

Exosomes-Derived Long Non-Coding RNA HOTAIR Reduces Laryngeal Cancer Radiosensitivity by Regulating microRNA-454-3p/E2F2 Axis

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Background: Studies have revealed exosomes are implicated in tumor microenvironment and tumorigenesis. Emerging evidence suggests long non-coding RNAs (lncRNAs) possess pivotal roles in laryngeal cancer progression. For this study, we aimed to find out the mechanism of exosomes and lncRNA HOTAIR in laryngeal cancer.

Methods: Laryngeal cancer cells-derived exosomes were initially extracted, separated and identified. Flow cytometry was applied to detect apoptosis to evaluate the effect of exosomes on cell radiosensitivity. Dual luciferase reporter gene assay, RNA pull-down and RNA immunoprecipitation assays were conducted to verify the interactions among HOTAIR, microRNA (miR)-454-3p and E2F2. The gain-and-loss functions of HOTAIR or miR-454-3p were carried out to explore their effects on TU212 and LLN cell viability, apoptosis and radiosensitivity. Levels of HOTAIR, miR-454-3p and E2F2 were detected after different treatments. An in vivo analysis was carried out in mice bearing laryngeal cancer xenografts.

Results: Laryngeal cancer-derived exosomes reduced laryngeal cancer cell radiosensitivity. HOTAIR expression was increased after cells were treated with exosome, and HOTAIR over-expression reduced laryngeal cancer cell radiosensitivity. Besides, HOTAIR worked as a competing endogenous RNA (ceRNA) of miR-454-3p to regulate E2F2 in laryngeal cancer cells. In vivo results were reproduced in in vivo studies, which demonstrated that HOTAIR knockdown reduced laryngeal cancer cell radiosensitivity by sponging miR-454-3p to silence E2F2.

Conclusion: Exosome-mediated HOTAIR acts as a ceRNA of miR-454-3p to regulate E2F2, thereby negatively regulating the radiosensitivity of laryngeal cancer cells. This study may offer novel insight into laryngeal cancer treatment.

Keywords: laryngeal cancer, long non-coding RNA HOTAIR, exosome, E2F2, microRNA-454-3p, radiosensitivity

Introduction

Head and neck malignant cancers take major responsibility for cancer-related mortality and morbidity, and its subset laryngeal cancer has the second-highest incidence and seriously threatens the life of patients.^{1,2} Patient-reported symptom questionnaires have noted that most laryngeal cancer patients experience voice, speech and swallowing afflictions, together with lowering quality of life and distress even after surgery or radiotherapy.³ The frontline treatments such as transoral laser microsurgery, open partial or total laryngectomy provide options alone or together with radiation or chemotherapy to enhance cure rate and keep the best quality of life for patient with laryngeal cancer.⁴ The tumor stage and size,

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radiotherapy fraction size and treatment duration are among the key factors related to local recurrence of laryngeal cancer after radiotherapy.⁵ Despite significant advances in surgery and radiotherapy over the last decades, the 5-year overall survival of locally advanced laryngeal cancer remains at a low level from 30% to 70%.⁶ Furthermore, elevation of the sensitivity of laryngeal cancer cells to radiation or chemotherapy is essential to improve the outcome of laryngeal cancer.^{7,8} So it is critical to find new ways to improve the radiosensitivity of laryngeal cancer and reduce the damage of normal tissues.

Exosomes, nanosized extracellular vesicles function as carriers because of their composition of lipid bilayer, proteins and RNAs,⁹ which can transport genetic materials derived from tumor cells to adjacent cells, contributing to tumor angiogenesis and metastasis.¹⁰ Long non-coding RNAs (lncRNAs) secreted from exosomes are regarded as potential biomarkers in several cancers.¹¹ A recent research indicates lncRNAs exert pivotal roles in the development of laryngeal squamous cell cancer (LSCC), the most common primary laryngeal cancer.¹² Previous studies have confirmed HOX transcript antisense intergenic RNA (HOTAIR) is highly expressed in LSCC cells¹³ and that HOTAIR knock-down could increase C33A cell radiosensitivity in human cervical cancer.¹⁴ Additionally, through database analysis and dual luciferase reporter gene assay, we found lncRNA HOTAIR could act as a competing endogenous RNA (ceRNA) of microRNA-454-3p (miR-454-3p) to further positively regulate E2F2. E2F transcription factors are major regulators of cell cycle, and E2F2 is involved for adult hepatocyte proliferation and liver regeneration.¹⁵ Interestingly, Molly E. Johnson et al have identified the involvement of Rb-E2F axis in head and neck squamous cell cancer.¹⁶ It has been reported that overexpressed miR-454-3p increases the radiosensitivity of renal carcinoma cells.¹⁷ In light of the above literatures, we may see some underlying connection of exosomes, HOTAIR and E2F2 in laryngeal cancer. Therefore, we carry out experiments to figure out the mechanism of exosomes transferring HOTAIR in laryngeal cancer cell radiosensitivity with the involvement of miR-454-3p/E2F2 axis.

Materials and Methods

Ethics Statement

This study was approved and supervised by the ethics committee of the First Hospital of Jilin University. All animal experiments in this study conform to the management of

local laboratory animals and the medical ethics committee of the First Hospital of Jilin University. Significant efforts were made to minimize both the number of animals used and their respective suffering.

Cell Culture

Laryngeal cancer cell lines TU212, LLN and TU686 (Cell Resource Center, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin in a 37°C incubator with 5% CO₂.

Isolation and Identification of Exosomes

The exosomes were isolated from laryngeal cancer cell line TU686 using differential ultracentrifugation. Serum was filtered with a 0.45 µm-polyvinylidene fluoride (PVDF) filter (Millipore, Billerica, MA, USA) to further eliminate cell debris. ExoQuick exosome precipitation solution (System Biosciences, Mountain View, CA, USA) was added to the supernatant and fully mixed, and then fixed for 30 mins at -4°C to precipitate the mixture. The exosomes were harvested after centrifugation at 15,000 g and 4°C for 30 mins, and preserved at -80°C after phosphate-buffered saline (PBS) suspension and precipitation.

After PBS washing, the exosome samples were pre-fixed in PBS (pH 7.4) with 2.5% glutaraldehyde for 2 hrs, and then fixed in PBS with 1% osmium tetroxide for another 2 hrs. The exosomes were incubated on a glow-discharged copper grid for 1 min, and stained with a drop of 2% phosphotungstic acid aqueous solution. Afterwards, the excess buffer was carefully drained from the edge of the copper grid with filter papers. The mesh was dyed with 2% uranyl acetate (pH 7.0) for 40 s. Then, samples were air-dried at room temperature and examined under transmission electron microscope (TEM) at 80 keV.

A total of 1×10^6 exosomes were re-suspended with PBS and added with 2 µL of fluorescent antibodies and homotypic control of the same volume, and incubated on the ice for 30 mins. Following washing with fluorescence-activated cell sorting buffer and fixing with 10% formalin, the positive rate of antigens was measured on the flow cytometer (MoFloAstrios EQ, Beckman Coulter Inc, CA, USA). Western blot analysis was used to detect the expression of CD63/CD81/tumor susceptibility gene 101 (TSG101). The antibodies used in this experiment were CD63 (1:1000,

ab59479), CD81 (1:1000, ab79559) and TSG101 (1:1000, ab30871) (Abcam, Cambridge, MA, USA).

