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Original article

Regulator of G protein signaling 20 contributes to radioresistance of non-small cell lung cancer cells by suppressing pyroptosis

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A R T I C L E I N F O

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A B S T R A C T

Objective: To investigate the potential role of the regulator of G protein signaling 20 (RGS20) in radioresistance of non-small cell lung cancer (NSCLC).

Methods: A total of 35 lung adenocarcinoma (LUAD) patients from The Cancer Genome Atlas (TCGA), who underwent radiotherapy, were enrolled and divided into radiosensitive ($n = 16$) and radioresistant ($n = 19$) groups based on clinical prognosis. The expression and prognosis of RGS20 were analyzed by Gene Expression Profiling Interactive Analysis (GEPIA) database. A radioresistant cell line (A549R) was constructed by irradiating A549 cells with 6 Gy X-rays for 10 fractions. Cell survival was measured by colony formation assay. The regulatory effect of RGS20 on pyroptosis were verified by LDH release and Western blot assay, and the underlying mechanism was investigated by transfecting RGS20 siRNA and applying a GSDMD inhibitor).

Results: A total of 2,181 differentially expressed genes (DEGs) were identified by analyzing the data of radiosensitive and radioresistant individuals from the TCGA-LUAD dataset. These DEGs were enriched in G alpha (z) signalling events analyzed by Reactome database. RGS20 exhibited significant upregulation among the DEGs, and its higher expression predicted poor prognosis in LUAD patients. In vitro, the expression of RGS20 protein was increased by irradiation in A549 cells, whereas it remained at much high levels in A549R cells regardless of irradiation. After irradiation, the expressions of pyroptosis-related proteins were significantly increased in A549 cells ($P < 0.05$), with no significant changes were observed in A549R cells. Treatment with LDC7559 significantly reduced LDH release ($P < 0.01$) and improved the survival rate of irradiated A549 cells ($P < 0.01$). Furthermore, knockdown of RGS20 gene in A549R cells significantly increased LDH release ($P < 0.001$) and enhanced radiosensitivity ($P < 0.01$), while LDC7559 administration reversed LDH release ($P < 0.01$) and radiation-induced cell death increased by siRGS20 ($P < 0.05$). Meantime, the increased expression level of GSDMD-NT was observed in A549 and A549R cells transfected with siRGS20 ($P < 0.05$).

Conclusion: RGS20 contributes to the radioresistance of NSCLC cells, which might be a potential target for NSCLC radiotherapy.

1. Introduction

Lung cancer remains a leading cause of cancer-related mortality globally, and non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all cases.[1] The majority of patients are diagnosed in advanced stages, missing the optimal window for surgical intervention.[2] For advanced-stage NSCLC patients, radiotherapy is one of the mainstream modalities. With advancements in treatment technologies, such as stereotactic radiotherapy, intensity modulated radiotherapy, proton and heavy ion radiotherapy, as well as ultra-high dose rate (FLASH) radiotherapy, NSCLC can be effectively managed, leading to improved overall survival rate of patients. However, tumor radioresistance limits the efficacy of radiotherapy, often resulting in local recurrence.

RGS20, a member of the regulator of G protein signaling (RGS) family that acts as GTPase-activating protein (GAP), accelerates GTP hydrolysis on the α-subunits of heterotrimeric G proteins and results in termination of signaling pathways downstream of G protein-coupled receptors (GPCRs).[3] Through its regulatory role in GPCR signaling, RGS20 has been

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implicated in the development of various malignant tumors, including oral squamous cell carcinoma[4], breast cancer[5,6], lung cancer[7], bladder cancer[8], and penile cancer.[9] However, despite its emerging role in cancer biology, the specific function of RGS20 in the radiosensitivity of NSCLC and its underlying molecular mechanism remain to be elucidated.

In this study, we identified RGS20 as a gene of interest by analyzing the data of patients undergoing radiotherapy from the TCGA-LUAD dataset and explored the relationship between its expression level and prognosis. The level of RGS20 expression and cellular pyroptosis were detected in A549 and A549R cells, and the effect of knockdown of RGS20 on radiosensitivity were investigated. Moreover, an inhibitor of pyroptosis was used to confirm the role of RGS20 in GSDMD-mediated pyroptosis.

