

LINC00261 Inhibits Esophageal Cancer Radioresistance By Downregulating microRNA-552-3p And Promoting DIRAS1

Baolong Yang

Second Affiliated Hospital of Xi'an Jiaotong University

Hongbing Ma (✉ mahongbing0530@163.com)

Second Affiliated Hospital of Xi'an Jiaotong University <https://orcid.org/0000-0003-0682-8741>

Yan Bian

Second Affiliated Hospital of Xi'an Jiaotong University

Research

Keywords: Esophageal cancer, Radioresistance, Long intergenic non-protein coding RNA 00261, microRNA-552-3p, DIRAS1

Posted Date: August 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-779476/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Esophageal cancer (EC) is a life-threatening tumor with a high increasing incidence rate. Long intergenic non-protein coding RNAs (LINC) are widely researched in EC. This study set out to investigate the role of LINC00261 in EC radioresistance.

Methods: Radioresistant EC cell lines TE-1-R and TE-5-R were established using TE-1 and TE-5 cells. LINC00261, microRNA (miR)-552-3p, and DIRAS1 expression in EC tissues and adjacent normal tissues and EC cells was evaluated. Then, survival fraction (SF), colony formation, apoptosis, and γ -H2AX expression were analyzed. The binding relation between LINC00261 and miR-552-3p and between miR-552-3p and DIRAS1 were detected. Eventually, xenograft transplantation was applied to confirm the effect of LINC00261 on EC radioresistance *in vivo*.

Results: LINC00261 and DIRAS1 were weakly expressed in EC tissues and cells, but miR-552-3p was overexpressed. For EC cells with X-ray radiation, overexpression of LINC00261 reduced SF and cell viability, strengthened γ -H2AX expression, and promoted apoptosis, which were all counteracted by miR-552-3p overexpression or DIRAS1 silencing. Mechanically, the binding relation between LINC00261 and miR-552-3p and between miR-552-3p and DIRAS1 were verified. In addition, LINC00261 overexpression suppressed tumor growth and reduced EC radioresistance *in vivo*.

Conclusion: LINC00261 could suppress EC radioresistance via the competing endogenous RNA network to sponge miR-552-3p and upregulate DIRAS1 transcription.

Background

Esophageal cancer (EC) is defined as one of the most prevalent and malignant tumors in the world, which brings about extremely high incidence and mortality rates and is characterized by geographic variability, high aggressiveness, and frustrating prognostic outcomes [1]. EC is multifactorial cancer that could be elicited by micronutrient deficiency, gastroesophageal diseases, smoking, drinking, obesity, hot food, excessive nitrosamines, and even poor socioeconomic status [2]. Presently, the known regimens for EC are endoscopic resection, minimally invasive esophagectomy, salvage esophageal resection, chemotherapy, and radiotherapy [3]. Although chemotherapy and radiotherapy are potent in delaying tumor growth and enhancing prognostic results, the toxicity promoted by chemoresistance or radioresistance imposes damage to patients with EC and hinders curative effect [4]. Therefore, our study is designed to elaborate on the underlying mechanism of EC radioresistance.

As an integrated part of the long non-coding RNA (lncRNA) family, long intergenic non-protein coding RNAs (LINC) are implicated in cancer oncogenesis, progression, treatment, and clinic consequences [5]. Importantly, LINC dysregulation affects EC initiation, development, metastasis, aggressiveness, cell renewal, and chemoradiotherapy resistance by recruiting complexes to chromatin, modulating gene expression, and regulating the interplay between RNAs and proteins [6]. LINC00261 is insufficiently expressed in a variety of cancers ranging from pancreatic cancer, colon cancer, and prostate cancer, and

it is responsible for cancer cell viability, mobility radioresistance, and poor prognosis [7–9]. Moreover, it has been reported that LINC00261 promotes EC cell sensitivity to drugs, and LINC00261 silencing results in strengthened EC growth and reduced apoptosis and sensitivity [10]. Mechanically, LINC00261 mediates tumorigenesis and cancer progression through the competing endogenous RNA (ceRNA) interaction [11, 12]. ceRNA interaction is composed of lncRNA, microRNAs (miRNAs), circular RNAs, mRNAs, and proteins and it renders critical effects on neoplasm development [13]. Thus, we are inspired to explore miRs involved in EC. miR-552 is consistently upregulated in a considerable number of cancers to affect cancer cell survival, transcription, mobility, dissemination, propagation, invasiveness, death and resistance, and sensibility to drugs [14]. A recent study unravels that miR-552-3p is a valuable diagnostic and prognostic biomarker in digestive tract malignancies including EC [15]. DIRAS family GTPase 1 (DIRAS1) is a member of the RAS superfamily and serves as an inhibitor in human cancers [16]. Essentially, DIRAS1 is identified as a putative inhibitor in EC [17]. In conclusion, this experiment is designed to find out the specific role of LINC00261 in EC radiosensitivity via the interaction with the miR-552-3p/DIRAS1 axis.

Materials And Methods

Ethics statement

This study was approved and supervised by the ethics committee of The Second Affiliated Hospital of Xi 'an Jiaotong University. Informed consent was received from all subjects. The protocol was also approved by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Xi 'an Jiaotong University and the *Guidelines for the Care and Use of Laboratory Animals* provisions of administration and usage of laboratory animals [18]. Significant efforts were made to minimize both the number of animals and their respective suffering.

Tissue collection

Endoscopic biopsy specimens (69 EC tissue specimens and 69 paracancerous tissues) from 69 EC patients (43 males and 26 females) diagnosed in The Second Affiliated Hospital of Xi 'an Jiaotong University from June 2016 to June 2018 were recruited in our experiment. The inclusion criteria included: I) no patients received anticancer treatment prior to tissue acquisition; II) no patients were complicated with other malignancies or chronic infectious diseases; III) no tissue lesions in paracancerous tissues; and IV) completed clinical information such as age, gender, medical history, and treatment plan. Exclusion criteria included: I) non-completed 3-year follow-up; II) accidental death due to other diseases; III) pregnancy, breastfeeding, or drug addiction; and IV) patients undergoing other types of medical treatment, such as participation in and withdrawal from the clinical experiment of other drugs or biological therapies. All EC patients received radiotherapy after surgery. Depending on the condition of patients, doses of 50–66 Gy were prescribed, and fractional doses of 2 Gy/d were administered once a day, 5 times a week for 6–7 weeks. Patients were treated with a Varian 23EX and a 120-leaf MLC 600C/D linear accelerator. The 69 EC specimens were divided into the radiation sensitive group (N = 47) and the radiation resistant group (N = 22) in accordance with the response evaluation criteria [19].

Cell culture

Human normal esophageal cell line (HEEC) and human EC cell lines (TE-1, TE-5, ECA109, and KYSE150) were obtained from Shanghai Yaji Biotechnology Co., Ltd. (Shanghai, China). The adherent cells were cultured in Roswell Park Memorial Institute-1640 medium (Wuhan Procell Life Technology Co., Ltd, Wuhan, Hubei, China) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin in a 37°C incubator with 5% CO₂ and saturated humidity. The medium was refreshed every other day and cells in the logarithmic growth phase were selected for subsequent experiments.

TE-1 and TE-5 cells were cultured in 6-well plates for 24 h and then infected with lentivirus (LV)-packaged overexpression of LINC00261 vector pcDNA3.1-LINC00261 (oe-LINC00261) or the empty vector pcDNA3.1-negative control (oe-NC) (GenePharma, Shanghai, China) at a multiplicity of infection of 50 pfu/cell, and transfected with miR-552-3p-mimic or mimic-NC, small interfering (si)-DIRAS1 or si-NC (GenePharma) using 200 ng Lipofectamine 2000 (Invitrogen Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

Establishment of radioresistance cell lines

TE-1 and TE-5 cells at the logarithmic growth phase passaged in the 25 cm² cell culture flask. When the adherent cells reached 50% confluence, they were irradiated with 2 Gy X-rays and cultured in a 37°C incubator with 5% CO₂. When cell confluence reached 90% (2–3 d), cells were isolated for passaging. When cell confluence reached 50% again, cells were irradiated with 2 Gy X-ray and incubated in a 37°C incubator with 5% CO₂. Cells were irradiated several times as described above, at a total dose of 50 Gy (25 × 2Gy), and the irradiated cells were named TE-1-R and TE-5-R. Throughout the process, the parent cells, namely TE-1, passaged in the same manner, but without X-ray irradiation.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The total RNA was extracted according to the instructions of TRIzol. Next, RNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). And then the complementary DNA was reverse transcribed using the RT kit (Fermentas Inc., Burlington, ON, Canada). RT-qPCR was carried out using Power SYBRgreen PCR master Mix (Life Technologies; Thermo Fisher Scientific, Waltham, MA, USA). The primers synthesized by Shanghai Sangon (Shanghai, China) are listed in Table 1. Gene relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequence of RT-qPCR