The exosome suspension was diluted to 1 mL in tris (pyrazolyl)methane for further analysis. ZetaView PMX 110 (Particle Metrix GmbH, Microtrac, Meerbusch, Germany) was used to determine the size and concentration of exosomes by Nanosight Tracking Analysis.

Cell Treatment

The exosomes isolated from TU686 cell line (TU686-exo) and the TU686-conditioned medium added with exosome inhibitor GW4869 (TU686-GW4869) were added to TU212 and LLN cell lines, respectively, and irradiated with different doses of X-IR (0, 2, 4, 6, 8 Gy).

The HOTAIR cDNA was cloned into mammalian expression vector pcDNA3.1 (Invitrogen Inc., Carlsbad, CA, USA). The small interfering RNA (si-RNA) of HOTAIR and negative control (NC) was purchased from Guangzhou RiboBio Co., Ltd (Guangzhou, Guangdong, China). While miR-454-3p mimic, miR-454-3p inhibitor and miR-NC were synthesized and designed by Thermo Fisher Scientific (Shanghai, China). Lipofectamine 2000 was applied for cell transfection as per manufacturer's instructions (Invitrogen Inc., Carlsbad, CA, USA).

Colony Formation Assay

About 500 cells were inoculated into a 6-well plate for 24 hrs, exposed to different doses of X-ionizing radiation (IR, 2, 4, 6 or 8 Gy) (The culture conditions of different transfected cells were the same without X-IR.), and then incubated at 37°C for 9–12 days until colony development, with the number of colony cells greater than 50 counted as

one cell clone. Colonies were stained in crystal violet dyeing and counted. The survival fraction = (number of colonies/number of cells plated)_{irradiated}/(number of colonies/number of cells plated)_{non-irradiated}.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

The one-step method of Trizol (Invitrogen, Carlsbad, CA, USA) was employed to extract total RNA, and extracted high-quality RNA was confirmed using ultraviolet analysis and formaldehyde denaturation electrophoresis. RT-qPCR was conducted based on instructions of RT-qPCR kit (mirVana™ qRT-PCR miRNA Detection Kit, AM1558, Thermo Fisher Scientific, Shanghai, China) with glyceraldehyde-3-phosphate dehydrogenase as an internal reference for lncRNA-HOTAIR and E2F2, and U6 as an internal reference for miR-454-3p. PCR primers were designed and synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China) (Table 1). The amplification and dissolution curves were confirmed after the reaction, and data were analyzed by $2^{-\Delta\Delta C_t}$ method.

Western Blot Analysis

The proteins in each group were extracted, and the concentration was determined as per the instructions of bicinchoninic acid kit (Thermo Scientific Pierce, Rockford, IL, USA). The extracted proteins were boiled at 95°C for 10 mins, and 30 µg of each protein sample was run on 10% w/v sodium dodecyl sulfate polyacrylamide gel electrophoresis from 80 v to 120 v. Afterwards, proteins were transferred into the PVDF membranes by wet transfer method at

Table 1 Primer Sequences of RT-qPCR

Genes	Primer Sequences
lncRNA-HOTAIR	F: 5'-GATCCGCCACATGAACGCCAGAGATTTTCAAGAAATCTCTGGGCGTTCATGTGGTTTTTTG-3' R: 5'-AATTCAAAAAACCACATGAACGCCAGAGATTTCTCTTGAAATCTCTGGGCGTTCATGTGGCG-3'
E2F2	F: 5'-TCCACAAACAGAACAGATG-3' R: 5'-TCAATTAATCAACAGGTCCCC-3'
GAPDH	F: 5'-GGGAGCCAAAAGGGTCAT-3' R: 5'-GAGTCCTTCCACGATACCAA-3'
miR-454-3p	F: 5'-TAGTGCAATATTGCTTATAGGGT-3' R: 5'-ACCCTATAAGCAATATTGCACTA-3'
U6	F: 5'-CGCTTCGGCAGCACATATAC-3' R: 5'-AATATGGAACGCTTCACGA-3'

Abbreviations: RT-qPCR, reverse transcription quantitative polymerase chain reaction; lncRNA-HOTAIR, long non-coding RNA HOX transcript antisense intergenic RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; miR-454-3p, microRNA-454-3p; F, forward; R, reverse.

100 mV for 45–70 mins. The membranes were sealed in 5% bovine serum albumin at room temperature for 1 hr, and incubated with primary antibodies at 4°C overnight: E2F2 (1:1000, ab235837) and β -actin (1:1500, ab8227) (both from Abcam Inc., Cambridge, MA, USA). Then, the membranes were washed in tris-buffered saline tween (TBST) 3 times, and incubated with secondary antibody labeled by horseradish peroxidase for 1 hr at room temperature. After TBST washing, proteins were visualized by enhanced chemiluminescence reagent and developed by Gel EZ imager (Bio-Rad Laboratories, CA, USA). The target band was analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

Single-cell suspension was prepared at a density of 1×10^5 cells/mL using logarithmic growth phase cells, and then cells were inoculated into a 96-well plate at 200 μ L/well. Under different doses of X-IR or different transfection conditions, each well was added with 20 μ L MTT solution (5 mg/mL) to detect cell viability. Afterwards, each well was added with 150 μ L dimethyl sulfoxide for 15 mins to fully dissolve crystals. The absorbance value at the wavelength of 490 nm (A490) was measured in the 6 wells to obtain the mean value by a microplate reader. Cell proliferation rate = (A490 in the experimental group – A490 in the blank group)/(A490 in the NC group – A490 in the blank group) \times 100%.

Flow Cytometry

After 48 hrs of transfection, the cells were washed with 0.01 mol/L PBS and centrifuged at 2500 g for 5 mins with the supernatant removed. Then, cells were suspended in $1 \times$ binding buffer, and cell concentration was adjusted to 1×10^6 cells/mL. A total of 500 μ L cell suspension, 5 μ L Annexin V-fluorescein isothiocyanate and 10 μ L propidium iodide (Invitrogen Inc., Carlsbad, CA, USA) were added into each tube, mixed, and incubated at room temperature for 10 mins, followed by detection on the flow cytometer (MoFloAstrios EQ).

Dual Luciferase Reporter Gene Assay

By searching the database (<http://starbase.sysu.edu.cn>), it was found that HOTAIR worked as a ceRNA of miR-454-3p to regulate E2F2, and HOTAIR and E2F2 both have binding sites

with miR-454-3p. HOTAIR fragment containing the binding site of miR-454-3p was cloned into the pmirGLO oligosaccharide enzyme vector (Promega, Madison, WI, USA), and the pmirGLO-HOTAIR-wild-type (Wt) reporting vector was constructed. The pmirGLO-HOTAIR-mutant type (Mut) was constructed with the mutant binding site of miR-454-3p based on pmirGLO-HOTAIR-Wt. The pmirGLO-E2F2-Wt and pmirGLO-E2F2-Mut vectors were constructed using the same methods. The constructed vectors were transfected into TU212 cells and then transfected with miR-454-3p and miR-NC, respectively. After 48 hrs, the luciferase activity was detected using dual luciferase reporter gene assay system (Promega, Madison, WI, USA), and the relative activity was calculated as the ratio of firefly luciferase activity to renilla luciferase activity.