2. Materials and methods

2.1. Bioinformatic analysis

The data of patients undergoing radiotherapy from the TCGA-LUAD dataset was downloaded from UCSC Xena.[10] We manually divided them into radiosensitive ($n = 16$, OS $>$ 24 months) and radioresistant ($n = 19$, OS $<$ 24 months) groups according to the overall survival. The Bioconductor package limma[11] was utilized to analyze the quantitative differentiation between two identified groups. The DAVID database[12] was employed to process Reactome pathways enrichment. GEPIA clinical database[13] was used to analyze the difference in RGS20 gene expression levels in LUAD tumor tissues and adjacent normal tissues, as well as the correlation between RGS20 expression and the overall survival of LUAD patients.

2.2. Cell culture and treatment

Human non-small cell lung cancer (NSCLC) of A549 cells were purchased from Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Yeason, Shanghai, China) and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO_2 in a humidified incubator (Thermofisher, Waltham, MA, USA). A549 cells were authenticated using STR profiling (Chinese Academy of Science, Shanghai, China).

LDC7559 is a specific-GSDMD inhibitor.[14] To inhibit cellular pyroptosis, A549 or A549R cells were w incubated with 5 μmol/L LDC7559 (MedChemExpress, Monmouth Junction, NJ, USA) for 6 h before irradiation.

2.3. Construction of radioresistant cell lines

To construct a stable radioresistant cell line, A549 cells in logarithmic phase were fractionally irradiated with 6 Gy X-rays once every week in general with a total dose of 60 Gy at a dose rate of 1 Gy/min (X-RAD 320, PXI Inc., North Branford, CT, USA; 12 mA, 2-mm aluminum filtration). One day before irradiation, 3×10^6 cells were seeded in a 100 mm culture dish. After each irradiation, the cells were passaged more than two times and confirmed to be viability enough for the subsequent irradiation. When the total dose reached 60 Gy, the survived and stable passage A549 cells became radioresistant A549 cells named as A549R cells.

2.4. Colony formation assay

The radiosensitivities of A549 and A549R cells were determined using a colony formation assay. After irradiation with different doses (0, 2, 4, or 6 Gy) of X-rays at a dose rate of 1 Gy/min (X-RAD 320), the cells were immediately trypsinized to generate a single cell suspension and plated in triplicate in 6-well plates at suitable density. The cells were cultured for two weeks and fixed with methanol for 20 min. After staining with crystal violet (Beyotime, Shanghai, China), the visible colonies containing at least 50 cells were counted. Besides, the survival curve was fitted with the single-hit multitarget model $SF = 1-(1-\exp(-k \times D))^N$ using GraphPad Prism 10 software (San Diego, CA, USA).

2.5. Western blot assay

Total cellular protein extraction was performed using RIPA buffer containing 100 mmol/L phenylmethanesulfonyl fluoride (PMSF, Beyotime) with a loading buffer (Beyotime) con-taining protease inhibitor. An equal amount of protein was subjected to 12.5% SDS-PAGE gel at a constant voltage and then transferred to a polyvinylidene difluoride (PVDF) mem-brane (0.45 μm, Millipore, Burlington, MA, USA) for further antibody binding reactions. After being blocked with 5% non-fat milk in Tris-buffered saline/Tween 0.05% (TBST) for 1.5 h, the PVDF membranes were incubated with a primary antibody of anti-RGS20 (1:1,000, ABclonal, Wuhan, China), anti-Caspase 1(1:1,000, Cell signaling Technology, Inc., Danvers, MA, USA), anti-GSDMD (1:500, Affinity, Changzhou, China) and anti-β Tubulin (1:1,000, ABclonal) at 4°C overnight. After incubated with anti-Rabbit secondary antibody (1:3,000, Beyotime) for 1.5 h, the proteins were finally detected using an ECL kit (Tanon, Shanghai, China) and analyzed using the ChemiDoc XRS system (Tanon).

2.6. Lactate dehydrogenase (LDH) release assay

LDH released to the extracellular medium was determined using LDH release kit (Beyotime). Briefly, A549 or A549R were seeded at a density of 1×10^5 cells in 12-well plate the night before irradiation. The level of LDH release was measured at 48 h post irradiation. Briefly, the LDH release reagent was added into the maximum enzyme activity control well for 1 h, then 1 ml of the culture supernatant was harvested and centrifuged for 5 min (400 *g*, 4°C). 120 μl of supernatant from each well was transferred into a 96-well plate for subsequent measurement. Fresh culture medium was used as blank control. The OD value was detected at a wavelength of 490 nm by the microplate reader (BioTek Synergy H1, Winooski, Vermont, USA).

