



LINC01140 Regulates Radiosensitivity of Nasopharyngeal Carcinoma Cells Through the ceRNA Network

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is one of the most lethal head and neck cancers, threatening the health of people across the globe, especially in East and Southeast Asia, the Arctic, the Middle East and North Africa. Long non-coding RNAs (lncRNA) have been reported to regulate multiple cancers, including NPC. However, the role of LINC01140 in NPC remains to be covered. In this study, we found that LINC01140 is downregulated in NPC cells. It was uncovered from functional assays that LINC01140 inhibits the proliferation and improves the apoptosis and radiosensitivity of NPC cells. The downstream mechanism by which LINC01140 exerted its functions was explored in subsequent. As proven by mechanism experiments, cytoplasmic LINC01140 positively regulated the expression of ZNF621 through competitively binding to miR-452-5p. ZNF621 can also enhance the radiosensitivity of NPC cells. To summarize, LINC01140 regulates the radiosensitivity of NPC cells through the competing endogenous RNA (ceRNA) mode. Our study aims to identify novel biomarkers for regulating the radiosensitivity of NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is a kind of head and neck cancer with a poor survival rate and possesses the highest metastasis rate compared with other head and neck cancers (1,2). NPC is especially prevalent in East and Southeast Asia, the Arctic, the Middle East and North Africa (3,4). The major risk factors for NPC include diet, life styles and viral infection. The intake of salted fish, alcohol and tobacco is found to be correlated with NPC incidence (5). Furthermore, Epstein-Barr virus (EBV) is also associated with the occurrence of NPC, and males are more likely to get infected with EBV-associated NPC (6). Recently, great progresses have been achieved in the treatment of NPC. The evolution of therapeutic methods like liquid biopsies, and minimally invasive surgery, significantly improves the overall survival of NPC patients (7). However, the recurrence and metastasis rate of NPC is still high. Therefore, it is urgent to identify novel biomarkers of NPC for the sake of in-time diagnosis and treatment.

Long non-coding RNAs (lncRNA) refer to the RNAs with over 200 nucleotides, and without the

capacity to code proteins. A large number of lncRNAs have been found to be associated with a variety of carcinomas; their aberrant expressions or mutations influence metastasis or tumorigenesis (8). NPC has been found to be regulated by lncRNA as well. For instance, lncRNA PVT1 has been reported to modulate NPC cells through the activation of the KAT2A acetyltransferase and the stabilization of HIF-1 α (9); lncRNA C22orf32-1 facilitates the proliferation, migration and invasion of NPC cells (10); NKILA suppresses the development of NPC cells via deactivating NF- κ B signaling pathway (11).

As the lncRNA we have studied, LINC01140, according to the previous study, acts as a tumor suppressor of bladder cancer (12). Moreover, it plays a regulatory role in breast cancer cells (13). However, there is no research on the role of LINC01140 in NPC. Radiotherapy is considered as the preferred treatment for NPC (14). In recent years, lncRNAs have been found to play a role in influencing the radiosensitivity of a variety of cancers (15,16). However, the effect of LINC01140 on the radiosensitivity of NPC cells remains largely

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unknown. Therefore, in this study, we aimed to explore the effect of LINC01140 on the radiosensitivity of NPC cells and its underlying mechanism in vitro.

Materials and methods

Cell culture

CNE2Z and CNE cells were procured from the Cell Culture Center, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, S18-1C3 cells were commercially acquired from China Center for Type Culture Collection (CCTCC), and NP69 cells were bought from American Type Culture Collection (ATCC). All the cell lines were cultured using RPMI 1640 medium, with the addition of 10% fetal bovine serum (FBS, 10270-106, Gibco, USA), 100 µg/mL penicillin and 100 µg/mL streptomycin under the condition of 5% CO₂ at 37 °C.

Vector construction

The overexpression vectors were constructed by inserting LINC01140 and ZNF621 into vectors, pcDNA3.1. The vector itself was used as negative control (NC). Short hairpin RNA (shRNA) targeting LINC01140 was commercially acquired from RiboBio (Nanjing, China).

qPCR

The total RNAs of CNE2Z, S18-1C3, CNE and NP69 cells were subjected to extraction via using Trizol (9108-1, Thermo Fisher, America). Then, the extracted total RNAs were reverse transcribed to cDNAs. All the samples were measured by qPCR via using ChamQ Universal SYBR qPCR Master Mix (Q711-02, Vazyme Biotech, Nanjing, China). The results were calculated via $2^{-\Delta\Delta C_t}$. The results were quantified in the form of mean ± standard deviation (SD). Bio-repeats were implemented in triplicate.

