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Knockdown of LINC00511 enhances radiosensitivity of lung adenocarcinoma via regulating miR-497-5p/SMAD3

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ABSTRACT

As the most common histological subtype of primary lung cancer, lung adenocarcinoma (LUAD) causes enormous cancer deaths worldwide. Radiotherapy has been frequently used in LUAD cases, and radiosensitivity is vital for LUAD therapy. This research sought to explore the genetic factors affecting radiosensitivity in LUAD and inner mechanisms. LINC00511, miR-497-5p, and SMAD3 expression in LUAD cells were detected via qRT-PCR and western blot. CCK-8 assays, colony formation, and flow cytometry assays were employed to explore the cell viability, apoptosis, and radiosensitivity in PC-9 and A549 cells. The targeting relationship between LINC00511, miR-497-5p, and SMAD3 was verified by dual luciferase reporter assay. Furthermore, xenograft experiments were performed for the in vivo verification. In conclusion, LINC00511 was overexpressed in LUAD cells, which downregulated downstream miR-497-5p expression and mediately led to SMAD3 activation. LINC00511 downregulation suppressed cell viability while enhanced apoptosis rate in LUAD cells. Also, LINC00511 and SMAD3 were overexpressed, while miR-497-5p was downregulated in LUAD cells exposed to 4Gy irradiation treatment. Moreover, LINC00511 inhibition could block SMAD3 expression and promoted the radiosensitivity both in vitro and in vivo. These findings uncover LINC00511 knockdown promoted miR-497-5p expression and subsequently led to lower SMAD3 level, which enhanced radiosensitivity in LUAD cells. LINC00511/miR-497-5p/SMAD3 axis could be of considerable potential to enhance radiosensitivity in LUAD.

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LINC00511; miR-497-5p; SMAD3; lung adenocarcinoma; radiosensitivity

Introduction

Lung cancer remains a major cause of cancer death globally and lung adenocarcinoma is the most prevalent histological subtype of primary lung cancer.[1] Radiotherapy is a frequently used oncological method for treating lung adenocarcinoma (LUAD), and its efficacy can profoundly influence LUAD prognosis.[2] Clinicians have long been dedicating to improving radiotherapy outcomes in LUAD, thus the response to radiotherapy in LUAD has aroused wide concern in an extensive body of research.

Long non-coding RNAs (lncRNA) are discovered through functional genomics and are crucial for regulating gene expression and affect varieties of biological activities as well as disease processes especially cancer.[3] Researchers have discovered that several dynamically regulated non-coding RNAs and their downstream genetic factors could affect radiotherapy efficacy in some malignancies, like cervical cancer and hepatocellular carcinoma.[4,5] Whether and how lncRNA-mediated targets and pathways could induce changes in LUAD cell sensitivity to radiotherapy remains unclear. Therefore, we aimed to explore lncRNA and relative signals that influence radiosensitivity in LUAD. In the previous researches on cancer field, LINC00511 was reported to exert carcinogenic effect.[6–9] In lung cancer, the oncogene role LINC00511 is also widely reported.[10–12] However, its relationship with radiosensitivity and the function role in regulating the radiosensitivity in LUAD is implicit and rarely studied. Considering that lncRNA can sponge miRNAs and further regulate downstream factors and signaling pathways, we did not only look into the impacts of LINC00511 expression on LUAD but also investigate its target, miR-497-5p. It is also widely observed that miR-497-5p inhibited lung cancer cells' malignant behavior.[13,14] Li *et al.* discovered miR-497-5p's role in radioresistance of breast cancer.[15] Nevertheless, miR-497-5p's contribution to lung cancer cells' radioresistance remains unexplored.

SMAD3 was also involved in this study as an affecting factor on LUAD cell fate. SMAD family has been considered as regulatory genes of malignancy progression in several cancers.[16] Nicholas *et al.* reported the overrepresented mutation of SMAD3 promoting colorectal carcinogenesis via activating TGF-β signaling pathway.[17] A newly published paper indicated SMAD3 gene suppressed radiosensitivity in LUAD.[18] Hence, lowering SMAD3 levels is essential for making the best use of cancer radiation therapy.

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Our research focused on LINC00511, the expression of which was predicted to fluctuate in LUAD, and its downstream genetic factors. Our research proved that LINC00511 was overexpressed in LUAD cells, which blocked the proliferation of malignant cells. Besides, knockout LINC00511 brought LUAD cell lines higher sensitivity to radiation. Thus, we explored the modulation and function of LINC00511/miR-497-5p/SMAD3 axis in LUAD with radiation treatments.

Results

LINC00511 suppression inhibited cell viability in LUAD cells

By analyzing the TCGA-LUAD data using GEPIA webtool (http://gepia2.cancer-pku.cn/), we found the higher LINC00511 expression in tumor samples in comparison with the normal ones (Figure 1a). Besides, by using the Kaplan–Meier analysis (http://www.kmplot.com), we found that