RNA Pull-Down Assay

At room temperature, biotinylated HOTAIR probes and NC probes were dissolved in wash/binding buffer, and incubated with streptavidin-coupled magnetic beads for 2 hrs. Next, the probes were added with cell lysates for 2 hrs to elute the RNA complex conjugated with magnetic beads, and the miR-454-3p expression was determined by RT-qPCR.

RNA Immunoprecipitation (RIP) Assay

RIP experiments were carried out according to the Magna RIP RNA binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) and Ago2 antibody (1:500, ab32381, Abcam Inc., Cambridge, MA, USA). The expression of lncRNA HOTAIR, miR-454-3p and E2F2 was analyzed by RT-qPCR with normal mouse antibody immunoglobulin G (IgG) (ab200699, Abcam Inc., Cambridge, MA, USA) as the NC.

Laryngeal Tumor-Bearing Mice

Fifteen specific pathogen-free BALB/c nude mice (4–6 week-old, 20 ± 2 g) (Beijing Vital River Laboratory Animal Technology Co., Ltd, Beijing, China, SCXK (Beijing) 2015-0001) were enrolled in this experiment. After feeding for 1 week, mice were numbered with body weight as a parameter, and were assigned into 3 groups using a random number table method. Then, mice were treated with TU212 injection, TU212 injection + X-IR, and TU212 injection + X-IR + exosome injection, respectively. Five mice were randomly selected in each group and subcutaneously injected with 2×10^6 TU212 cells into the right leg. When the tumor diameter reached about 5 mm, the tumors were

irradiated with a single dose of 12 Gy of X-IR, and the collected exosomes were injected via the tail vein ($2 \sim 3 \mu\text{g}/\text{mouse}$) every 3 days. Tumor size was measured every 3 days after X-IR for 2 days, and the volume was determined by the formula $(\text{length} \times \text{width})^2 \times 0.5$. Twenty-one days after X-IR irradiation, the tumors were taken out and weighed. Some of tumors were ground into homogenate to detect the apoptotic rate and the rest were embedded in paraffin and sectioned.

Hoechst 33342 Staining

The tumors were treated properly, washed with PBS, fixed with 4% formaldehyde and stained with $10 \mu\text{g}/\text{mL}$ Hoechst 33342 at room temperature for 15 mins. After PBS washing, the sections were observed and recorded under a fluorescence microscope.

Statistical Analysis

SPSS 21.0 (IBM Corp., Armonk, NY, USA) was applied for data analysis. Kolmogorov–Smirnov test showed whether the data were in normal distribution. The results were expressed as mean \pm standard deviation. Comparisons between the two groups were analyzed with *t*-test, among multiple groups were analyzed with one-way analysis of variance (ANOVA) or two-way ANOVA, and pairwise comparisons after ANOVA were conducted by Tukey's multiple comparisons test. *p* Value was obtained by two-tailed test and $p < 0.05$ indicated significant difference.

Results

Separation and Identification of Exosomes

The first step to evaluate the mechanism of exosomes in the radiosensitivity of laryngeal cancer cells was to separate and identify the exosomes. The exosomes collected from laryngeal cancer cells TU686 were observed by TEM (Figure 1A). The specific surface marker proteins of exosome CD63, CD81 and TSG101 were detected using flow cytometry (Figure 1B). Western blot analysis was applied to detect levels of CD63, CD81 and TSG101 with TU686 cells treated with GW4869, exosome inhibitor as the control (Figure 1C). The size and concentration of exosomes were assessed by Nanosight Tracking Analysis (Figure 1D). TEM showed the size of exosomes was about 100 nm. Flow cytometry and Western blot analysis observed CD63, CD81 and TSG101 were all positive. Nanosight Tracking Analysis found the peak

size of exosomes was $108.3 \pm 16.1 \text{ nm}$ and the concentration of exosomes was $4.0 \times 10^6 \text{ particles}/\text{mL}$. In short, exosomes were separated successfully.

Laryngeal Cancer-Derived Exosomes Reduces Cell Radiosensitivity

After separation and identification, exosomes were extracted and incubated with laryngeal cancer cell lines TU212 and LLN together with GW486-treated TU686 cells and different doses of X-IR to assess the mechanism of exosomes in laryngeal cancer cell radiosensitivity. As shown in Figure 2A, the radiosensitivity of TU212 and LLN cell lines after TU686-exosome treatment was reduced (both $p < 0.05$). The apoptotic rate of laryngeal cancer cells TU212 and LLN irradiated with 8 Gy of X-IR was greatly reduced by exosomes (both $p < 0.05$; Figure 2B). That is to say, laryngeal cancer-derived exosomes reduce cell radiosensitivity.

HOTAIR Overexpression Reduces Laryngeal Cancer Cell Radiosensitivity

As mentioned above, exosomes reduce laryngeal cancer cell radiosensitivity, while HOTAIR has an abnormal expression in laryngeal cancer tissues.¹⁸ In order to verify our hypothesis that HOTAIR could be involved in laryngeal cancer cell radiosensitivity, HOTAIR expression in TU686 cells before and after exosome and GW4869 treatment was measured. HOTAIR expression in TU686 cells treated with GW4869, an exosome inhibitor, was noticeably lower than that in TU686 cells treated with exosomes ($p < 0.05$, Figure 3A). Besides, RT-qPCR found HOTAIR expression in TU212 and LLN cells was increased markedly after exosome treatment (both $p < 0.05$; Figure 3B).

The gain-and-loss function of HOTAIR demonstrated that transfection of pcDNA3.1-HOTAIR or si-HOTAIR into laryngeal cancer cells TU212 and LLC could increase or decrease HOTAIR expression (Figure 3C). Colony formation assay showed HOTAIR overexpression substantially increased TU212 and LLN cell colonies and si-HOTAIR decreased cell colonies (Figure 3D). After HOTAIR silencing, the apoptotic rate of TU212 and LLN cells under X-IR was increased significantly ($p < 0.05$; Figure 3E). Exosome treatment or overexpression of HOTAIR could reduce the apoptotic rate of laryngeal cancer cells. It can be concluded that HOTAIR is a key factor affecting the radiosensitivity of laryngeal cancer cells.