Western blot

The total proteins of CNE2Z cells were isolated. Next, the total proteins were separated using SDS/PAGE gel and then transferred onto the polyvinylidene fluoride (PVDF) membranes (T2234, Invitrogen, America). Next, the membranes were blocked using 5% skim milk at room temperature for 2 hours. Afterward, the membranes were incubated with the primary anti-ZNF621 (SAB4301769, Sigma-

Aldrich, America) and anti-β-actin (ab8226, Abcam, British) overnight at 4 °C. After being washed five times for 6 minutes per time, these membranes were subjected to incubation with the secondary antibodies (ab150077, Abcam, British) for one hour at room temperature. Lastly, the levels of proteins were visualized and recorded. β-actin was considered as the internal reference. Bio-repeats were implemented in triplicate.

RIP assay

RIP assays were conducted in CNE2Z cells by use of Imprint® RNA Immunoprecipitation Kit (RIP-12RXN, Sigma-Aldrich, America). Cells were lysed by the use of RIPA buffer. The cell lysate was then subjected to incubation with magnetic beads of anti-Ago2 (ab186733, Abcam, British), and anti-IgG (ab133470, Abcam, British). The precipitated RNAs were extracted for the analysis of qPCR. Bio-repeats were implemented in triplicate.

Dual-luciferase reporter assay

The assay was conducted using the Dual-Luciferase Reporter Gene Assay Kit (RG027, Beyotime, Shanghai, China). The full-length sequence or mutated sequence of LINC01140 was subcloned into pmirGLO vectors to create pmirGLO-LINC01140-Wt or pmirGLO-LINC00941-Mut. Likewise, the 3' UTR sequence or mutated sequence of ZNF621 was subcloned into pmirGLO vectors to construct pmirGLO-ZNF621-Wt or pmirGLO-ZNF621-Mut. CNE2Z cells were cultured into the 96-well plates. Then, pmirGLO-LINC01140/ZNF621-Wt or pmirGLO-LINC00941/ZNF621-Mut was co-transfected with mimics NC or miR-452-5p mimics. The vectors themselves were used as the negative controls. A microplate photometer (51119770DP, Thermo Fisher) was used to assess the luciferase activity. Bio-repeats were implemented in triplicate.

FISH assay

The FISH assay was conducted utilizing Fluorescence in situ hybridization probe kit (C10910, RiboBio, Guangzhou, China). CNE2Z and S18-1C3 cells were subjected to fixation in 4% PFA for 15 min. Subsequently, the cells were subjected to permeabilization using 0.5% TritonX-100 for 25 min at 4 °C. Next, Digoxigenin (DIG)-labeled target gene

probe or control probe mix was prepared to be incubated with the cells for 4 hours at 55 °C. Then the signal was detected by utilizing Hoechst-conjugated Anti-Digoxin Antibodies. A confocal microscope (LSM800, Zeiss) was used to obtain images. DAPI was used to counterstain nuclei of CNE2Z and S18-1C3 cells. Bio-repeats were implemented in triplicate.

RNA pulldown assay

LINC01140 was subjected to biotinylation using Pierce™ RNA3' End Desthiobiotinylation Kit (20163, Thermo Fisher, America). The structure buffer was added into the 1µg of biotin-labeled LINC01140. Afterward, Bio-LINC01140 was heated at 95°C for 2 min, then treated with an ice bath for 3 min, and left for 30 min at room temperature for the sake of denaturing. Subsequently, biotin-labeled and denatured RNA was co-incubated with streptavidin beads for 2 hours at 4°C. Three groups (input, Bio-NC, Bio-LINC01140) were set up to prepare the cell lysate of CNE2Z cells. Then, the magnetic beads with Bio-LINC01140 were then subjected to incubation with the cell lysate. The biotin-coupled RNA complex was pulled down by the magnetic beads and the total RNAs were isolated using TRIzol. Lastly, qPCR was used to detect the enrichment of miR-4676-3p, miR-452-5p, miR-200c-3p, miR-200b-3p, miR-29b-3p and miR-429. Bio-repeats were implemented in triplicate.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 assay was carried out using a CCK-8 kit (M4839, ABMOLE, America) based on the protocol of the manufacturer. Transfected and irradiated CNE2Z and S18-1C3 cells were plated into 96-well culture plates for the experiments. Next, all the cells underwent 12, 24, 36, 48 and 60 hours of incubation, followed by the addition of 100 µl CCK-8 solution to each well. Then the solution and the cells were incubated for another 4 hours. The absorbance was evaluated at the wavelength of 450 nm. Bio-repeats were implemented in triplicate.