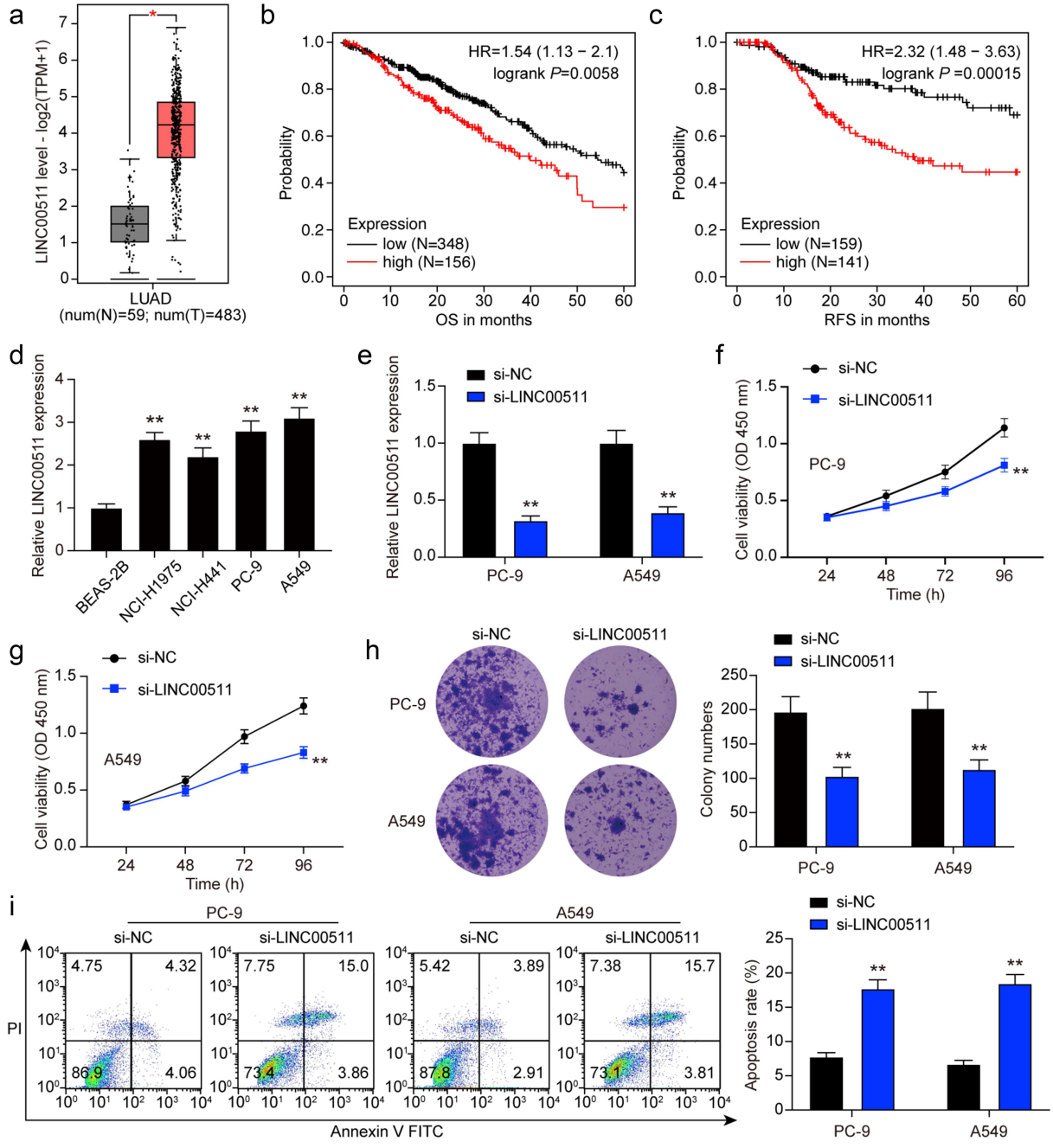

Figure 1. Upregulated LINC00511 accelerated tumorigenesis in LUAD. (a) LINC00511 expression is up-regulated in tumor samples than in the normal samples in TCGA data by GEPIA webtool. (b-c) OS (b) and RFS (c) in LUAD. OS: overall survival; RFS: disease-free survival. (d) LINC00511 expression in LUAD cell lines. (e) Transfection of si-NC or si-LINC00511 to PC-9 and A549 cell. LINC00511 downregulations in two LUAD cell lines via si-LINC00511 treatment were realized. (f-g) Cell viability of PC-9 (f) and A549 (g) cells with or without si-LINC00511 treatment. LUAD cell viability was suppressed after LINC00511 expression being inhibited. (h) Colony formation of PC-9 and A549 cells treated with si-NC or si-LINC00511. The colony number in LINC00511-transfected LUAD cells was less than negative control group. (i) Flow cytometry results indicate an upregulation of LUAD cells apoptosis occurred when LINC00511 expression was blocked. *$P < .05$, **$P < .01$, compared with the normal group or si-NC group.

LUAD patients with high expression of LINC00511 had short overall survival rate (Figure 1b) and recurrence-free survival rate (Figure 1c) in comparison with LUAD patients with low expression of LINC00511. Additionally, LINC00511 expression in LUAD cell lines was detected. It was found that that LINC00511 level was higher in the four LUAD cells than in the normal lung epithelial cell-line BEAS-2B (Figure 1d). According to the results, PC-9 and A549 cells with higher LINC00511 expression were selected for the following experiments.

Next, we examined whether LINC00511 knockdown affected PC-9 and A549 cells' viability. qRT-PCR results revealed that transfection with si-LINC00511 decreased LINC00511 expression, suggesting the successful knockdown of LINC00511 in PC-9 and A549 cells (Figure 1e & Figure S1A). CCK-8 and colony formation assay presented a suppression of cell viability in LINC00511-downregulated group (figure 1f-h). Flow cytometry outcomes indicate an upregulation of apoptosis rate when LINC00511 expression was suppressed (Figure 1i). Therefore, LINC00511 expression is higher in LUAD and LINC00511 downregulation inhibit LUAD cells' cell viability.

Irradiation promoted LINC00511 expression in LUAD, and LINC00511 inhibition enhanced irradiation anti-tumor effects in vitro

The expression of LINC00511 in PC-9 and A549 cells after IR treatment (at the dose of 0–8 Gy) was detected. Results revealed that LINC00511 was up-regulated in LUAD cells after IR treatment in a dose-dependent manner (Figure 2a). In addition, after 4 Gy IR treatment, LINC00511 level in PC-9 and A549 cells was apparently elevated with the increase in time (Figure 2b). These results suggested thatLINC00511 level is promoted by irradiation treatment. Thus, we investigate whether knockdown of LINC00511 affect radiosensitivity in LUAD cells. Cell survival was suppressed along with the escalating radiation dose (Figure 3a). Meanwhile, LINC00511 knockdown resulted in a remarkable decline of survival fractions (Figure 3b). Moreover, cell viability was decreased while apoptosis rate went up in PC-9 and A549 after si-LINC00511 or 4 Gy radiation treatment, and these effects were enhanced with the combination of si-LINC00511 and radiation treatment (Figure 3c-e). DNA damage repair was observed by γ-H2AX staining, which indicated that in PC-9 and A549, LINC00511 knockdown sparked delayed DNA DSBs repair but enhanced DNA damage (figure 3f). All these indicate that LINC00511 knockdown could potentiate LUAD cells' radiosensitivity.

LINC00511 sponged miR-497-5p in LUAD

Next, as lncATLAS (https://lncatlas.crg.eu/) predicts, LINC00511 was mainly distributed in cytoplasm (Figure 4a), which was further confirmed in PC-9 and A549 (Figure 4b). As starBase webtool presents, there are complementary sites between LINC00511 and miR-497-5p "seed sequence" (Figure 4c). To confirm this, dual-luciferase reporter assay was performed. In PC-9 and A549 cells, as was demonstrated in transfection efficiency evaluation, miR-497-5p expression went up significantly ensuing miR-497-5p mimic transfection in comparison with miR-NC-transfected cells (Figure 4d). Next, in dual-luciferase reporter assay, miR-497-5p overexpression notably decrease LINC00511-Wt reporter's luciferase activity, however, that of LINC00511-Mut reporter was barely influenced (Figure 4e), suggesting LINC00511 can bind with miR-497-5p as predicted. RIP assay result also confirmed LINC00511 could sponge miR-497-5p (figure 4f). We also detected the miR-497-5p expression in LUAD cells. It was found that miR-497-5p expression was lower in LUAD cells as compared with BEAS-2B cells (Figure 4g). Besides, in PC-9 and A549 cells after irradiation treatment, miR-497-5p expression was reduced in a time□dependent manner (Figure 4h). Additionally, qRT-PCR results presented that LINC00511 knockdown induced a notable miR-497-5p expression elevation (Figure 4i). Functional analyses unveiled miR-497-5p overexpression decreased cell viability while boosted apoptosis rate of PC-9 and A549 cells. Moreover, miR-497-5p overexpression enhanced the radiosensitivity in PC-9 and A549 cells (Figure 5a-e).

LINC00511/miR-497-5p regulated the SMAD3 expression in LUAD cells

TargetScan online database (http://www.targetscan.org/) was used to predict target genes of miR-497-5p. SMAD3 was a candidate. Based on the bind site presented in Figure 6a, dual-luciferase reporter assay was carried out. It turned out that SMAD3-Wt reporter luciferase activity in PC-9 and A549 cells was markedly downregulated via miR-497-5p overexpression

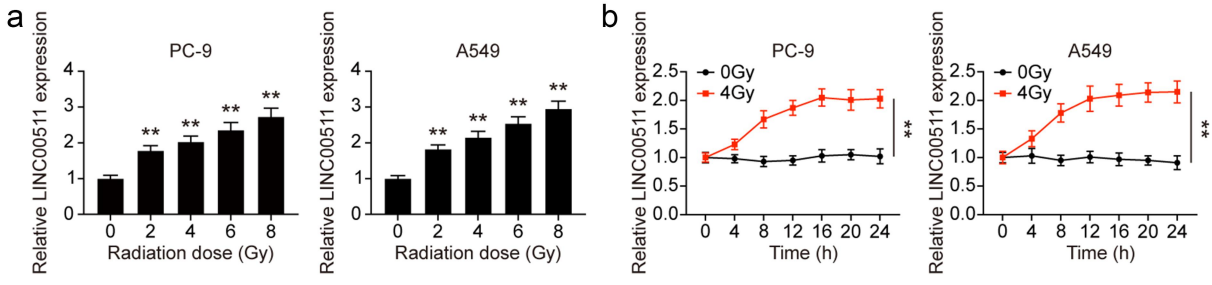

Figure 2. Upregulated LINC00511 in LUAD cells after irradiation treatment. (a) 24 h later, LINC00511 expression in PC-9 and A549 cells treated with different doses of IR was detected by qRT-PCR. (b) LINC00511 expression in PC-9 and A549 cells treated with 4 Gy IR was detected every 4 hours for 24 hours by qRT-PCR. $^{**}P < .01$, compared with 0 Gy group.

