Original Article

LncRNA KCNQ1OT1 enhances the radioresistance of lung squamous cell carcinoma by targeting the miR-491-5p/TPX2-RNF2 axis

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Background: Lung cancer, especially lung squamous cell carcinoma (LUSC), is one of the most common malignant tumors worldwide. Currently, radiosensitization research is a vital direction for the improvement of LUSC therapy. Long non-coding RNAs (lncRNAs) can be novel biomarkers due to their multiple functions in cancers. However, the function and mechanism of lncRNA KCNQ1OT1 in the radioresistance of LUSC remain to be elucidated.

Methods: The clonogenic assay was employed to determine the radioresistance of SK-MES-1R and NCI-H226R cells. Real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot were conducted for the detection of gene expression. Cell proliferation was determined by the methyl thiazolyl tetrazolium (MTT) assay, colony formation assay, and 5-ethynyl-2'-deoxyuridine (EdU) staining, and cell apoptosis was assessed by flow cytometry. The relationships between genes were also evaluated by applying the luciferase reporter and radioimmunoprecipitation (RIP) assays.

Results: Radioresistant LUSC cells (SK-MES-1R and NCI-H226R) had strong resistance to X-ray irradiation, and lncRNA KCNQ1OT1 was highly expressed in SK-MES-1R and NCI-H226R cells. Moreover, knockdown of lncRNA KCNQ1OT1 prominently suppressed proliferation, attenuated radioresistance, and accelerated the apoptosis of SK-MES-1R and NCI-H226R cells. More importantly, we verified that miR-491-5p was a regulatory target of lncRNA KCNQ1OT1, and Xenopus kinesin-like protein 2 (TPX2) and RING finger protein 2 (RNF2) were the target genes of miR-491-5p. The rescue experiment results also demonstrated that miR-491-5p was involved in the inhibition of cell proliferation and the downregulation of TPX2 and RNF2 expression mediated by lncRNA KCNQ1OT1 knockdown in SK-MES-1R and NCI-H226R cells.

Conclusions: LncRNA KCNQ1OT1 was associated with the radioresistance of radioresistant LUSC cells, and the lncRNA KCNQ1OT1/miR-491-5p/TPX2-RNF2 axis might be used as a therapeutic target to enhance the radiosensitivity of radioresistant LUSC cells.

Keywords: Lung squamous cell carcinoma (LUSC); long non-coding RNA KCNQ1OT1 (lncRNA KCNQ1OT1); miR-491-5p; Xenopus kinesin-like protein 2 (TPX2); RING finger protein 2 (RNF2)

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Introduction

Lung cancer is still a common malignant tumor worldwide, with high morbidity and mortality (1). Non-small cell lung cancer (NSCLC) accounts for 80–85% of lung cancer cases (2). According to histopathological classification, NSCLC can be further divided into lung adenocarcinoma, lung squamous cell carcinoma (LUSC), and large cell carcinoma (3). Currently, more than half of newly diagnosed cancer patients require radiation therapy, which can be combined with surgery, chemotherapy, or molecular targeted therapies (4). Radiation therapy has played a vital role in the treatment of patients with metastatic disease (5). It has been proven that the biological basis of radiation therapy is the effect of ionizing radiation on biological cells (6). Radiation-sensitive patients means that when radiation doses are limited to levels that maximize therapeutic effects, reduce normal tissue cell death, prevent excessive inflammation, and preserve stem cell populations, the irradiated normal tissues of patients can resist permanent damage and do not exhibit clinically relevant adverse effects (7). However, according to statistics, 60–70% of NSCLC patients have received radiotherapy, while the radiotherapy effect and prognosis of NSCLC are still poor due to the constraints of radiation resistance and other factors (8). It is worth noting that the exact mechanism of LUSC cell radioresistance has not been elucidated. Therefore, how to improve the radioresistance of LUSC cells has become a new approach and strategy for LUSC treatment.

Long non-coding RNAs (lncRNAs) are a class of linear RNA molecules that have no transcriptional function or protein coding potential, and are mainly produced by RNA polymerase II/I (9). LncRNAs have been confirmed to regulate gene expression through genomic imprinting, transcriptional regulation, and chromatin modification (10,11). Studies have also demonstrated that lncRNAs can be involved in cell proliferation, apoptosis, differentiation, chromatin remodeling, migration, and invasion, among other processes (12,13). At present, lncRNAs have been proven to be crucial in almost all diseases, such as tumors (12), nervous system disorders (14), and cardiovascular system diseases (15). Therefore, lncRNAs can be applied as potential diagnostic markers and new drug targets for diseases. Recent research revealed that lncRNA KCNQ1OT1 was closely associated with the processes of multiple diseases including cancers (16-18), ischemia reperfusion (19), and diabetic nephropathy (20), among others. Interestingly, It was discovered that lncRNA KCNQ1OT1 was upregulated in lung cancer compared with the normal tissues and the upregulation of lncRNA KCNQ1OT1 reduced the survival rate of NSCLC patients (18). Besides, lncRNA KCNQ1OT1 has also been revealed to regulate the cisplatin resistance of cancer (21). However, the role and mechanism of lncRNA KCNQ1OT1 in radiation therapy for LUSC remain unclear.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that can mediate mRNA transcription and degradation through binding to the complementary 3'-untranslated region (3'-UTR) (22,23). Research has shown that miRNAs are involved in cell proliferation, cell cycle, apoptosis, oncogenesis, and differentiation (24,25). In cancer tissues, miRNAs play the roles of oncogenes or tumor suppressor genes (26). In the diagnosis and therapy of clinical tumors, miRNAs can be used as early diagnostic indicators, effective prognostic indicators, and new treatment targets for lung cancer (27,28). Recent research has demonstrated that lncRNAs can competitively bind miRNAs with the targeted regulatory genes to induce cancer cell progression (29). However, the potential miRNAs and target genes of lncRNA KCNQ1OT1 have not been explored in radiation therapy for LUSC.

In this study, we generated radioresistant cells (SK-MES-1R and NCI-H226R cells) through X-ray irradiation, and further verified the correlation of lncRNA KCNQ1OT1 with the radioresistance of LUSC cells. Moreover, we disclosed the potential role and regulatory mechanism of lncRNA KCNQ1OT1 in the radioresistance of radioresistant LUSC cells. We present the following article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1261/rc).

Methods

Cell lines

LUSC cell lines (SK-MES-1 and NCI-H226) and HEK293 cells were purchased from American Type Culture Collection (Manassas, USA). SK-MES-1 cells were incubated in minimum Eagle's medium (MEM, Gibco; Thermo Fisher Scientific, Inc.; Shanghai, China), NCI-H226 cells were grown in RPMI-1640 medium (Gibco), and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies; Thermo Fisher Scientific, Inc.; Shanghai, China). All the media were supplemented with 10% fetal bovine serum (FBS, HyClone; Logan, USA) and all cells were incubated at 37 ℃ with 5% CO_2.