2.7. Small interfering RNA (siRNA) transfection

A549 and A549R cells were transfected with RGS20-specific siRNA (siRGS20, target sequence: 5′-GAG AAG UGA UCA ACA GAA ATT-3′) and its negative control (siNC target sequence: 5′-ACG UGA CAC GUU CGG AGA ATT-3′) at a final concentration of 20 pmol/μl using RFect transfection agent (Changzhou Bio-generating Biotechnology Co., LTD, Changzhou, China) according to the manufacturer's instruction. After 48 h of culture, the transfection efficiency was evaluated by Western blot assay.

2.8. Statistical analysis

The experimental data were analyzed using GraphPad Prism10 software (San Diego, CA, USA). Results were expressed as the mean ± standard error mean (SEM) for at least three independent experiments. Statistical significance was evaluated using Student's *t*-test between two groups and using one-way analysis of variance (ANOVA) for more than two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of gene expression differences in radiotherapy response

To explore the key genes potentially participating in NSCLC radioresistance, we downloaded the data of patients undergoing radiotherapy from the TCGA-LUAD dataset and divided them into radiosensitive (OS $>$ 24 months) and radioresistant (OS $<$ 24 months) groups according to

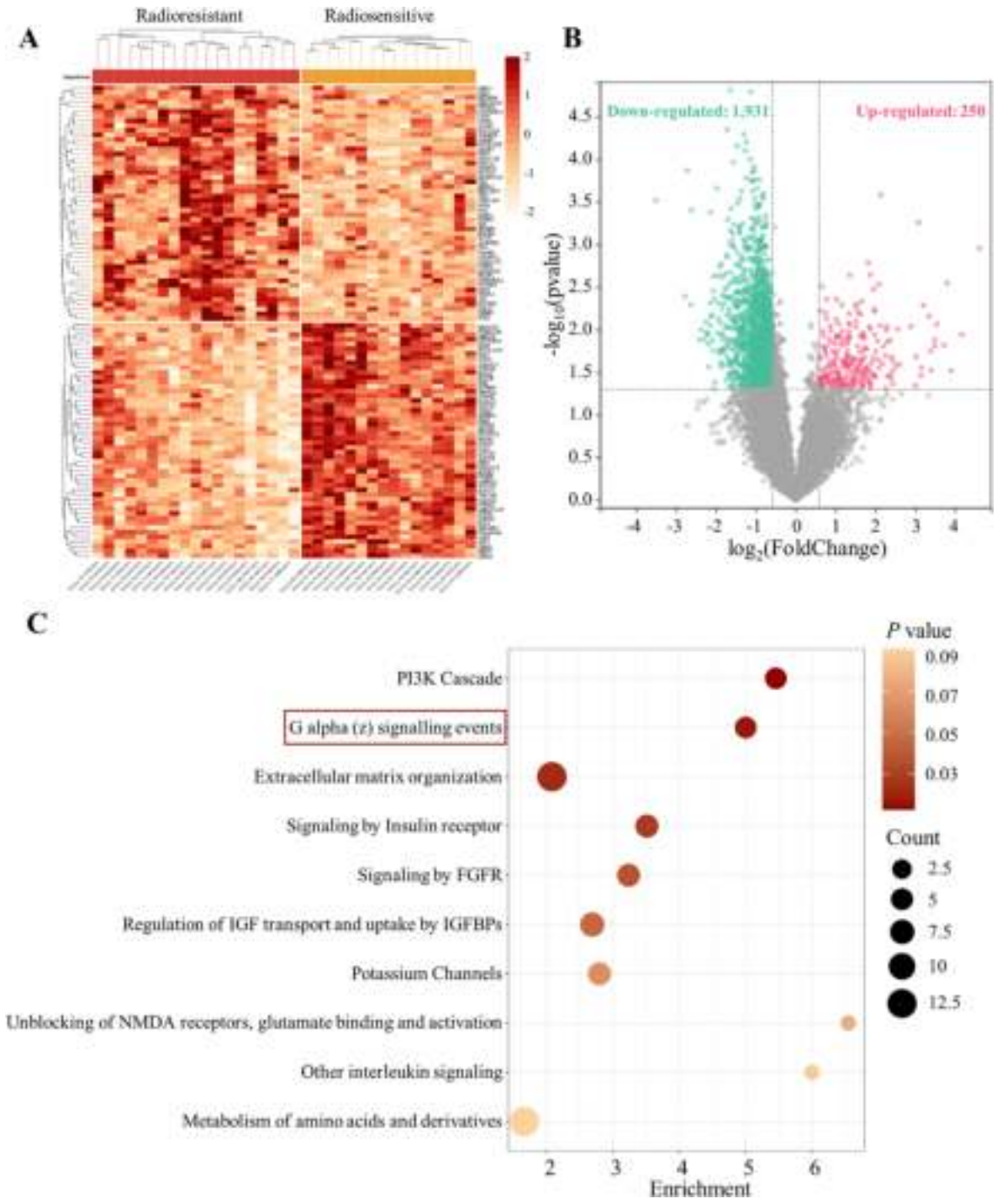

