Carbon ion irradiation suppresses angiogenic response in human lung adenocarcinoma cells mediated by LINC00167/miR-663a/TGF-β1 axis

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Abstract

Background

Radiotherapy plays an important role in numerous tumor clinical treatments and over 65% of cancer patients need to accept radiotherapy all over the world. However, tumor angiogenesis and metastasis induced by conventional photon radiotherapy adversely impact the survival of patients, and limit the clinical radiotherapy efficiency. Heavy-ion radiotherapy has attracted wide attention in recent years because of its excellent physical property and outstanding tumor control rate, however, the underlying gene expression regulation mechanism response to heavy-ion irradiation remains elusive.

Methods

RNA-sequencing (RNA-seq) and public database analysis were employed to identify the differential molecular changes in lung adenocarcinoma cells exposed to both X-ray and carbon ion (C-ion) irradiation. The expression of the identified LINC00167 was verified by real-time quantitative PCR in different lung cancer cell lines and pulmonary bronchial epithelial cell lines. The content of serum vascular endothelial growth factor (VEGF) and transforming growth factor beta 1 (TGF-β1) of 8 lung cancer patients who received X-ray or carbon ion radiotherapy were detected by ELISA experiment. Loss-of-function and gain-of-function experiments were performed to explore the biological roles of LINC00167 and miR-663a in lung cancer cell angiogenesis and metastasis. Comprehensive biochemical and biological techniques were utilized to explore the functions of LINC00167 in tumor angiogenesis and metastasis induced by different radiation types.

Results

In this study, we confirmed that LINC00167 was highly expressed and induced by X-ray irradiation in lung cancer cells. Moreover, increased LINC00167 expression was positively correlated with tumor angiogenesis and metastasis caused by conventional photon radiotherapy. LINC00167 worked as a sponge of miR-663a to positively regulate the expression of TGF-β1 and the downstream VEGF signaling and then promoted the tumor angiogenesis and metastasis of lung cancer cells. LINC00167 could strengthen the pro-angiogenesis and metastasis ability of lung cancer cells. Photon radiation-induced LINC00167 promoted angiogenesis both in vitro and in vivo.

Conclusion

Our data suggest that LINC00167/miR-663a/TGF-β1 axis is involved in the differential angiogenic response of lung adenocarcinoma cells exposed to X-ray or C-ion irradiation, providing the molecular mechanisms underlying the suppressed angiogenic response induced by carbon ion radiotherapy.
Introduction

According to the latest world cancer data published in 2021, lung cancer remains the leading cause of cancer death. In 2020, the number of new cases of lung cancer is about 2.2 million (11.4%) and new deaths are about 1.8 million (18.0%)\[1\]. Globally, the burden of lung cancer in incidence and mortality rate is increasing rapidly, and radiotherapy is one of the main therapies and plays an important role in lung cancer treatment. Over 50% of cancer patients have the requirements for at least one course of radiotherapy in their treatment process, and the requirements are still gradually increasing with the number of cancer patients\[2–6\]. In recent decades of years, conventional radiotherapy employing photon radiation has achieved rapid development and various new technologies have emerged, including intensity-modulated radiation therapy (IMRT), volumetric-modulated arc therapy (VMAT), image-guided radiation therapy (IGRT), etc, which have made radiotherapy more precise and safe. However, despite these advantages, photon radiation-induced tumor angiogenesis and metastasis severely inhibit patients’ survival and have become a key obstacle to the improvement of photon therapy efficacy\[7\]. In recent years, the prevalence of proton and heavy ion radiotherapy dramatically increased the tumor control rate and patients’ survival rate\[8, 9\]. It has been reported that proton and heavy ion radiotherapy exhibit better efficacy and fewer adverse reactions compared with photon radiotherapy in the treatment of different stages of NSCLC\[8, 10\].

Benefitting from the Bragg peak, proton and heavy ion radiation deposit more energy into tumor tissues and protects the normal tissues on the particle track. Meanwhile, heavy ion radiation has an excellent killing effect for hypoxia tumors, which is obviously better than conventional photon radiation that relied on oxygen free radicals to kill tumor cells. More importantly, several studies have suggested that proton and heavy-ion radiation suppress tumor angiogenesis and metastasis compared with photon radiation \[11–14\]. Akino et al. reported that X-ray irradiation of lower doses promoted cell proliferation, migration and invasion; whereas carbon ion irradiation suppressed the migration and invasion of A549 cells without enhancing cell proliferation. Meanwhile, the carbon ion irradiation caused different gene expression profile from X-ray irradiation, like inhibiting the expression of ANLN, which is responsible for cell migration and invasion and its expression was not changed by X-ray irradiation \[15\]. Similarly, Kamlah et al. reported that 6 Gy X-ray irradiation significantly increased blood vessel density, whereas carbon ions (2 Gy) irradiation has no promotive effects on it, indicating the therapeutic advantage of carbon ions compared with X-rays\[16\]. Yamada et al. reported the gratifying inhibition effect of carbon-ion radiotherapy (CIRT) in recurrence, lung metastasis, liver metastasis, and lymph node metastasis of rectal cancer\[13\]. Tinganelli et al. summarized in a review that the carbon ions induced the down-regulation of motility-associated genes, which were generally upregulated by photon radiotherapy, indicating that carbon ion radiotherapy has the advantage for tumor angiogenesis and metastasis inhibition\[14\]. However, the potential molecular mechanism underlying the heavy ion-induced inhibition of tumor angiogenesis and metastasis remains unclear.

To clarify the potential mechanisms, we pay attention to long non-coding RNAs (lncRNAs), which are a class of non-coding transcripts that consist of over 200 nucleotides \[17\]. Long intergenic non-coding
RNAs (lincRNAs) are IncRNAs longer than 200 nucleotides that don’t overlap annotated coding genes [18]. More and more studies have suggested that lncRNAs participated in various biological processes, such as metabolism, immunity, tumorigenesis, cardiovascular diseases, nervous system disorders, and many other diseases [17, 19–21]. However, the functions of lncRNAs in the regulation of differential cellular response to different radiation types have not been demonstrated yet.

In this study, we aimed to explore the molecular mechanism involved in radiation-induced tumor angiogenesis and metastasis and tried to explain the more optimized tumor control rate of carbon ions compared with X-rays from a new insight. We found that LINC00167 was highly expressed in lung cancer cell lines and positively correlated with tumor cells’ abilities of pro-angiogenesis, migration, and invasion. Here, we confirmed that LINC00167 promoted TGF-β1 expression in lung cancer cells by sponging miR-663a. Collectively, our data suggested that LINC00167/miR-663a/TGF-β1 axis plays an important role in radiation-induced lung cancer angiogenesis and metastasis and may serve as a novel diagnostic marker of lung cancer and anti-angiogenesis treatment target in conventional photon radiotherapy.

**Methods And Materials**

**Cell culture**

A normal human bronchial epithelium cell line (BEAS-2B) and non-small cell lung cancer cell lines (A549, H1299, HCC827, and Calu-1) were all purchased from American Type Culture Collection (Rockville, MD, USA). BEAS-2B cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% FBS, 1% penicillin sodium, and 100 µg/mL streptomycin. A549, H1299, and Calu-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin sodium, and 100 µg/mL streptomycin. HCC827 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 1% penicillin sodium, 100 µg/mL streptomycin, 1 mM sodium pyruvate solution, and 2.5 mg/mL glucose. All above cells were incubated at 37°C temperature with 5% (v/v) CO₂ in a cell incubator (Thermo Fisher Scientific, Wilmington, DE, USA).