	Forward Primer (5'-3')	Reverse Primer (5'-3')
LINC00261	CGTAGTGATAGTCCCGTGCC	TGGCAAGACGGATGGAAACA
miR-552-3p	GCCGAGGCGGAGCGCCTG	CTCAACTGGTGTCTGTGGA
DIRAS1	CAGGGTATCTGGGGAATGGC	GTCCATAATGAGAGGCAGGGA
U6	CGCACTTTACGGCTACCTCT	GCGACAAGGGAAGGGAACAA
GAPDH	GCATCTTCTTGTGCAGTGCC	TACGGCCAAATCCGTTTACA
Note: RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LINC00261, long intergenic non-protein coding RNA; miR, microRNA; DIRAS1, DIRAS family GTPase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase		

Cell counting kit-8 (CCK-8) method

Cell proliferation in different groups was evaluated in accordance with the instructions of the CCK-8 kit (Dojindo Molecular Technologies, Rockville, MD, USA). Cells (3×10^3 cells/well) were seeded into 96-well plates overnight until cells were adherent. Afterwards, cells were irradiated using 0, 2, 4, 6, and 8 Gy X-ray, respectively. Cell viability was assessed 72 h after irradiation. Subsequently, cells were cultured with CCK-8 solution (10 $\mu\text{g}/\text{mL}$, Dojindo) in a 37°C humidified incubator with 5% CO_2 . The optical density at the wavelength of 450 nm was detected using a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony formation assay

The suspension containing cells (2×10^3) was seeded into 6-well plates followed by the X-ray irradiation at 4 Gy on cells. Cells were cultured for 14 days till the appearance of colonies. Then colonies were subjected to a 15 min-fixation with 4% methyl alcohol and a 30 min-staining by 1% Giemsa (AppliChem, Darmstadt, Germany), and followed by the counting of the number of colonies containing more than 50 cells. Each procedure was independently committed 3 times.

Flow cytometry

Cells (3×10^5 cells/well) were seeded in 6-well plates and then irradiated with 4 Gy X-ray for 24 h when cell density reached 60%. Cells were collected in 1.5 mL EP tubes and centrifuged at $2000 \times g$ at 4°C to discard the supernatant. Cells were then suspended with 500 μL binding buffer and cultured with 5 μL Annexin V-fluorescein isothiocyanate (FITC) at 4°C for 30 min. Afterwards, cells were incubated with 5 μL propidium iodide for 5 min at room temperature. Additionally, apoptosis was detected using the Annexin-V-FITC assay kit (K201-100, BioVision Inc., Mountain View, CA, USA) and flow cytometry (version 10.0, FlowJo, FACS Calibur™, BD, USA).

Immunofluorescent staining

Immunofluorescent staining was performed in compliance with the standard protocols. Briefly, cells were fixed in 4% paraformaldehyde, permeated using 0.2% Triton X-100, and blocked using 5% bovine serum albumin at room temperature for 1 h. Then, cells were incubated with rabbit anti-human γ -H2AX antibody (1:250, ab81299, Abcam Inc., Cambridge, MA, USA) overnight at 4°C, cultured with fluorescent staining-coupled secondary antibody (1:1000, ab6759, Abcam) for 1 h, followed by the staining with 4', 6-diamidino-2-phenylindole. Finally, cells were photographed and observed under a Zeiss microscope (Zeiss Inc, AG, Oberkochen, Germany).

Bioinformatics

Subcellular localization of LINC00261 was predicted via the IncLocator (http://www.csbio.sjtu.edu.cn/bioinf/IncLocator/?tdsourcetag=s_pcqq_aiomsg). The target genes downstream LINC00261 and miR-552-3p and their corresponding binding sites were predicted through databases including LncBase v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted), Starbase (<http://starbase.sysu.edu.cn/index.ph>), LNCRNASNP2 (<http://bioinfo.life.hust.edu.cn/IncRNASNP#!/>), RNA22 (<https://cm.jefferson.edu/rna22/Precomputed/>), miRTarBase (<http://miRTarBase.cuhk.edu.cn/>), and miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>).

Fractionation of nuclear and cytoplasmic RNA

The Cytoplasmic and Nuclear RNA purification kit (Norgenbiotek Corporation, Thorold, ON, Canada) was employed for the subcellular fractionation of RNA from TE-1 cells according to the manufacturer's instructions. Cytoplasmic and nuclear fractions were detected by RT-qPCR.

Dual-luciferase reporter gene assay

Wild type (WT) and mutant type (MUT) of LINC00261 and DIRAS1 fragments containing the binding sites of miR-552-3p were constructed and cloned into the psiCHECK-2 vector (Hanbio Biotechnology Co., Ltd, Shanghai, China). The constructed dual-luciferase reporter plasmids were co-transfected with mimic-NC or miR-552-3p-mimic into 293T cells. After 48 h, cells were collected and evaluated using the dual-luciferase reporter gene assay system (Promega, Madison, WI, USA).

RNA pull-down assay

TE-1 cells were digested using trypsin and lysed in RNA-binding protein immunoprecipitation (RIP) lysis buffer. The lysis buffer was divided into several aliquots and frozen at -80°C. Next, 1 μ g biotin-labeled RNA and 500 μ L structure buffer were added into EP tubes using the RNA-protein magnetic bead sedimentation kit (Pierce, Rockford, IL). Cells were immersed in water at 95°C for 2 min, soaked in ice for 3 min, cultured with 50 μ L fully resuspended beads at 4°C overnight with the supernatant discarded, centrifuged at 12 *g* for 3 min, rinsed 3 times with 500 μ L RIP cleaning solution and mixed with 10 μ L cell lysate for 1 h. In addition, protein in the incubated bead-RNA-protein mixture was eluted. Protein concentration was determined using the bicinchoninic acid method. RNA expression was determined by RT-qPCR.

Xenografts tumors in nude mice

Thirty-six male BALB/c nude mice (4–6 week old, Charles River, Beijing, China) were raised in 12-h light/dark cycles at 25°C and 60% humidity and had free access to standard food and sterile water. TE-1-R cells (0.2 mL, 1×10^7 cells) infected with LV-LINC00261 or LV-NC were subcutaneously injected into the back of nude mice. After 7 d, the tumor of mice was exposed to 4 Gy of X-ray radiation. Tumor volume was calculated every 7 days as tumor volume = (length \times width²)/2. Pentobarbital sodium (150 mg/kg) was intraperitoneally injected into mice about 35 d after the injection to euthanize the mice. Tumors were removed and weighed. The tumor tissues from 6 mice in each group were applied for RT-qPCR and the remaining 6 mice were used for immunohistochemistry (IHC).

IHC

The expression of γ -H2AX and Ki67 in tumors was evaluated via IHC. Tissue sections (4 μ m) were subjected to dehydration, rehydration, antigen recovery, endogenous peroxidase exhaustion, and non-specific signal blockage. Subsequently, tissue sections were detected by anti- γ -H2AX (1:1000, ab124781, Abcam) and anti-ki67 (1:200, ab16667, Abcam) at 4°C, and then cultured with enzyme-labeled secondary (1:500, ab150113, Abcam) at room temperature for 1 h. Afterwards, tissue sections were observed using the 2,4-diaminobutyric acid substrate, stained using hematoxylin, and then imaged.

Statistical analysis

GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis. The measurement data were presented as mean \pm standard deviation. The *t*-test was appointed for comparison analysis between two groups and one-way or two-way analysis of variance (ANOVA) was appointed for comparison analysis among multiple groups, and Tukey's multiple comparisons test was used for post-test of data. The *p* value was attained using a two-tailed test and a value of *p* < 0.05 indicated a significant difference.

Results

LINC00261 is poorly expressed in EC

To figure out the role of LINC00261 in EC radioresistance, TCGA was applied to reveal that LINC00261 was downregulated in EC (Fig. 1A). Detection on the EC and paracancerous tissues found that LINC00261 was downregulated in EC tissues (*p* < 0.05; Fig. 1B). Patients undergoing treatment were divided into the radiation sensitive group and the radiation resistant group according to the evaluation standards. Our experiment found that the radiation resistant group had lower LINC00261 expression than the radiation sensitive group (*p* < 0.05; Fig. 1C). Next, LINC00261 expression in 4 different EC cell lines was detected, and it was revealed that LINC00261 was weakly expressed in all of them (*p* < 0.05; Fig. 1D).

TE-1 cells, which had the lowest LINC00261 expression, and TE-5, which had the highest LINC00261 expression were selected for further experiments.

LINC00261 overexpression boosts EC cell radiosensitivity

To investigate the role of LINC00261 in EC radioresistance, LINC00261 expression in radioresistant and parental EC cell lines was evaluated. Compared with TE-1 and TE-5 cells, TE-1-R and TE-5-R cells showed reduced LINC00261 expression ($p < 0.05$; Fig. 2A). LINC00261 expression was upregulated in radioresistant cell lines ($p < 0.05$; Fig. 2B). Then we found that compared with the parental cells, the radioresistant cells had a higher survival fraction (SF) under different radiation doses of X-ray, and cells with oe-LINC00261 treatment had further lower SF ($p < 0.05$; Fig. 2C). Radiation of 4 Gy X-ray, under which SF of parental cells was about 50%, was selected in the subsequent performance. In TE-1-R and TE-5-R cells under 4 Gy X-ray radiation and treated with oe-LINC00261, colony formation was decreased ($p < 0.05$; Fig. 2D), γ -H2AX expression was elevated ($p < 0.05$; Fig. 2E), and apoptosis was expedited ($p < 0.05$; Fig. 2F), suggesting that LINC00261 overexpression could improve EC cell radiosensitivity.