Table 1 The sequences of primers used in the RT-qPCR assay

ID	Sequence (5'-3')
GAPDH	Forward: TGTTCGTCATGGGTGTGAAC
GAPDH	Reverse: ATGGCATGGACTGTGGTCAT
LncRNA KCNQ1OT1	Reverse: GACCTGGCAGTCTCAAAAGC
LncRNA KCNQ1OT1	Forward: CACTGGGGCAGTCACCTAAT
U6	Forward: CTTCGGCAGCACATATAC
U6	Reverse: GAACGCTTCACGAATTTGC
miR-491-5p	Forward: GGAGTGGGGAACCCTTCC
miR-491-5p	Reverse: GTGCAGGGTCCGAGGT

LncRNA, Long non-coding RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-qPCR, real-time quantitative polymerase chain reaction.

X-ray irradiation

SK-MES-1 and NCI-H226 cells were cultured to achieve 90% confluence and then exposed to 0, 2, 4, 6, 8, and 10 Gy 6 Mv X-rays at room temperature with a radiation distance of 100 cm and a radiation area of 10 cm × 10 cm for 4 h. After cell passage, the procedure was repeated. The surviving cells were the radioresistant cells (SK-MES-1R and NCI-H226R). After 8 passages, cells were used for the subsequent experiments.

Cell transfection

Control, lncRNA KCNQ1OT1 siRNAs (si#1 and si#2), miR-491-5p mimics, and anti-miR-491-5p were obtained from Shanghai Integrated Biotech Solutions Co., Ltd. (Shanghai, China). SK-MES-1R and NCI-H226R cells were transfected with above recombinants at a concentration of 40 nM using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.; Shanghai, China) in line with the experimental instructions.

Clonogenic assay

The 4 kinds of cell lines were inoculated in 6-well plates with 200, 200, 400, 800, 1,600, 2,000, and 4,000 cells per well, respectively. Then, cells were exposed to 0, 2, 4, 6, 8, and 10 Gy 6 Mv X-rays. After 14 days, 4% paraformaldehyde was applied to fix the cells and Wright-Giemsa (Shanghai yuan Mu Biotechnology Co., Ltd.; Shanghai, China) was adopted to stain the cells. Cell clones were observed and counted with at least 50 cells. Based on previous research (30), the surviving fraction was also counted.

RT-qPCR analysis

According to the kit instructions, total RNA was extracted by the TRIzol method (Invitrogen), and cDNA was obtained by reverse transcription using the Bestar™ qPCR RT kit (DBI Bioscience, Shanghai, China). The levels of lncRNA KCNQ1OT1 and miR-491-5p in cells were examined by RT-qPCR analysis with SYBR green master mix (Thermo Fisher Scientific). Primer sequences are exhibited in *Table 1*.

MTT assay

The treated SK-MES-1R and NCI-H226R cells (4,000 cells/well) were administered in 96-well plates and cultured at 37 ℃ for 0, 12, 24, 48, and 72 h. At the set time point, 20 μL MTT reagent (cat. no. M5655) was added to each well and the cells were incubated for an additional 4 h. After dissolution with dimethyl sulfoxide (DMSO, cat. no. D8418), the 96-well plates were placed under an automatic microplate reader to detect the optical density (OD) at 490 nm.

Colony formation assay

After digestion and counting, the treated SK-MES-1R and NCI-H226R cells were seeded in 6-well plates with 400 cells/well. After incubation for 12 days, cells were fixed using methanol for 10 min and dyed with crystal violet for 10 min. After washing, the cells were air dried, and the number of clones was taken and counted.

EdU staining

Cell proliferation was monitored by the EdU assay kit (Life Technologies). EdU solution (10 μM) was added to the cells in 24-well plates, and cells were cultured for 2 h at 37 ℃. After fixation using 4% formaldehyde for 20 min, the EdU-stained cells were examined. Next, the cells were treated with Hoechst 33342 for 20 min, and the results were visualized under a fluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometry

The treated SK-MES-1R and NCI-H226R cells were collected and washed using phosphate-buffered saline (PBS). The cell suspension was then added with Annexin-V-

fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BD Biosciences, San Jose, USA) for 15 min. Apoptotic cells were determined using a FACS Calibur Flow cytometer (BD Bioscience).

Western blot

The treated SK-MES-1R and NCI-H226R cells were harvested, and total proteins were extracted using RIPA buffer. The protein concentration was determined by the BCA method (Beyotime Biotechnology, China). Protein samples (40 μg) in each group were subjected to electrophoresis for 2 h and transmembrane treatment for 1 h, and then sealed in 5% non-fat milk for 2 h. Subsequently, the membranes were cultivated with primary antibodies at 4 ℃ overnight, followed by the cultivation with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1,000, cat. no. ab6802, Abcam, Shanghai, China) for 1 h. The immunochemical detection was conducted using the ECL system (Thermo Fisher Scientific). The primary antibodies were Bax (1:1,000, cat. no. 5023, Cell Signaling Technology, Shanghai, China), Bcl2 (1:1,000, cat. no. ab196495, Abcam), cleaved caspase-3 (1:1,000, cat. no. 9664, Cell Signaling Technology), Xenopus kinesin-like protein 2 (TPX2; 1:1,000, cat. no. ab32795, Abcam), RING finger protein 2 (RNF2; 1:1,000, cat. no. ab101273, Abcam), and β-actin (1:5,000, cat. no. ab179467, Abcam).

Anti-AGO2 radioimmunoprecipitation (RIP) assay

As reported by a previous study (31), we also used the Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Billerica, USA) to conduct the anti-AGO2 RIP assay. Extracts of the treated SK-MES-1R and NCI-H226R cells in RIP buffer were incubated with normal rabbit IgG (Proteintech Group, Inc.; Wuhan, China) and AGO2 antibodies (Cell Signaling Technology), which were combined with magnetic beads. We isolated the immunoprecipitated RNAs and examined genes using RT-qPCR.

Luciferase assay

We constructed the wild-type and mutant lncRNA KCNQ1OT1, TPX2, and RNF2 with potential miR-491-5p binding sites using the pMIR-REPORT plasmids (Promega Biotechnology Co., Ltd.; Beijing, China). HEK293T cells (1×10^5 cells/well) were inoculated in a 24-well plate and co-transfected with luciferase plasmids, miR-491-5p mimics, and miRNA control for 48 h. Luciferase activity was confirmed using a dual luciferase reporter assay system (Promega).