**Cell irradiation**

Cells were seeded in a T25 flask with an amount of 1×10⁶ and slightly shook the flasks to separate cells. Then the flasks were placed in a cell incubator overnight to allow the cells to adhere. The cells in X-ray irradiation group were irradiated by a RS 2000 X-ray Biological Irradiator (Rad Source Technologies, Suwanee, GA, USA) (160 kVp, 1.12 Gy/min), while the cells in the carbon ion irradiation group were irradiated by a carbon beam generated by Heavy Ion Medical Accelerator in Chiba (HIMAC) of National Institute of Radiological Science (NIRS, Japan) with a dose-averaged LET of 80 keV/µm within the spread-out Bragg peak (SOBP) (290 MeV/u). After 24 h, the total RNAs, protein, and conditional medium of irradiated cells were collected and used for the following experiments.
RNA sequencing and ceRNA network construction

X-ray or C-ion irradiated cells were lysed by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and quickly frozen into −80°C at 24 h post-irradiation. The RNA samples were sent to Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China) for RNA sequencing. In detail, after the total RNA was extracted from the sample, the ribosomal RNA (rRNA) was removed to maximize the retention of all coding RNA and ncRNA. The obtained RNA was randomly broken into short fragments, and then the first strand of cDNA was synthesized with random hexamers using the fragmented RNA template. Then the buffer solution, dNTPs (dUTP replace dTTP), RNase H, and DNA polymerase I were added to synthesize the second strand of cDNA, then synthesized cDNA is purified by a QiaQuick PCR kit and eluted by EB buffer solution. After end repair, base A adding, and sequencing connector adding, the second strand was degraded by UNG (Uracil-N-Glycosylase) enzyme. The fragment size was filtered by agarose gel electrophoresis and then following PCR amplification. Finally, the constructed sequencing library was sequenced with Illumina HiSeqTM 4000. In the data analysis program, the FPKM (fragments per kilobase of exon model per million mapped fragments) of the genes was used for filtration. Genes with FPKM over or equal to 1.0 (p < 0.05) were selected and the differentially expressed genes were chosen by DEseq2[22] with R studio software. The differentially expressed genes with fold change larger than 2.0 (p < 0.05) were selected for the following analysis. The heatmap, volcano plot and KEGG pathway enrichment analysis were operated with the R packages (ggplot2, limma, pheatmapD) [23].

Given that we focused on the ceRNA mechanism caused by irradiation, we firstly predicated the miRNAs that could bind to LINC00167 by using two different and independent online tools, RNAhybrid (minimal free energy, -e -30) [24] and ENCORI [25], which are based on miRBase. Finally, three miRNAs, miR-663a, miR-6787-5p, and miR-6816-5p were chosen for downstream analysis. Then, LINC00167, miR-663a, miR-6787-5p, miR-6816-5p, and the mRNAs up-regulated by irradiation were selected to construct the ceRNA network. Cytoscape software was performed for ceRNA network construction [26].

Gene set enrichment analysis (GSEA)

First, we calculated Spearman correlations between LINC00167 and each protein-coding mRNA in our RNA-seq results using the function cor.test in R. Next, we performed gene set enrichment analysis (GSEA) [27] by ranking the mRNAs according to their correlation coefficients. By the way, we found that the TGF-β signaling pathway appeared in the HALLMARKER gene sets and KEGG gene sets in the MSigDB[28] database at the same time, and only 15.7% of the genes in the two overlapped. In order to ensure the robustness of the analysis results, we integrated the above two pathways as the TGF-β signaling pathway including 121 genes.

Cell transfection, lentivirus packing and infection

The lentivirus overexpressing LINC00167 and matched negative control lentivirus were purchased from Shanghai Genechem Co., LTD. All shRNAs, miRNA mimics, miRNA inhibitors, and their matched negative controls were designed and produced by RiboBio (Guangzhou, China). The transfection of these shRNAs,
miRNA mimics, and miRNA inhibitors was operated by using Lipofectamine™3000 reagent (Invitrogen, USA) following the manufacturer’s instructions. The knock-down lentiviruses were created by HEK293T cells which were transfected with shRNAs lentiviral vectors and packaging plasmids (psPAX2, pMD2-VSVG) by using Lipofectamine™3000 reagent (Invitrogen, USA) following the manufacturer’s instructions. Forty-eight hours after the transfection, the media containing lentivirus particles were collected and filtered with a 0.22 µm filter, and then lung cancer cells were infected with these media for 72 h and selected with 2 µg/ml puromycin (Invitrogen). The sequences of shRNAs and siRNAs used in our experiments were listed in Supplementary Materials: Table S5.

**Western blotting**

The cell lysate was collected using RIPA lysing buffer (Beyotime, Shanghai, China). Then BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to quantify the protein concentration. All protein samples were denatured for 5 min at 100°C. The equal amounts of proteins were separated by 10% SDS-PAGE and 0.22 mm polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) was used for protein transfer. The membranes were blocked with 5% Bovine Serum Albumin (BSA, Beyotime, Shanghai, China) at room temperature for 1 h and then incubated with primary antibodies at 4°C overnight. After washing with PBST for several times, the prepared membranes were immersed in horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. The ECL kit (Millipore, Bedford, MA, USA) and a polychromatic fluorescence chemiluminescence imaging analysis system (Alpha Innotech, San Leandro, CA, USA) were used to detect the band signal on the membranes. GAPDH was used as the internal control. Antibodies against the following proteins were used, TGF-β1 (3711S, Cell Signaling Technology, Beverly, MA, USA), VEGF (19003-1-AP, Proteintech, Chicago, IL, USA), and GAPDH (5174, Cell Signaling Technology, Beverly, MA, USA). Anti-rabbit IgG (A0208, Beyotime, China) and anti-mouse IgG (A0216, Beyotime, China) were used as secondary antibodies.

**RNA reverse transcription and real time quantitive PCR (RT-qPCR)**

Total RNA was collected by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturers’ instructions. The PrimeScript RT Reagent Kit (Takara Shuzo Co., Kusatsu, Shiga, Japan) was used for lncRNAs and mRNAs reverse transcription. For miR-663a, miR-6816-5p, miR-6787-5p, and U6 RT-qPCR, the primers for RT and qPCR were designed and synthesized by RiboBio (Guangzhou, China). The cDNA amplification was operated by using SYBR Green qPCR Master Mix (Life Technologies, Grand Island, NY, USA) on a Vii7A real-time PCR system (Life Technologies, Foster City, CA, USA). GAPDH and U6 were selected to be the internal controls and all the data were analyzed with the relative quantification \(2^{-\Delta\Delta CT} \) method. The primer sets used are listed in Supplementary Materials: Table S5.