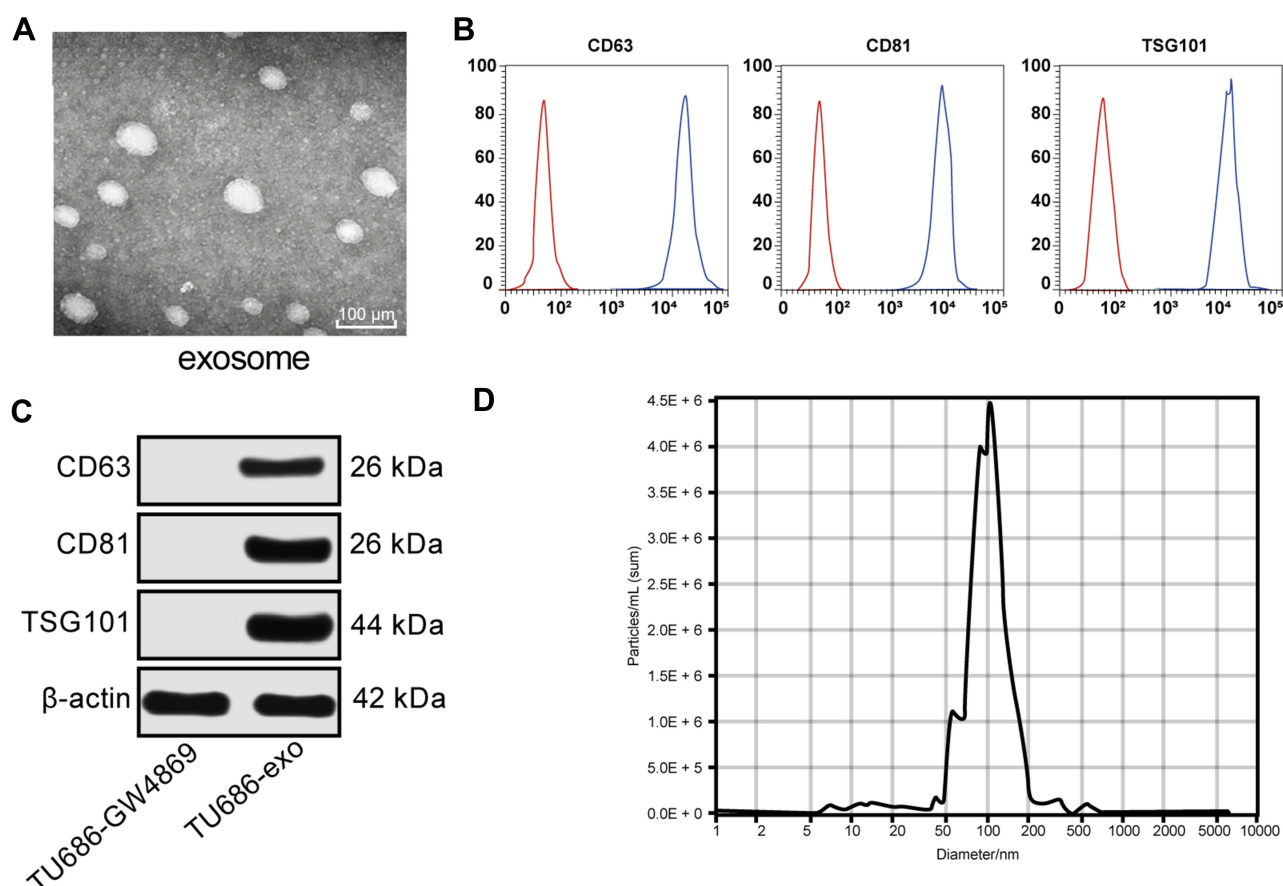


Figure 1 Separation and identification of exosomes. **(A)** Representative image of exosome separation under TEM, and TEM showed the size of exosomes was about 100 nm. **(B)** Specific surface marker proteins CD63, CD81 and TSG101 of exosome detected by flow cytometry and confirmed in positive. **(C)** Expression of specific surface marker proteins in TU686 cells treated with GW4869 detected by Western blot analysis. **(D)** Size and concentration of exosomes assessed by Nanosight Tracking Analysis, and showed peak size of exosomes was 108.3 ± 16.1 nm and the concentration of exosomes was 4.0×10^6 particles/mL. Repetitions = 3.

HOTAIR Acts as a ceRNA to Modulate E2F2 by Sponging miR-454-3p in Laryngeal Cancer

A former study infers lncRNAs can work as ceRNAs to absorb miRs and regulate target genes,¹⁹ which triggered us to speculate that HOTAIR may exert similar mechanisms to regulate target genes to influence radiosensitivity of laryngeal cancer cells. Through database searching (<http://starbase.sysu.edu.cn>), we found both HOTAIR and E2F2 had binding sites with miR-454-3p ([Supplementary Figure 1A](#)). In order to determine the ceRNA relationship among HOTAIR/miR-454-3p/E2F2 axis, the dual luciferase reporter vectors related to HOTAIR, E2F2 and miR-454-3p were constructed, respectively. We found HOTAIR and miR-454-3p had a targeted binding site ($p < 0.05$; [Figure 4A](#)), and E2F2 is the direct target of miR-454-3p ($p < 0.05$; [Figure 4B](#)). Then, we detected significant enrichment of miR-454-3p in HOTAIR by RNA pull-down assay and RT-qPCR ([Figure 4C](#)). The immunoprecipitation of HOTAIR

and Ago2 was performed in TU212 cells. Compared with the IgG group, the co-precipitation of HOTAIR and Ago2 increased ([Figure 4D](#)). After silencing HOTAIR, RT-qPCR and Western blot analysis ([Figure 4E](#) and [F](#)) showed that miR-454-3p expression was increased while E2F2 level decreased relatively. But in case of HOTAIR overexpression, miR-454-3p expression was decreased while E2F2 level increased relatively (all $p < 0.05$). Similarly, after the transfection of miR-454-3p mimic or miR-454-3p inhibitor, the expression of E2F2 and miR-454-3p showed an opposite trend ([Figure 4G](#)). These experiments indicated HOTAIR could act as a ceRNA to modulate E2F2 through absorbing miR-454-3p.

Overexpression of miR-454-3p Enhances Radiosensitivity of TU212 Cells

After determining the ceRNA relationship among HOTAIR/miR-454-3p/E2F2 axis, we adjusted miR-454-3p expression in TU212 and LLC cells to detect its effect on laryngeal