LINC00261 targets miR-552-3p

To clarify the downstream mechanism of LINC00261, the subcellular localization of LINC00261 was predicted through the IncLocator website, which unveiled that LINC00261 was localized in the cytoplasm (Fig. 3A). Then, fractionation of nuclear and cytoplasmic RNA analysis verified that LINC00261 was expressed at the cytoplasm (Fig. 3B), indicating that LINC00261 might modulate EC cell activities via the ceRNA network. Thus, the LncBase v.2, Starbase, and LNCRNASNP2 online websites were employed to search the downstream targets of LINC00261 to take the intersection (Fig. 3C), from which miR-552-3p was noticed. It was reported that miR-552-3p was overexpressed in EC and could function as a diagnostic marker [15]. The binding site between LINC00261 and miR-552-3p was predicted by the Starbase, and the binding relation between them was validated by dual-luciferase reporter gene assay and RNA pulldown assay ($p < 0.05$; Fig. 3D-E). Next, miR-552-3p expression in EC tissues was detected and it was found that miR-552-3p expression was increased in EC tissues ($p < 0.05$; Fig. 3F), and it was further revealed that miR-552-3p was negatively related to LINC00261 ($r = -0.751$, $p < 0.001$; Fig. 3G). Then, the detection of miR-552-3p expression in 4 kinds of EC cell lines found that miR-552-3p showed an increased expression in EC cells as compared with that in HEEC cells ($p < 0.05$; Fig. 3H). Furthermore, miR-552-3p expression was upregulated in TE-1-R and TE-5-R cells as compared with that in parental cells ($p < 0.05$; Fig. 3I). When LINC00261 was overexpressed, miR-552-3p expression was downregulated ($p < 0.05$; Fig. 3J).

miR-552-3p overexpression neutralizes the promotive effect of LINC00261 on EC cell radiosensitivity

The miR-552-3p-mimic or mimic-NC was transfected into TE-1-R cells ($p < 0.05$; Fig. 4A) to perform a collaborative experiment with oe-LINC00261. Under 4 Gy X-ray radiation, compared with the oe-LINC00261 group, the miR-552-3p-mimic group showed decreased γ -H2AX expression ($p < 0.05$; Fig. 4B), elevated colony formation ($p < 0.05$; Fig. 4C), and reduced apoptosis ($p < 0.05$; Fig. 4D). In general, miR-552-3p overexpression neutralized the promotive effect of LINC00261 on EC cell radiosensitivity.

miR-552-3p targets DIRAS1

Next, the downstream regulation mechanism of miR-552-3p was investigated. Starbase, RNA22, miRTarBase, and miRWalk online websites predicted the downstream target genes of miR-552-3p to obtain the intersection (Fig. 5A), and DIRAS1 was noticed. DIRAS1 acted as an inhibitor of esophageal squamous cancer [20]. The binding site between miR-552-3p and DIRAS1 was predicted by the Starbase website, and the binding relation between them was verified by dual-luciferase reporter gene assay ($p < 0.05$; Fig. 5B). Besides, DIRAS1 expression was lower in EC tissues than that in the paracancerous tissues ($p < 0.05$; Fig. 5C) and DIRAS1 was positively correlated with LINC00261 ($r = 0.611$, $p < 0.001$) but negatively correlated with miR-552-3p ($r = -0.747$, $p < 0.001$, Fig. 5D). Besides, DIRAS1 mRNA was poorly expressed in EC cells as compared with that in HECC cells ($p < 0.05$; Fig. 5E), and DIRAS1 mRNA was poorly expressed in TE-1-R and TE-5-R cells as compared with that in parental cells ($p < 0.05$; Fig. 5F). LINC00261 overexpression led to an elevated DIRAS1 expression; while miR-552-3p overexpression resulted in the decrease of DIRAS1 ($p < 0.05$; Fig. 5G).

DIRAS1 depletion partially annuls the promotive role of LINC00261 overexpression in EC cell radiosensitivity

si-DIRAS1 or si-NC was transfected into TE-1-R cells ($p < 0.05$; Fig. 6A) to perform collaborative experiments with oe-LINC00261. Under 4Gy X-ray radiation, TE-1-R cells in the si-DIRAS1 group showed decreased γ -H2AX expression ($p < 0.05$; Fig. 6B), elevated colony formation ($p < 0.05$; Fig. 6C), and reduced apoptosis ($p < 0.05$; Fig. 6D). In conclusion, DIRAS1 depletion partially annulled the promotive effect of LINC00261 on EC cell radiosensitivity.

LINC00261 overexpression enhances EC radiosensitivity in vivo

EC radiosensitivity was studied in the *in vivo* model. TE-1-R cells with stable oe-LINC00261 were injected into mice. After the treatment of 4 Gy X-ray radiation, mice with LINC00261 overexpression had declined tumor volume and weight ($p < 0.05$; Fig. 7A-B), reduced miR-552-3p expression ($p < 0.05$; Fig. 7C), elevated DIRAS1 mRNA expression ($p < 0.05$; Fig. 7D), upregulated γ -H2AX expression, and downregulated Ki67 expression ($p < 0.05$; Fig. 7E). The above results indicated that LINC00261 enhanced EC radiosensitivity by competitively binding to miR-552-3p to upregulate DIRAS1 transcription.

Discussion

As a kind of life-threatening cancer, EC is correlated with ascending morbidity and unsatisfying overall survival rate despite the improved therapeutic interventions for EC [4]. EC could be effectively alleviated by radiotherapy but radioresistance therefrom could sabotage EC treatment and even escalate EC malignancy [21]. LINC00261 could serve as a tumor inhibitor in various cancers including EC by

controlling cellular survival, death, development, and chemoresistance as well as promoting prognostic consequences [22]. It was safe to postulate that LINC00261 is a benign indicator of EC. Hence, we tried to discuss the underlying interplay of LINC00261 in EC radioresistance. This paper may provide novel insights into EC amelioration.

LncRNAs participate in EC progression by mediating tumor-associated axis, therapeutic efficiency, and cell radioresistance and chemoresistance [23]. LINC00261 expression was decreased in pancreatic adenocarcinoma and catalyzed EC cell aggressiveness and migration [24]. Initially, this experiment unveiled that LINC00261 was weakly expressed in EC tissues and cells. To investigate the role of LINC00261 in EC radioresistance, LINC00261 expression in radioresistant and parental EC cells was evaluated, and it was noticed that LINC00261 expression was further reduced in radioresistant cells. When LINC00261 expression was upregulated in radioresistant cells via oe-LINC00261 treatment, SF was reduced, colony formation was decreased, γ -H2AX expression was elevated, and apoptosis was expedited. Similarly, LINC00261 ablation was found in colon carcinoma and drug-resistant cells, while overexpressing LINC00261 resulted in quenched cell biological behaviors, encouraged apoptosis, and enhanced drug-sensibility [25]. When treated by effective medicine, EC malignancy was partially repressed and radiosensitivity was improved, which was in parallel with elevated γ -H2AX expression, indicating that γ -H2AX might predict alleviated EC and is related to improved radiosensitivity of EC [26]. Apoptosis is a process related to cell death and self-renewal and apoptosis deficiency exacerbates cancer development as it augments tumor growth and cell resistance to therapies [27]. LINC00261 overexpression inhibited EC growth and enhanced apoptosis and sensitivity to 5-fluorouracil [10]. In conclusion, LINC00261 overexpression could improve EC cell radiosensitivity. We also performed the *in vivo* experiments. TE-1-R cells with stable oe-LINC00261 were injected into mice. After the treatment of 4 Gy X-ray radiation, mice with LINC00261 overexpression had declined tumor volume and weight, upregulated γ -H2AX expression and downregulated Ki67 expression. The above results indicated that LINC00261 enhanced EC radiosensitivity in EC cells.

miRs could be involved in cell radioresistance of different carcinomas by participating in the ceRNA network [28]. To clarify the downstream mechanism of LINC00261, the subcellular localization of LINC00261 was predicted and analyzed, which unveiled that LINC00261 was localized in the cytoplasm, indicating that LINC00261 might modulate EC cell activities via the ceRNA network. The downstream targets were predicted, and miR-552-3p was noticed. miR-552-5p served as a dangerous indicator for esophageal squamous cell carcinoma (ESCC) prognosis [29], suggesting that miR-552-5p was detrimental to EC. miR-552-3p showed an increased expression in EC cells, and miR-552-3p expression was upregulated in TE-1-R and TE-5-R cells as compared with that in parental cells. Furthermore, miR-552-3p could be a target gene of LINC00261. Then, the miR-552-3p-mimic was transfected into EC radioresistant cells with oe-LINC00261 treatment, after which γ -H2AX expression was decreased, cell colony was facilitated, and apoptosis was quenched. Overexpressing miR-552 elicited colony formation, enhanced cancer cell dissemination, and limited radiosensitivity in colorectal cancer [30]. Essentially, miR-552 exhaustion discouraged cell mobility but augmented apoptosis in hepatocellular carcinoma [31]. In

general, miR-552-3p overexpression neutralized the promotive effect of LINC00261 on EC cell radiosensitivity.