**Tube formation assays**

A549 and H1299 cells were seeded in Φ60 mm dishes with an amount of 1×10^5. When the cell density reached 60%-70%, X-ray or carbon ion irradiation was performed. The conditional medium collected from
irradiated cells and recombinant human TGF-β1 protein (PeproTech, Rocky Hill, NJ, USA) was used for tube formation assay. The pre-cooling Matrigel (Corning, New York, NY, USA) was plated in 96-well plates with 50 µL/well and put into a 37°C incubator to solidify for 30 min. Then, the HUVEC cells were digested by trypsin and collected by centrifuge (1000 rpm, 5 min), and then resuspended with the above collected conditional media and seeded in 96-well plates with an amount of 2×10^4/well. The images of the HUVEC tubes were taken by Leica inverted phase-contrast microscope (Leica, Wetzlar, Germany) at different time points and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Wound healing assays**

The transfected or infected lung cancer cells were seeded in six-well plates and grown up to 90% confluence. Then a 200 µL pipette tip was used to make linear scratch wounds for monolayer cells and washed the wells with sterile PBS three times to remove the detached cells. The wound images were taken at 0 h and 24 h by Leica inverted phase-contrast microscope (Leica, Wetzlar, Germany) and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA). The ratio of decreased wound area at 24 h to 0 h in each group was calculated to indicate the cell migration ability.

**Transwell assays**

The 24-well plates containing transwell chambers (NEST, Wuxi, China) were used to test the invasive ability of cells. 60 µL Matrigel (Corning, New York, NY, USA) was added into the upper chamber and the upper chamber was plated in a 37°C incubator to solidify for 30 min. And then 5×10^4 cells in 200 µL serum-free cell culture medium were added to the upper chamber, while the down chamber was soaked in 600 µL medium with 10% FBS. After incubation for 36 h, the remnant cells in the upper chamber were wiped off with a cotton swab slightly, while the invasive cells in the down chamber were fixed with 75% ethanol for 15 min and followed by 0.1% crystal violet staining for 20 min. Finally, an inverted phase-contrast was employed microscope to take images of five random fields of view and the number of invasive cells was analyzed by ImageJ software.

**Dual luciferase reporter assay**

The potential binding sites of miR-663a with LINC00167 and TGF-β1 3’ UTR were predicted by ENCORI. The wild-type and mutant sequences were inserted into pmiRRB-reporter Dual-luciferase vectors (RiboBio, Guangzhou, China). These vectors were named LINC00167-WT, LINC00167-Mut, TGF-β1-WT, and TGF-β1-Mut, respectively. They were co-transfected with miR-663a mimics or negative control (miR-NC) into A549 or 293T cells. Luciferase activity after transfection was determined by using the Dual-Luciferase Reporter Gene Assay Kit (Bytotime, Shanghai, China) according to the manufacturer’s instructions.

**RNA fluorescence in situ hybridization (FISH)**

To determine the localization of LINC00167 in lung cancer cells, we performed the FISH assay with Cy3-labeled LINC00167 probes (RiboBio, Guangzhou, China) and Fluorescent In Situ Hybridization Kit (RiboBio, Guangzhou, China) according to the manufacturer’s instructions. A laser scanning confocal microscope (Olympus, Tokyo, Japan) was used to acquire the cell’s images.
RNA immunoprecipitation (RIP)

Biotin-labeled LINC00167 probe, miR-663a mimics and miR-NC was synthesized by RiboBio (Guangzhou, China). A549 cells that transfected with miR-663a mimics or miR-NC were fixed with 1% formaldehyde for 10 min and lysed with Radio Immunoprecipitation Assay (RIPA) buffer (Bytotime, Shanghai, China). Then, the lysate was sonicated and centrifuged to acquire the supernatant. 50 µL supematant was collected as input and the remaining part was incubated with LINC00167 specific probes-streptavidin dynabeads (M-280, Invitrogen) mixture at 30°C for over 12h. Next, the lncRNAs-probes-dynabeads mixture was washed and added with 200 µL lysis buffer containing proteinase K. Eventually, TRizol reagent (Invitrogen, CA, USA) was used to isolate the RNA from the mixture and RT-qPCR analysis was performed.

Enzyme-linked immunosorbent assay (ELISA) of clinical serum specimens

Serum specimens from lung cancer patients who accepted X-ray or carbon ion radiotherapy were kindly provided by Prof. Lin Kong from Shanghai Proton and Heavy Ion Center (SPHIC). Mouse serum specimens were collected from treated mice at the end of the treatment. The protein content of TGF-β1 and VEGF in these serum specimens were quantied by Enzyme-Linked Immunosorbent Assay using Human/Mouse/Rat TGF-β1 ELISA Kit (Multi Sciences, Hangzhou, China), Mouse VEGF ELISA Kit (Multi Sciences, Hangzhou, China), and Human VEGF ELISA Kit (Multi Sciences, Hangzhou) respectively.

Nude mice tumorigenesis and treatment

The male BABL/c nude mice aged 6–8 weeks were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. The mice were raised in animal rooms with a specific pathogen-free (SPF) environment. The animal experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee, Soochow University. For mice subcutaneous tumor model construction, $5 \times 10^6$ A549 cells were injected into the right flank of the mice. After 18 days, when the tumor size reached approximately 100 mm$^3$, then the mice were irradiated with 5 Gy X-rays on the tumor sites, and keeping the other part of the body was shielded by lead sheets. All of the mice were divided into IR and NoIR groups, the NoIR group included 7 little groups which were named Ctrl, pcDNA3.1, pcDNA3.1(+), pcDNA3.1(+)+ TGF-β1-In, si-NC, si-LINC00167, si-LINC00167 + TGF-β1, while the IR group included 4 little groups which were named as IR, IR + si-NC, IR + si-LINC00167, IR + TGF-β1-In. The pcDNA3.1, pcDNA3.1(+), and TGF-β1-In mean the empty vector plasmids, LINC00167 overexpression plasmids, and TGF-β1 Inhibitor respectively. The si-NC and si-LINC00167 mean negative control siRNAs and LINC00167 siRNAs that designed and synthesized for animal experiments by RiboBio (Guangzhou, China). On the fifth day after irradiation, each mouse was intratumorally injected with pcDNA3.1 (5 µg, 25 µL), pcDNA3.1(+) (5 µg, 25 µL), si-NC (0.625 nmoL, 25 µL), si-LINC00167 (0.625 nmoL, 25 µL), TGF-β1-In (2 µg/mL, 25 µL), and TGF-β1 (100 ng/µL, 25 µL) respectively according to the grouping situation. Meanwhile, from the 20th day on, the mice were given intratumoral injections once every five days until all eight rounds of injections were finished on the 55th day. The body weight and tumor volume of each mouse were also recorded once every five days. Tumor
volume was calculated by the formula \( \pi/6 \times a \times b^2 \), where \( a \) means the long diameter and \( b \) means the short diameter of each solid tumor. On the 56th day, all of the mice were sacrificed for tumor and serum harvesting. The sequences of the siRNAs used in animal experiments were shown in Supplementary Materials: Table S4.