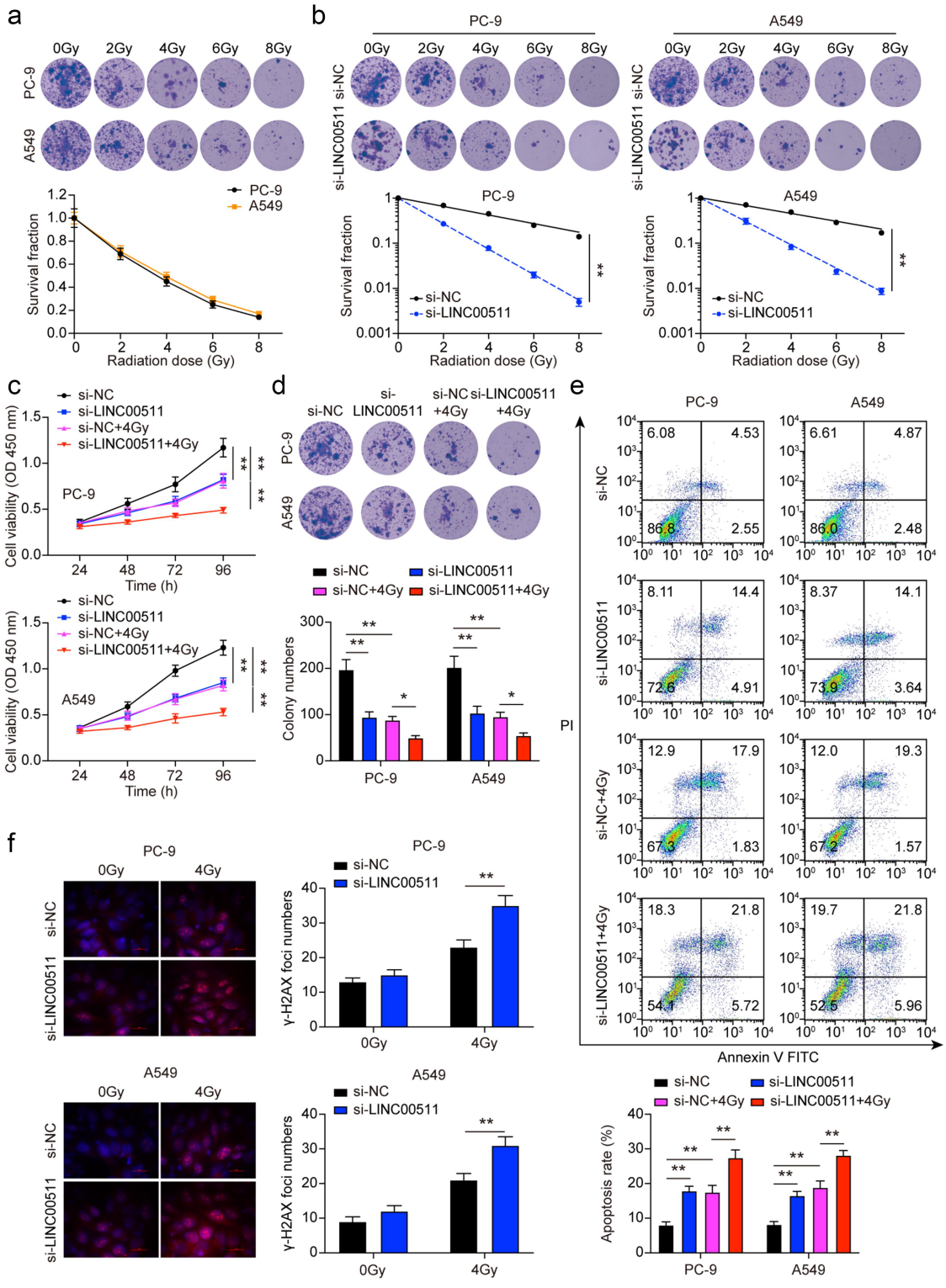

Figure 3. LINC00511 knockdown potentiated radiosensitivity of LUAD cells. (a) Survival fraction of PC-9 and A549 cells exposed to gradient dose of radiation. LUAD cell survival stepped down along with the radiation dose rising. (b) Survival fraction of PC-9 and A549 cells at different LINC00511 levels under gradient IR. The decline of LUAD cell survival via radiation treatment intensified further when LINC00511 expression was blocked. (c) Cell viability of PC-9 and A549 cells under different treatments. Radiation treatment and si-LINC00511 both decreased cell viability in two cell lines. And the combination of radiation treatment and si-LINC00511 transfection further inhibited LUAD cell viability compared to the groups with single treatment. (d) Colony formation of PC-9 and A549 cells in different groups. Colony numbers in si-LINC00511 group as well as si-NC+4 Gy radiation group were both smaller than the normal control. Si-LINC00511 + 4 Gy radiation presented additional suppression in colony formation. (e) Apoptosis rate of PC-9 and A549 cells with different treatments. Based on the flow cytometry, the apoptosis was activated in si-LINC00511-transfected or radiation-treated LUAD cells. In the group treated with si-LINC00511 and 4 Gy radiation, the apoptosis activation was further promoted. (f) γ-H2AX staining of PC-9 and A549 cells under different treatments. Cells treated by si-LINC00511 exhibited enhanced DNA damage in malignant cells treated by radiation. *$P < .05$, **$P < .01$.