Colony formation assay

Transfected CNE2Z and S18-1C3 cells were plated into 6-well plates. After 24 hours, the cell colonies were washed with PBS (C10010500BT, Gibco, USA), treated with 4% paraformaldehyde for fixation and then dyed with 0.5% crystal violet. Lastly, the number

of colonies was counted for comparison. Bio-repeats were implemented in triplicate.

Comet assay

CNE2Z and S18-1C3 cells are embedded in agarose and then subjected to lysis. Then electrophoresis was conducted to analyze the cells. In the course of electrophoresis, the damaged DNA migrated from the nuclei to the anode. The measurement of the migrated cell was conducted to analyze the extent of damaged DNA.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

CNE2Z and S18-1C3 cells were rinsed in ice-cold PBS and then permeabilized for 15 min. Then the cells were stained with TUNEL solution with the counterstaining of DAPI. A confocal microscope (LSM800, Zeiss) was used to analyze the TUNEL-positive cells.

Statistical analyses

Every experiment was conducted in triplicate. All the data were presented as means ± SD. Comparisons between only two groups were analyzed using student's t-test, and one-way ANOVA as well as two-way ANOVA were applied for comparison between multiple groups. A p-value less than 0.05 were considered as statistical significance.

Results and discussion

LINC01140 inhibits the proliferation and promotes the apoptosis of NPC cells

To explore the association of LINC01140 with NPC, we used qPCR to detect the expression of LINC01140 in human nasopharyngeal carcinoma cell lines (CNE2Z, S18-1C3 and CNE) and human immortalized nasopharyngeal epithelial cell line (NP69), finding that LINC01140 was downregulated in CNE2Z, S18-1C3 and CNE cells compared with NP69 cells (Fig. 1A). Due to their relatively low expression of LINC01140, CNE2Z and S18-1C3 cells were selected for our research. Next, we assessed the efficiency of pcDNA3.1-LINC01140 in CNE2Z and S18-1C3 cells using qPCR (Fig. 1B). Subsequently, a series of functional experiments were conducted to evaluate the effects of LINC01140 on the proliferation and apoptosis of NPC cells. The results of CCK-8 and

colony formation assays demonstrated that LINC01140 overexpression led to the decline in the proliferative capacity of CNE2Z and S18-1C3 cells (Fig. 1C-D). TUNEL assay showed that upregulated LINC01140 promoted the apoptosis of CNE2Z and

S18-1C3 cells as evidenced by the increase of TUNEL-positive cells (Fig. 1E). Taken together, LINC01140 inhibits the proliferation and promotes the apoptosis of NPC cells.