Statistical analysis

Measurement data was presented as mean ± standard deviation (SD) from 3 replications. The statistical significance was confirmed using SPSS software 21.0 (SPSS Inc., Chicago, USA) with Student's *t*-test. $P<0.05$ indicated a significant difference. All experiments were independently repeated in triplicate and all experimental data were biologically repeated in triplicate.

Results

LncRNA KCNQ1OT1 was highly expressed in radioresistant LUSC cells

To explore the possible relationship between LUSC cell radiosensitivity and lncRNA KCNQ1OT1, the parental LUSC cells (SK-MES-1 and NCI-H226) and the radioresistant LUSC cells (SK-MES-1R and NCI-H226R) were exposed to different levels of X-ray irradiation. The results from the clonogenic assay showed that SK-MES-1R and NCI-H226R cells displayed greater resistance to X-ray exposure than their parental cells (*Figure 1A*,*1B*). Subsequently, our data from RT-qPCR demonstrated that lncRNA KCNQ1OT1 expression was notably increased in SK-MES-1R and NCI-H226R cells relative to their respective parental cells (*Figure 1C*). Furthermore, we demonstrated that lncRNA KCNQ1OT1 was significantly expressed in the cytoplasm of SK-MES-1R and NCI-H226R cells, as well as in the nucleus (*Figure 1D*,*1E*). Consequently, we validated that lncRNA KCNQ1OT1 was prominently upregulated in the SK-MES-1R and NCI-H226R cells, especially in the nucleus.

Knockdown of lncRNA KCNQ1OT1 significantly repressed the proliferation of radioresistant LUSC cells

To further elucidate the impact of lncRNA KCNQ1OT1 on radioresistant LUSC cells, lncRNA KCNQ1OT1 expression was knocked down by siRNAs in SK-MES-1R, NCI-H226R, and the parental cells. As presented in *Figure 2A*, lncRNA KCNQ1OT1 was significantly downregulated in the knockdown group versus the control group, indicating the effective transfection of siRNAs in each group. Next,

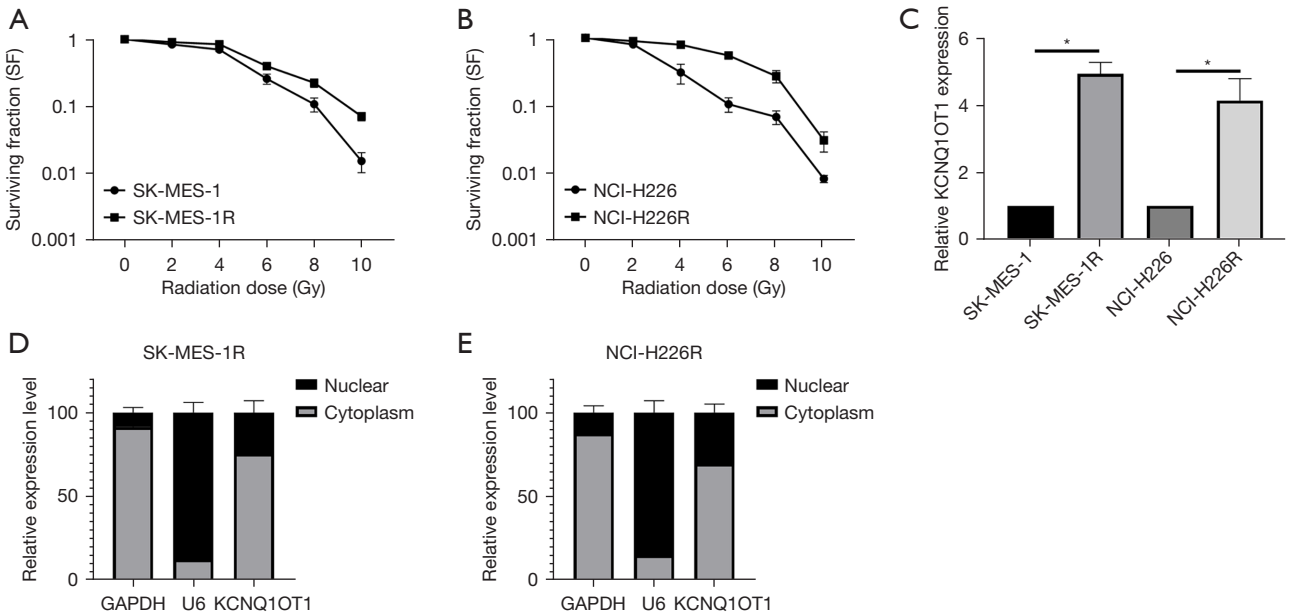

Figure 1 LncRNA KCNQ1OT1 was highly expressed in radioresistant LUSC cells. (A) The clonogenic assay was adopted to examine the radioresistance of SK-MES-1 and SK-MES-1R cells, which were exposed to 0, 2, 4, 6, 8, and 10 Gy X-rays. (B) The radioresistance of NCI-H226 and NCI-H226R cells was also determined via the clonogenic assay. (C) RT-qPCR analysis of lncRNA KCNQ1OT1 expression in the parental LUSC cells (SK-MES-1 and NCI-H226) and the radioresistant LUSC cells (SK-MES-1R and NCI-H226R). (D,E) The level of lncRNA KCNQ1OT1 was identified by applying RT-qPCR in the nucleus and cytoplasm of SK-MES-1R and NCI-H226R cells. *, P<0.05. LncRNA, long non-coding RNAs; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LUSC, lung squamous cell carcinoma.

cell proliferation was examined by conducting the MTT assay, colony formation assay, and EdU staining. The MTT results demonstrated that the proliferation capacities of SK-MES-1R and NCI-H226R cells were dramatically weakened in the knockdown group compared to the control group (*Figure 2B,2C*). Also, the experimental results of the colony formation assay showed that silencing lncRNA KCNQ1OT1 resulted in a significant reduction in the proliferation of SK-MES-1R and NCI-H226R cells (*Figure 2D*). Similarly, the results of EdU staining also revealed that the proliferation of SK-MES-1R and NCI-H226R cells could be significantly inhibited by lncRNA KCNQ1OT1 knockdown (*Figure 2E*). On the whole, we demonstrated that silencing lncRNA KCNQ1OT1 has a significant inhibitory effect on radioresistant LUSC cell proliferation.