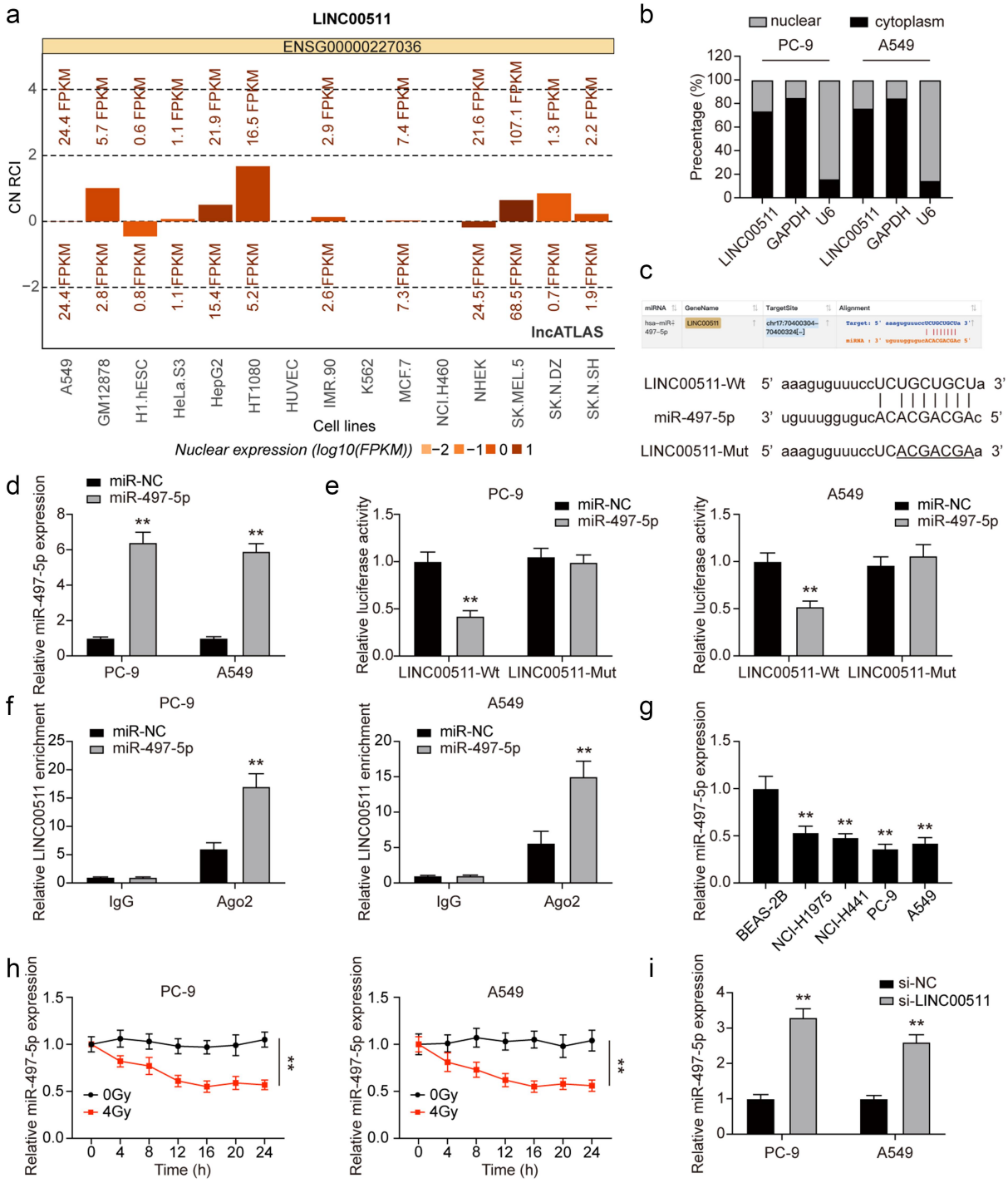

Figure 4. Cytoplasmic LINC00511 was an endogenous sponge of miR-497-5p in LUAD. (a) LINC00511 information in lncATLAS. LINC00511 was predicted to locate in cytoplasm. (b) Subcellular location of LINC00511. Both in PC-9 and A549 cells, LINC00511 was identified to be located in cytoplasm. (c) Targeting relationship between LINC00511 and miR-497-5p. miR-497-5p was predicted binding to LINC00511. (d) Transfection of miR-NC or miR-497-5p to PC-9 and A549 cells. miR-497-5p expression was successfully upregulated in two LUAD cell lines after transfection. (e) Luciferase assays in PC-9 and A549. (f) LINC00511 enrichment in PC-9 and A549. Luciferase assays and miRNA array testing both proved miR-497-5p binds to LINC00511. (g) miR-497-5p expression in LUAD cell lines. (h) miR-497-5p expression in PC-9 and A549 cells treated with 4 Gy IR was detected every 4 hours for 24 hours by qRT-PCR. (i) MiR-497-5p expression in PC-9 and A549 cells transfected with si-NC or si-LINC00511. LINC00511 upregulation in LUAD cells inhibited miR-497-5p expression. $^{**}P < .01$, compared with the NC group or 0 Gy group.

mediation, while the SMAD3-Mut reporter luciferase activity was not changed (Figure 6b), verifying that miR-497-5p can bind with SMAD3 3′-UTR via putative-binding sites. Besides, SMAD3 expression was higher in LUAD cells than in BEAS-2B cells (Figure 6c). And irradiation treatment promoted SMAD3 expression in a time□dependent manner (Figure 6d-e). We also detected the SMAD3 expression changes in PC-3 and A549 cells with LINC00511 knockdown or miR-497-5p overexpression. The results showed a significant reduction of SMAD3 mRNA and protein expression was observed ensuing LINC00511 suppression or miR-497-5p overexpression (Figure 6e-h). All these demonstrated that LINC00511 could serve as a ceRNA of miR-497-5p to sequester miR-497-5p from SMAD3, leading to miR-497-5p decrease as well as SMAD3 increase in LUAD cells.

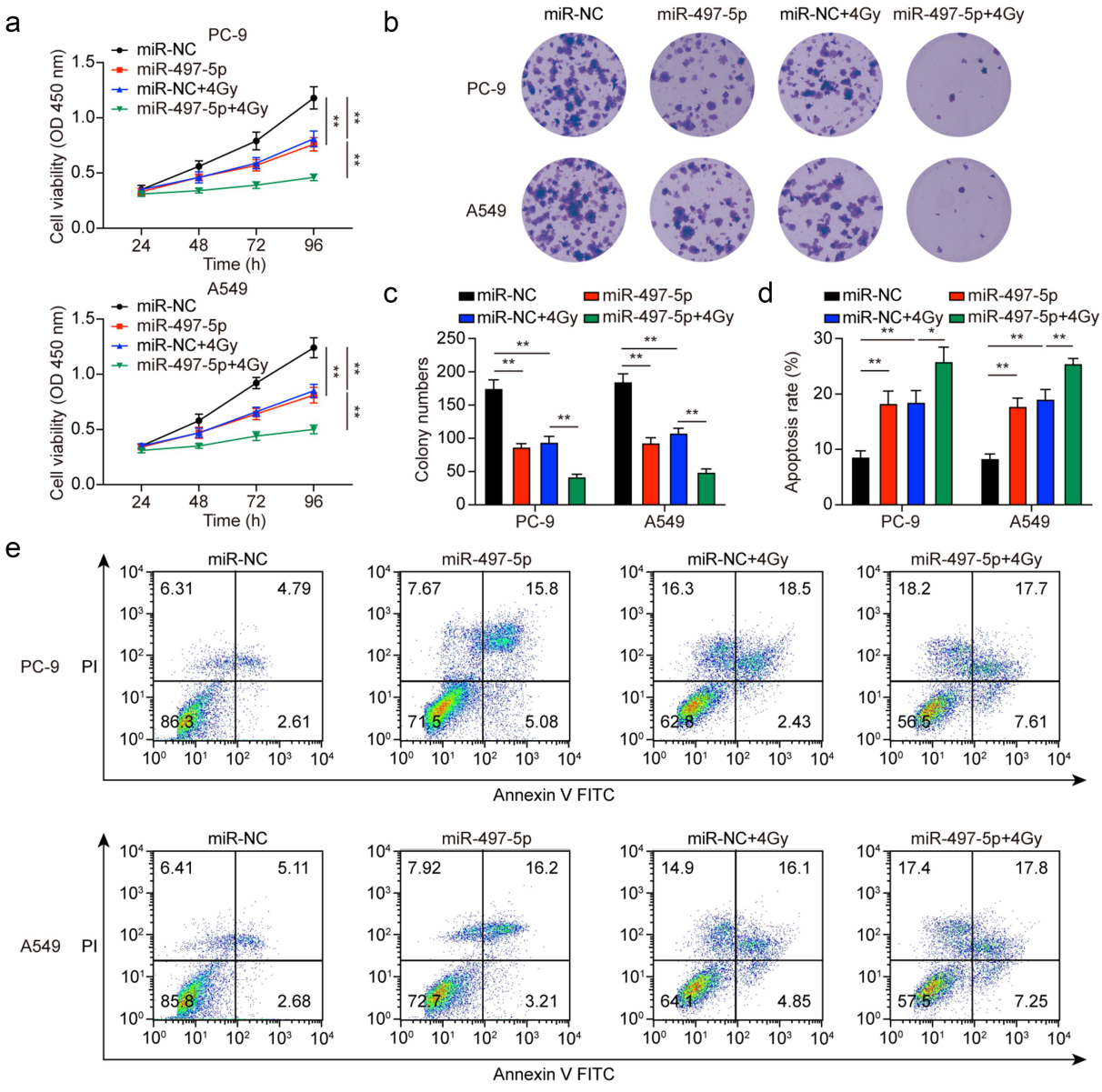

Figure 5. miR-497-5p overexpression promoted the radiosensitivity of LUAD cells. (a) Cell viability of PC-9 and A549 cells under different treatments. Radiation treatment and miR-497-5p both decreased cell viability in two cell lines. The combination of radiation treatment and miR-497-5p transfection further inhibited PC-9 and A549 cells viability compared to the groups with single treatment. (b-c) Colony formation of PC-9 and A549 cells in different groups. Colony numbers in miR-497-5p or 4 Gy radiation treated LUAD cells were both lower than the normal control. And the miR-497-5p+4 Gy radiation presented additional suppression in colony formation. (d-e) Apoptosis rate of PC-9 and A549 cells with different treatments. Flow cytometry results reveal that the apoptosis was activated in miR-497-5p group or 4 Gy radiation group. In miR-497-5p+4 Gy radiation group, apoptosis activation was further enhanced. *$P < .05$, **$P < .01$.