**Hematoxylin & Eosin Stain (H&E) and Immunohistochemistry (IHC)**

The dissected tumor tissues were fixed with Formalin and embedded in paraffin, then processed into sections. Hematoxylin and Eosin stain was operated with Hematoxylin-eosin (HE) Stain Kit (Solarbio, Beijing, China) following the manufactures instruction. The subcutaneous tumor sections were subjected to immunohistochemistry staining with a standard protocol. After deparaffinization and antigen retrieval, the tumor sections were incubated overnight at 4°C with antibodies of CD31 (CST, 77699), VEGF (Proteintech, 19003-1-AP), TGF-β1 (CST, 3711) and Ki-67 (CST, 9449), respectively. After incubation, the sections were washed with PBST (0.025% Triton X-100 in PBS) and incubated with secondary antibody (PV-6001, PV-6002, ZSGB-BIO, Beijing, China) at 37°C for 30 min. Finally, DAB color rendering, dehydration, and neutral balsam sealing were operated and the slides were scanned by the DMS-10 Digital Pathological Section Scanner System (Dmetrix, Suzhou, China) to acquire high-quality pictures. The positive rate of target protein in tissues was analyzed by the ImageJ plugin IHC-profiler [29].

**Statistical analysis**

The survival curve data was downloaded from the OncoLnc database (www.oncolnc.org) and constructed with the Kaplan-Meier method, and the significance of the group difference was analyzed by log-rank test. The differences between experimental and control groups were analyzed by Student \( t \)-test. Each experiment was repeated at least three times and all the results are expressed as mean ± standard deviation (SD). Two-tailed \( p \)-values were used in statistical analyses and \( p \)-value of less than 0.05 was considered to be statistically significant.

**Results**

**X-ray radiotherapy induced the upregulation of TGF-β1 and LINC00167 in NSCLC.**

To screen potential genes that respond to X-ray or C-ion irradiation in lung cancer, we collected the RNA samples of irradiated A549 cells and performed a high-throughput transcriptome sequencing analysis. Then, the differentially expressed lncRNAs and mRNAs were filtered and analyzed with R language. Figure 1A-D shows the heatmap and volcano plot of the differentially expressed lncRNAs induced by X-ray or C-ion irradiation. For upregulated lncRNAs responsive to X-ray, we performed Hazard Ratio (HR) evaluation according to data from the online database lncAR (https://lncar.renlab.org) to select the genes related to NSCLC survival. Among 46 X-ray responsive up-regulated lncRNAs (Fig. 1B&1E&1F), LINC00167 was the only lncRNA with HR > 1.0 and \( p < 0.05 \), which means that it is significantly negatively related to
NSCLC prognosis (Table S1). However, compared with X-ray irradiation, LINC00167 is not involved in the differentially expressed IncRNAs that are responsive to carbon-ion irradiation. (Fig. 1C&D).

As shown in Fig. 1G, the relative expression levels of LINC00167 in both lung adenocarcinoma (LUAD) and squamous carcinoma (LUSC) are much higher than in matched normal peritumoral tissues. Meanwhile, the overall survival (OS) rate of lung cancer patients is negatively correlated with the LINC00167 and TGF-β1 expression, which indicates that LINC00167 may function as an oncogene (Fig. 1H). Next, to explore the potential functional changes of irradiated cells, we performed KEGG pathway enrichment analysis for radiation-responsive mRNAs with the R package. The enrichment results indicated that the TGF-β1 signaling pathway was one of the significantly up-regulated pathways in X-ray irradiated cells, while not in C-ion irradiated cells (Fig. S1E&S1F). Furthermore, to explore the biological processes affected explicitly by LINC00167 after irradiation, we performed GSEA analysis for the differentially expressed mRNAs which were probably affected by LINC00167 after X-ray or C-ion radiation (the details of the analysis were shown in Materials and methods section). The results showed that high expression of LINC00167 may promote the upregulation of the TGF-β signaling pathway in X-ray irradiated cells, while the GSEA analysis results of carbon-ion induced up-regulated mRNAs didn’t exhibit significant enrichment in the TGF-beta signaling pathway (Fig. 1I&1J).

These data indicated that X-ray-induced LINC00167 and TGF-β1 upregulation may be related to the poor prognosis of NSCLC patients, and the curtailed function of carbon ion irradiation on LINC00167 and TGF-β1 promotion may contribute to its better radiotherapy effect.

**LINC00167 and TGF-β1 are highly-expressed in NSCLC cells and correlated with differential tumor angiogenesis and metastasis induced by X-ray or carbon ion irradiation.**

Angiogenesis was not only required for tumor growth but also required for tumor dissemination[30, 31]. Many studies have reported that traditional photon radiotherapy can activate tumor angiogenesis, besides its capability in killing the tumor cells[7, 32]. However, patients who received carbon ion radiotherapy were reported to have decreased metastasis and recurrence rates[12, 13]. TGF-β1 and its downstream target VEGF play important roles in tumor angiogenesis and metastasis process.

To verify the relationship between irradiation and the tumor pro-angiogenesis ability, we collected the medium of A549 cells which were irradiated with X-rays or carbon ion beams, and these conditional media (CM) were used for HUVEC incubation followed by tube formation assay to test the pro-angiogenesis ability. The data implied that CM from X-ray irradiated cells can induce a much higher level of tubulogenesis than the CM from carbon ion irradiated cells (Fig. 2A&2B). Next, to verify if the angiogenesis-related proteins were changed by irradiation, we measured the protein levels of TGF-β1 and VEGF in X-ray or carbon ion irradiated A549 cells, indicating that X-rays induced more TGF-β1 and VEGF protein expression compared with carbon ions (Fig. 2C&2D).

To further explore the correlation between radiation-induced LINC00167 and TGF-β1 expression, A549 cells were exposed to 2 Gy X-rays (160 kVp, 1.12 Gy/min) and 2 Gy carbon ions (290 MeV/u, 1.22
Gy/min), respectively, and followed with RT-qPCR analysis. At 24 h post-irradiation, the LINC00167 expression of irradiated cells was upregulated obviously, however, the TGF-β1 upregulation induced by carbon ion irradiation was slight (Fig. 2G). According to Fig. 2H, the relative expression level of LINC00167 in X-ray irradiated (2 Gy) A549 cells gradually increased after radiation, while TGF-β1 increased first but decreased after the peak. Next, we predicted the localization of LINC00167 in cells through an online tool (IncLoactor) and confirmed its subcellular distribution with the FISH experiment. The results show that LINC00167 was localized in both the nucleus and cytoplasm and more in the cytoplasm than in the nucleus (Fig. 2I-K).

The relative expression of TGF-β1 in NSCLC patients was acquired from InCAR, and it shows tumor tissues have higher TGF-β1 levels related to normal adjuvant tissues. Furthermore, we evaluated the serum TGF-β1 and VEGF protein levels in patients who received X-ray or carbon ion radiotherapy with ELISA assay. We found that patients who received X-ray radiotherapy presented higher TGF-β1 and VEGF protein levels than those who received carbon ion radiotherapy (Fig. 2M and O, Table S2). Besides, through analyzing the survival data from the online database (Oncolnc), we found that the patients with higher TGF-β1 and VEGF levels presented poor prognoses.

All these data suggested that X-rays can induce more angiogenesis than carbon ions in lung cancer radiotherapy, and LINC00167 may play an essential role in this process.

**LINC00167 is required for angiogenesis and metastasis in NSCLC cells.**

To verify the pro-angiogenesis and pro-metastasis ability of LINC00167 in lung cancer cells, we constructed the LINC00167-overexpressing lentivirus and the negative control lentiviral vector. A549 and H1299 cells were infected with the two vectors to establish the negative control cell model (LV-NC) and overexpression model (LV-LINC00167). Next, we used quantitative PCR experiments to verify the LIN00167 expression in cell models, and conditional mediums from A549 (A549-CM) and H1299 (H1299-CM) cells were collected for HUVEC tube formation assay to test the pro-tubulogenesis ability of LINC00167. The results show that the up-regulation of LINC00167 obviously promoted the pro-angiogenesis ability of both two cell lines (Fig. 3A-D).