Silencing of lncRNA KCNQ1OT1 suppressed radioresistance and induced the apoptosis of radioresistant LUSC cells

Furthermore, we adopted MTT assay to assess the role of lncRNA KCNQ1OT1 silencing in the radioresistance of SK-MES-1R and NCI-H226R cells. The results showed that silencing of lncRNA KCNQ1OT1 significantly repressed cell growth in SK-MES-1R and NCI-H226R cells, and X-ray irradiation could also lead to a decrease in the survival rate of SK-MES-1R and NCI-H226R cells (*Figure 3A,3B*). Additionally, the flow cytometry data showed that silencing of lncRNA KCNQ1OT1 could significantly increase the number of apoptotic cells after treatment with 4 Gy X-ray irradiation (*Figure 3C,3D*). Meanwhile, we also found that in X-ray-treated SK-MES-1R and NCI-H226R cells, the levels of Bax and cleaved caspase-3 were dramatically elevated, and the level of Bcl2 was markedly lowered in the lncRNA KCNQ1OT1 silencing group compared to the control group (*Figure 3E,3F*). Overall, we uncovered that lncRNA KCNQ1OT1 knockdown markedly reduced the radioresistance of radioresistant SK-MES-1R and NCI-H226R cells.

MiR-491-5p was a regulatory target of lncRNA KCNQ1OT1

More and more evidence has shown that lncRNAs may

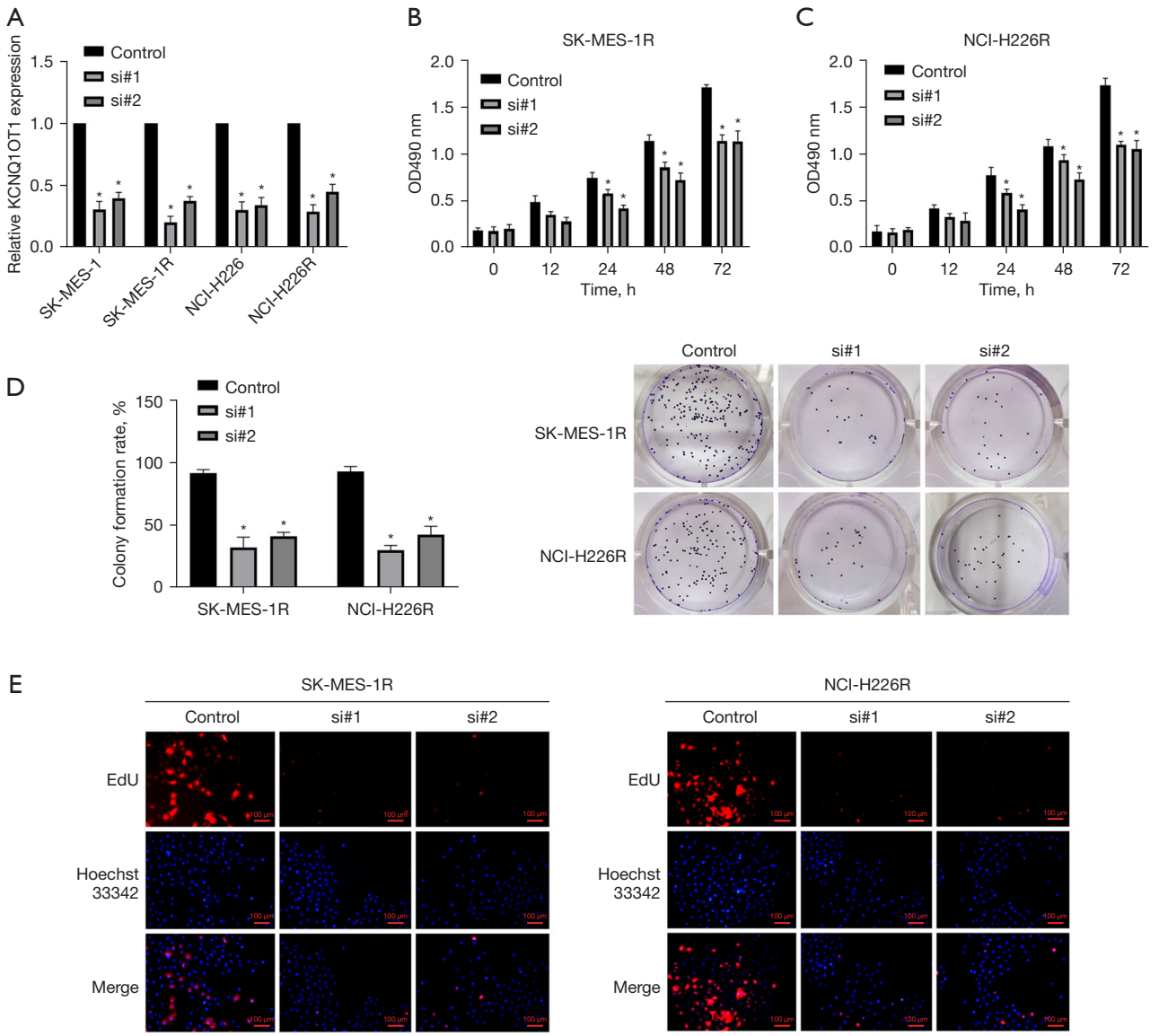

Figure 2 Knockdown of lncRNA KCNQ1OT1 significantly repressed the proliferation of radioresistant LUSC cells. (A) LncRNA KCNQ1OT1 was silenced through the transfection of siRNAs into SK-MES-1, SK-MES-1R, NCI-H226, and NCI-H226R cells, and the silencing efficacy of lncRNA KCNQ1OT1 was examined using RT-qPCR. (B,C) After lncRNA KCNQ1OT1 knockdown, proliferation was examined by the MTT assay in SK-MES-1R and NCI-H226R cells. (D) The colony formation assay was used to confirm the impact of lncRNA KCNQ1OT1 knockdown on the proliferation of SK-MES-1R and NCI-H226R cells (crystal violet staining, ×1). (E) EdU staining of lncRNA KCNQ1OT1-silenced SK-MES-1R and NCI-H226R cells. Magnification, ×100; scale bar =100 μm. *, P<0.05. LncRNA, long non-coding RNAs; LUSC, lung squamous cell carcinoma; OD, optical density.

play the role of miRNA sponges, regulating the binding of miRNAs to the target mRNAs (32,33). We used a bioinformatics tool (LncBase Predicted v.2) to predict the target miRNAs of lncRNA KCNQ1OT1, and screened 4 potential target miRNAs including miR-491-5p, miR-133b, miR-15a, and miR-7. Firstly, we discovered that only miR-491-5p was prominently downregulated in SK-MES-1R and NCI-H226R cells compared with their respective parental cells (*Figure 4A,4B*). Secondly, we performed the anti-AGO2 RIP assay to monitor whether