SMAD3 suppression reinforced the radiosensitivity of LUAD cells

To further explore SMAD3′s role in LUAD, si-SMAD3 and its negative control si-NC were synthesized. qRT-PCR and western blot displayed si-SMAD3 transfection led to a marked SMAD3 expression drop in PC-9 and A549 (Figure 7a-b& Figure S1B). Radiation clonogenic survival assay suggested SMAD3 knockdown resulted in a marked fall of cell survival fractions in PC-9 and A549 cells exposed to radiation (Figure 7c). Moreover, SMAD3 knockdown enhanced radiation treatment-induced apoptosis (Figure 7d-e). In other words, SMAD3 knockdown promoted LUAD cells' radiosensitivity.

LINC00511 regulated the radiosensitivity in LUAD cells via miR-497-5p/SMAD3

Above results suggested that LINC00511 could affect miR-497-5p and SMAD3 expressions in LUAD cells. Fluctuations of these genetic factors influenced LUAD cell abilities. To profile a complete regulation system of LINC00511/miR-497-5p/SMAD3 in LUAD, PC-9 and A549 cells were treated with si-NC, si-LINC00511, si-LINC00511+ anti-miR-497-5p, or si-LINC00511+ SMAD3. In comparison with the si-NC group, si-LINC00511 transfection induced a significantly decline in SMAD3 expression, which was reversed with additional anti-miR-497-5p or SMAD3 treatment (Figure 8a-b). CCK-8 assay unfolded LINC00511 downregulation further suppressed

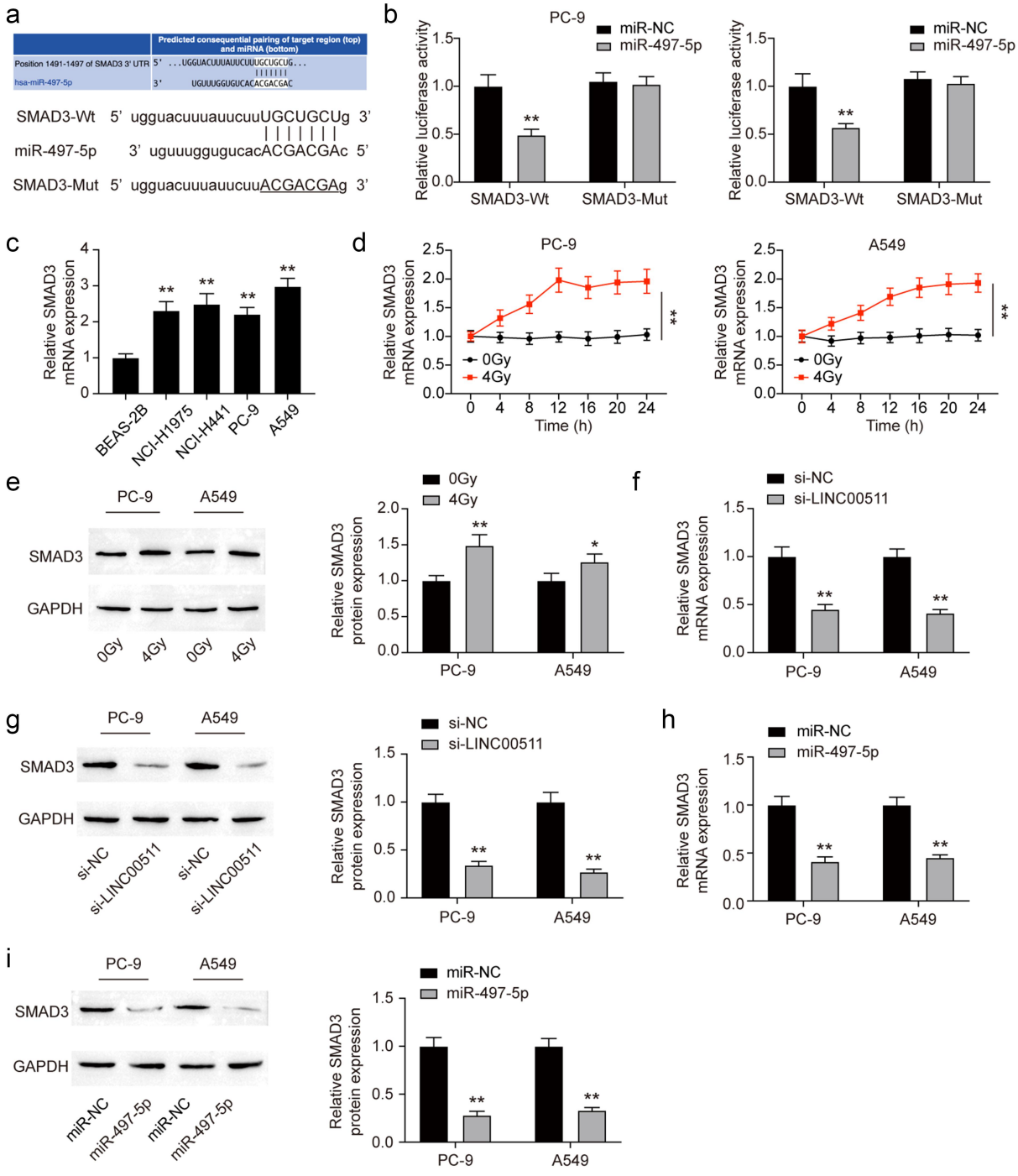

Figure 6. LINC00511 regulated SMAD3 expression via miR-497-5p. (a) Targeting relationship between miR-497-5p and SMAD3. (b) Luciferase assays in PC-9 and A549 cells. SMAD3 binding to miR-497-5p was confirmed in LUAD cells. (c) SMAD3 expression in LUAD cell lines. (d) SMAD3 expression in PC-9 and A549 cells treated with 4 Gy IR was detected every 4 hours for 24 hours by qRT-PCR. (e) 24 hours later, SMAD3 expression in PC-9 and A549 cells treated with 4 Gy IR was detected by western blot. (f-g) 48 hours later, the mRNA (f) and protein (g) level of SMAD3 in PC-9 and A549 cells transfected with si-LINC00511 was detected by qRT-PCR and western blot. (h-i) 48 hours later, the mRNA (h) and protein (i) level of SMAD3 in PC-9 and A549 cells transfected with miR-497-5p mimics was detected by qRT-PCR and western blot. *$P < .05$, **$P < .01$, compared with the NC group.

radiation-treated PC-9 and A549 cells' viability, and anti-miR -497-5p or SMAD3 overexpression treatment could restore the benefit effect (Figure 8c). Colony formation in two cell lines treated with radiation presented the same phenomenon as cell viability changes (Figure 8d). Flow cytometry in PC-9 and A549 revealed that apoptosis triggered by radiation was further promoted via si-LINC00511 transfection, which was repressed with additional anti-miR-497-5p or SMAD3 treatment (Figure 8e). Therefore, LINC00511/miR-497-5p/SMAD3 axis was proved to modulate the radiosensitivity in LUAD cells. Through blocking LINC00511, miR-497-5p expression was enhanced and mediately downregulated SMAD3 level, which was capable to further enhance radiation treatment effects in LUAD cells.