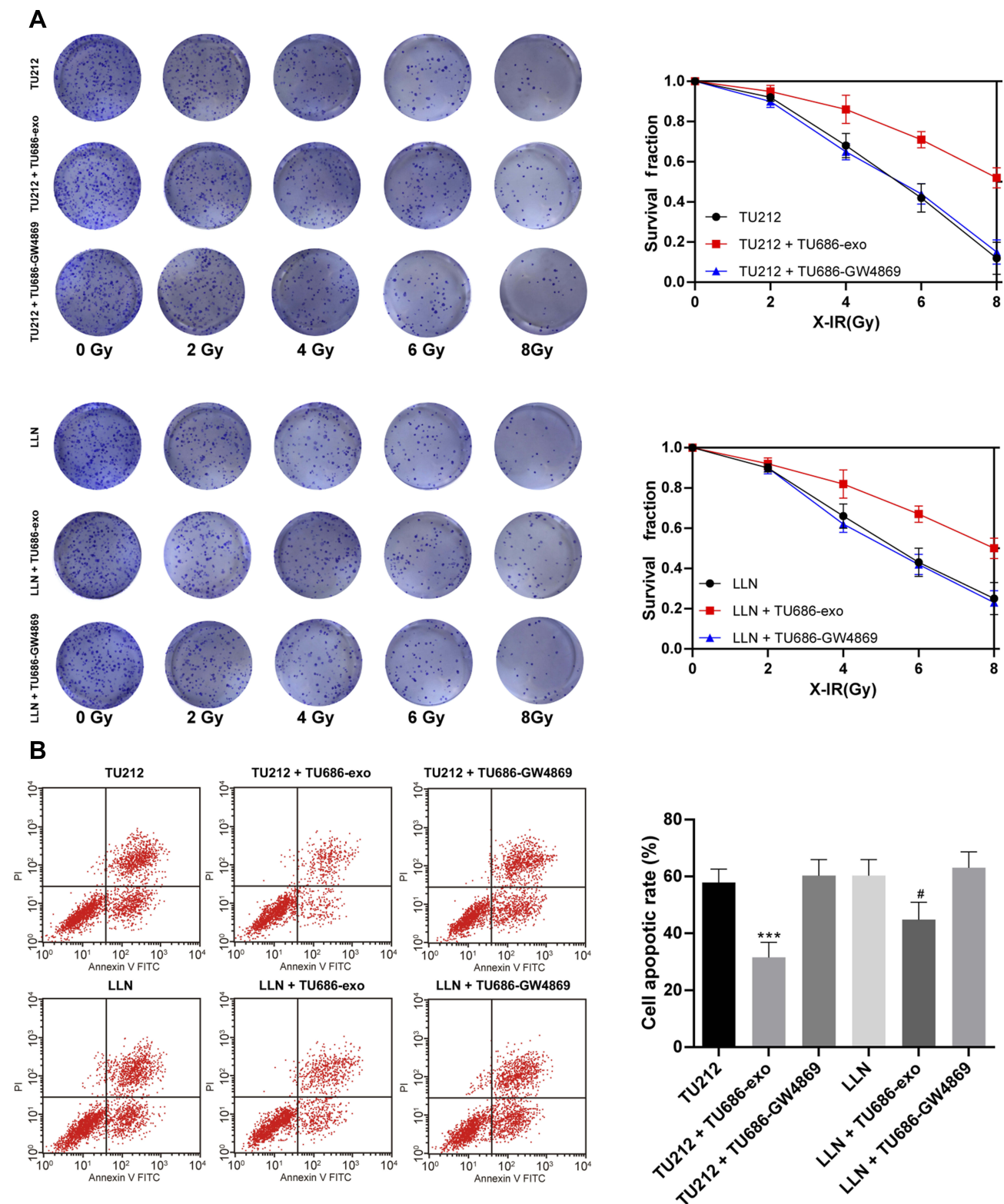


Figure 2 Laryngeal cancer-derived exosomes reduce laryngeal cancer cell radiosensitivity. **(A)** Representative images of cell colony formation ability before and after exosome treatment and fraction survival at different doses of X-IR. ** $p < 0.01$, *** $p < 0.005$. **(B)** Cell apoptosis after exosome treatment at condition of 8 Gy of X-IR detected by flow cytometry; compared to TU212 cells, *** $p < 0.005$; compared to LLC cells, # $p < 0.01$. Data were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used for post hoc tests. Repetitions = 3.

cancer cell radiosensitivity. miR-454-3p mimic and miR-454-3p inhibitor were transfected into TU212 and LLC

cells and irradiated with different doses of X-IR. With the increase of X-IR doses, TU212 and LLC cell viability was

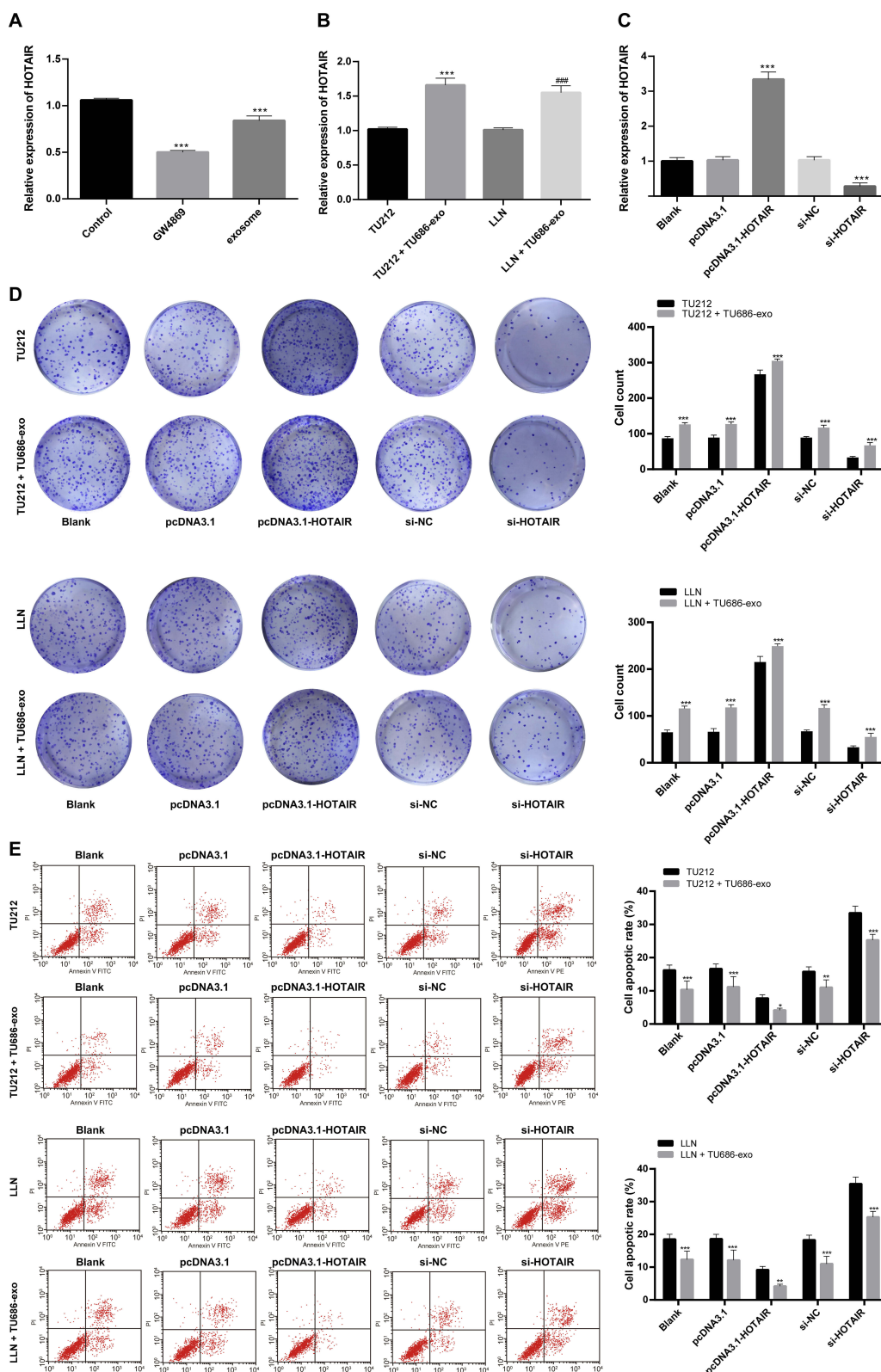


Figure 3 HOTAIR overexpression reduces laryngeal cancer cell radiosensitivity. **(A)** Relative expression of HOTAIR in laryngeal cancer cells treated with exosomes or GW4869 detected by RT-qPCR; compared to the control group, *** $p < 0.005$. **(B)** Relative expression of HOTAIR in laryngeal cancer cell lines before and after exosome treatment detected by RT-qPCR; compared to TU212 cells, *** $p < 0.001$; compared to LLC cells, #### $p < 0.001$. **(C)** Relative expression of HOTAIR in TU212 and LLC cells after restoration and depletion of HOTAIR detected by RT-qPCR; compared with the blank group, *** $p < 0.001$. **(D)** Cell counts and colony formation ability in laryngeal cancer cells after different transfection measured by colony formation assay; compared to TU212 or LLC cells, *** $p < 0.001$. **(E)** Relative cell apoptotic rate before and after exosome treatment and transfection detected by flow cytometry; compared to TU212 or LLC cells, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data in panels A, B and C were analyzed by one-way ANOVA, while panels D and E were analyzed by two-way ANOVA. Tukey's multiple comparisons test was used for post hoc tests. Repetitions = 3.