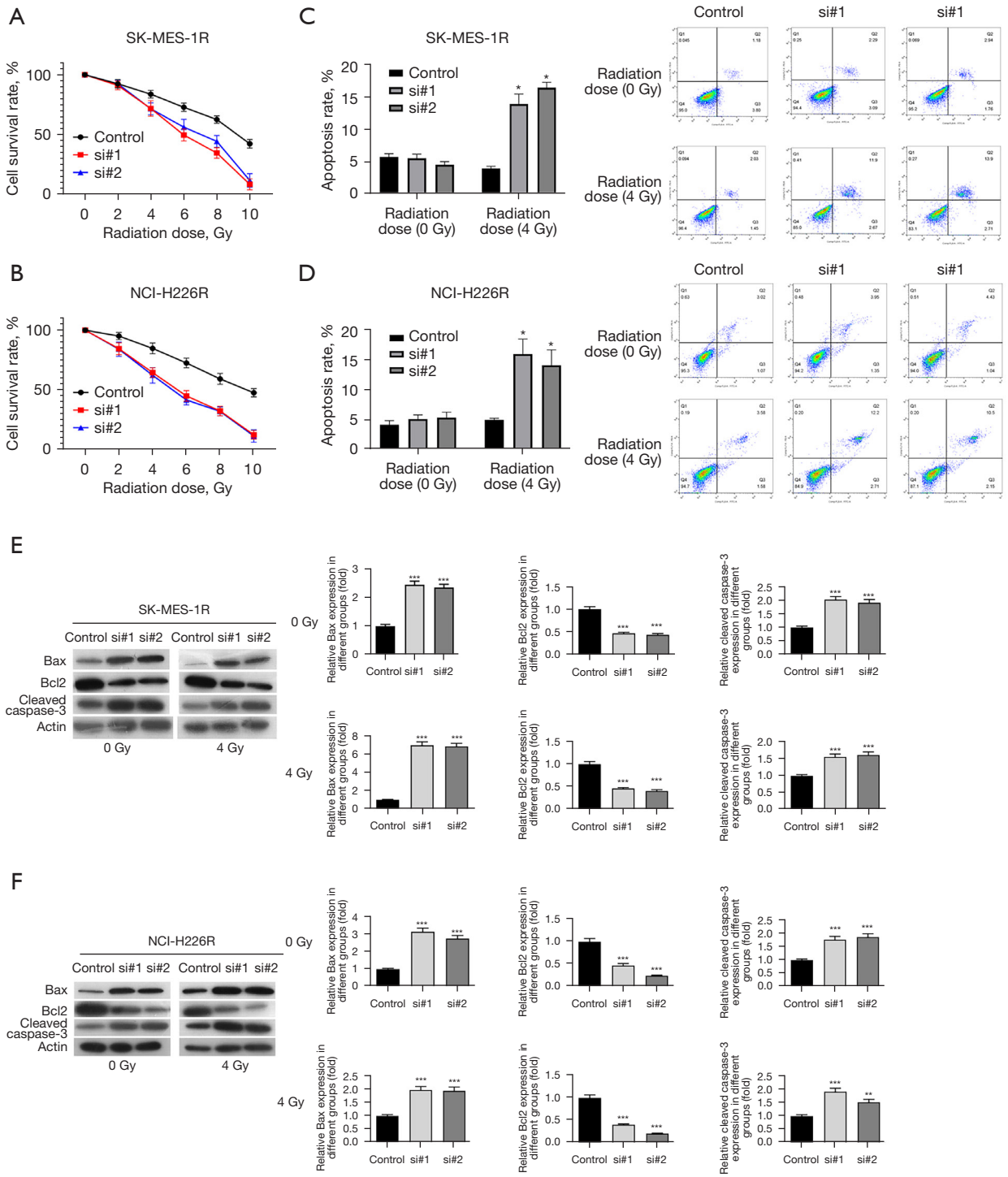

Figure 3 Silencing of lncRNA KCNQ1OT1 markedly suppressed the radioresistance and induced the apoptosis of radioresistant LUSC cells. (A,B) SK-MES-1R and NCI-H226R cells were exposed to 0, 2, 4, 6, 8, and 10 Gy X-rays, and the cell survival rate was evaluated by applying the MTT assay. (C,D) After treatment and without treatment of 4 Gy X-rays, the apoptosis rate was estimated using a flow cytometer in lncRNA KCNQ1OT1-silenced SK-MES-1R and NCI-H226R cells. (E,F) Western blot was conducted to assess the influence of lncRNA KCNQ1OT1 knockdown on the expression of Bax, Bcl2, and cleaved caspase-3 in SK-MES-1R and NCI-H226R cells. β-actin acted as the internal reference. *, P<0.05, **, P<0.01, ***, P<0.001. LncRNA, long non-coding RNAs; LUSC, lung squamous cell carcinoma.

