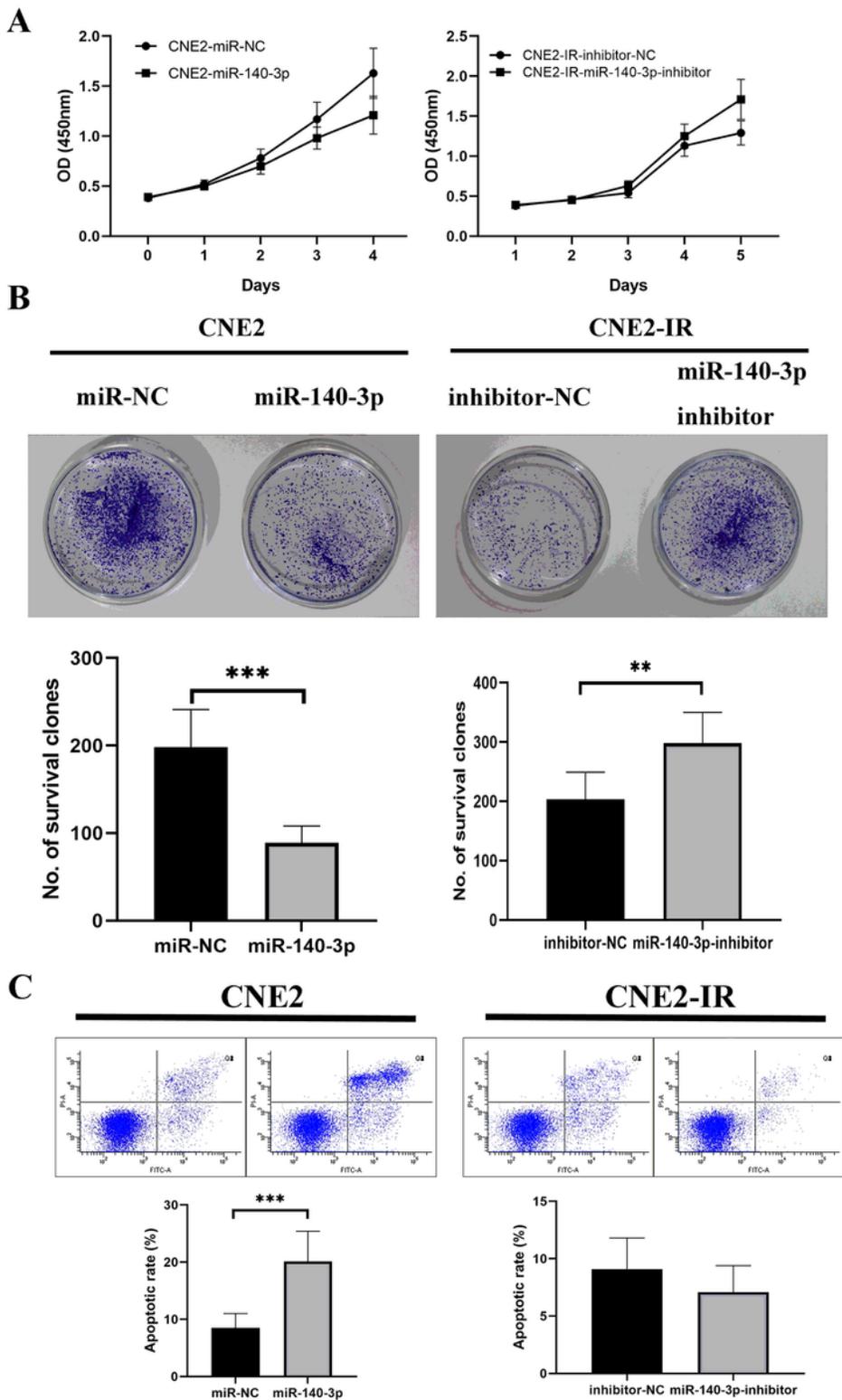


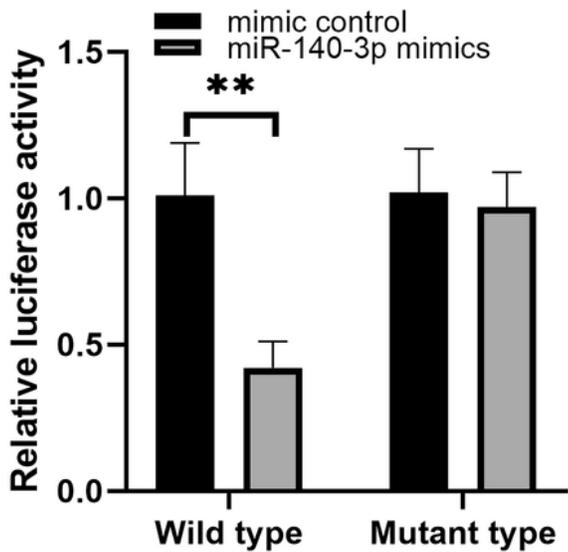
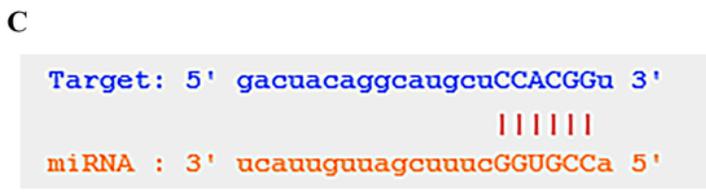
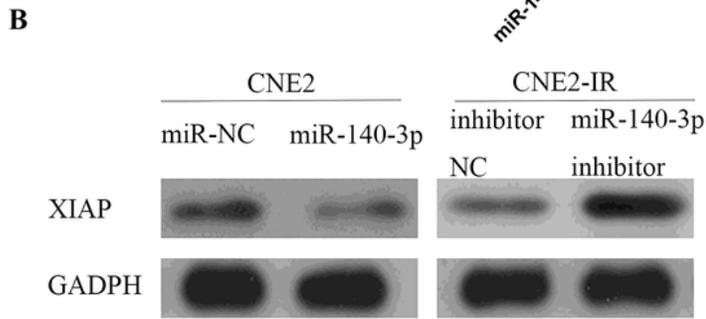
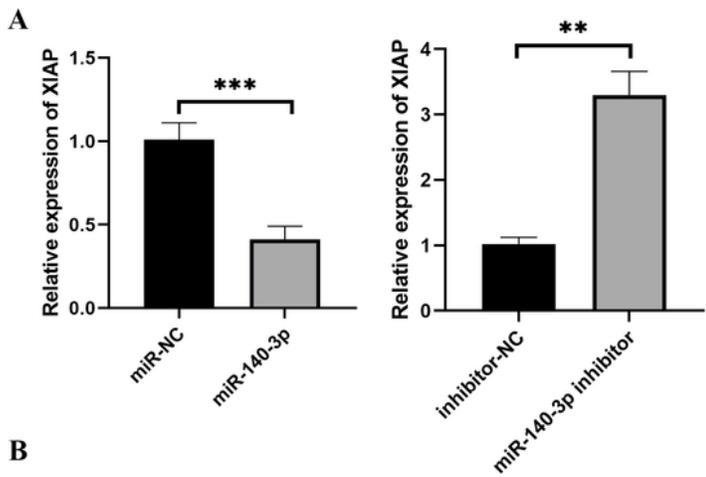
Figure 1

MiR-140-3p is downregulated in radioresistant NPC and negatively correlates to the prognosis of NPC. qPCR assays indicated that miR-140-3p was decreased in CNE2-IR cells (A), NPC tissue samples (B) and radioresistant NPC tissue samples (C) compared with CNE2, NNM tissue samples, and radiosensitive NPC tissue samples, respectively. (D) The patient of low miR-140-3p exhibited poor overall survival demonstrating by Kaplan-Meier survival analysis. \*\*\*Stands for  $P < 0.001$ .