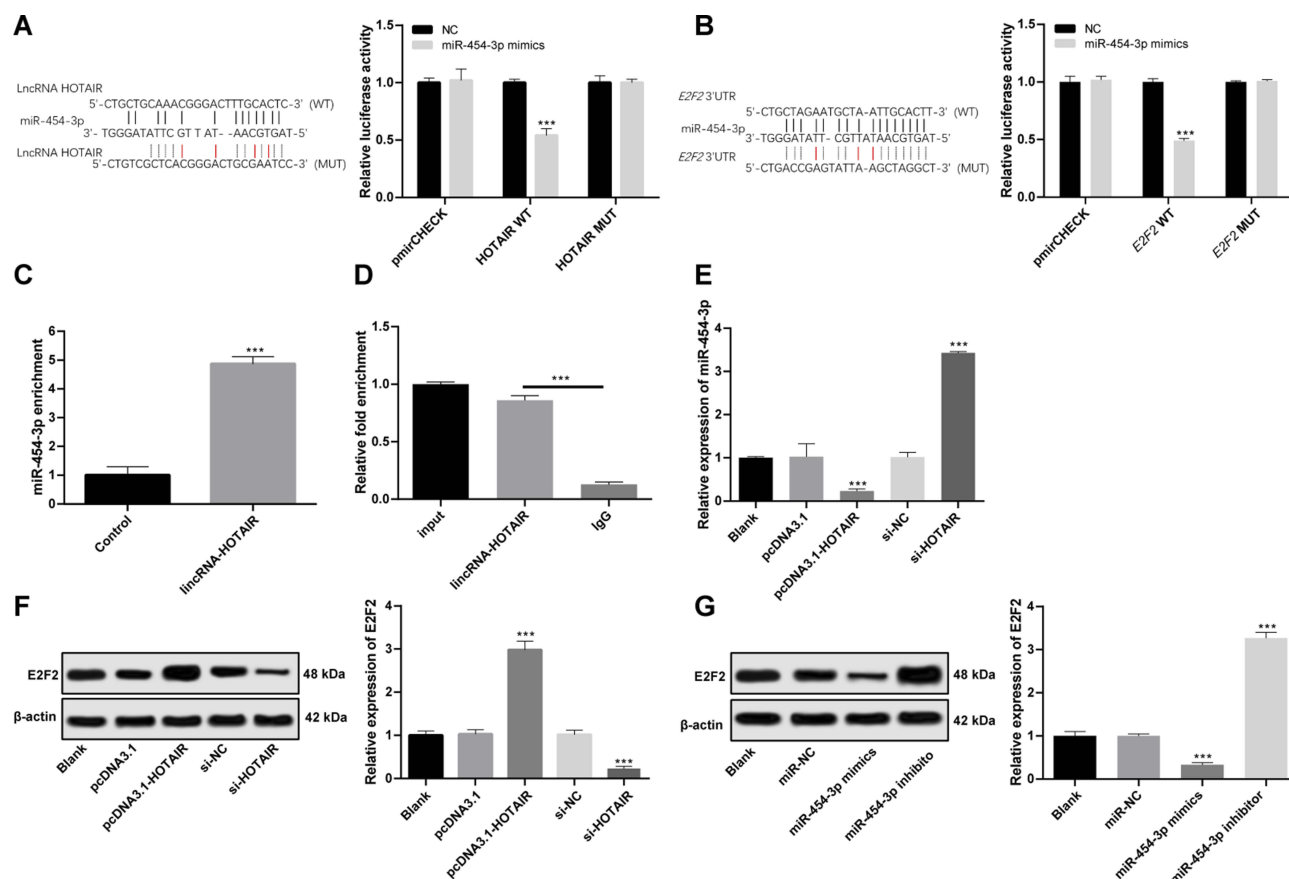


Figure 4 HOTAIR acts as a ceRNA to monitor E2F2 by sponging miR454-3p in laryngeal cancer. **(A)** Dual luciferase reporter gene assay of HOTAIR and miR-454-3p, *** $p < 0.001$. **(B)** Dual luciferase reporter gene assay of E2F2 and miR-454-3p, *** $p < 0.001$. **(C)** miR-454-3p enrichment tested by RNA pull-down assay, *** $p < 0.001$. **(D)** Co-precipitation of HOTAIR and Ago2 detected by RIP assay, *** $p < 0.001$. **(E)** Relative expression of miR-454-3p after HOTAIR overexpression and silencing detected by RT-qPCR; compared to the blank group, *** $p < 0.001$. **(F)** Relative expression of E2F2 after HOTAIR overexpression and silencing detected by Western blot analysis; compared to the blank group, *** $p < 0.001$. **(G)** Relative expression of E2F2 in cells transfected with miR-454-3p mimic or miR-454-3p inhibitor; compared with the blank group, *** $p < 0.001$. Data in panels A and B were analyzed by two-way ANOVA, while panels C-G were analyzed by one-way ANOVA. Tukey's multiple comparisons test was used for post hoc tests. Repetitions = 3.

decreased notably ($p < 0.05$; Figure 5A). Laryngeal cancer cells with overexpressed miR-454-3p were extremely sensitive to IR, while inhibited miR-454-3p obviously decreased the sensitivity of laryngeal cancer cells to IR. At this time, adding laryngeal cancer-derived exosomes to cells could significantly increase the cell viability in each group (Figure 5B) and significantly reduce apoptosis (Figure 5C). In conclusion, miR-454-3p expression positively regulates laryngeal cancer cell radiosensitivity.

Exosome-Mediated HOTAIR Reduces Radiosensitivity of TU212 Cells

After determining the effects of exosomes-mediated HOTAIR on radiosensitivity of laryngeal cancer cells in vitro, we subsequently conducted in vivo experiments to further confirm these results. TU212 cells were injected into mice. The tumor volume of mice treated with TU212 was increased

significantly 21 days later, the tumor volume of mice treated with TU212 injection + X-IR grew slowly ($p < 0.05$, Figure 6A, Supplementary Figure 1B), while that of mice treated with TU212 injection + X-IR + exosome was in between. The tumors were weighed (Figure 6B) and detected for apoptosis rate (Figure 6C). The tumor sections were stained with Hoechst 33342 staining and observed after embedding in paraffin (Figure 6D). It could be seen that the apoptotic rate of TU212 cells was increased significantly under X-IR ($p < 0.05$), while exosomes-mediated HOTAIR inhibited the killing effect of X-IR on TU212 cells, indicating exosomes-mediated HOTAIR could lessen the sensitivity of tumor cells to IR.