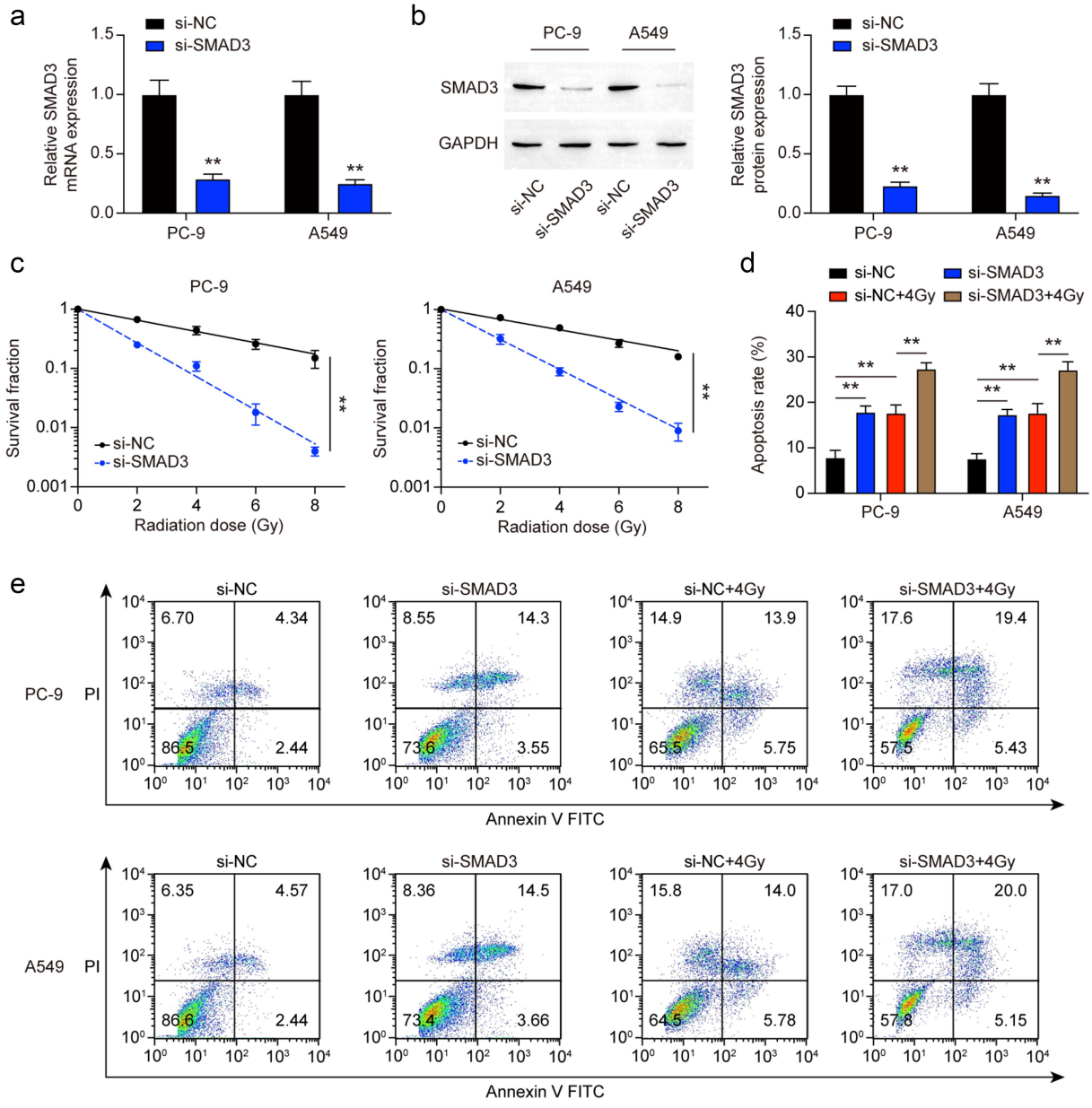

Figure 7. SMAD3 suppression enhanced the radiosensitivity of LUAD cells. (a) SMAD3 mRNA expression in PC-9 and A549 cells treated with si-NC or si-SMAD3. (b) Western blot of SMAD3 in cells with or without si-SMAD3 treatment. (c) PC-9 and A549 cells' survival fraction at different SMAD3 levels under gradient radiation treatment. The decline of LUAD cell survival via radiation dose rising was expanded after SMAD3 downregulation. (d-e) Apoptosis rate of PC-9 and A549 cells under different treatments. In accordance with flow cytometry results, apoptosis was activated in si-SMAD3-transfected or 4 Gy radiation-treated LUAD cells. In the group treated with the combination of si-SMAD3 and 4 Gy radiation, cell apoptosis was further promoted. $^{**}P < .01$, compared with the NC group.

LINC00511 knockdown enhanced radiosensitivity of LUAD xenograft tumors

To investigate whether LINC00511 could exert regulating impacts on LUAD *in vivo*, xenograft tumors were established in nude mice. Tumor volume and weight were suppressed by sh-LINC00511 or 4 Gy radiation treatment, and the suppression was strengthened in the group treated by sh-LINC00511 + 4 Gy radiation combination (Figure 9a-c). Ki-67 level was also found to be reduced dramatically in sh-LINC00511 + 4 Gy group (Figure 9d). Also, qRT-PCR assay presented a noticeable fall of LINC00511 expression and an obvious climb of miR-497-5p level in 4 Gy radiation-treated LUAD xenograft tumors after introducing sh-LINC00511 (Figure 9e). In western blot outcomes, SMAD3 protein expression in LUAD xenograft tumors with LINC00511 knockdown and 4 Gy radiation treatment significantly shrunk (figure 9f). Therefore, LINC00511 downregulation led to SMAD3 inhibition and enhanced the radiosensitivity in LUAD xenograft tumors.

Discussion

This study observed an upregulation of LINC00511 in LUAD, and LINC00511 inhibition suppressed the cell viability in LUAD cells, which coincides with previous reports implying LINC00511's oncogenic role. Lu *et al.* reported that LINC00511 could result in breast cancer tumorigenesis.[19] Another study by Sun *et al.* reported LINC00511 could promote gastric cancer cell growth.[20] Xue *et al.* reported that