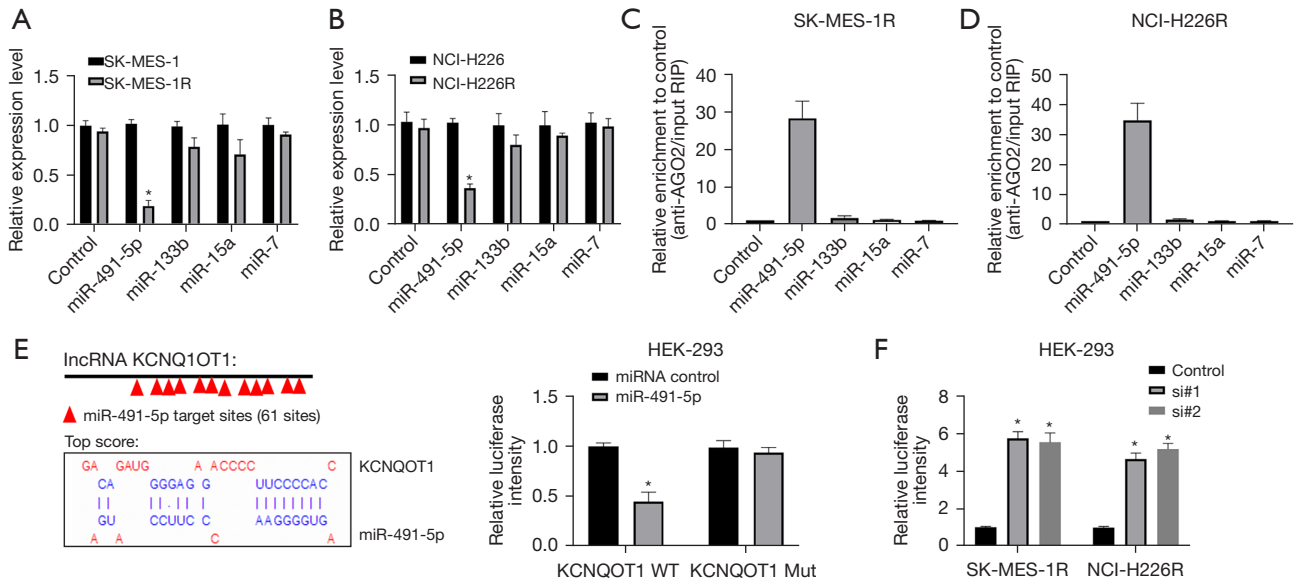

Figure 4 MiR-491-5p was a regulatory target of lncRNA KCNQ1OT1. (A,B) The potential target miRNAs of lncRNA KCNQ1OT1 were predicted through LncBase Predicted v.2, and the levels of miR-491-5p, miR-133b, miR-15a, and miR-7 were validated by RT-qPCR. (C,D) The anti-AGO2 RIP assay was conducted in SK-MES-1R and NCI-H226R cells overexpressing 4 miRNAs, then the level of lncRNA KCNQ1OT1 in anti-AGO2 was monitored by RT-qPCR. (E) The binding site between miR-491-5p and lncRNA KCNQ1OT1 was illustrated, and the luciferase assay was carried out in 293T cells with wild-type or mutant lncRNA KCNQ1OT1 and miR-491-5p mimics. (F) The expression level of miR-491-5p in lncRNA KCNQ1OT1-knockdown SK-MES-1R and NCI-H226R cells was validated by RT-qPCR. *, P<0.05. LncRNA, long non-coding RNAs; WT, wild type; Mut, mutant.

lncRNA KCNQ1OT1 could directly interact with these 4 miRNAs. The data indicated that lncRNA KCNQ1OT1 could be specifically enriched in miR-491-5p-overexpressed SK-MES-1R and NCI-H226R cells (*Figure 4C*,*4D*). In addition, we constructed the wild-type and mutant lncRNA KCNQ1OT1 plasmids, which were co-transfected into HEK-293 cells. The results uncovered that the luciferase activity was substantially reduced in HEK-293 cells with the co-transfection of miR-491-5p and wild-type lncRNA KCNQ1OT1, while the luciferase activity was not affected in the lncRNA KCNQ1OT1 mutant co-transfection group (*Figure 4E*). We also found that silencing of lncRNA KCNQ1OT1 could notably upregulate miR-491-5p in SK-MES-1R and NCI-H226R cells (*Figure 4F*). As a whole, miR-491-5p was an inhibitory target of lncRNA KCNQ1OT1.

MiR-491-5p inhibited proliferation and downregulated TPX2 and RNF2 in radioresistant LUSC cells

We further predicted the target genes of miR-491-5p using bioinformatics analysis, and discovered that TPX2 and RNF2 might be the potential target genes of miR-491-5p. The luciferase assay showed that miR-491-5p reduced only the luciferase activity of wild-type TPX2, while the luciferase activity of mutant TPX2 was not responsive to miR-491-5p overexpression (*Figure 5A*). We also demonstrated that miR-491-5p significantly attenuated the luciferase activity of wild-type RNF2, but did not affect the luciferase activity of mutant RNF2 (*Figure 5B*). Furthermore, the colony formation assay elucidated that overexpression of miR-491-5p led to the inhibition of proliferation in SK-MES-1R and NCI-H226R cells (*Figure 5C*). Similarly, inhibition of miR-491-5p could result in the enhancement of proliferation in SK-MES-1R and NCI-H226R cells (*Figure 5D*). Western blotting analysis demonstrated that overexpression of miR-491-5p significantly lowered TPX2 and RNF2 expression, and inhibition of miR-491-5p markedly elevated TPX2 and RNF2 expression in SK-MES-1R and NCI-H226R cells (*Figure 5E*,*5F*). Overall, we demonstrated that miR-491-5p could significantly prevent proliferation and reduce TPX2 and RNF2 expression in SK-MES-1R and NCI-H226R cells.