Discussion

Although radiotherapy is the standard treatment due to its better larynx preservation, nearly 50% of patients with laryngeal cancer do not respond to radiotherapy.⁸ It has been

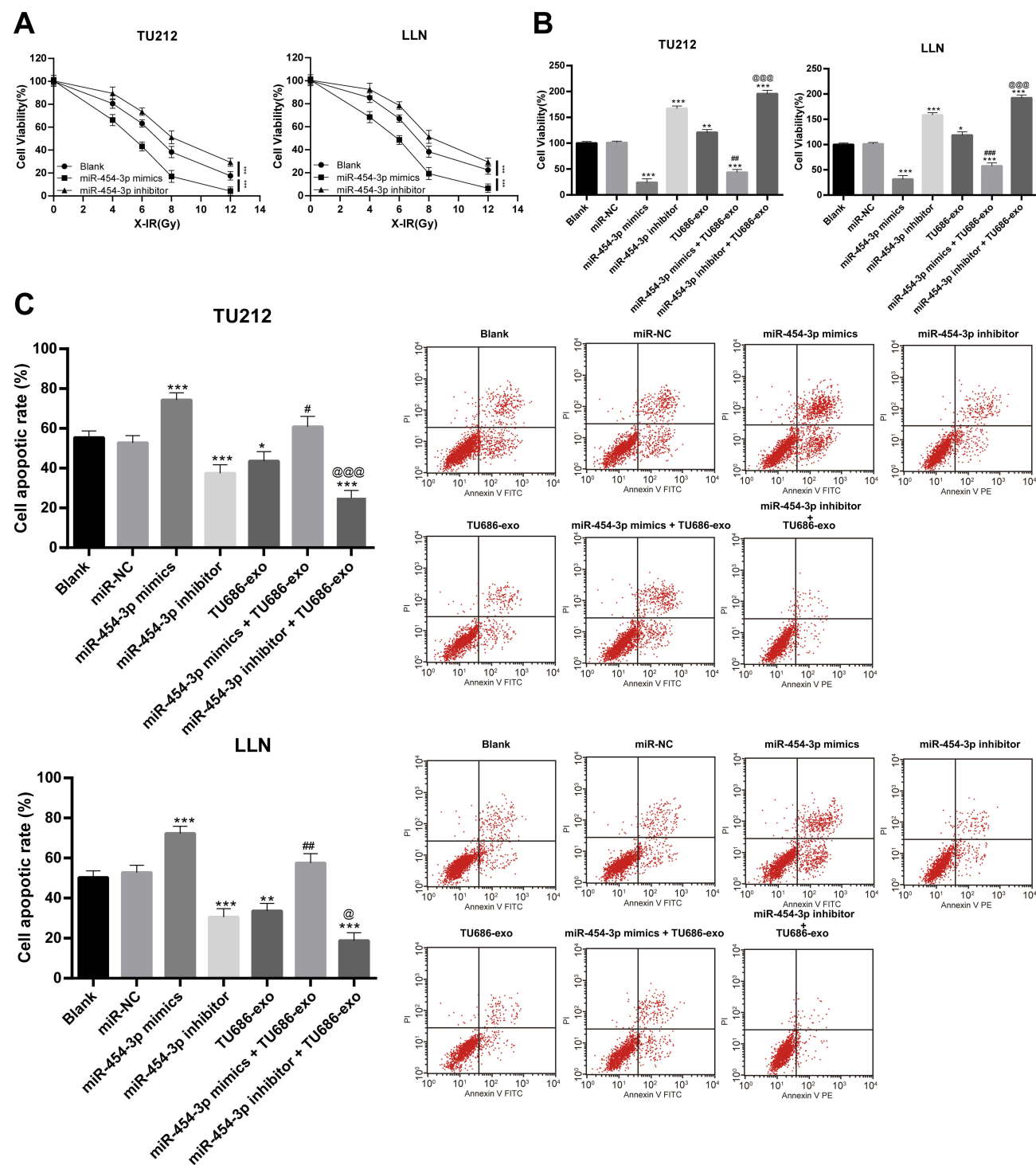


Figure 5 Overexpression of miR-454-3p enhances radiosensitivity of laryngeal cancer cells. **(A)** TU212 and LLC cell viability after transfection with miR-454-3p mimic and miR-454-3p inhibitor at different doses of IR detected by MTT assay, $***p < 0.001$. **(B)** Laryngeal cancer cell viability after 8 Gy of IR radiation and exosome treatment detected by MTT assay; compared with the blank group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; compared with the miR-454-3p mimic group, $###p < 0.01$, $####p < 0.001$; compared with the miR-454-3p inhibitor group, $@@@p < 0.001$. **(C)** Laryngeal cancer cell apoptosis after 8 Gy of IR radiation and exosome treatment detected by flow cytometry; compared with the blank group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; compared with the miR-454-3p mimic group, $*p < 0.05$, $###p < 0.01$; compared with the miR-454-3p inhibitor group, $@p < 0.05$, $@@@p < 0.001$. Data were analyzed by two-way ANOVA. Tukey's multiple comparisons test was used for post hoc tests. Repetitions = 3.

well established for the importance of the ceRNA network in lncRNA-mRNA interaction in tumorigenesis of cancers.²⁰ Hence, the present study was designed to explore the specific

ceRNA network among HOTAIR, miR-454-3p and E2F2 in laryngeal cancer cell radiosensitivity. Consequently, we found that exosome-mediated HOTAIR functioned as