Fig. 1. RNA-seq analysis of the paired radiosensitive and radioresistant LUAD patients in TCGA datasets. A. Heatmap of DEGs and normalized gene expression values were rescaled from −1 to 1. B. Volcano plot of the genes. Red dots represent upregulated genes, green dots represent downregulated genes, and gray dots represent no significant change genes. $P < 0.05$ is considered statistically significant with the threshold of fold change >1.5 or fold change <0.6667 (i.e., |log2 fold change| > 0.585). C. Ten Reactome pathways enriched by the DEGs.

overall survival. RNA-seq of tumors from these patients were analyzed. A cluster analysis of differentially expressed genes (DEGs) between the radiosensitive and radioresistant groups was performed (Fig. 1A), and a volcano plot was displayed (Fig. 1B), which showed that the gene expression profiles of these two groups were significantly different. In total, 250 genes were upregulated, and 1,931 genes were downregulated in the radioresistant group compared with the radiosensitive group. (Fig. 1B). A Reactome pathway enrichment analysis of DEGs was performed, revealing the top ten enrichment pathways (Fig. 1C). Notably, the DEGs exhibited a significant enrichment score in G alpha (z) signalling events ($P < 0.05$).

3.2. Relationship between RGS20 and the survival of LUAD patients

The Regulator of G protein Signaling (RGS) family is well known for its critical role in negatively regulating GPCRs signaling cascades.[3] However, their contribution to radiosensitivity remains largely unexplored. RGS20, a significantly upregulated gene, was found to be