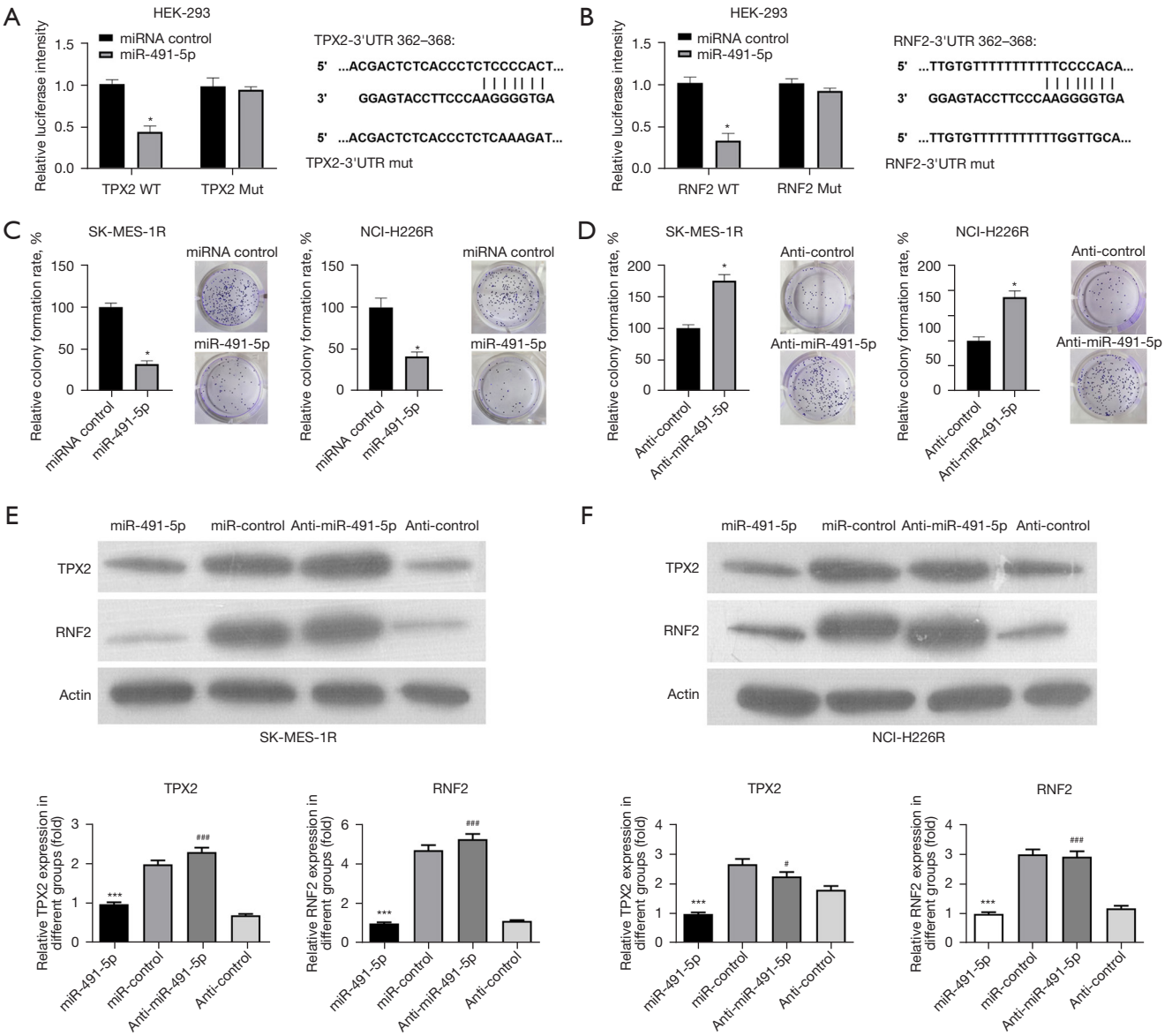

Figure 5 MiR-491-5p inhibited proliferation and downregulated TPX2 and RNF2 expression in radioresistant LUSC cells. (A) Schematic illustration of the binding site between miR-491-5p and TPX2, and the targeted regulation was evaluated by the luciferase assay. *, P<0.05 *vs.* miRNA control. (B) The binding site between miR-491-5p and RNF2 was exhibited, and the targeted regulation was identified by the luciferase assay. *, P<0.05 *vs.* miRNA control. (C,D) The proliferation was monitored through the colony formation assay in miR-491-5p-overexpressed or -inhibited SK-MES-1R and NCI-H226R cells (crystal violet staining, ×1). *, P<0.05 *vs.* miRNA control or anti-control. (E,F) The regulatory effects of miR-491-5p on TPX2 and RNF2 were identified by Western blot in SKMES-1R and NCI-H226R cells. ***, P<0.001 *vs.* miR-control. $^{\#}$, P<0.05; $^{\#\#\#}$, P<0.001 *vs.* anti-control. LUSC, lung squamous cell carcinoma; UTR, untranslated region; Mut, mutant.

Knockdown of lncRNA KCNQ1OT1 markedly prevented radioresistant LUSC cell proliferation by targeting miR-491-5p to regulate TPX2 and RNF2

Subsequently, we performed rescue experiments to explore the impact of lncRNA KCNQ1OT1/miR-491-5p on the proliferation or TPX2 and RNF2 expression in radioresistant LUSC cells. LncRNA KCNQ1OT1 siRNAs and anti-miR-491-5p were adopted to transfect SK-MES-1R and NCI-H226R cells. The colony formation

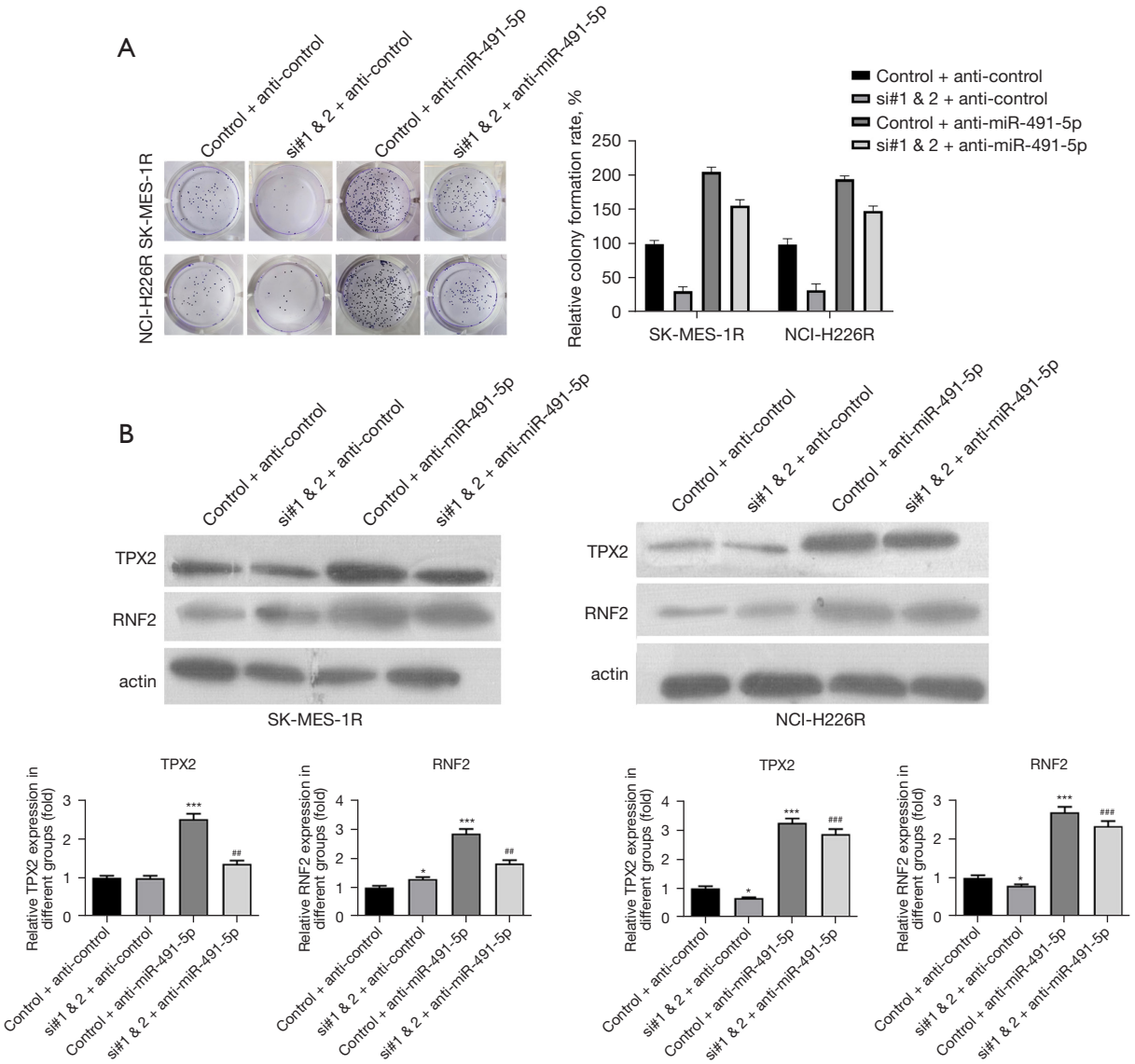

Figure 6 Knockdown of lncRNA KCNQ1OT1 markedly prevented radioresistant LUSC cell proliferation by targeting miR-491-5p to regulate TPX2 and RNF2. SK-MES-1R and NCI-H226R cells were transfected with lncRNA KCNQ1OT1 siRNAs and/or anti-miR-491-5p. (A) The colony formation assay was applied to assess the influence of lncRNA KCNQ1OT1 and miR-491-5p on cell proliferation (crystal violet staining, ×1). (B) The expression changes of TPX2 and RNF2 were tested by applying Western blot in the treated SK-MES-1R and NCI-H226R cells. ***, P<0.001 *vs.* control + anti-control. ##, P<0.01; ###, P<0.001 *vs.* si#1 & 2 + anti-control. LncRNA, long non-coding RNAs; LUSC, lung squamous cell carcinoma.