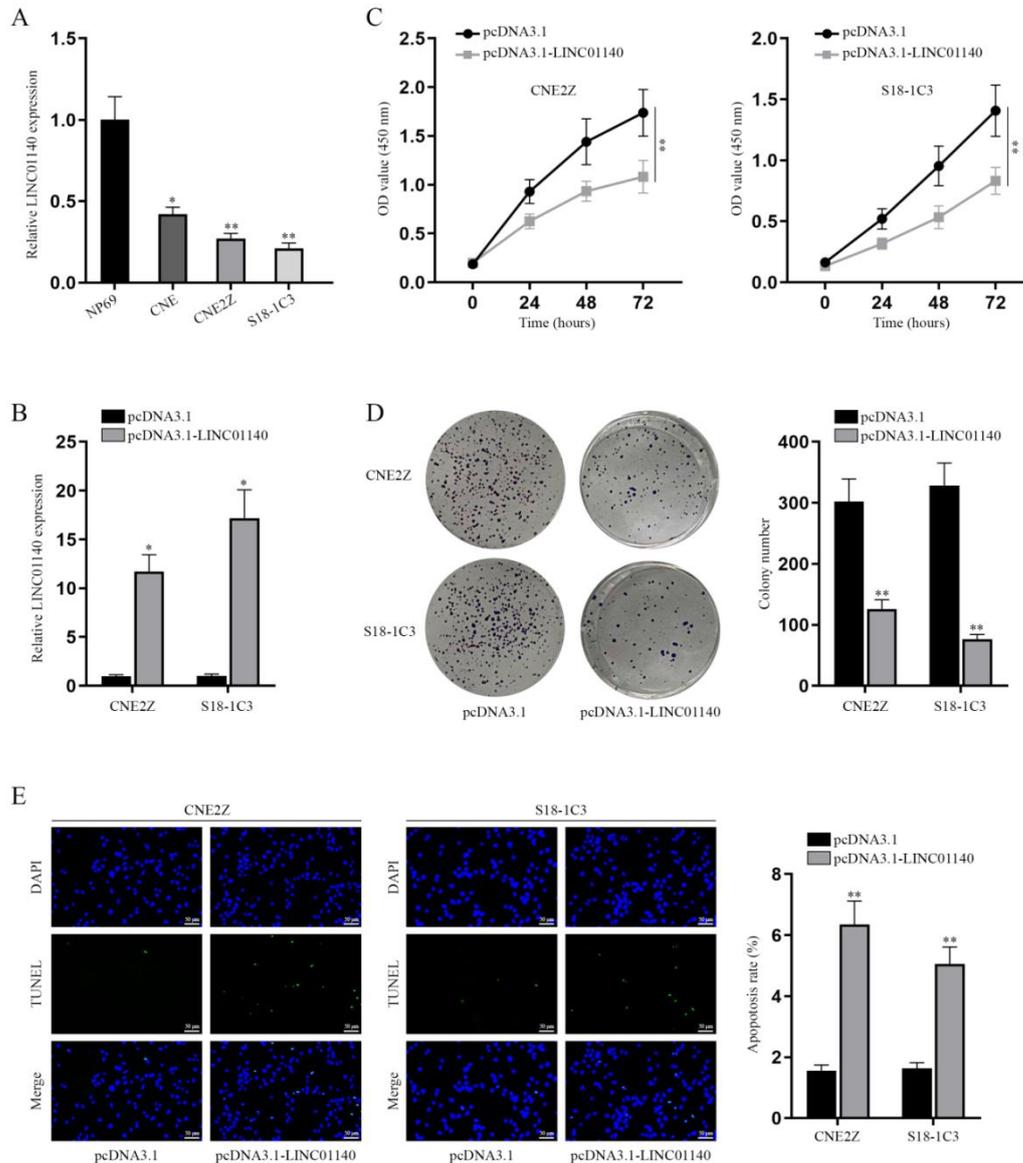


Figure 1. LINC01140 inhibits the proliferation and promotes the apoptosis of NPC cells.

(A) The expression of LINC01140 was detected by qPCR in CNE, CNE2Z, S18-1C3 and NP69 cells (One-way ANOVA, Tukey). (B) The efficiency of pcDNA3.1-LINC01140 in CNE2Z and S18-1C3 cells was evaluated using qPCR (Student's t-test). (C-D) CCK-8 and colony formation assays were used to detect the proliferation of CNE2Z and S18-1C3 cells after the overexpression of LINC01140 (Student's t-test). (E) TUNEL assay was used to detect the apoptosis of CNE2Z and S18-1C3 cells after the overexpression of LINC01140 (Student's t-test). * $P < 0.05$, ** $P < 0.01$.

LINC01140 enhances the radiosensitivity of NPC cells in vitro

In recent years, lncRNAs have been found to play regulatory roles in influencing the radiosensitivity of a variety of cancers. As radiotherapy is the preferred treatment for NPC, we probed into the effect of

LINC01140 on the radiosensitivity of NPC cells. Firstly, we performed a CCK-8 assay to detect the OD value of CNE2Z and S18-1C3 cells 48 hours after the treatment of the X-ray. We compared the differences in OD value between the pcDNA3.1 group and pcDNA3.1-LINC01140 group and found that the

difference was most obvious in NPC cells at the dose of 8 Gy (Fig. 2A). Therefore, we selected 8 Gy as the treatment conditions for cells in the follow-up experiments (17). Afterward, we performed a colony formation assay to assess the proliferation of irradiated NPC cells after the overexpression of LINC01140. It showed the inhibitory effect of irradiation on survival fraction, and this effect was enhanced by LINC01140 overexpression (Fig. 2B). The results of CCK-8 and colony formation assays showed that LINC01140 can enhance the sensitivity of NPC cells to ionizing radiation. Subsequently, a comet assay was conducted to detect the DNA damage of irradiated CNE2Z and S18-1C3 cells after the overexpression of LINC01140. The results showed that the percentage of the tail moment and tail DNA in the pcDNA3.1-LINC01140 group were significantly higher than those of the control group, indicating that LINC01140 could inhibit the repair of DNA damage, thus aggravating the DNA damage of NPC cells caused by radiation (Fig. 2C). Then we conducted a TUNEL assay and found out that X-ray irradiation increased the number of apoptotic cells, and LINC01140 enhanced the effect of X-ray (Fig. 2D). Taken together, LINC01140 enhances the radiosensitivity of NPC cells in vitro.