Thereafter, the downstream mechanism of miR-552-3p was investigated, and DIRAS1 was noticed. DIRAS1 is a well-acknowledged cancer inhibitor as its overexpression consolidates the effect of anti-tumor drugs [32]. Besides, DIRAS1 could inhibit ESCC progression [33]. DIRAS1 was poorly expressed in EC cells, and it was downregulated in TE-1-R and TE-5-R cells as compared with that in parental cells. Besides, DIRAS1 could be a target gene of miR-552-3p. To figure out the effect of DIRAS1 on EC radiosensitivity, si-DIRAS1 was transfected into EC radioresistant cells with oe-LINC00261 treatment and it was unveiled that γ -H2AX expression was discouraged, colony formation was elevated, and apoptosis was restricted. DIRAS1 contributed to repressed colony formation and cell activities in ESCC [20]. DIRAS1 silencing facilitated tumor growth, augmented cancer cell survival and metastasis, and suppressed apoptosis [34]. In conclusion, DIRAS1 depletion partially annulled the promotive effect of LINC00261 on EC cell radiosensitivity.

Conclusion

In a word, this experiment revealed that LINC00261 functioned as a ceRNA to competitively bind to miR-552-3p and upregulate DIRAS1 expression to repress EC radioresistance (Fig. 8). These findings suggested a therapeutic implication for EC alleviation. Currently, this research only focused on the role of the LINC00261/miR-552-3p/DIRAS1 ceRNA network in EC radioresistance. We will further seek out the possible upstream mechanism of LINC00261 and other downstream mechanisms. The experimental revelation and realistic application into medical practice require extensive validation. We hope this experiment could contribute to EC research.

Abbreviations

EC: esophageal cancer; LINC: long intergenic non-protein coding RNAs; miR: microRNA; SF: survival fraction; lncRNAs: long non-coding RNA; ceRNA: competing endogenous RNA; DIRAS1: DIRAS family GTPase 1; LV: lentivirus; oe: overexpression ; NC: negative control; si: small interfering; RT-qPCR: reverse transcription quantitative polymerase chain reaction; CCK-8: cell counting kit-8; FITC: fluorescein isothiocyanate; WT: wild type; MUT: mutant type; RIP: RNA-binding protein immunoprecipitation; IHC: immunohistochemistry; ANOVA: analysis of variance; ESCC: esophageal squamous cell carcinoma

Declarations

Ethics approval and informed consent

This study was approved and supervised by the ethics committee of The Second Affiliated Hospital of Xi'an Jiaotong University. Informed consent was received from all subjects. The protocol was also approved by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Xi'an

Jiaotong University and the *Guidelines for the Care and Use of Laboratory Animals* provisions of administration and usage of laboratory animals. Significant efforts were made to minimize both the number of animals and their respective suffering.

Consent for publication

All images and tabular data in this article are publishable

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Competing interests

All authors declare that there is no conflict of interests in this study.

Funding

Not applicable

Authors' contributions

guarantor of integrity of the entire study: BY; study concepts: HM; study design: YB; definition of intellectual content: BY; literature research: BY; clinical studies BY, HM, YB; experimental studies BY, HM, YB; data acquisition BY, HM, YB; data analysis BY, HM, YB; statistical analysis BY, HM, YB; manuscript preparation BY; manuscript editing BY; manuscript review BY

Acknowledgments

We thank all of members in our team for the excellent work.

References

1. Domper Arnal MJ, et al. Esophageal cancer: Risk factors, screening and endoscopic treatment in Western and Eastern countries. *World J Gastroenterol.* 2015;21(26):7933-7943.
2. Uhlenhopp DJ, et al. Epidemiology of esophageal cancer: update in global trends, etiology and risk factors. *Clin J Gastroenterol.* 2020;13(6):1010-1021.
3. Sohda M, et al. Current Status and Future Prospects for Esophageal Cancer Treatment. *Ann Thorac Cardiovasc Surg.* 2017;23(1):1-11.
4. Huang FL, et al. Esophageal cancer: Risk factors, genetic association, and treatment. *Asian J Surg.* 2018;41(3):210-215.

5. Sun DE, et al. Emerging Roles of Long Noncoding RNA Regulator of Reprogramming in Cancer Treatment. *Cancer Manag Res.* 2020;12:6103-6112.
6. Su M, et al. Long non-coding RNAs in esophageal cancer: molecular mechanisms, functions, and potential applications. *J Hematol Oncol.* 2018;11(1):118.
7. Eke I, et al. The lncRNAs LINC00261 and LINC00665 are upregulated in long-term prostate cancer adaptation after radiotherapy. *Mol Ther Nucleic Acids.* 2021;24:175-187.
8. Liu S, et al. Methylation-mediated LINC00261 suppresses pancreatic cancer progression by epigenetically inhibiting c-Myc transcription. *Theranostics.* 2020;10(23):10634-10651.
9. Zhou Z, et al. Expression and Clinical Significance of Long-chain Noncoding RNA LINC00261 in Colon Cancer. *Clin Lab.* 2019;65(12).
10. Lin K, et al. Long noncoding RNA LINC00261 induces chemosensitization to 5-fluorouracil by mediating methylation-dependent repression of DPYD in human esophageal cancer. *FASEB J.* 2019;33(2):1972-1988.
11. Chen T, et al. Linc00261 inhibits metastasis and the WNT signaling pathway of pancreatic cancer by regulating a miR5525p/FOXO3 axis. *Oncol Rep.* 2020;43(3):930-942.
12. Yan D, et al. LINC00261 suppresses human colon cancer progression via sponging miR-324-3p and inactivating the Wnt/beta-catenin pathway. *J Cell Physiol.* 2019;234(12):22648-22656.
13. Qi X, et al. ceRNA in cancer: possible functions and clinical implications. *J Med Genet.* 2015;52(10):710-718.
14. Zou Y, et al. miR-552: an important post-transcriptional regulator that affects human cancer. *J Cancer.* 2020;11(21):6226-6233.
15. Lai CH, et al. Study on miRNAs in Pan-Cancer of the Digestive Tract Based on the Illumina HiSeq System Data Sequencing. *Biomed Res Int.* 2019;2019:8016120.
16. Li X, et al. The mechanisms of DIRAS family members in role of tumor suppressor. *J Cell Physiol.* 2019;234(5):5564-5577.
17. Zheng R, et al. Methylation of DIRAS1 promotes colorectal cancer progression and may serve as a marker for poor prognosis. *Clin Epigenetics.* 2017;9:50.
18. Jones-Bolin S. Guidelines for the care and use of laboratory animals in biomedical research. *Curr Protoc Pharmacol.* 2012;Appendix 4:Appendix 4B.
19. Yanagawa M, et al. Evaluation of response to neoadjuvant chemotherapy for esophageal cancer: PET response criteria in solid tumors versus response evaluation criteria in solid tumors. *J Nucl Med.*

2012;53(6):872-880.

20. Zhu YH, et al. Downregulation of the novel tumor suppressor DIRAS1 predicts poor prognosis in esophageal squamous cell carcinoma. *Cancer Res.* 2013;73(7):2298-2309.
21. Fei Z, et al. Artesunate enhances radiosensitivity of esophageal cancer cells by inhibiting the repair of DNA damage. *J Pharmacol Sci.* 2018;138(2):131-137.
22. Zhang M, et al. LINC00261: a burgeoning long noncoding RNA related to cancer. *Cancer Cell Int.* 2021;21(1):274.
23. Ghafouri-Fard S, et al. Expression profile of lncRNAs and miRNAs in esophageal cancer: Implications in diagnosis, prognosis, and therapeutic response. *J Cell Physiol.* 2020;235(12):9269-9290.
24. Dorn A, et al. LINC00261 Is Differentially Expressed in Pancreatic Cancer Subtypes and Regulates a Pro-Epithelial Cell Identity. *Cancers (Basel).* 2020;12(5).
25. Wang ZK, et al. Long non-coding RNA LINC00261 sensitizes human colon cancer cells to cisplatin therapy. *Braz J Med Biol Res.* 2017;51(2):e6793.
26. Wu X, et al. Telomerase antagonist imetelstat inhibits esophageal cancer cell growth and increases radiation-induced DNA breaks. *Biochim Biophys Acta.* 2012;1823(12):2130-2135.
27. Wong RS. Apoptosis in cancer: from pathogenesis to treatment. *J Exp Clin Cancer Res.* 2011;30:87.
28. Wu K, et al. microRNA-10b confers cisplatin resistance by activating AKT/mTOR/P70S6K signaling via targeting PPARgamma in esophageal cancer. *J Cell Physiol.* 2020;235(2):1247-1258.
29. Shi X, et al. Genome-wide analysis of lncRNAs, miRNAs, and mRNAs forming a prognostic scoring system in esophageal squamous cell carcinoma. *PeerJ.* 2020;8:e8368.
30. Kwak B, et al. MicroRNA-552 links Wnt signaling to p53 tumor suppressor in colorectal cancer. *Int J Oncol.* 2018;53(4):1800-1808.
31. Ma Y, et al. Downregulation of miR-552 in hepatocellular carcinoma inhibits cell migration and invasion, and promotes cell apoptosis via RUNX3. *Exp Ther Med.* 2019;18(5):3829-3836.
32. Verma SP, et al. Sodium butyrate induces cell death by autophagy and reactivates a tumor suppressor gene DIRAS1 in renal cell carcinoma cell line UOK146. *In Vitro Cell Dev Biol Anim.* 2018;54(4):295-303.
33. Bergom C, et al. The Tumor-suppressive Small GTPase DiRas1 Binds the Noncanonical Guanine Nucleotide Exchange Factor SmgGDS and Antagonizes SmgGDS Interactions with Oncogenic Small GTPases. *J Biol Chem.* 2016;291(12):6534-6545.