Given that angiogenesis is essential for tumor metastasis[30, 33–35], we wondered whether overexpression or knockdown of LINC00167 would affect the cell metastasis ability of lung cancer cell lines. Furthermore, the migration and invasion ability of cell models was also tested. The results show that the migration rate (Fig. 3E-H) and the number of invasive cells (Fig. 3I-J) were all remarkably increased in LV-LINC00167. Next, to further confirm the function of LINC00167 in vitro, we constructed shRNAs targeting LINC00167 (shLINC00167#1 and shLINC00167#1) and transfected them into A549 and H1299 cells (Fig. 3M). Twenty-four hours post-transfection, the conditional mediums of A549 and H1299 cells were also collected for HUVECs co-incubation, and the pro-tubulogenesis ability of A549-CM or H1299-CM was all decreased (Fig. 3N-P). Similarly, after silencing LINC00167, the cell migration rate (Fig. 3Q-T) and invasion ability (Fig. 3U-X) were all impaired remarkably.
All these data suggested that LINC00167 may be involved in the regulation of angiogenesis and dissemination of lung cancer cells.

**LINC00167 regulates the expression of TGF-β1.**

TGF-β1 has been proven to target VEGF and play an essential role in the angiogenesis process[36–38]. According to the previous RNA-seq analysis, just like LINC00167, TGF-β1 was also found to be activated by X-ray irradiation, but not carbon ion beams. So, we explored the potential ceRNA regulation mechanism between them and found some miRNAs that can form a ceRNA network with LINC00167 and TGF-β1 mRNA. Firstly, the ENCORI online database was used to filter potential miRNAs that can form ceRNA networks with LINC00167 and TGF-β1, and ten potential miRNAs were found. Next, we used RNAhybrid online tool to filter the miRNAs with the lowest minimal free energy (<-160 kcal/mol) when binding with LINC00167 (Fig. S2). Finally, as shown in Fig. 4A, three miRNAs (hsa-miR-663a, hsa-miR-6787-5p, and hsa-miR-6816-5p) were chosen for further study. The IncRNA-miRNA-mRNA ceRNA network was constructed with Cytoscape [26] and the flowchart of the ceRNA network construction was shown in Fig. 4C. Next, we performed RT-qPCR for LINC00167 silencing cells to detect the relative expression of the three miRNAs and the results show that the miR-663a was the most significantly upregulated miRNA (Fig. 4D).

Next, to verify the regulation of LINC00167 for TGF-β1 and angiogenesis, we used lentivirus to construct stable cell lines overexpressing LINC00167, and then the relative expression of TGF-β1 and VEGF in the cells was tested through RT-qPCR and Western blotting experiments. As shown in Fig. 4E-J, the up-regulation of LINC00167 (LV-LINC00167) in the two cell lines both increased the expression of TGF-β1 and VEGF on both transcriptional and translational levels. Besides, when the cells were treated with 1 ng/mL TGF-β1 for 24 h, the VEGF expression was obviously promoted (Fig. 4F).

To further confirm the pro-angiogenesis function of LINC00167 after irradiation, we performed shRNA transfection to silence the expression of LINC00167 in A549 and H1299 cells. The data shows that down-regulation of LINC00167 inhibited the mRNA expression of TGF-β1 and VEGF, while X-ray irradiation and TGF-β1 treatment (1 ng/mL) rescued this inhibition effect (Fig. 4K&4L). Similarly, as shown in Fig. 4M-P, the silencing of LINC0167 also curtailed the protein expression of TGF-β1 and VEGF, while X-ray irradiation can recover the inhibition effect.

According to the above results, we confirmed that LINC00167 regulates the expression of TGF-β1 and VEGF, which can promote angiogenesis.

**MiR-663a inhibited angiogenesis and metastasis in NSCLC cells**

MiR-663a is one of the microRNAs which has been reported to negatively regulate TGF-β1 expression and influence tumor angiogenesis[39, 40]. Next, to explore the function of miR-663a for tumor angiogenesis in NSCLC cells by targeting TGF-β1, we employed miR-663a inhibitors or mimics to down-regulate or up-
regulate its levels in A549 and H1299 cells. As shown in Fig. 5A, compared with the NC group, the miR-663a expression of the inhibitor group was decreased by 75.01% in A549 cells ($p < 0.01$) and by 65.83% in H1299 cells ($p < 0.001$). Conversely, the mimics increased their levels by 4.30 times in A549 cells ($p < 0.001$) and by 3.75 times in H1299 cells ($p < 0.01$). Next, we measured the TGF-β1 and VEGF mRNA expression in A549 or H1299 cells transfected with miR-663a inhibitors, mimics, and NC vectors. The results show that down-regulation of miR-663a can significantly promote TGF-β1 and VEGF mRNA expression, but up-regulation of miR-663a has the opposite effect (Fig. 5B&5C). The conditional medium of A549 and H1299 cells transfected with miR-663a inhibitors or mimics were collected and used for HUVEC tube formation assay treatment. The results indicated that down-regulation of miR-663a promoted the angiogenesis level while up-regulation of miR-663a inhibited it in A549 and H1299 cells (Fig. 5D&5E). Similarly, the protein expression TGF-β1 and VEGF in A549 and H1299 cells were also up-regulated by miR-663a inhibitor and down-regulated by miR-663a mimics (Fig. 5F&5I).

Besides, we further detected the migration and invasion ability in miR-663a inhibitors or miR-663a mimics transfected cells. The results remain consistent with the angiogenesis test, the down-regulation of miR-663a obviously promoted the migration (Fig. 5J-5M) and invasive (Fig. 5N-5Q) of A549 and H1299 cells, while the up-regulation of miRNA could curtail this promotion effect.

These data illustrated that miR-663a can negatively regulate the pro-angiogenesis and metastasis ability of NSCLC cells.

**LINC00167 promotes tumor angiogenesis and metastasis by positively regulating TGF-β1 expression through sponging miR-663a.**