LINC01140 regulates ZNF621 expression via competitively binding to miR-452-5p in NPC cells

Next, the molecular mechanism of LINC01140 in regulating the radiosensitivity of NPC cells was explored. We first conducted a FISH assay and discovered that LINC01140 was mainly located in the cytoplasm of CNE2Z and S18-1C3 cells (Fig. 3A). Hence, we speculated that LINC01140 plays its regulatory role through ceRNA mode. To verify our speculation, we designed an Anti-Ago2 RIP assay and detected the enrichment of LINC01140 in RNA-induced silencing complex (RISC) in CNE2Z cells, where miRNAs exert their functions (Fig. 3B). The results of the RIP assay prove the ceRNA mode of LINC01140. Next, we screened the potential target miRNAs of LINC01140 via the utilization of the starBase database. Under the condition of CLIP-Data (≥ 1), pan-Cancer (≥ 4), we selected 5 candidates, miR-4676-3p, miR-452-5p, miR-200c-3p, miR-200b-3p, miR-29b-3p and miR-429 (Fig. 3C). Subsequently, an RNA pulldown assay was conducted

in CNE2Z cells by biotinylated LINC01140 to pull down the candidates. The results showed that only miR-452-5p was enriched in the Bio-LINC01140 group, indicating the combination between miR-452-5p and LINC01140 (Fig. 3D). Then we mutated the binding site of LINC01140 and performed an RNA pulldown assay as well, further verifying the combination between miR-452-5p and LINC01140 (Fig. 3E). To find out the target mRNA of miR-452-5p, we used the starBase database to screen the potential mRNAs interacting with miR-452-5p under the condition of CLIP-Data (≥ 1), pan-Cancer (≥ 10), and programNum (≥ 4). We screened out 11 candidates, RHOA, DAG1, ZNF621, SMAD2, TIMP2, NKIRAS1, VEZF1, PRKAR2B, KLF12 and ANKS1B (Fig. 3F). Then, we used qPCR to detect the expression of these candidates after the overexpression of LINC01140. The results demonstrated that only ZNF621 expression was significantly increased (Fig. 3G). Moreover, ZNF621 was reported to exert an inhibitory effect on cervical cancer (18). Therefore, we selected ZNF621 for the follow-up experiments. Then we used a luciferase reporter assay to confirm the interaction between miR-452-5p and ZNF621, miR-452-5p and LINC01140 (Fig. 3H). Afterward, we conducted rescue experiments to figure out the mechanism among LINC01140, miR-452-5p and ZNF621. Via the results of qPCR and western blot assays, we found out that miR-452-5p could restore the enhanced expression of ZNF621 caused by LINC01140 overexpression, indicating that LINC01140 can regulate ZNF621 expression via miR-452-5p (Fig. 3I-J). Taken together, LINC01140 regulates ZNF621 expression via competitively binding to miR-452-5p in NPC cells.

ZNF621 boosts the radiosensitivity of NPC cells in vitro

The experiments shown in Figure 3 confirmed that LINC01140 can regulate the expression of ZNF621, but it's unclear whether ZNF621 can regulate the radiosensitivity of NPC cells. Therefore, we designed experiments to determine the effect of ZNF621 on the radiosensitivity of NPC cells. We first detected the expression of ZNF621 in human nasopharyngeal carcinoma cell lines (CNE2Z and S18-1C3) and human immortalized nasopharyngeal epithelial cell

line (NP69) and found out the downregulation of ZNF621 in CNE2Z and S18-1C3 cells (Fig. 4A). Then we used qPCR to evaluate the overexpression efficiency of pcDNA3.1-ZNF621 (Fig. 4B). Subsequently, we conducted CCK-8, colony

formation, comet and TUNEL assays to confirm the capacity of ZNF621 to enhance the inhibitory effect of X-ray irradiation on CNE2Z and S18-1C3 cells (Fig. 4C-F). Taken together, ZNF621 boosts the radiosensitivity of NPC cells in vitro.