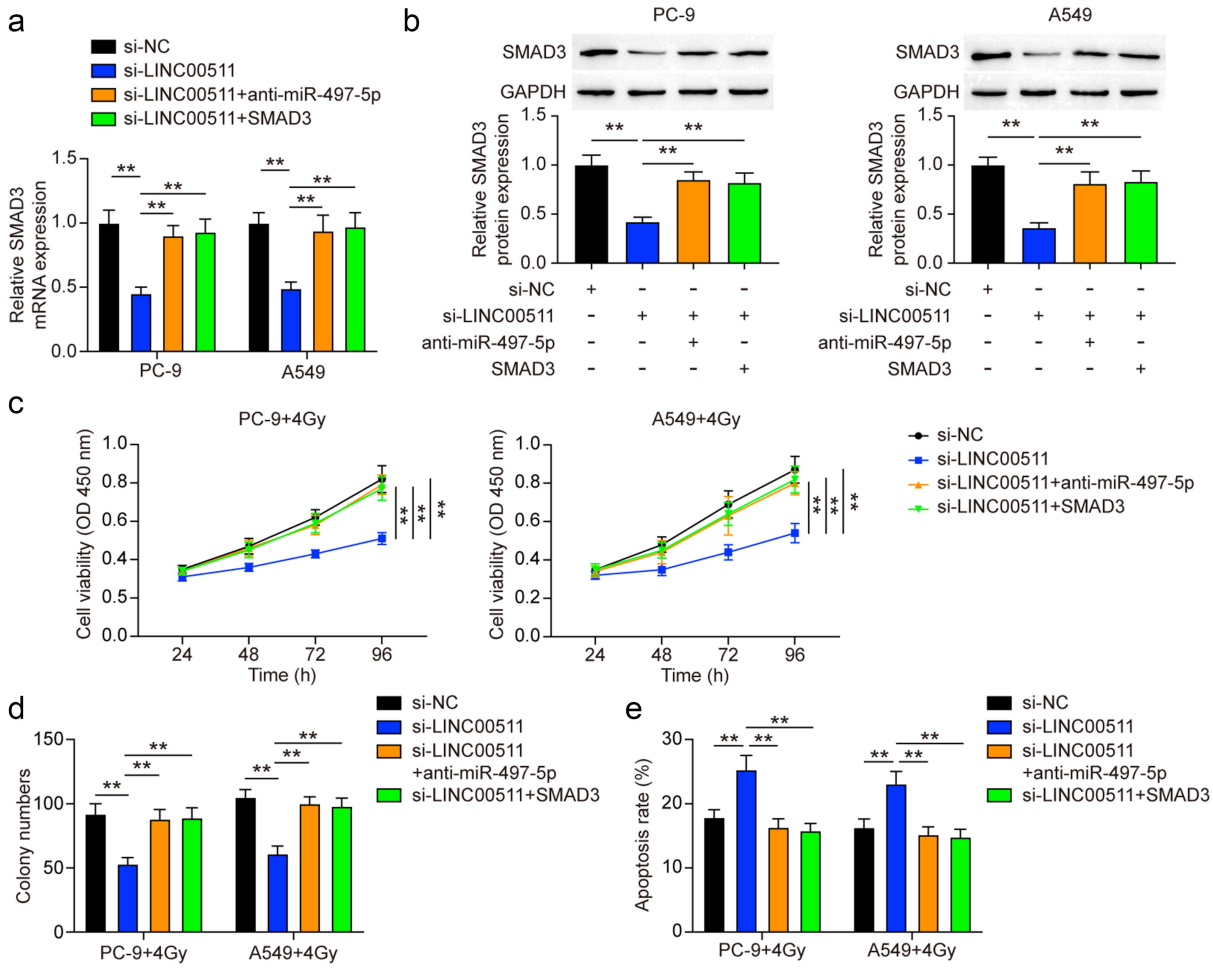

Figure 8. LINC00511 inhibition activated miR-497-5p and mediately downregulated SMAD3, which further promoted radiosensitivity in LUAD cells. (a) SMAD3 mRNA expression in PC-9 and A549 cells of different groups. (b) SMAD3 protein expression in PC-9 and A549 cells with different treatments. Compared to the NC, si-LINC00511 transfection induced a sharp decline in SMAD3 expression, which was reversed with additional anti-miR-497-5p or SMAD3 treatment. (c) Cell viability of 4 Gy radiation-treated PC-9 and A549 cells with different treatments. Downregulated LINC00511 decreased LUAD cell viability, and along with the prolonged radiation time the decreasing was more significant, which was reversed with miR-497-5p inhibition or SMAD3 activation. (d) Colony formation of 4 Gy radiation-treated PC-9 and A549 cells with different treatments. LINC00511 downregulation suppressed colony formation of IR-treated LUAD cells while additional miR-497-5p inhibition or SMAD3 activation reactivated it. (e) Apoptosis rate of 4 Gy radiation-treated PC-9 and A549 cells in different groups. 4 Gy radiation-triggered apoptosis was further promoted via si-LINC00511 transfection, which was repressed with additional anti-miR-497-5p or SMAD3 treatment. $^{**}P < .01$, compared with the NC group.

LINC00511 could be a contributor to lung cancer.[11] More importantly, we investigated the impact of LINC00511 in LUAD's radiosensitivity. The results disclosed that LINC00511 inhibition could improve radiosensitivity of LUAD cells. Similarly, Chen *et al.* discovered that LINC00511 knockdown promotes thyroid carcinoma cells' radiosensitivity.[21] Besides, our study also indicated that LINC00511 loss intensified IR-mediated antitumor effects in LUAD xenograft tumors. Based on our findings, LINC00511 inhibition could be promising adjuvant strategy for radiotherapy.

Over the last few years, lncRNAs in cytoplasm has emerged as competing endogenous RNAs in regulating cancer development by sponging certain miRNA to release miRNAs-targeted mRNAs from degradation at post-transcriptional level.[22,23] LINC00511 was proved to be a cytoplasmic lncRNA in LUAD cells. In the investigation of downstream mechanisms, LINC00511 serves as a sponge for miR-497-5p to induce SMAD3 expression in LUAD. As a typical miRNA, miR-497-5p's contribution to suppressing tumor has been elaborated in many studies.[24–27] Previous studies also revealed miR-497-5p promoted radiosensitivity of breast cancer cells,[15] cervical cancer cells,[28] and esophageal squamous cell carcinoma cells.[29] Moreover, bioinformatics analysis suggested SMAD3 as a possible miR-497-5p target. Belonging to the SMAD family, SMAD3 is critical for transcription in the TGF-β–SMAD signaling pathway. What's more, SMAD3 engaged in numerous biological processes such as cell proliferation and differentiation, tumorigenesis, angiogenesis, embryogenesis, and bone formation.[30,31] Abnormal upregulation/activation of SMAD3 expression is observed in lung cancer.[32,33] Also, knockdown of SMAD3 is reported to enhance the radiosensitivity of lung adenocarcinoma.[18]

Our research discovered that LINC00511 knockdown promoted miR-497-5p expression and subsequently decreased SMAD3 level both *in vitro* and *in vivo*. Through functional analyses, it was discovered miR-497-5p overexpression sapped cell viability and facilitated cell apoptosis in LUAD. We also disclosed miR-497-5p overexpression could promote LUAD cells' radiosensitivity, as underpinned by the decline of cell

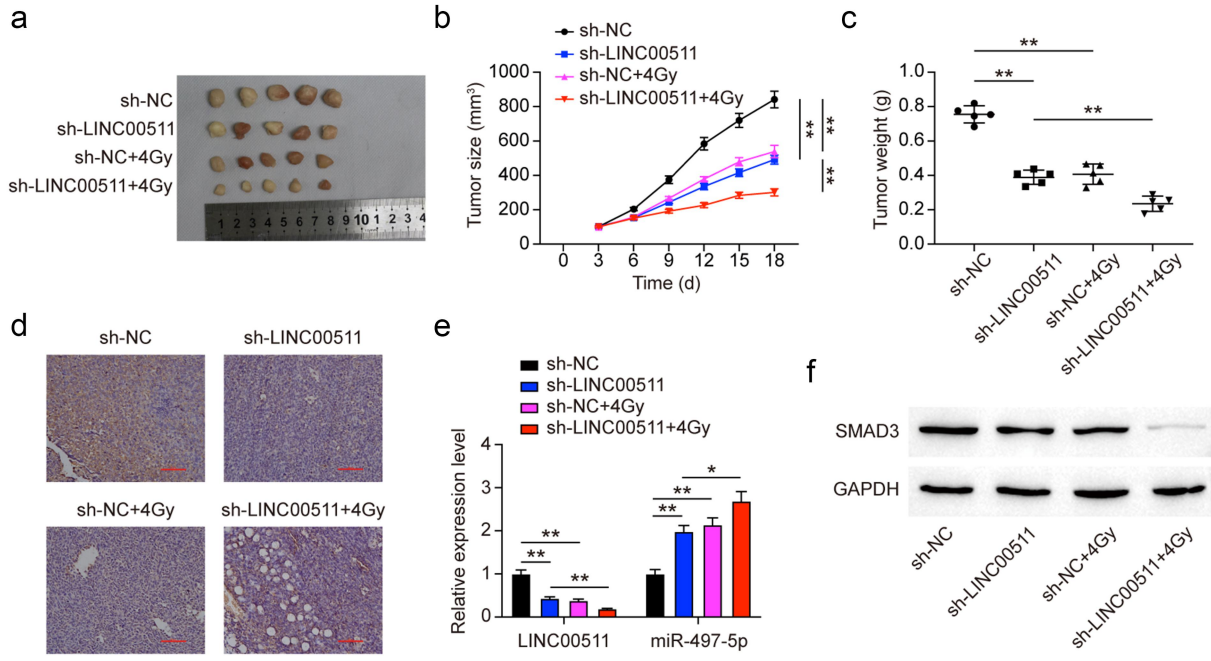

Figure 9. LINC00511 downregulation suppressed SMAD3 expression and enhanced radiation *in vivo*. (a) Tumor images of LUAD xenograft tumor from different group. (b) Tumor size monitoring with a caliper at day 3, 6, 9, 12, 15, and 18 after injection. (c) Tumor weight measurement at day 18 after injection. (d) IHC staining of Ki67 in LUAD xenograft tumors. (e) qRT-PCR assay used for determining LINC00511 and miR-497-5p expression in LUAD xenograft tumors. (f) Analyze SMAD3 expression in LUAD xenograft tumors via western blot. $^{**}P < .01$, compared with the NC group.

survival fraction and cell viability, as well as cell apoptotic rate growth in radiation-exposed cells ensuing miR-497-5p upregulation. In addition, restoration experiments showed that miR-497-5p inhibition nullified LINC00511 inhibition on cell survival, viability, and apoptosis in LUAD cells exposed to radiation, indicating that miR-497-5p regulated LINC00511's radiosensitivity.