Figures

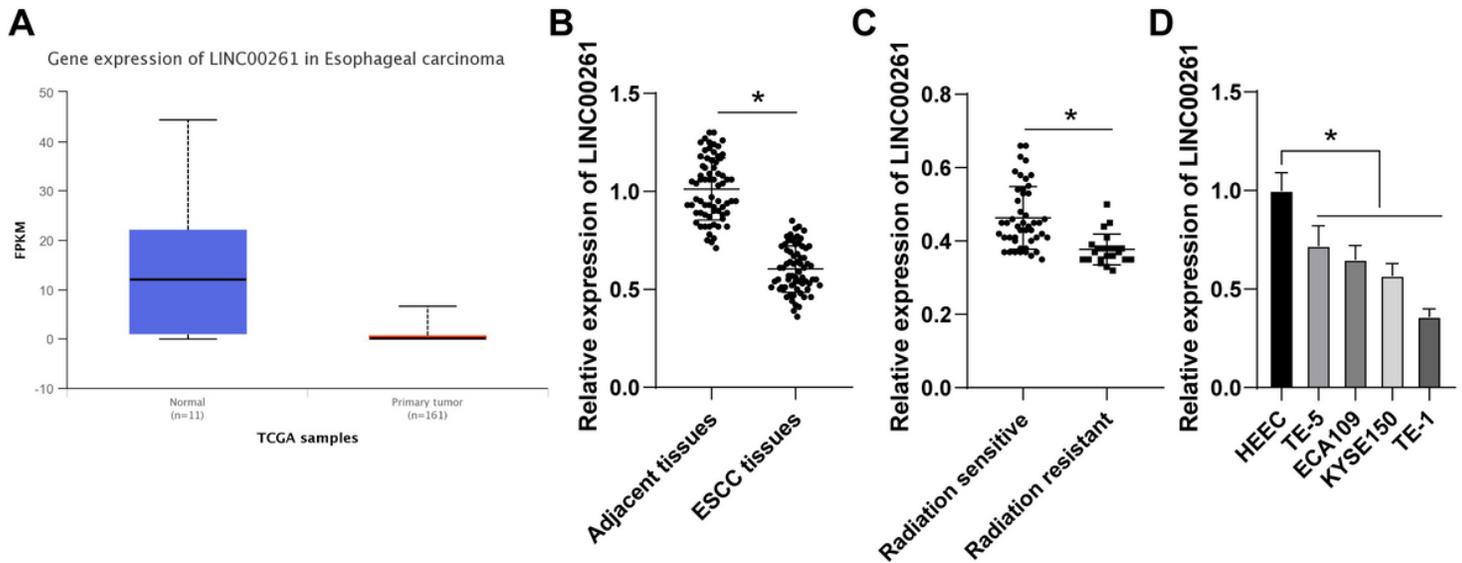


Figure 1

LINC00261 is poorly expressed in EC. EC and paracancerous tissues were harvested from EC patients. A, LINC00261 expression was analyzed by TCGA. B, LINC00261 expression in EC tissues was tested by RT-qPCR (N = 69). C, LINC00261 expression in the radiation sensitive group (N = 47) and the radiation resistant group (N = 22) was assessed. D, LINC00261 expression in normal and EC cells was measured by RT-qPCR. The independent cell experiment was repeated 3 times. The measurement data were presented as mean \pm standard deviation. The independent t-test was appointed to analyze data in panels B and C. One-way ANOVA was appointed to analyze data in panel D. Tukey's multiple comparisons test was employed for the post hoc test. * p < 0.05.

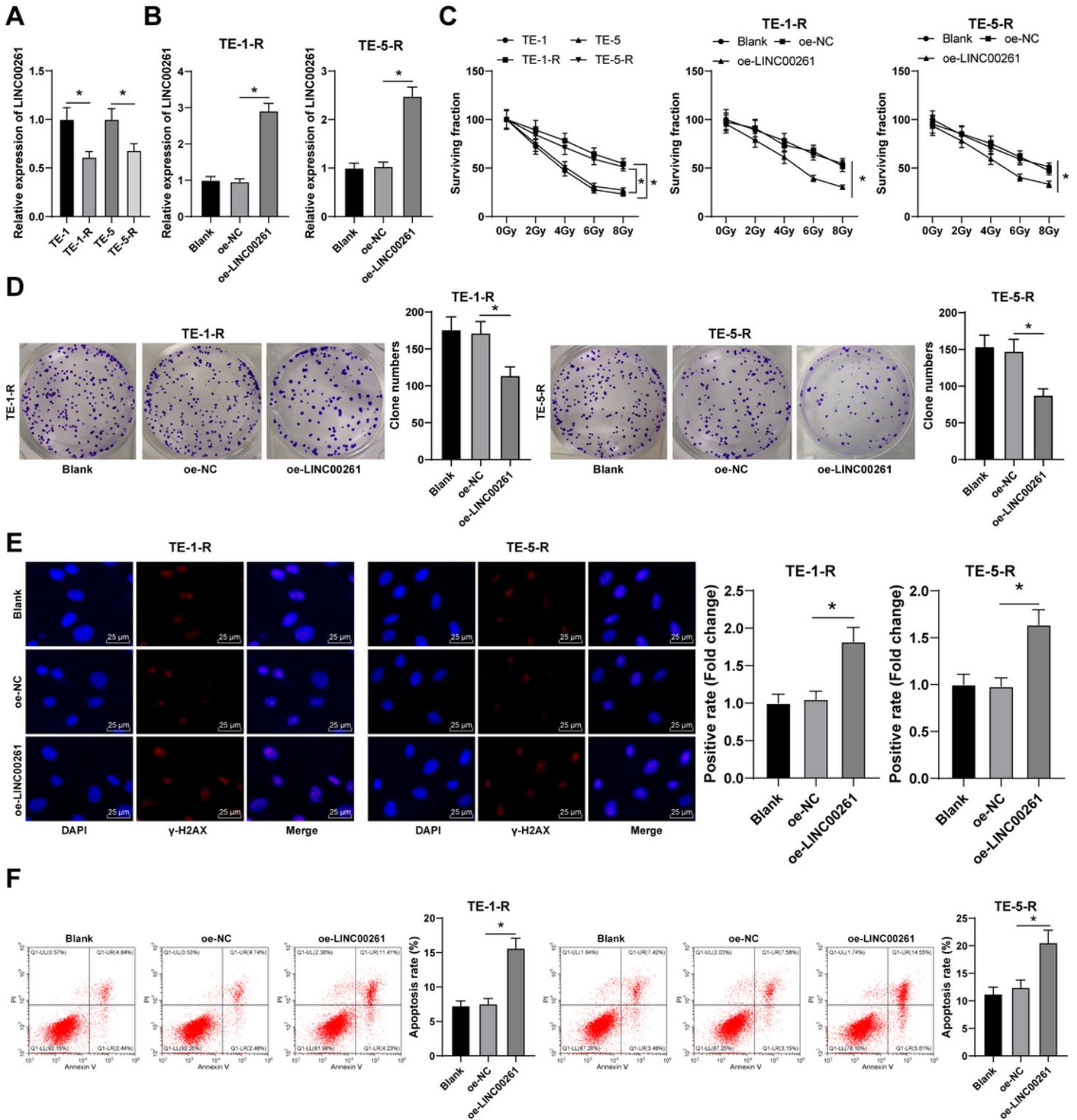
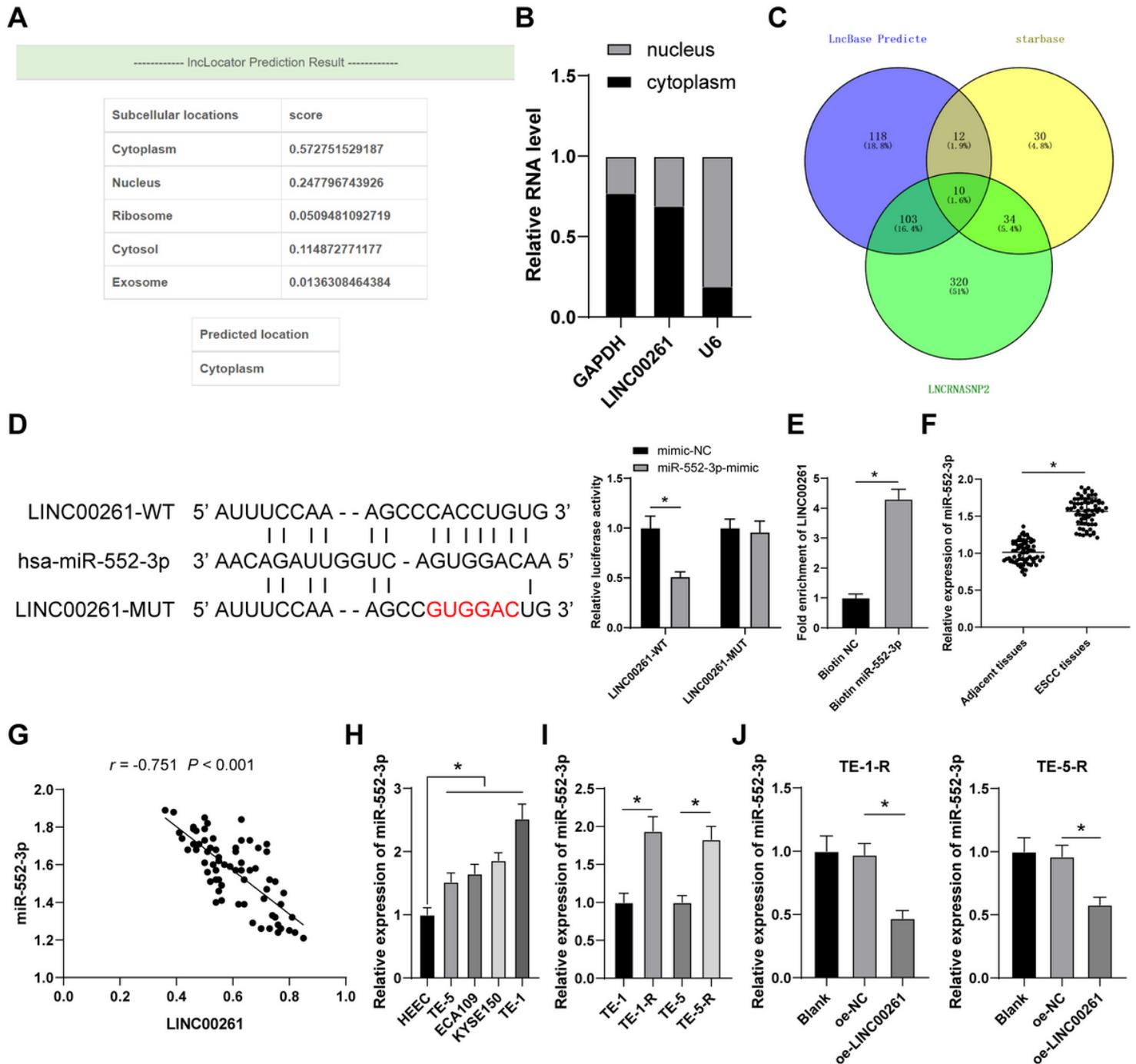