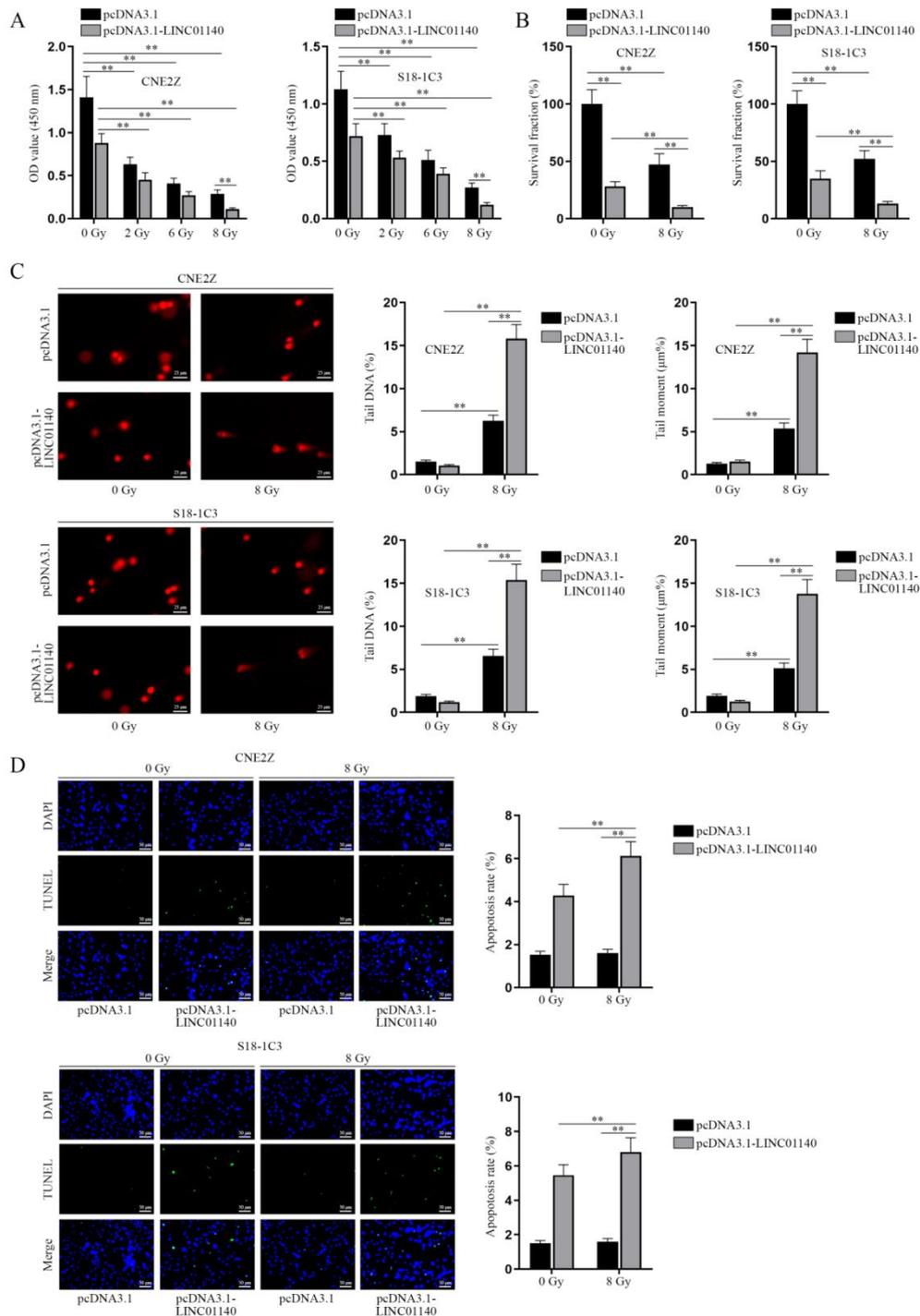


Figure 2. LINC01140 enhances the radiosensitivity of NPC cells in vitro.