**Figure 2**

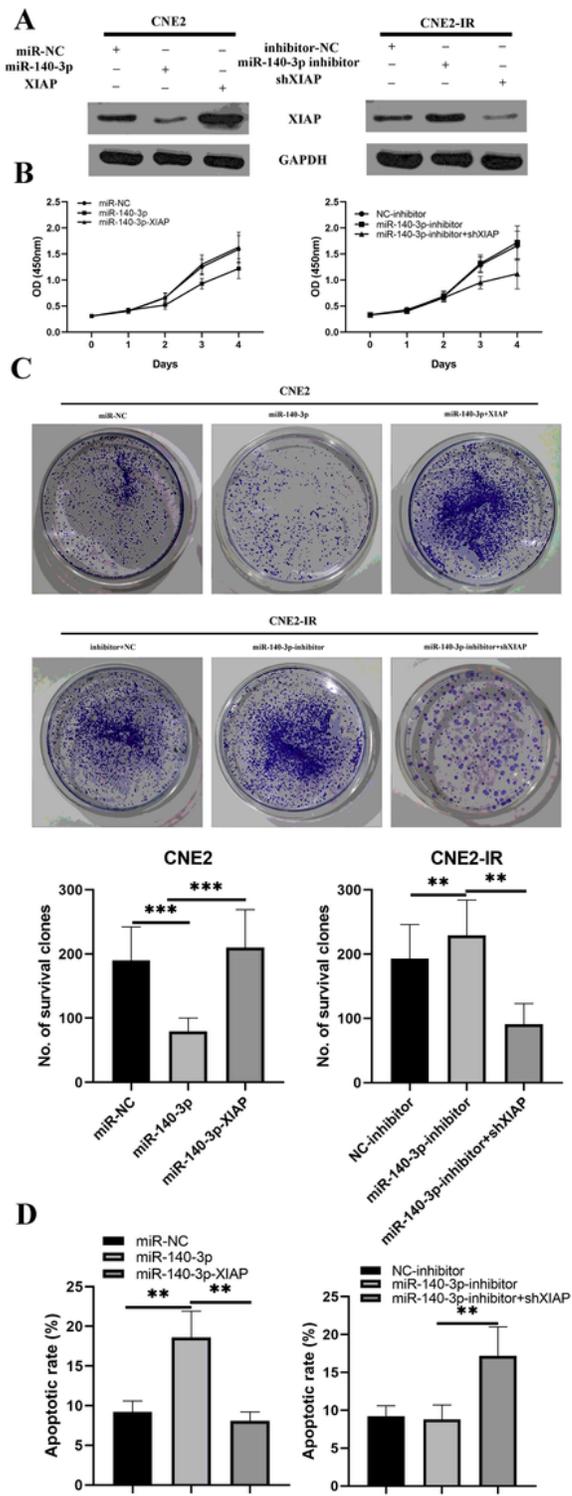
MiR-140-3p improves NPC radiosensitivity in vitro. Ectopic expression of miR-140-3p reduced the cell viability (A, left panel), survival clones (B, left panel), and non-apoptotic cell rates (C, left panel) of CNE2 under 4Gy irradiation. Accordingly, overexpression of miR-140-3p inhibitor promoted the cell viability (A, right panel), survival clones (B, right panel), and non-apoptotic cell rates (C, right panel) of CNE2-IR under 4Gy irradiation.



**Figure 3**

Mir-140a could directly target XIAP in NPC. qPCR and Western blot assays showed that miR-140-3p overexpression could inhibit XIAP mRNA (A, left panel) and XIAP protein (B, left panel) level, while miR-140-3p inhibitor overexpression could upregulate XIAP mRNA (A, right panel,) and XIAP protein (B, right panel) level. (C) The relative luciferase activity of CNE2 transfected with wild type XIAP reporter plasmid

was significantly decreased, whereas, the no inhibitory effect was not presented in CNE2 transfected with mutant type XIAP reporter plasmid.



**Figure 4**

Mir140-3p promotes NPC radiosensitivity via directly targeting XIAP in vitro. (A) Western blot showed that miR-140-3p overexpression notably reduced XIAP expression in CNE2 cells which could be rescued by ectopic expression of XIAP (left panel). Accordingly, overexpression of miR-140-3p inhibitor significantly

promoted XIAP in CNE2-IR cells, which could be inhibited by specific small interfering RNA targeting XIAP (right panel). Upon 4 Gy irradiation, CCK-8, clone survival, and apoptosis assays demonstrated that miR-140-3p overexpression significantly impaired cell viability (B, left panel), decreased cell survival (C, upper panel), and promoted apoptosis (D, left panel), and these effects could be antagonized by ectopic XIAP in CNE2 cells; while, miR-140-3p inhibitor overexpression remarkably reinforced cell viability (B, right panel), increased cell survival (C, lower panel), and inhibited apoptosis (D, right panel), and these effects could be rescued by sh-RKIP in CNE2-IR cells.