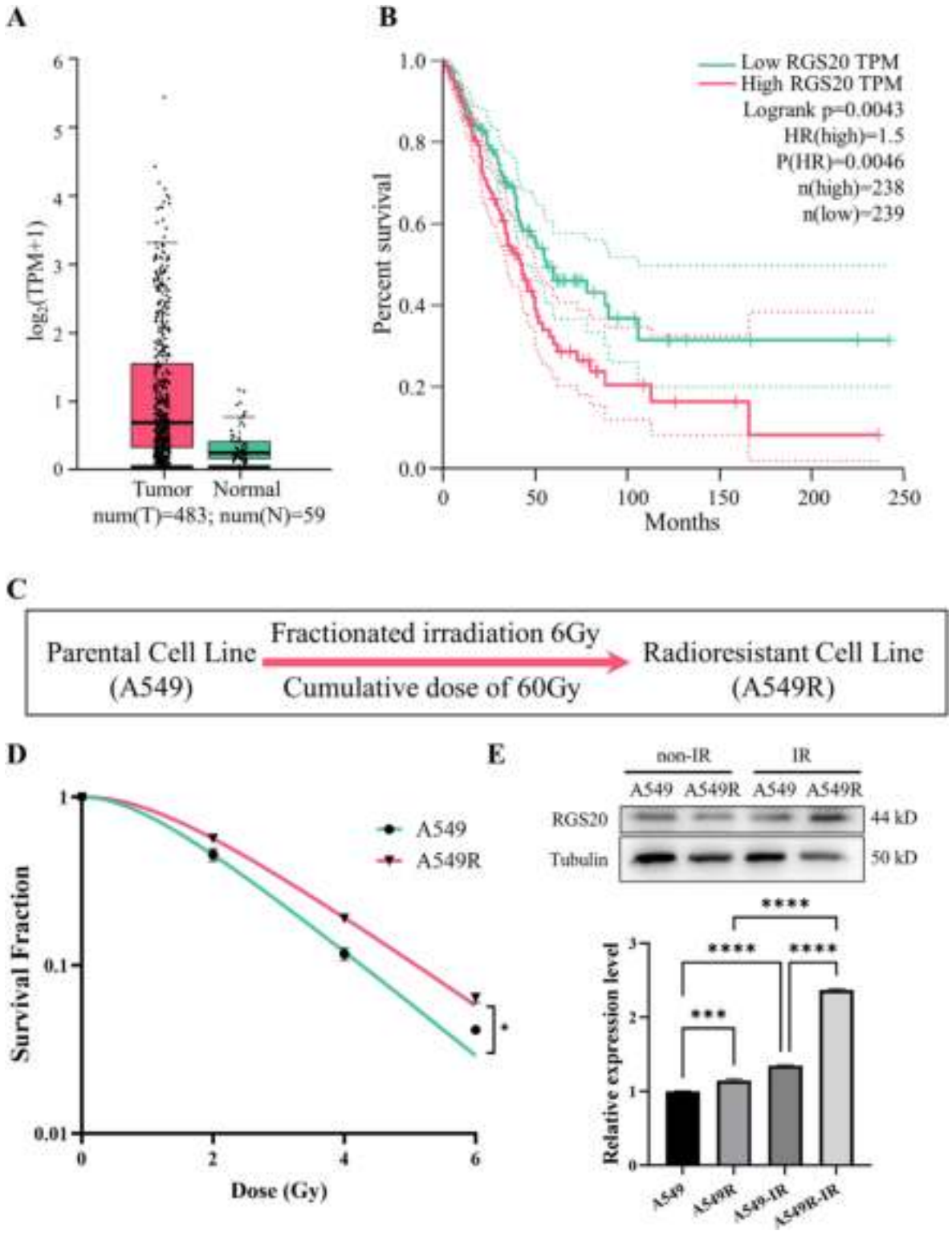

Fig. 2. The effect of RGS20 gene on the prognosis of LUAD and the expression of RGS20 in radioresistant A549R cells and its parental cells. A. The level of RGS20 in LUAD tumor tissue and adjacent normal tissue. B. The correlation between the level of RGS20 and the overall survival (OS) of LUAD patients. C. Pattern of the establishment of A549R radioresistant cell line. D. Survival curves of A549 and A549R cells after X-ray irradiation, $^{*}P < 0.05$. E. Western blot assay of RGS20 and its relative expression level in A549 and A549R cells. $^{***}P < 0.001$; $^{****}P < 0.0001$.

involved in the pathway of G alpha (z) signaling events. GEPIA online database analysis showed that the expression level of RGS20 gene in LUAD tumor tissues was higher than that in adjacent normal tissues (Fig. 2A). Meanwhile, LUAD patients with high expression levels of RGS20 exhibited significantly lower overall survival (OS) compared to those with low expression levels of RGS20 (Fig. 2B). These findings suggested that RGS20 might enhance radioresistance of NSCLC.