(A) CCK-8 assay was used to detect the OD value of irradiated CNE2Z and S18-1C3 cells before and after the overexpression of LINC01140 at a dose of 0 Gy, 2 Gy, 6 Gy and 8 Gy respectively (Two-way ANOVA, Tukey). (B-D) Colony formation, comet and TUNEL were conducted to analyze the inhibitory effect of irradiation on CNE2Z and S18-1C3 cells before and after the overexpression of LINC01140 (Two-way ANOVA, Tukey). **P<0.01.

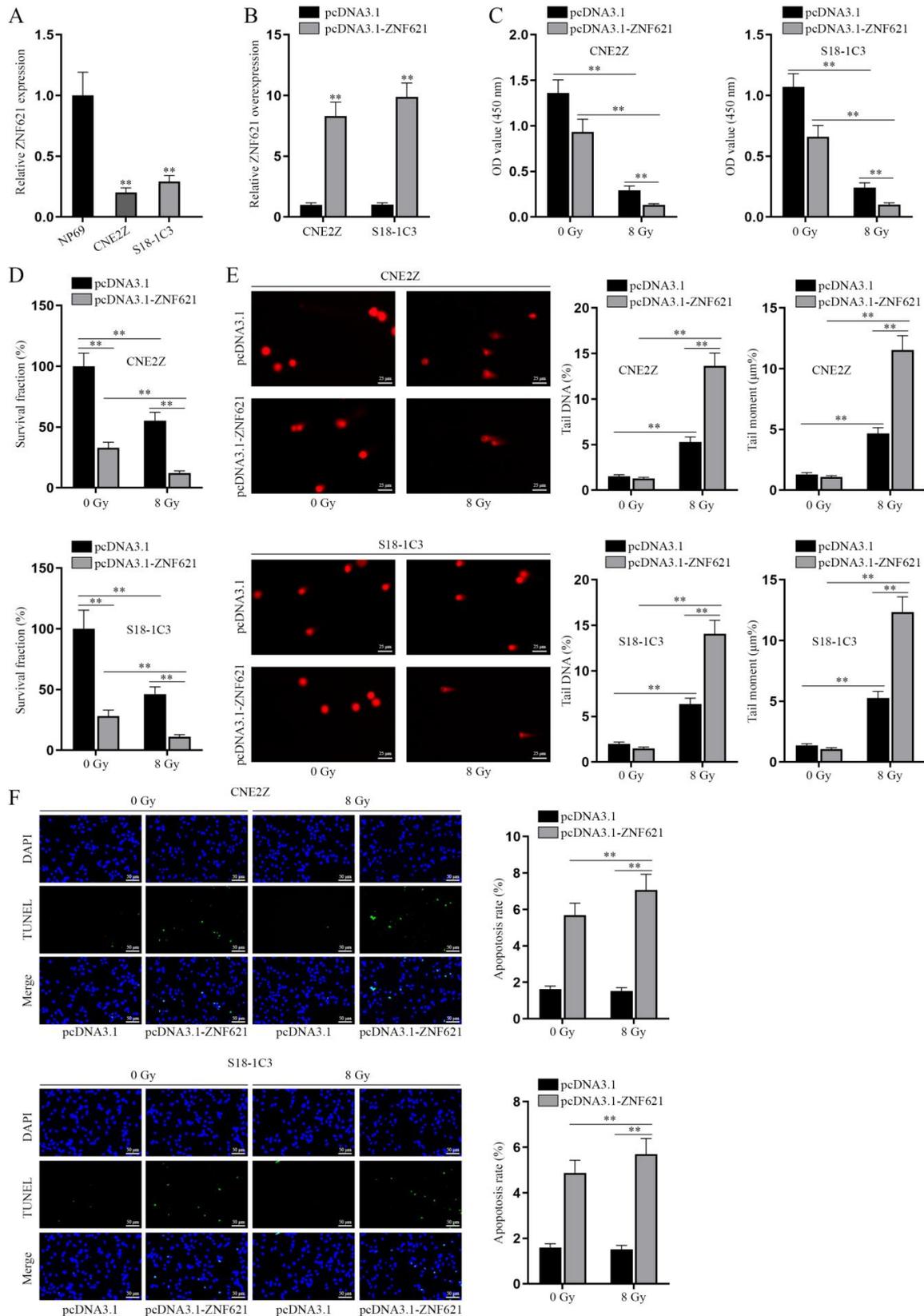


Figure 4. ZNF621 boosts radiosensitivity of NPC cells *in vitro*.