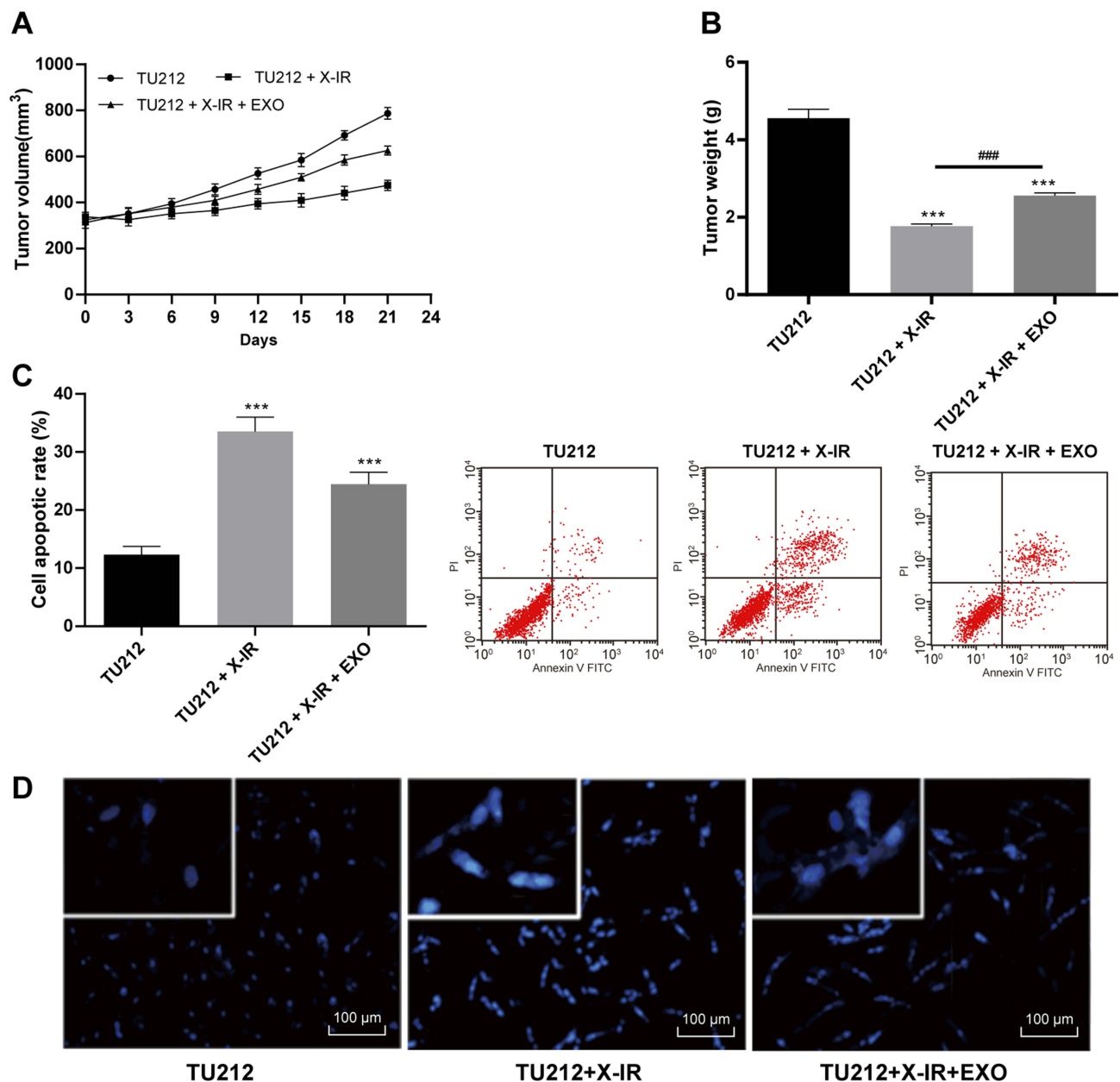


Figure 6 Exosomes-mediated HOTAIR reduces radiosensitivity of TU212 cells. **(A)** Tumor volume [(length × width)² × 0.5] under different treatment with TU212 injection, X-IR or exosome. **(B)** Tumor weight under different treatment with TU212 injection, X-IR or exosome; compared with mice injected with TU212, ****p* < 0.001; in pair-wise comparison, ###*p* < 0.001. **(C)** Cell apoptotic rate under different treatment with TU212 injection, X-IR or exosome detected by flow cytometry; compared with mice injected with TU212, ****p* < 0.001. **(D)** Representative images of Hoechst 33342 staining Morphological evaluation of Hoechst 33342 staining for detection of apoptosis in mouse tumor sections; Following staining, cells presenting nuclear fragmentation were counted as apoptotic cells. Data were analyzed by two-way ANOVA. Tukey's multiple comparisons test was used for post hoc tests. *n* = 5.

ceRNA through miR-454-3p to regulate E2F2, and negatively regulate radiosensitivity of laryngeal cancer cells.

The initial finding in this study revealed that laryngeal cancer-derived exosomes reduced cell apoptosis and radiosensitivity. Tumor cells release abundant exosomes, which facilitate the progress of tumors via communication between tumors and surrounding matrix tissues, and activation of proliferation and angiogenesis signaling pathways.²¹ The inhibitory role of cancer-derived exosomes in cell apoptosis was

also revealed in bladder cancer cells.²² Mutschelknaus L and his colleagues noted that squamous head and neck cancer-derived exosomes enhanced cell survival and radioresistance after IR exposure,²³ which was in agreement with our results. Besides, markedly upregulated HOTAIR expression was observed in laryngeal cancer cells after exosome treatment. Of particular note, high HOTAIR expression was associated with exosome amounts in hepatocellular carcinoma and HOTAIR promoted exosome production by transporting

multivesicular bodies to plasma membranes.²⁴ Similarly, a former study indicated HOTAIR was overexpressed in LSCC.¹³ Importantly, overexpression of HOTAIR in LSCC tissues was verified to positively associate with T stage, pathological grade and lymphatic metastasis of LSCC.¹⁸ Therefore, high expression of HOTAIR might be a diagnostic marker for laryngeal cancer.

In the following experiments, we silenced HOTAIR expression to explore the specific effects on laryngeal cancer cell viability, apoptosis and radiosensitivity. The validated evidence showed that after HOTAIR silencing, cell colonies were reduced, the apoptotic rate of TU212 cell and radiosensitivity under X-IR were increased dramatically. Consistent observation of HOTAIR knockdown inhibiting cell proliferation and viability was found in head and neck squamous cell carcinoma,²⁵ revealing the protective roles of HOTAIR silencing in malignancy. Another study clarified HOTAIR silencing repressed proliferation and invasion, and reinforced apoptosis and radiosensitivity in colorectal cancer.²⁶ Meanwhile, HOTAIR silencing inhibited autophagy and raised apoptosis and sensitivity to cisplatin in oral squamous cell carcinoma.²⁷ Most importantly, siRNA-mediated HOTAIR knockout led to the inhibited invasion and induced apoptosis of Hep-2 cells, and blocked the growth of LSCC xenografts.¹³

Furthermore, the results obtained from this study supported that HOTAIR acted as a ceRNA to modulate E2F2 by sponging miR-454-3p in laryngeal cancer, and overexpressed miR-454-3p enhanced radiosensitivity of TU212 cells. The similar ceRNA regulatory network involving HOTAIR was also revealed by Zheng Mei et al in gastric cancer, in which HOTAIR myeloid 1 acted as a ceRNA to regulate Disks Large Homolog-Associated Protein 1 by sponging miR-148a in head and neck tumor cells.²⁸ It was previously reported that serum levels of miR-21 and HOTAIR in exosomes were noticeably correlated with clinical characteristics of LSCC, and might be potential diagnostic tools for LSCC patients.²⁹ A former research had proved the association of single-nucleotide polymorphisms of E2F1 and E2F2 with the initiation and susceptibility of head and neck squamous cell carcinoma.³⁰ The promotion effect of overexpressed miR-454-3p on radiosensitivity was also observed in renal carcinoma cells.¹⁷ More recent evidence suggested a role for miR in the radiosensitivity of laryngeal cancer. Xu et al showed that miR-24 was down-regulated in LSCC and its overexpression was able to enhance radiosensitivity in LSCC by targeting X-linked inhibitor of apoptosis protein.³¹

In a word, we provided compelling evidence to state that tumor-secreted exosomes reduced cell radiosensitivity, while

lncRNA HOTAIR silencing strengthened the radiosensitivity of laryngeal cancer cell by functioning as a ceRNA to down-regulate E2F2 through sponging miR-454-3p. This study may offer a new perspective for further understanding of laryngeal cancer and finding new targets for effective therapies. Analysis of abovementioned results may give the chance to modify some clinical attitudes and create the background for future clinical investigation and application in this field. Thus, more studies in radiosensitivity of laryngeal cancer cell are required in the future to develop clinical values based on results obtained from this study.

Data Availability Statement

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

Disclosure

The authors declare no conflicts of interest in this work.

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