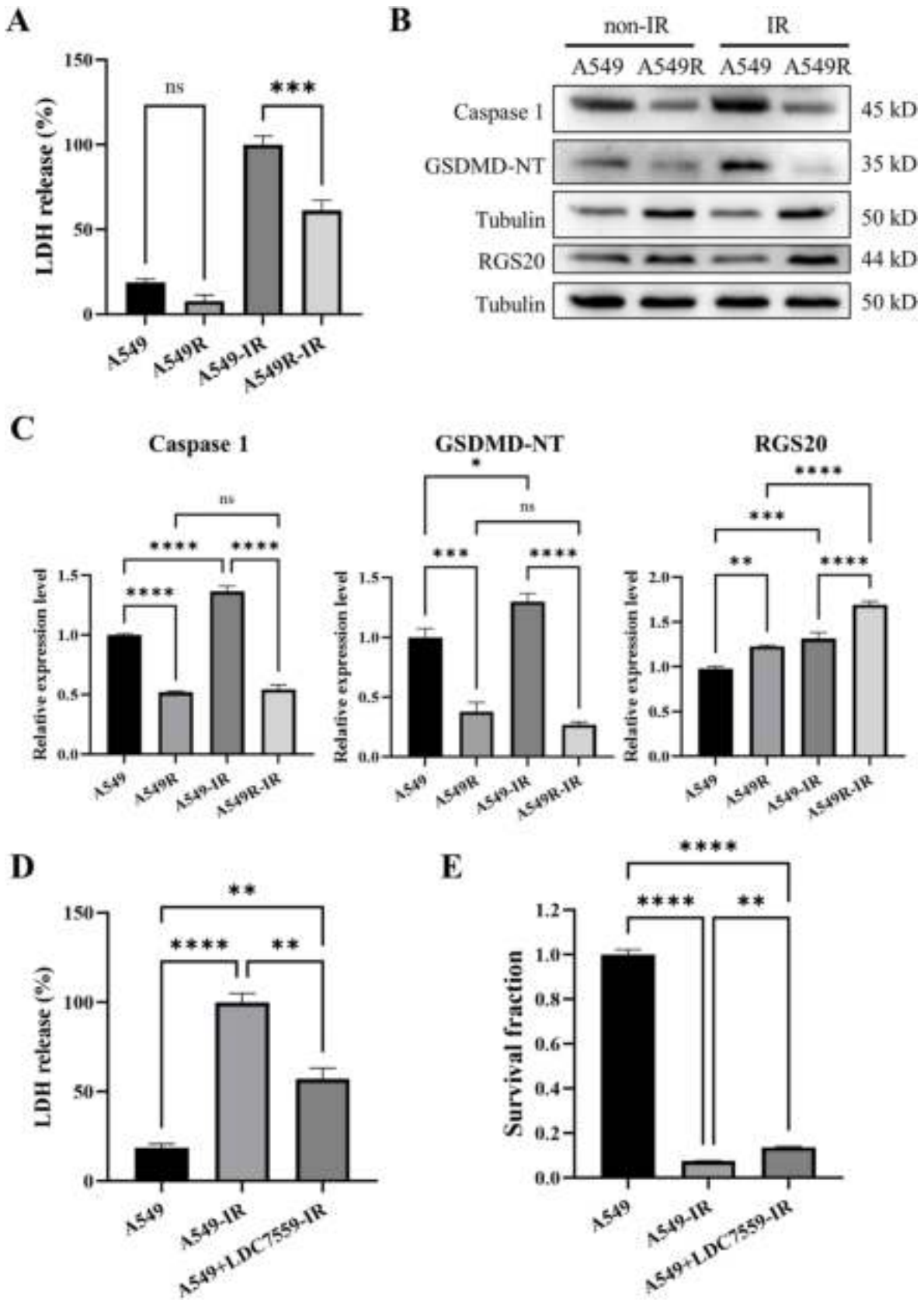

Fig. 3. The role of pyroptosis in radioresistance. A. The levels of released LDH in A549 and A549R cells were detected at 48 h after IR. B–C. Western blot assay of Caspase 1, GSDMD-NT and RGS20 proteins and their relative expression levels in A549 and A549R cells at 6 h after IR. D. A549 cells were incubated with 5 μmol/L LDC7559 at 6 h before IR. After 48 h of IR, the levels of released LDH were detected as indicated. E. Clonogenic survivals of A549 cells under above treatments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; ns, no significance.