(A) qPCR evaluated the expression of ZNF621 in CNE2Z, S18-1C3 and NP69 cells (One-way ANOVA, Tukey). (B) qPCR showed the overexpression efficiency of pcDNA3.1-ZNF621 in CNE2Z and S18-1C3 cells (Student's t-test). (C-F) CCK-8, colony formation, comet and TUNEL assays were conducted to analyze the inhibitory effect of radiation on CNE2Z and S18-1C3 cells before and after the overexpression of ZNF621 (Two-way ANOVA, Tukey). **P<0.01.

LncRNAs have been reported to participate in the regulation of multiple cancer cells, including NPC cells. For example, lncRNAs PVT1 (9), C22orf32-1 (10) and NKILA (11) have been found to regulate the progression of NPC. In our study, we firstly used qPCR and found that LINC01140 was downregulated in human nasopharyngeal carcinoma cell lines (CNE2Z, S18-1C3 and CNE) compared to human immortalized nasopharyngeal epithelial cell line (NP69). CCK-8, colony formation and TUNEL assays demonstrated that LINC01140 suppressed the proliferation, and promoted the apoptosis of NPC cells.

LncRNA MALAT1 influences the radiotherapy efficiency on cervical carcinoma (15); PVT1 increases the radiosensitivity in non-small cell lung cancer via sequestering miR-195 (16); PTPRG-AS1 regulates the sensitivity of NPC cells to radiotherapy (17). In our study, we conducted a CCK-8 assay to find out that the effect of LINC01140 was more obvious in the irradiated cells at a dose of 8 Gy. Then colony formation, comet and TUNEL assay bespoke that the effect of irradiation on the proliferation, apoptosis and DNA damage repair of NPC cells was enhanced by LINC01140.

Numerous studies have shown that lncRNAs function as ceRNAs to regulate the development of various cancers, including NPC. For instance, FAM225A facilitates the progression of NPC cells by targeting miR-1275/ITGB3 (19); SNHG1 enhances the expression of NUA1K1 via competitively binding to miR-145a-5p in NPC cells (20); ZNRD1-AS1 promotes cell invasion and migration of NPC via regulating the miR-335/ROCK1 axis (21). In our study, we used starBase to screen out the potential target miRNAs and mRNAs. We used an RNA pulldown assay to verify the interaction between miR-452-5p and LINC01140. Luciferase reporter assay was conducted to evaluate the interaction between miR-452-5p and LINC01140, miR-452-5p and ZNF621. After finding out the targets, we performed rescue experiments to prove that LINC01140 regulates ZNF621 expression via competitively binding to miR-452-5p in NPC cells.

Lastly, we conducted experiments to clarify the effect of ZNF621 on the radiosensitivity of NPC cells. In previous studies, mRNAs have been found to regulate the radiosensitivity of cancer cells. For

example, CDC25A downregulation enhances the radiosensitivity of non-small cell lung cancer cells (22); berberine increases the sensitivity of osteosarcoma to radiotherapy by regulating mRNA Rad51 (23); depleted CCAR1 strengthens the radiosensitivity of pelvic malignancies (24). In our study, we performed qPCR and found that ZNF621 was low-expressed in NPC cells. Afterward, CCK-8, colony formation, comet and TUNEL assays were conducted to confirm the effect of ZNF621 on the inhibitory functions of irradiation in vitro. A previous study reported that PVT1 mediates cell proliferation, apoptosis and radioresistance in NPC via miR-515-5p/PIK3CA axis (25), which had a similar functional study to our current study. However, we elucidated the DNA repair during radiotherapy. More importantly, we unveiled a novel ceRNA pathway in NPC. A genome-wide association study (GWAS) may be needed in this regard (26), so we will conduct it in our future research works.

In conclusion, LINC01140 regulates the radiosensitivity of NPC cells by targeting the miR-452-5p/ZNF621 axis. Our study offers insight into biomarkers for radiosensitization in NPC. However, the lack of clinicopathological analysis undermines the stringency of our study. In the future, we will correlate the effectiveness of radiotherapy in treating NPC patients with LINC01140 expression, further probing into the clinical value of LINC01140.

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Interest conflict

The authors declare that they have no conflict of interest.

Author Contributions

Jia Li, Yan Li: Conceptualization, Methodology, Project Administration, Investigation, Supervision. Liang Liu, Dan Wu, Tao Yang: Data Curation, Resources, Software, Validation, Visualization. Yuwei Fan: Writing – Original Draft Preparation, Writing – Review & Editing

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