Figure 2

LINC00261 overexpression boosts EC cell radiosensitivity. Radioresistant cell lines TE-1-R and TE-5-R were established, and cells were infected by oe-LINC00261, with cells infected by oe-NC as control. A and B, LINC00261 expression in cells was assessed by RT-qPCR. C, SF in each group under 0, 2, 4, 6, and 8 Gy X-ray radiation was measured by the CCK-8 method. D, colony formation of cells under 4 Gy X-ray radiation was determined by colony formation assay. E, γ -H2AX expression of cells under 4 Gy X-ray

radiation was determined by immunofluorescent staining. F, apoptosis of cells under 4 Gy X-ray radiation was determined by flow cytometry. The independent experiment was repeated 3 times. The measurement data were presented as mean \pm standard deviation. One-way ANOVA was appointed to analyze data in panels A, B, D, E, and F. Two-way ANOVA was appointed to analyze data in panel C. Tukey's multiple comparisons test was employed for the post hoc test. * $p < 0.05$.



LNCRNASNP2 websites. D and E, the binding relation between LINC00261 and miR-552-3p was verified by dual-luciferase reporter gene assay (D) and pull-down assay (E). F, miR-552-3p expression in EC tissues was measured by RT-qPCR. G, the correlation analysis of LINC00261 and miR-552-3p. H and J, miR-552-3p expression in EC cells was determined by RT-qPCR. The independent experiment was repeated 3 times. The measurement data were presented as mean \pm standard deviation. The independent t-test was appointed to analyze data in panels E and F. One-way ANOVA was appointed to analyze data in panels H, I, and J. Two-way ANOVA was appointed to analyze data in panel D. Tukey's multiple comparisons test was employed for the post hoc test. * $p < 0.05$.

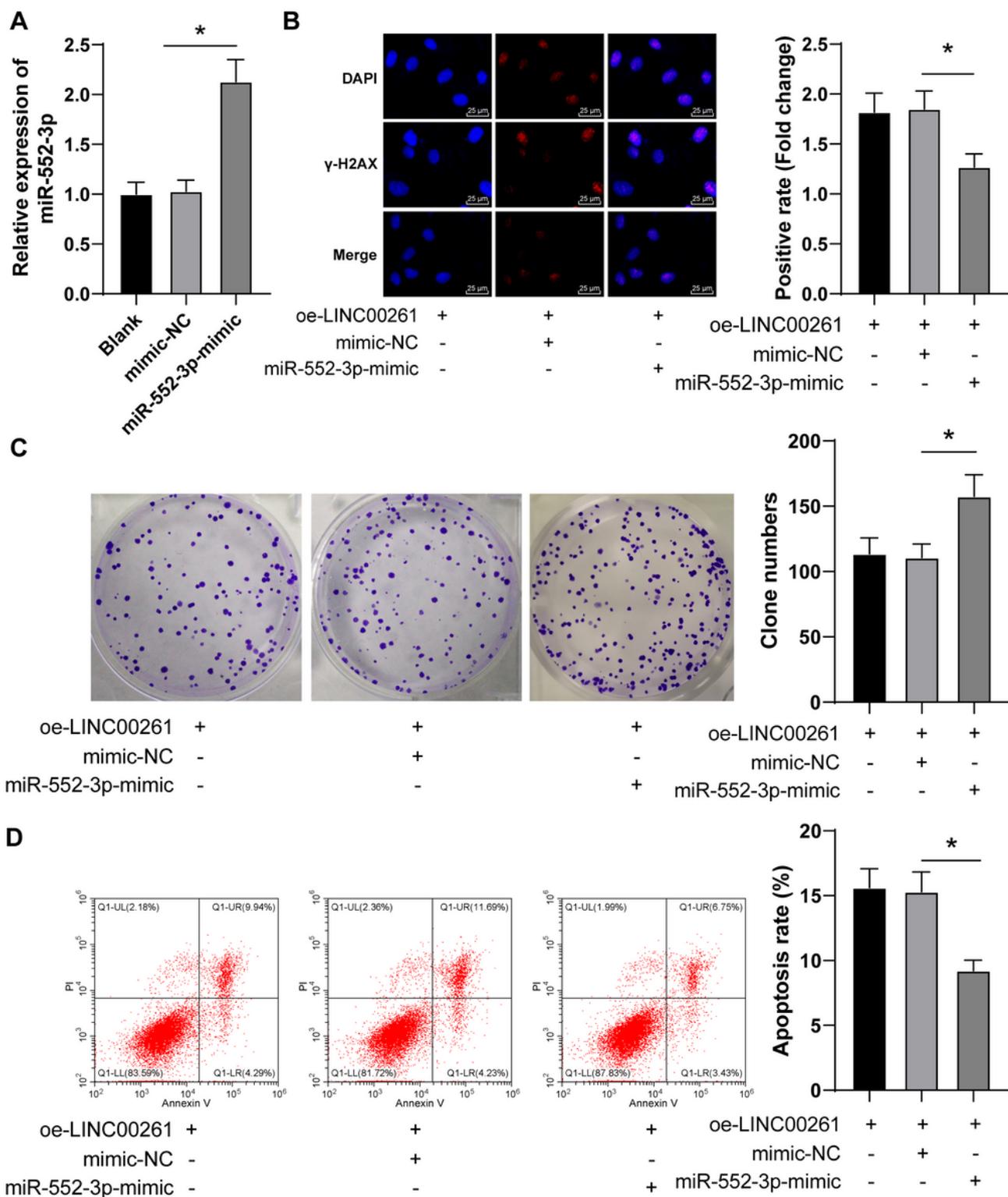


Figure 4

miR-552-3p overexpression neutralizes the promotive effect of LINC00261 on EC cell radiosensitivity. miR-552-3p-mimic was transfected into TE-1-R cells, with mimic-NC as control. A, transfection efficiency was examined by RT-qPCR, and the collaborative experiment of miR-552-3p-mimic and oe-LINC00261 was conducted under 4 Gy X-ray radiation. B, γ -H2AX expression in cells was determined by immunofluorescent staining. C, colony formation of cells was measured by colony formation assay. D,

apoptosis of cells was determined by flow cytometry. The independent experiment was repeated 3 times. The measurement data were presented as mean \pm standard deviation. One-way ANOVA was appointed to analyze data in panels A, B, C, and D. Tukey's multiple comparisons test was employed for the post hoc test. * $p < 0.05$.