Moreover, this research unveiled the positive regulation of SMAD3 expression by LINC00511 in LUAD cells. In addition, SMAD3 knockdown triggered diminishing cell viability as well as growing cell apoptotic rate in LUAD. SMAD3 deficiency elevated LUAD cells' radiosensitivity. There was a recent study corresponding to our research which demonstrated that knockdown of SMAD3 raised the proportion of G2/M phase cells and LUAD's radiodensity.[18]

In conclusion, this research discovered that via regulation of miR-497-5p/SMAD3, LINC00511 depletion suppressed LUAD tumorigenesis *in vitro*. Moreover, LINC00511 knockdown could trigger LUAD cells' radiosensitivity via upregulation of miR-497-5p and downregulation of SMAD3. This research sheds light on the role of LINC00511 in regulating LUAD tumorigenesis and radioresistance. It also highlights LINC00511′s potential in the prognosis, treatment, and radioresistance prevention of LUAD.

Materials and methods

Cell lines and cell culture

Human normal lung epithelial cell-line BEAS-2B and 4 LUAD cell lines (NCI-H1975; NCI-H441; PC-9; A549) were obtained from Shanghai iCell Bioscience Inc. BEAS-2B were seeded in DMEM medium supplemented with 10% fetal bovine serum (FBS, Gibco, Waltham, MA) and 1% Pen/Strep solution. NCI-H1975, NCI-H441 and PC-9 cells were cultivated in RPMI 1640 medium with 10% FBS and 1% Pen/Strep solution. A549 cells were cultivated in F-12 K medium with 10% FBS and 1% Pen/Strep solution. All above cells were incubated in a 5% CO_2 humidified environment under 37°C.

Cell transfection

To analyze the role of LINC00511 in LUAD, two cell lines (PC-9 and A549) were separated into two groups, the negative control one (NC, transfected with si-NC) and the experimental one transfected with si-LINC00511. Moreover, miR-497-5p mimics and miR-497-5p inhibitor (anti-miR-497-5p) was applied for promoting the miR-497-5p expression and inhibiting the miR-497-5p expression. SMAD3 level was modulated via si-SMAD3 or SMAD3 overexpression vector. All the transfections were conducted with Lipofectamine 2000 (Invitrogen). After 48 hours of transfection, the cells were harvested for subsequent experiments.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated with the TRIzol reagent (Invitrogen, CA, USA), followed by the cDNA synthesis with the reverse transcription kit (Ribobio, Guangzhou, China). In compliance with manufacturer protocol, the qRT-PCR analysis was performed with the SYBR Green PCR Master Mix (Takara), followed by the $2^{-\Delta\Delta Ct}$ method. The expression of the gene was relative to GAPDH (for lncRNA and mRNA) or U6 (for miRNA). Prime sequences were shown in Table S1.

Western blot

Total protein was extracted by RIPA lysis buffer (Beyotime, Shanghai, China) and quantified using a BCA kit (Beyotime). Then, 20 μg protein samples were separated by SDS-PAGE and transferred to PVDF membranes. After adding 5% skimmed milk, the membranes were incubated with the primary antibodies (anti-SMAD3, ab40854, 1:2000; anti-GAPDH, ab9485, 1:2500) at 4°C overnight, and the secondary antibody at 37°C for 1 hour. The blots were then visualized with the enhanced chemiluminescence regent (Millipore, Billerica, MA, USA).

Colony formation assay and X-ray radiation treatment

Transfected cells were seeded into 6-well plates with 2000 cells per well and incubated overnight in 5% CO_2 prior to exposure to 0, 2, 4, 6, or 8 Gy of X-ray radiation. One week later, cell colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet solution (Sigma-Aldrich) for 15 minutes respectively. Lastly, positive colonies with over 50 cells were recorded.

CCK-8 assay

A Cell Counting Kit-8 kit was utilized for cell viability identification. Before exposed to sham or 4 Gy radiation, cells were cultivated at a density of 5×10^3 cells per well in 96-well plates. After 24 hours, 10 μL CCK-8 solution was supplemented to each well and underwent 4-hour incubation at 37°C. Cell viability was subsequently assessed by measuring the absorbance at 450 nm.

Flow cytometry apoptosis analysis

Flow cytometry assay is conducted to determine the cell apoptosis with different treatments. Transfected PC-9 and A549 cells were put into six-well plates and went through 24-hour incubation and then exposed to sham or 4 Gy of radiation. At 24 h post irradiation, cells were double-stained with the Annexin V-FITC/PI detection kit. Eventually, a flow cytometry (BD Biosciences) was employed to measure the cell apoptosis rate.

γ-H2AX staining

To observe whether and how LINC00511 could affect DNA repairing in LUAD cells, a γ-H2AX staining kit was utilized. Cells were firstly resuspended in culture medium to 5×10^5 cell/mL. Next, 100 μL of cell suspension was put into each well and went through incubation at 37°C with 5% CO_2 for one night. After medium getting removed, cells were fixed with 100 μL of 3.7% Formaldehyde/PBS, washed with PBS, added with 100 μL ice-cold 90% methanol, and washed again. 200 μL blocking buffer was added to cells for 30 minutes. Then the wells got aspirated and supplemented with 100 μL of antibody against phospho-Histone H2AX (ab2893; Abcam) for 1 hour at room temperature. After that, secondary antibody was added for incubation at room temperature for 1 hour. DAPI was employed to perform nuclei staining. After final wash, stained cells were viewed by fluorescent microscope.

Luciferase reporter assay

LINC00511-Wt, SMAD3-Wt and their respective mutations (LINC00511-Mut, SMAD3-Mut) were synthesized by GenePharma (Shanghai, China) and inserted into the pmirGLO dual-luciferase plasmid (Promega, Madison, WI, USA). Next, the constructed vectors were introduced to PC-9 and A549 cells using Lipofectamine 2000. In accordance with manufacturer protocol, after 48-hour transfection, relative luciferase activity was examined with a Dual-luciferase Reporter Assay Kit (Promega).

Xenograft assay

Animal experiments were performed under institutional procedures and guidelines. Twenty male BALB/c nude mice (5-week-old) were randomly divided to four groups with five in each one. Thereinto 2 groups were injected approximately 5×10^6 PC-9 cells treated by sh-LINC00511, while the other 2 groups were injected sh-NC-transfected PC-9 cells as negative control. After that, one of sh-LINC00511 groups was treated with 4 Gy radiation, so was sh-NC groups (given 5-consecutive-day 4 Gy radiation until tumors grew into the average 100 mm^3 size). The tumor size measurement was conducted every 3 days using the formula: 0.5×(shortest tumor diameter)^2×longest tumor diameter. The mice were sacrificed at 18 days after inoculation. Each group's final tumor size and weight were measured and recorded. All animal experiments have obtained the approval of the Animal Care and Use Committee of the Qujing Affiliated Hospital of Kunming Medical University under Grant [number kmmu2021594].

Immunohistochemistry

All tissues were cut into 4-μm sections, which went through incubation with an anti-Ki67 antibody (1:200, Abcam) at 4°C overnight. Next, biotinylated secondary antibodies were incubated for 1 h at room temperature and visualized with diaminobenzidine substrate (Sigma-Aldrich, St. Louis, MO, USA). An Olympus microscope was used to take immunohistochemistry (IHC) images.

Statistical analysis

All assays involved were independently repeated for at least 3 times. Data from three replications were averaged, analyzed by GraphPad Prism version 9.0 (GraphPad Software, CA, USA) and reported as mean value ± standard deviation (SD). *T*-test was utilized to determine differences between the groups while those among three groups or more were evaluated by one-way ANOVA. *P* value under 0.05 was deemed as statistically significant.

Declarations

Ethics approval and consent to participate

Our investigation has obtained approval from the Qujing Affiliated Hospital of Kunming Medical University under [number kmmu2021594].

Disclosure statement

The authors report there are no competing interests to declare.

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