Given that lncRNAs can function as sponges of their interacting miRNAs, we predicted the binding sites between miR-663a and LINC00167 or TGF-β1. As shown in Fig. 6A&6B, the potential binding site between LINC00167 and miR-663a or between TGF-β1 mRNA and miR-663a are predicted by the TargetScanHuman database (www.targetscan.org)[41]. Next, we performed a dual-luciferase reporter assay to verify the interaction through co-transfection of miR-663a mimics and wild-type or mutant luciferase reporter plasmids (LINC00167-Wt, LINC00167-Mut, TGF-β1 Wt and TGF-β1 Mut) in 293T or A549 cells. The data shows that compared with the miR-NC and LINC00167-Wt co-transfection group, the luciferase activity was reduced by 25.11% in 293T cells ($p < 0.001$) and by 26.42% in A549 cells ($p < 0.001$) in miR-663a mimics and LINC00167-Wt co-transfection group (Fig. 6C-D). Similarly, compared with the miR-NC and TGF-β1-Wt co-transfection group, the luciferase activity was reduced by 31.98% in 293T cells ($p < 0.001$) and by 37.47% in A549 cells in miR-663a mimics and TGF-β1-Wt co-transfection group (Fig. 6E-F). However, in the miR-663a mimics and LINC00167-Mut co-transfection group or miR-663a mimics and TGF-β1-Mut co-transfection group, the luciferase activity didn't change (Fig. 6C-F). Besides, we further performed RIP (RNA Immunoprecipitation) experiment to confirm the binding between LINC00167 and miR-663a. The anti-Ago2 antibodies or control IgG were used to pull down LINC00167 in A549 cells which were transfected with miR-663a mimics or miR-NC. Then, the LINC00167 level was tested by RT-qPCR and the results showed that LINC00167 pulled down by anti-Ago2 antibodies
was specifically enriched in miR-663a mimics transfected cells but not miR-NC cells (Fig. 6G), meaning that miR-663a can directly bind to LINC00167. Then, we silenced or overexpressed LINC00167 in A549 cells and performed RT-qPCR to measure the miR-663a expression, and the results shows that silencing of LINC00167 promoted the miR-663a expression, while up-regulation of LINC00167 inhibited the miR-663a expression (Fig. 6H&6I).

To verify the participation of miR-663a in the process of LINC00167-induced angiogenesis, A549 and H1299 cells were firstly infected with LINC00167 silencing lentivirus (shLINC00167) and the negative control lentivirus (shNC), and then cells were transfected with miR-663a inhibitor or mimics. As shown in Fig. 6J-6L, the silencing of LINC00167 inhibited the mRNA and protein expression of TGF-β1 and VEGF, and the co-transfection of miR-663a mimics can strengthen this inhibition effect. Conversely, the transfection of miR-663a inhibitors would rescue the TGF-β1 and VEGF inhibition induced by LINC00167 silencing. Next, we collected the conditional mediums of cells and performed a tube formation assay. The data indicated that the transfection of miR-663a mimics in shLINC00167 cells showed decreased pro-angiogenesis ability, while miR-663a inhibitors’ transfection increased the pro-angiogenesis ability of the CMs (Fig. 6M-O). Similarly, the migration speed and the number of invasive cells were both significantly increased when miR-663a were inhibited in shLINC00167 cells, while the transfection of miR-663a mimics could obviously suppress the cell migration and invasion (Fig. 6P-6V).

These in vitro data indicated that LINC00167 might promote tumor angiogenesis and metastasis by regulating TGF-β1 expression through sponging miR-663a.

**LINC00167 is a potential therapeutic target for NSCLC radiotherapy**

To explore the auxiliary therapeutic effect of LINC00167 silencing for radiotherapy in vivo, we established the subcutaneous tumor mouse model by injecting A549 cells into the right flank of the BABL/c nude mice. As shown in Fig. 7A, 18 days post-injection, when the tumor size reached about 100 mm³, the mice were regrouped before treatment to make each group approximately equal in mean tumor size. Then, the tumor sites of the irradiation-treated groups were irradiated with 5 Gy X-rays while keeping the other part of the mouse body shielded by lead sheets. After irradiation, we operated drug intra-tumor injection on mice once every five days. The drugs included LINC00167 overexpression plasmids (pcDNA3.1(+), 50 ng×8), LINC00167 small interfering RNA (si-LINC00167, 0.625 nmol×8), TGF-β1 (100 ng/µL, 25 µL×8), and TGF-β1-Inhibitor (2 µg/mL, 25 µL×8). As shown in Fig. 7B&7C, the tumor growth speed and tumor size were obviously increased in the pcDNA3.1(+) group, while the TGF-β1-In combination treatment delayed the tumor growth. In the si-LINC00167 group, the tumor volume and weight all decreased relative to the si-NC group, while the TGF-β1 injection rescued this inhibition effect for tumor growth (Fig. 7B-7D). Among the four irradiation-treated groups, both si-LINC00167 and TGF-β1-In treatment remarkably delayed tumor growth. Besides, during the 40 days of treatment, no mouse died and the body weight of the mice didn't change significantly, indicating that the irradiation and drug injection has no severe side effects on the whole body (Fig. S1).
At the end of the treatment, mouse blood was collected and the serums were separated through centrifuging. As shown in Fig. 7E, the secreted TGF-β1 and VEGF protein levels were measured by ELISA experiment, and the data shows that up-regulation of LINC0067 induced by pcDNA3.1(+) increased the content of TGF-β1 and VEGF in peripheral blood, while the down-regulation of LINC00167 induced by si-LINC00167 significantly decreased the TGF-β1 and VEGF secretion. Interestingly, the four X-ray irradiation-treated groups exhibited increased TGF-β1 and VEGF levels, which was consistent with most reports. Besides, the treatment of TGF-β1 increased the TGF-β1 and VEGF in serums, while TGF-β1-In treatment decreased the secretion.

The dissected tumor tissues were processed into sections and performed with IHC and H&E stain. As shown in Fig. 7F, the expression of angiogenesis markers, such as TGF-β1, VEGF, and CD31 were all increased in the pcDNA3.1(+) group, whereas decreased in the si-LINC00167 group. The treatment of TGF-β1-In curtailed the increased angiogenesis level induced by up-regulation of LINC00167 in the pcDNA3.1(+) + TGF-β1-In group or by X-ray irradiation in the irradiation-treated group. Conversely, the TGF-β1 treatment rescued the inhibition of angiogenesis induced by si-LINC00167 in si-LINC00167 + TGF-β1 group. The down-regulation of LINC00167 also curtailed the angiogenesis caused by irradiation (Fig. 7G-I). In H&E stained sections, the microvessel density (MVD) was analyzed by ImageJ and the results indicated that upregulation of LINC00167 increased while si-LINC00167 treatment decreased the MVD in tumor tissues, with or without irradiation (Fig. 7K).

These data suggested that the silencing of LINC00167 in tumor tissues can inhibit the tumor angiogenesis levels obviously, indicating the potential of LINC00167 in clinical photon radiotherapy to function as a tumor angiogenesis inhibitor.

**Discussion**

Traditional photon radiotherapy is still the most wildly applied radiotherapy for tumor treatment[1]. However, radiation also kills the normal cells on its path. To protect the normal tissues and organs during radiotherapy and kill the tumor cells as efficiently as possible, fragmentation of radiation was always performed. That means not all tumor cells could be killed at one fraction of radiotherapy because of the insufficient dose of radiation, this window period between fractions gives the survivable tumor cells a chance to metastasize. Wild-Bode et al. have reported that the migration and invasion capacity of glioma cells were activated after 6 Gy γ-radiation[42]. Cheng et al. reported that sublethal doses of radiation may strengthen the invasion ability of hepatocellular carcinoma cells through the PI3K/Akt/NF-kappaB signal pathway[43]. Many other researchers have reported similar results[44–46]. Therefore, while killing the cancer cells, traditional photon radiotherapy may push cancer cells to acquire aggressive ability when the dose is sublethal. This causes recurrence and metastasis and makes tumor dissemination after photon radiotherapy an adverse factor for patients’ prognosis. Focusing on this problem, many researchers are trying to explore the underlying mechanisms of photon radiation-induced tumor metastasis. Bhardwaj et al. reported that radiation-induced EMT (Epithelial-Mesenchymal Transition) promoted migration and invasion through induction of the c-Met[47]. Juntermanns et al. reported that high-dose of radiation (20
Gy) can damage the tumor’s vascular obviously and induce the hypoxic condition of the tumor microenvironment[48], and then hypoxia inducible factor 1 (HIF-1α) incurred in the hypoxic condition can activate migratory and angiogenic genes such as VEGF, MMP-2, and Vimentin [49–51]. Other studies reported that radiation induces tumor metastasis through cytokine/chemokine signaling[52] or mitochondrial damage[53, 54].