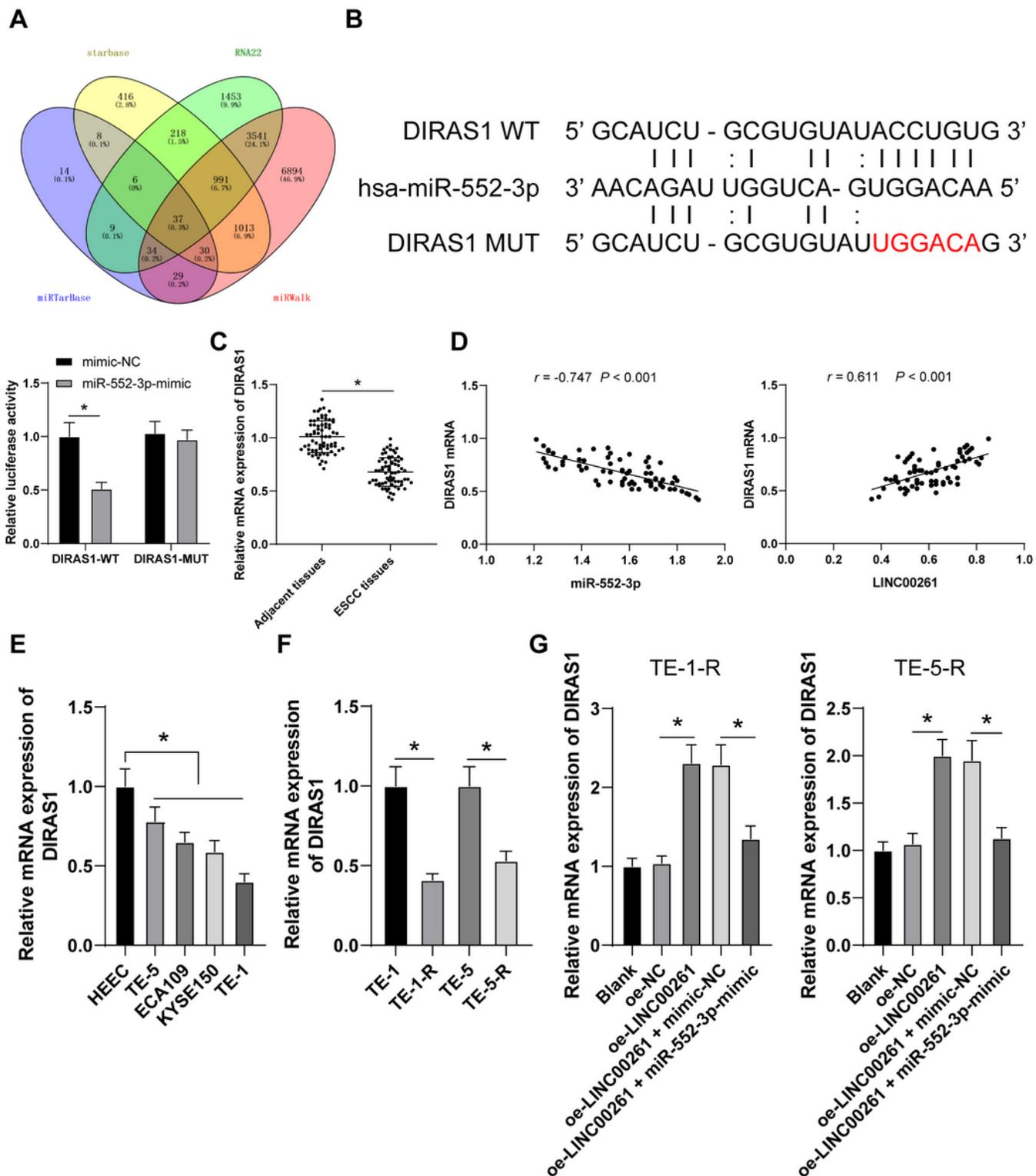


Figure 5

miR-552-3p targets DIRAS1. A, target genes downstream of miR-552-3p were analyzed by Starbase, RNA22, miRTarBase, and miRWalk websites. B, the binding relation between miR-552-3p and DIRAS1 was predicted by dual-luciferase reporter gene assay. C, DIRAS1 expression in EC tissues was detected by RT-qPCR. D, the correlation analysis of miR-552-3p and DIRAS1. E and G, DIRAS1 expression in EC cells was verified via RT-qPCR. The independent experiment was repeated 3 times. The measurement data were presented as mean \pm standard deviation. The t-test was appointed to analyze data in panel C. One-way ANOVA was appointed to analyze data in panels F and G. Two-way ANOVA was appointed to analyze data in panel B. Tukey's multiple comparisons test was employed for the post hoc test. * $p < 0.05$.

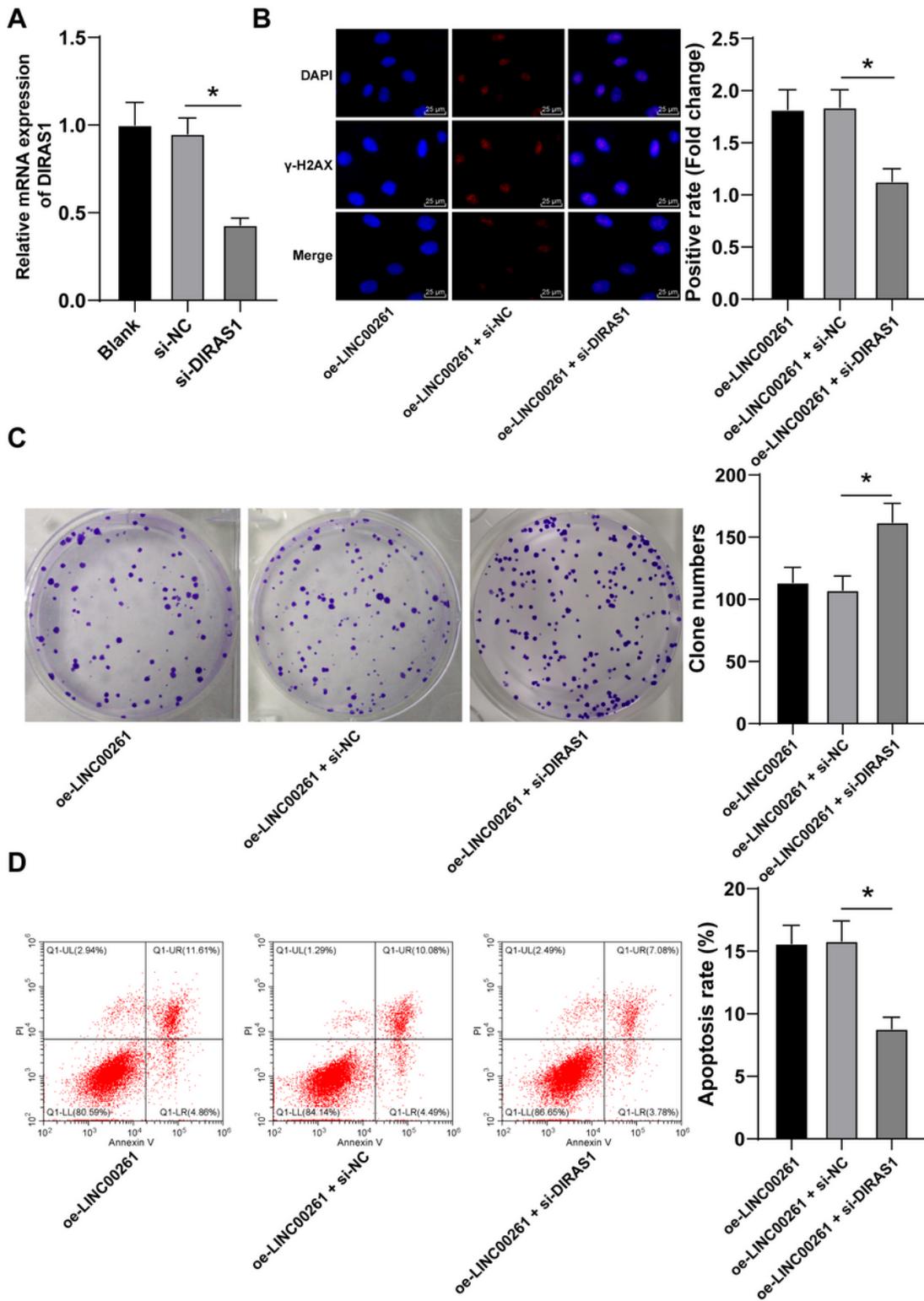


Figure 6

DIRAS1 depletion partially annuls the promotive role of LINC00261 overexpression in EC cell radiosensitivity. si-DIRAS1 was transfected into TE-1-R cells, with si-NC as control. A, transfection efficiency was examined by RT-qPCR, and the collaborative experiment of si-DIRAS1 and oe-LINC00261 was conducted under 4 Gy X-ray radiation. B, γ -H2AX expression in cells was determined by immunofluorescent staining. C, colony formation of cells was measured by colony formation assay. D,

apoptosis of cells was determined by flow cytometry. The independent experiment was repeated 3 times. The measurement data were presented as mean \pm standard deviation. One-way ANOVA was appointed to analyze data in panels A, B, C, and D. Tukey's multiple comparisons test was employed for the post hoc test. * $p < 0.05$.