assay revealed that lncRNA KCNQ1OT1 knockdown dramatically repressed proliferation, and inhibition of miR-491-5p markedly accelerated proliferation. Meanwhile, co-transfection of lncRNA KCNQ1OT1 siRNAs and anti-miR-491-5p offset this effect in SK-MES-1R and NCI-H226R cells (*Figure 6A*). Western blot results demonstrated that lncRNA KCNQ1OT1 knockdown significantly downregulated TPX2 and RNF2 expression, inhibition of miR-491-5p notably upregulated TPX2 and RNF2 expression, and co-transfection of lncRNA KCNQ1OT1 siRNAs and anti-miR-491-5p further enhanced the expression of TPX2 and RNF2 in SK-MES-1R and NCI-H226R cells (*Figure 6B*). Collectively, these data demonstrated that lncRNA KCNQ1OT1 knockdown

prevented the proliferation of SK-MES-1R and NCI-H226R cells by miR-491-5p to regulate TPX2 and RNF2.

Discussion

Lung cancer has been considered to be one of the biggest contributors to cancer-related deaths worldwide (34). As a vital subtype of lung cancer, the incidence of LUSC is also increasing yearly (35). At present, radiotherapy has become one of the crucial methods of LUSC treatment because it can inhibit tumor growth and induce cell apoptosis. Increasing the radiosensitivity of tumor cells has become the most effective strategy for LUSC radiotherapy (36). However, the progression of LUSC is a multifactorial and multi-step process, and the carcinogenesis mechanism is still not very clear. At present, there is no effective biological target to increase radiosensitivity. In recent years, more and more evidence has confirmed that lncRNAs can participate in tumor progression through multiple pathways (37,38). Over the past decade, significant efforts have been made to bring ncRNA-based therapies to clinical use, some of which have received FDA approval. However, trial results to date have been contradictory, with some studies reporting effective results and others showing limited efficacy or toxicity (39,40). More important, studies have found that lncRNAs are involved in regulating the radiosensitivity of tumors (41,42). For instance, LINC02532 contributes to the radiosensitivity of clear cell renal cell carcinoma (43). LINC00958 could suppress radiosensitivity in colorectal cancer (44), and lncRNAs, such as PVT1 (45) and TUG1 (46), can also enhance radiosensitivity by promoting the apoptosis of tumor cells. Therefore, investigating the mechanism of lncRNAs in LUSC can lay the foundation for the improvement of radiosensitivity.

According to the literature, lncRNA KCNQ1OT1 has also been proven to be involved in the progression of multiple diseases, such as osteolysis (47), fracture healing (48), myocardial ischemia/reperfusion injury (49), atrial fibrillation (50), and diabetic cardiomyopathy (51). Furthermore, research has shown that lncRNA KCNQ1OT1 is also related to the progression of acute promyelocytic leukemia (52), ovarian cancer (17), bladder cancer (53), and NSCLC (18). Moreover, studies demonstrated that lncRNA KCNQ1OT1 was involved in oxaliplatin-resistant colon cancer (16), methotrexate-resistant colorectal cancer (54), and cisplatin-resistant tongue cancer (21). In our study, we established radioresistant cells (SK-MES-1R and NCI-H226R cells) through X-ray irradiation. LncRNA KCNQ1OT1 was notably upregulated in SK-MES-1R and NCI-H226R cells. Moreover, we verified that knockdown of lncRNA KCNQ1OT1 has a significant inhibitory effect on the proliferation and radioresistance of radioresistant LUSC cells. Knockdown of lncRNA KCNQ1OT1 also had a significant promotive effect on the apoptosis of radioresistant LUSC cells. Therefore, we demonstrated that lncRNA KCNQ1OT1 could dramatically enhance the radioresistance and induce the malignant behaviors of LUSC.

MiRNAs are a group of crucial regulatory factors in cancers, and are closely related to the progression of NSCLC (55). Studies have discovered that lncRNAs can serve as "sponges" of miRNAs to reduce miRNA abundance, thus alleviating the inhibition effect of miRNAs on the downstream target genes (56,57). Research has shown that the lncRNA/miRNA/mRNA axis can play vital roles in regulating the biological behaviors and radiosensitivity of various cancer cells, such as hepatocellular carcinoma (58), pancreatic cancer (59), lung adenocarcinoma (60), nasopharyngeal carcinoma (61), and ovarian cancer (62), among others. We also predicted the target miRNAs of lncRNA KCNQ1OT1 through a bioinformatics tool, and miR-491-5p, miR-133b, miR-15a, and miR-7 were screened as the potential target miRNAs of lncRNA KCNQ1OT1. Several studies have confirmed that miR-491-5p can function as a tumor suppressor in many types of cancers, such as osteosarcoma (63), colorectal cancer (64), gastric cancer (65), prostate cancer (66), nasopharyngeal carcinoma (67), and NSCLC (68). After experimental verification, we also discovered that miR-491-5p was a regulatory target of lncRNA KCNQ1OT1, and miR-491-5p could also be markedly upregulated by lncRNA KCNQ1OT1 knockdown.

Furthermore, through bioinformatics and experimental validation, we proved that TPX2 and RNF2 might be the potential target genes of miR-491-5p. As a microtubule-associated protein, TPX2 is essential for microtubule formation and can regulate many crucial biological processes (69,70). The aberrant expression of TPX2 has a vital relationship with the progression of human malignant tumors (71,72). RNF2, a member of the polycomb gene family, is a ubiquitin ligase with ring structure (73). Recent research has also confirmed that RNF2 is highly expressed in human malignant tumors, and is associated with the proliferation, invasion, and prognosis of tumors (74,75). Another study showed that silencing of RNF2 could promote

the radiosensitivity of NSCLC (76). In our study, we also revealed that miR-491-5p could markedly downregulate TPX2 and RNF2 expression in radioresistant LUSC cells. In addition, knockdown of lncRNA KCNQ1OT1 could also markedly inhibit the proliferation of LUSC through miR-491-5p to regulate TPX2 and RNF2.

Conclusions

We demonstrated that lncRNA KCNQ1OT1 could markedly induce the radioresistance of LUSC by directly targeting miR-491-5p to reduce TPX2 and RNF2 expression. Therefore, we uncovered that the lncRNA KCNQ1OT1/miR-491-5p/TPX2 or RNF2 axis is correlated with the radioresistance of LUSC cells. In the future, this conclusion will be further validated in clinical and *in vivo* samples.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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