Given these radiation-induced metastasis mechanisms, kinds of adjuvant therapy have been developed in recent years. Vorinostat has been proven to inhibit HIF-1α transcription and activity, which caused the metastasis decrease of several cancer types[55]. Bevacizumab is an FDA-approved monoclonal antibody targeting VEGF and has been applied in clinical radiotherapy to inhibit angiogenesis[56]. Nevertheless, the adjuvant treatment is not sufficient for clinical radiotherapy because of drug resistance and some patients are not sensitive to these drugs. Nowadays, the currency of heavy ion radiotherapy may be a good solution for this clinical problem because heavy ions can effectively reduce tumor metastasis possibility. Zhang et al. reported that compared with X-rays, carbon ion irradiation can effectively inhibit the invasion, migration, and EMT of B16F10 cells through the inhibition of extracellular matrix cleavage to enhance cell adhesion[57]. However, the exact molecular mechanism underlying the pro-metastasis difference between photon and heavy ion radiotherapy remains unclear.

Numerous studies suggest that IncRNAs and microRNAs form competing for endogenous RNA relationships (ceRNA) and regulate the target mRNAs and this mechanism has been reported to participate in the development process and tumorigenesis of various tumors [58–60]. Besides, some studies have suggested that the ceRNA network may work in tumor angiogenesis and metastasis of primary tumors[61, 62]. However, the question of whether and how the ceRNA participates in radiation-induced tumor angiogenesis and metastasis is unanswered. In addition, TGF-β1 is a kind of secreted protein that has been studied for many years, and it has been confirmed to participate in numerous cancer cell biological processes, including immune-suppressing, stemness, metastasis, angiogenesis, drug/radioresistance, and so on[63–71]. The function of TGF-β1 in radiation biology has also been explored. Researchers have found that TGF-β1 plays an important role in radiation-induced fibrosis, DNA damage, tumor metastasis, inflammation, and cellular radiosensitivity[63, 72–76]. Besides, TGF-β1 has also been found to function in migration and invasion of cancer cells induced by photon radiation[77, 78]. However, the TGF-β1 response to radiations of different quality has not been clarified.

In this study, we focused on the differential epigenetic regulation induced by X-ray and carbon ion irradiation. Based on the RNA-seq and bioinformatics analysis, we commented that TGF-β1 was involved in LINC00167-induced angiogenesis. Besides, according to acquired results, miR-663a can function as a tumor inhibitor by regulating TGF-β1 expression[39]. TGF-β1 is reported to promote VEGF expression which controls the formation of new blood vessels and functions as an essential mediator in tumor angiogenesis [36–38]. So, we proposed the hypothesis that LINC00167 curtails the competing binding of miR-663a to TGF-β1 and further promoted the VEGF expression through sponging miR-663a. Then, through comprehensive in vitro and in vivo experiments, we confirmed this hypothesis. Our research indicated that LINC00167 binds to miR-663a and forms a ceRNA regulation network with TGF-β1 mRNA.
Upon stimulation of photon irradiation, but not carbon ion irradiation, the LINC00167/miR-663a/TGF-β1 axis plays the role of regulating tumor angiogenesis and metastasis by targeting VEGF.

In conclusion, our work identified LINC00167 functioning in tumor angiogenesis and metastasis induced by irradiation. The inhibition of the LINC00167/miR-663a/TGF-β1 axis has the potential to become an auxiliary strategy for traditional photon radiotherapy by counteracting tumor angiogenesis and metastasis.

**Declarations**

**Ethics approval**

The animal experiment was approved by the Ethics Committee of Soochow University.

**Consent for publication**

All of the patients provided signed, informed consent before the use of these clinical samples for research purposes.

**Competing interests**

The authors declared there are no conflicts of competing interests in this work.

**Author Contributions**

H. Huang, W. Hu, T.K. Hei, and G. Zhou developed the concept of this work, designed the experiment project, and wrote the manuscript. H. Huang, Y. Xu, J. Nie, and Z. Guo operated the molecular and biological experiments. H. Huang, Wanshi Li, and Yidan Song performed the bioinformatic analysis of the RNA-seq results. All authors have read and agreed to the published version of the manuscript.

**Availability of data and materials**

All data generated or analyzed during this work are included in this article and its Additional files.

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Figures
Radiation-induced high expression of LINC00167 in NSCLC cells. The X-ray (2 Gy, 1.12 Gy/min) or C-ion (2 Gy) irradiated NSCLC tumor cells and un-irradiated cells were collected and subjected to RNA-seq analysis. **A&D**, Heat map shows the differentially expressed lncRNAs of X-ray (A) or C-ion (D) irradiated NSCLC tumor cell samples ($p < 0.05$, FC > 2.0). **B&C**, Volcano plot displays the significantly up-regulated and down-regulated lncRNAs that as described in (A and D) after irradiation. Blue and red dots indicated
genes that were significantly changed ($p<0.05$, FC $>2.0$). E, Pie chart shows the percentage of up-regulated and down-regulated IncRNAs analyzed by the RNA-seq results. F, Flowchart shows the process that finding the clinical-relevant IncRNAs in NSCLC. G, The relative expression of LINC00167 in NSCLC tumor and normal tissues was retrieved from lnCAR online database. H, The correlation between prognosis of NSCLC patients and LINC00167 expression was retrieved from the OncoLnc database. I&J, GSEA results show the TGF-β signaling pathway enrichment of the differentially expressed mRNA affected by LINC00167 in X-ray (I) or carbon ion (J) irradiated NSCLC tumor cells.

Figure 2

X-rays induced higher angiogenesis levels than carbon ions in NSCLC cells. A&B, The conditional medium of irradiated A549 cells was collected at different time points after irradiation and used for the HUVEC tube formation assay. C&D, The protein level of TGF-β1 and VEGF were measured 24 h after irradiation. E&F, The relative expression of LINC00167 (E) and TGF-β1 mRNA (F) in lung bronchial epithelial cells
(BEAS-2B) and non-small cell lung cancer cells (A549, H1299, Calu-1, and HCC827) were measured by RT-qPCR. G, The A549 cells were irradiated by 2 Gy X-rays (160 kVp, 1.12 Gy/min) and 2 Gy carbon ion (290 MeV/u, 1.22 Gy/min), respectively, and the relative expression of LINC00167 and TGF-β1 mRNA was measured by RT-qPCR. H, The relative expression of LINC00167 and TGF-β1 mRNA changed with time after exposure to 2 Gy X-ray. I, The LINC00167 subcellular distribution was predicted by lncLoactor (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator). J&K, Localization of LINC00167 in A549 and H1299 cells was detected by FISH. Nuclear was stained with DAPI (blue) and LINC00167 was marked by Cy3-labeled LINC00167-specific probes (red). Scale bar = 20 μm. L, The relative expression of TGF-β1 in NSCLC tumor and normal tissues was retrieved from lnCAR online database. M&O, The TGF-β1 and VEGF protein content in serum from patients who accepted X-ray or carbon ion radiotherapy was tested by ELISA. N&P, The correlation between prognosis of NSCLC patients and TGF-β1 expression (N) or VEGF expression (P) was retrieved from the OncoLnc database. All above experiments were repeated at least three times, and data is exhibited as mean ± standard deviation (SD). *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
Figure 3