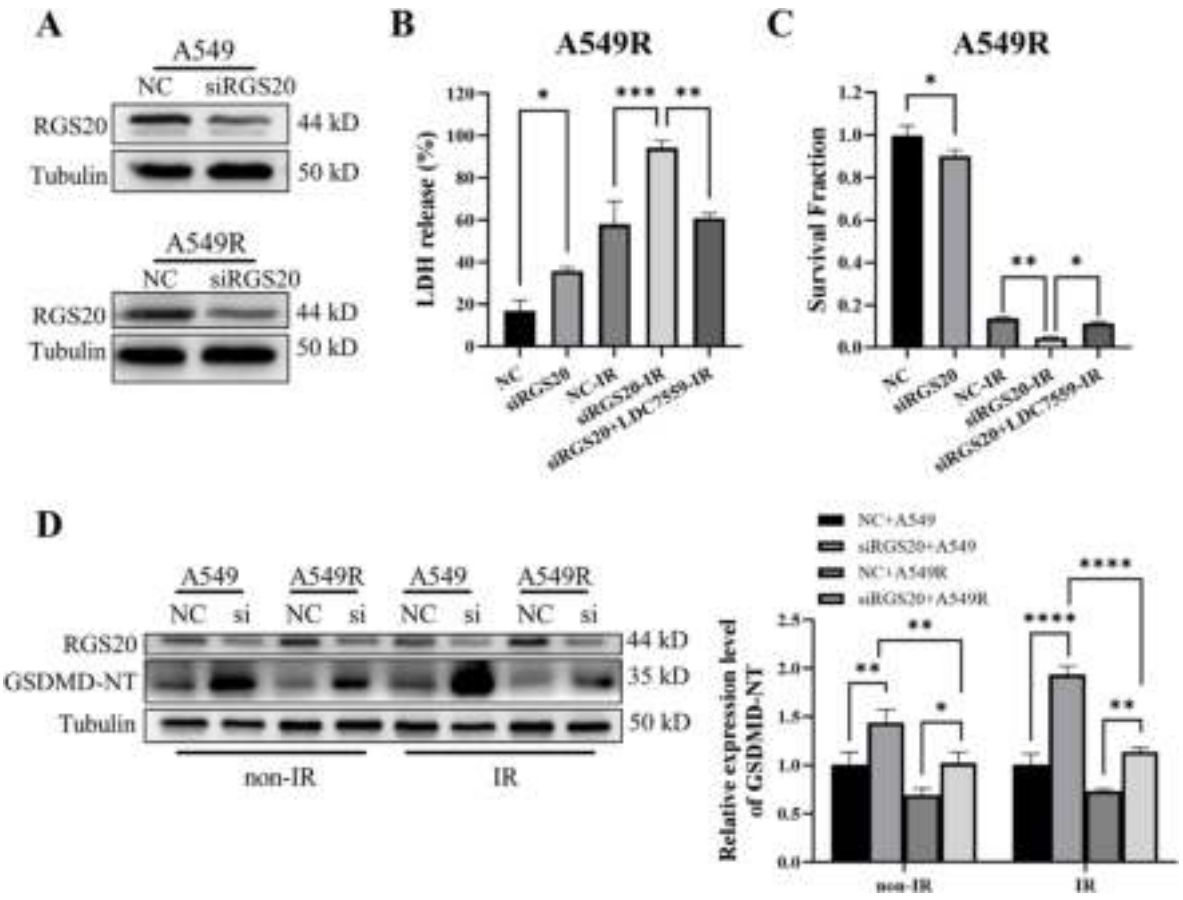

Fig. 4. Effect of siRGS20 on the radiosensitivity mediated by pyroptosis. A. Western blot assay was used to detect the knockdown efficiency of siRGS20 on A549 and A549R cells. B. After 48 h of IR, the levels of released LDH were detected in A549R cells transfected with NC or siRGS20 and A549R cells transfected with siRGS20 pretreated with 5 μmol/L LDC7559 at 6 h before IR. C. Clonogenic survivals of A549R cells under above treatments. D. Western blot assay of GSDMD-NT and RGS20 proteins and the relative expression level of GSDMD-NT in A549 or A549R cells at 6 h after IR as indicated. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

3.3. RGS20 gene expression response to radiation in NSCLC cells

To investigate the relationship of RGS20 and NSCLC radioresistance, we established the radioresistant A549R cells by fractionally irradiating A549 cells with a total dose of 60 Gy (Fig. 2C), and confirmed a significant increase in radioresistance of A549R compared to its parental A549 cells (Fig. 2D). As shown in Fig. 2E, RGS20 protein expression in A549 cells was lower than that in radioresistant cells A549R after irradiation. Therefore, RGS20 may be involved in the acquisition of radiation-induced radioresistance of A549 cells.