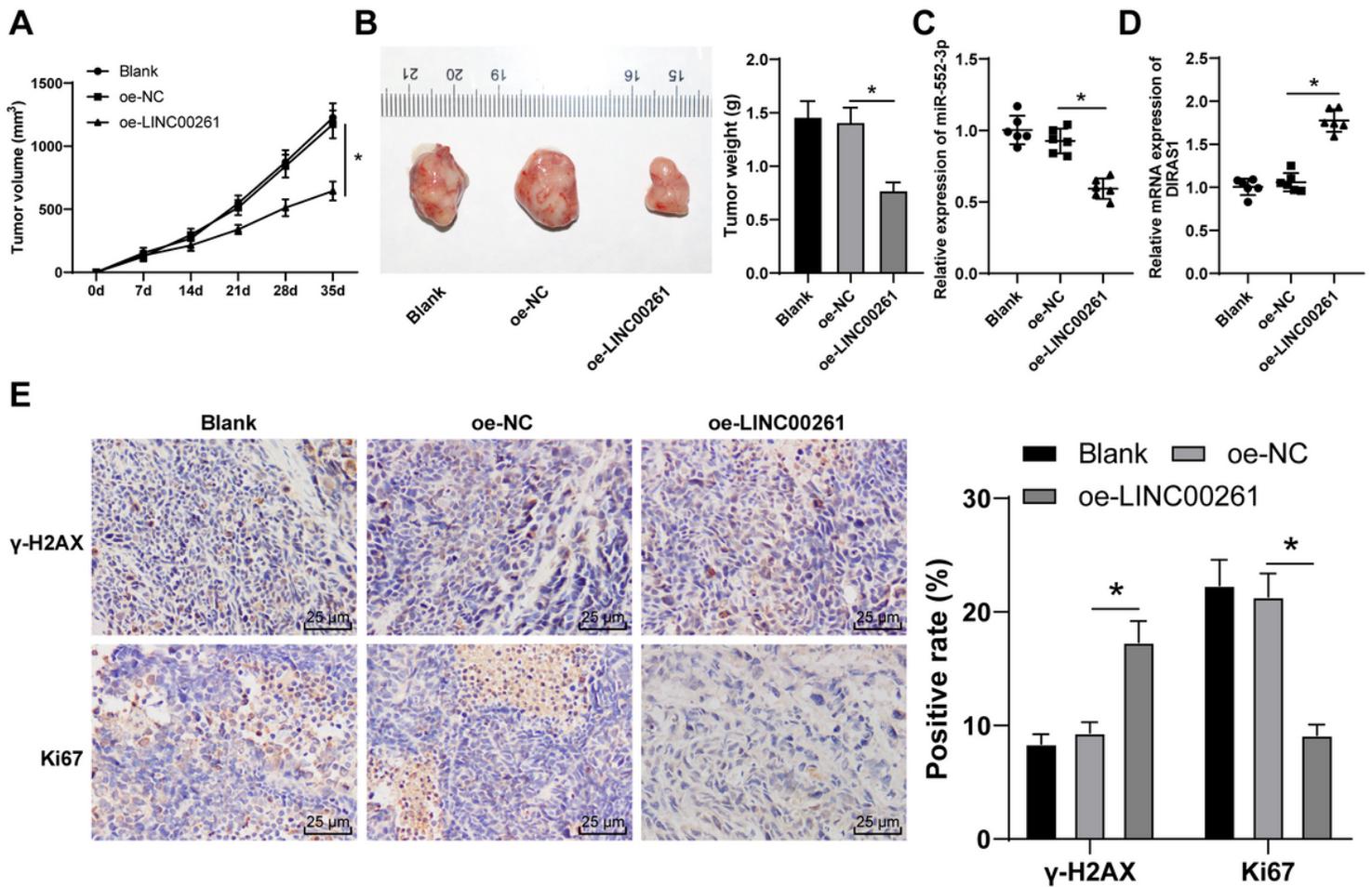


Figure 7

LINC00261 overexpression enhances EC radiosensitivity in vivo. TE-1-R cells with stable oe-LINC00261 were injected into mice. Tumor volume (A) and weight (B) were measured. C and D, miR-552-3p (C), and DIRAS1 mRNA (D) expression in mice tumor tissues was detected by RT-qPCR. E, γ -H2AX and Ki67 expressions in tumors were measured by IHC. A-B, N = 12; C-D, N = 6. The measurement data were presented as mean \pm standard deviation. One-way ANOVA was appointed to analyze data in panels B, C, and D. Two-way ANOVA was appointed to analyze data in panels A and E. Tukey's multiple comparisons test was employed for the post hoc test. * $p < 0.05$.

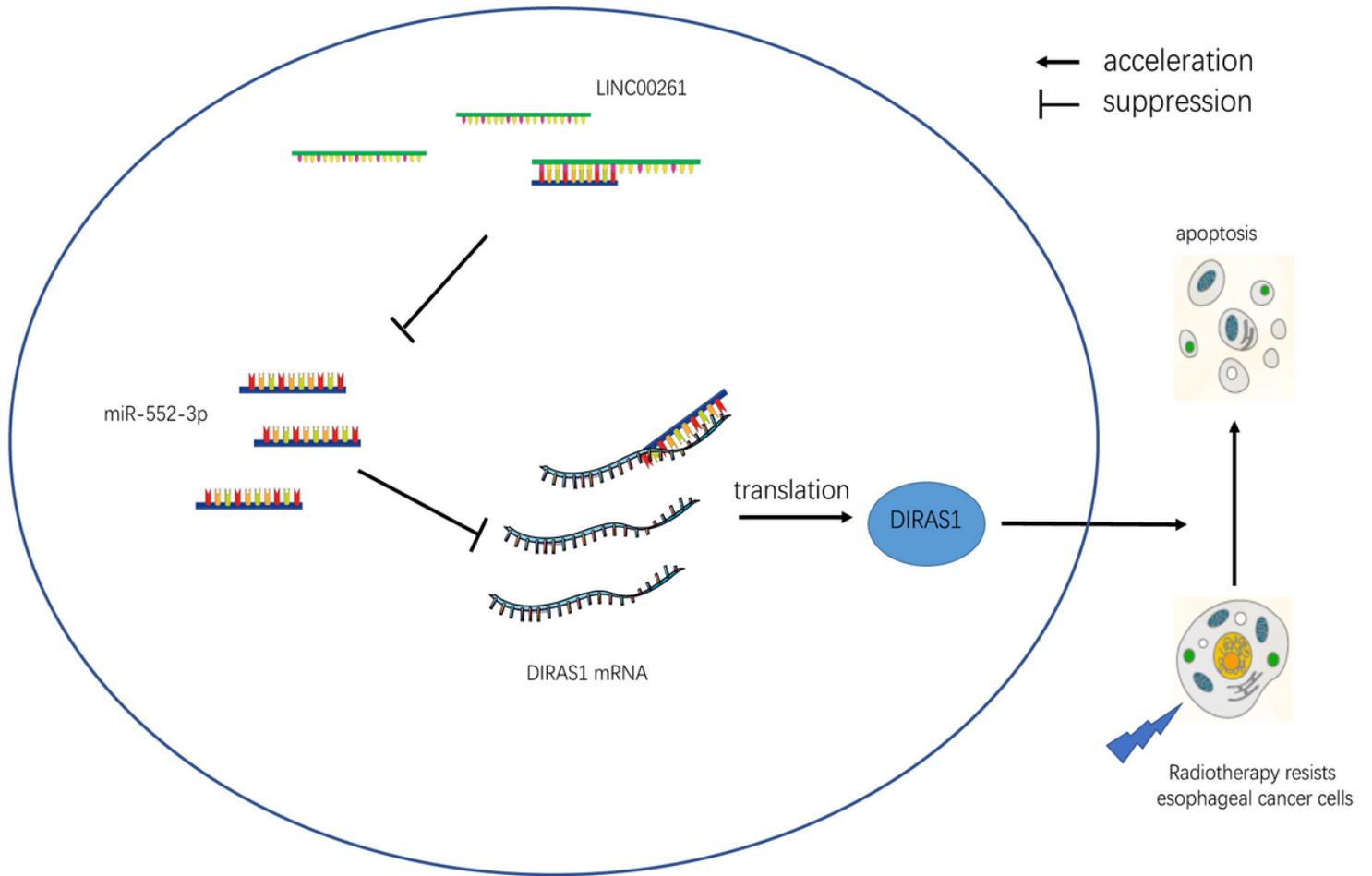


Figure 8

The mechanism of the LINC00261/miR-552-3p/DIRAS1 ceRNA network in EC radioresistance. LINC00261 functioned as a ceRNA to competitively bind to miR-552-3p and upregulate DIRAS1 expression to repress EC radioresistance.