LINC00167 promoted angiogenesis and metastasis in A549 and H1299 cells in vitro. A–L, A549 and H1299 cells were infected with negative control lentivirus (LV-NC) or LINC00167-overexpressing lentivirus (LV-LINC00167) and followed by RT-qPCR analysis (A), tube formation assay (B), wound healing assay (E&G), and transwell assay (I&K). Scale bar = 200 μm. Then, quantification of the number of meshes per field (C&D), relative cell migration rate (F&H), and the number of invasive cells (J&L) were performed. M–X,
A549 and H1299 cells were transfected with negative control shRNAs (shNC) or shRNAs targeting LINC00167 (shLINC00167#1, shLINC00167#2), respectively, and then subjected to RT-pPCR analysis (M), tube formation assay (N), wound healing assay (Q&S), and transwell assay (U&W). Scale bar = 200 μm. Then, quantification of the number of meshes per field (O&P), relative cell migration rate (R&T), and the number of invasive cells (V&X) were performed. All above experiments were repeated three times independently and the data are exhibited with mean ± standard deviation (SD). *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

Figure 4

The LINC00167 regulates TGF-β1 expression in NSCLC cells. A, The miRNAs that could bind to LINC00167 were selected by using two independent online tools, RNAhybrid (minimal free energy, -e -30) and ENCORI. B, The LINC00167-miRNA-mRNA ceRNA network was shown and the green, yellow, and blue nodes represent LINC00167, miRNAs, and mRNAs, respectively. C, The flowchart shows the process of
selecting clinically relevant genes and miRNAs that could form a ceRNA network with LINC00167 in NSCLC. **D&E**, A549 and H1299 cells were infected with negative control lentivirus (LV-NC) or overexpression lentivirus (LV-LINC00167), and then the relative expression of TGF-β1 (D) and VEGF mRNA (E) was tested by RT-qPCR. **F-I**, The protein expression of TGF-β1 and VEGF in A549 (F&G) and H1299 (H&I) cells was detected by western blotting. **J&K**, A549 and H1299 cells were transfected with negative control shRNAs (shNC) and knockdown shRNAs (shLINC00167) and then were exposed to 2 Gy X-rays. Twenty-four hours after irradiation, the relative expression of TGF-β1 and VEGF mRNA in A549 (J) and H1299 (K) cells were tested by RT-qPCR, while the TGF-β1 and VEGF protein levels of the irradiated cells were tested by western blotting as shown in **L-O**. All above experiments were repeated three times, and data are exhibited as mean ± standard deviation (SD). *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
Figure 5

miR-663a regulates the angiogenesis and metastasis of NSCLC cells. **A-C**, A549 and H1299 cells were transfected with negative control miRNA (NC), miR-663a inhibitors, or miR-663a mimics, respectively, and RT-qPCR was performed to quantify the relative expression of miR-663a (**A**), TGF-β1 mRNA (**B**), and VEGF mRNA (**C**) in transfected cells. **D&E**, The conditional medium (CM) of the abovementioned transfected cells was collected and used for HUVEC cells treatment. Then, the tube formation assay was operated to...
examine the pro-angiogenic ability of the CM. Scale bar = 200 μm. F-I, The protein expressions of TGF-β1 and VEGF in transfected A549 cells (F&G) and H1299 cells (H&I) were tested by Western blotting. J-M, The migration ability of transfected cells was tested by wound healing assay. Scale bar = 200 μm. N-Q, The invasive ability of transfected cells was tested by transwell assay. Scale bar = 200 μm. All above experiments were repeated three times, and data is exhibited as mean ± standard deviation (SD). *, p < 0.05; **, p < 0.01; and *** p < 0.001.
LINC00167 regulates tumor angiogenesis and metastasis through sponging miR-663a in NSCLC cells. **A&B**, The binding sites between miR-663a and wild type (WT) LINC00167 (A) or wild type TGF-β1 mRNA 3'UTR (B), and their mutant sequence (Mut) were also presented. **C&D**, The LINC00167 wild type (LINC00167-WT) or mutant (LINC00167-Mut) luciferase reporter vectors were transfected into 293T (C) and A549 (D) cells respectively. Then cells were transfected with negative control miRNA (miR-NC) or miR-663a mimics again and subjected to dual-luciferase assay. **E&F**, The same dual-luciferase assay as in C&D was performed to test the interaction between miR-663a and TGF-β1 mRNA. **G**, The anti-AGO2 RIP experiment was operated in A549 cells transfected with miR-NC or miR-663a mimics and followed by LINC00167 enrichment analysis with RT-qPCR. **H**, A549 and H1299 cells were transfected with negative control shRNAs (shNC) and knockdown shRNAs (shLINC00167), and then the relative expression of miR-663a was tested by RT-qPCR. **I**, A549 and H1299 cells were infected with negative control lentivirus (LV-NC) or overexpression lentivirus (LV-LINC00167) stably and the relative expression of miR-663a was tested. **M-O**, The shNC, shLINC00167, NC, miR-663a inhibitor, and miR-663a mimics were co-transfected into A549 cells and H1299 cells. Then the conditional medium of A549 cells (A549-CM) and H1299 cells (H1299-CM) were collected and used for HUVEC tube formation assay to test the pro-angiogenic ability. Scale bar = 200 μm. **P&S**, The migration ability of the abovementioned co-transfected A549 (P&Q) and H1299 cells (R&S). Scale bar = 200 μm. **T-V**, The invasive ability of mentioned co-transfected A549 and H1299 cells. Scale bar = 200 μm. All above experiments were repeated three times, and data is exhibited as mean ± standard deviation (SD). *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
Figure 7

Silencing of LINC00167 can decrease X-ray-induced tumor angiogenesis *in vivo*. **A**, Graphic illustration of subcutaneous A549 tumor model construction (SC Injection), X-ray irradiation, and drug treatment. (Created with BioRender.com) **B**, Photograph of dissected tumors at the end of treatment as described in (A). **C**, The subcutaneous tumor growth curve during the 40 days of treatment. **D**, The weight of dissected tumors measured at the end of treatment as described in (A). **E**, The content of TGF-β1 and VEGF protein...
in the serum of mice at the end of treatment as described in (A) was tested by ELISA. F, The dissected tumor tissues were processed into sections and then performed with H&E or IHC stain. The graphs of these sections were taken by the DMS-10 Digital Pathological Section Scanner System. Scale bar = 100 μm. G-K, The quantification results of CD31, Ki67, TGF-β1, VEGF, and MVD of dissected tumor tissues were analyzed with ImageJ software. The yellow arrow indicated microvessels in tumor sections. *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

Figure 8

Mechanistic model of X-ray irradiation-induced tumor angiogenesis and metastasis through LINC00167/miR-663a/TGF-β1 axis in NSCLC.

Supplementary Files

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