3.4. Inhibition of pyroptosis enhanced radioresistance of NSCLC cells

Ionizing radiation induces cellular pyroptosis of tumor cells.[15] Recent studies have revealed that GPCRs can act as damage-associated molecular pattern (DAMP)-sensing receptors.[16] DAMPs facilitate pyroptotic cell rupture through the caspase-mediated activation of pore-forming gasdermin (GSDM) proteins.[17] Accordingly, RGS20 may contribute to tumor radioresistance by regulating pyroptosis. We firstly determined whether pyroptosis was involved in the radioresistance of A549R cells. LDH assay revealed that irradiation-induced release of LDH from A549 cells was significantly higher that from A549R cells (Fig. 3A). Furthermore, the expressions of pyroptosis-related proteins, Caspase 1 and GSDMD-NT, were markedly increased in A549 cells after 6 Gy X-ray irradiation, while they had no significant differences in A549R cells with and without irradiation (Fig. 3B and C). Meanwhile, the expression of RGS20 in A549 cells was increased after irradiation, whereas it had a much high level in A549R cells after irradiation. To further investigate whether suppressing pyroptosis could mitigate radiation-induced cell death, we administered LDC7559, a newly selective GSDMD inhibitor[14], to A549 cells before irradiation. It was observed that LDC7559 significantly decreased LDH release (Fig. 3D) and enhanced the survival in irradiated A549 cells (Fig. 3E). Conclusively, the suppression of pyroptosis may elucidate the radioresistance of A549R cells exhibiting high expression of RGS20.

3.5. Knockdown of RGS20 enhanced radiosensitivity by promoting pyroptosis

To investigate the relationship between RGS20 and the radioresistance of NSCLC cells, we transfected A549 and A549R cells with siRGS20 and assessed the knockdown efficiency through Western blot assay (Fig. 4A). LDH release was significantly elevated by siRGS20 in A549R cells, and it even had high levels after irradiation (Fig. 4B). Besides, treatment with LDC7559 reversed the LDH release increased by siRGS20 in the irradiated A549R cells. Meanwhile, clonogenic assay demonstrated that RGS20 knockdown notably increased the radiosensitivity of A549R cells (Fig. 4C), indicating that RGS20 gene contributed to the radioresistance of A549R cells. The mitigation of radioresistance by siRGS20 could be rescued by LDC7559. As expected, the expression level of GSDMD-NT was increased by inhibiting RGS20 and it was higher in A549 cells than A549R cells (Fig. 4D). In light of these findings, RGS20 was involved in tumor radioresistance by suppressing cellular pyroptosis.

4. Discussion

RGS proteins selectively bind with G-protein α subunits to enhance GTP hydrolysis rate thus dampening signal transduction initiated by GPCRs, which is known as their canonical functions.[3] Accumulating evidence has indicated that RGS proteins have both tumor-suppressive and oncogenic functions depending on the specific RGS protein and the context of cancer.[18] As reported, some RGS proteins including RGS5[19] and RGS6[20] negatively regulated the progression of lung cancer, whereas RGS20 protein promoted the carcinogenesis of lung cancer.[7] Besides, the increased level of RGS20 was found to be correlated with tumor progression and unfavorable clinical outcome in oral squamous cell carcinoma[4], breast cancer[5,6], bladder cancer[8] and penile cancer.[9] Surprisingly, the function of RGS20 in tumor radiosensitivity remains unconfirmed. Our study demonstrated the contribution of RGS20 to tumor radioresistance for the first time.

Radiotherapy exerts anti-tumor effects based on direct DNA damage and other indirect pathways resulting in cell death, such as apoptosis[21], ferroptosis[22], and immunogenic cell death.[23] Pyroptosis has emerged as a newly discovered form of cell death during radiotherapy. Mechanistically, radiotherapy induces cell death, which releases various DAMPs.[23] DAMPs facilitate pyroptotic cell rupture through the caspase-mediated activation of pore-forming GSDM proteins.[24] Moreover, several GPCRs can be activated by DAMPs[25], suggesting that RGS proteins (the negative modulator of GPCR signaling pathway) may be potential to regulate pyroptosis in radiotherapy. GSDMD, the key protein that causes pyroptosis, is cleaved by caspase-1 into two parts: the C-terminal and N-terminal of GSDMD. The traditional view held that the N-terminal structure binds to the cell membrane to form membrane pores, producing cell pyroptosis and releasing cell contents.[15] A new study[26] reported that full-length GSDMD induced pyroptosis by a mechanism that reversible palmitoylation was a checkpoint for pore formation by both GSDMD-NT and intact GSDMD. Our results showed that the expression levels of Caspase 1 and GSDMD-NT were significantly lower in A549R cells compared to the parental A549 cells. Additionally, inhibiting RGS20 increased GSDMD-NT expression to induce pyroptosis thereby alleviating radioresistance. Combining radiotherapy with therapeutic strategies targeting RGS20 and pyroptosis may improve treatment outcomes. Moreover, pyroptosis has dual capability of inducing tumor cell death and stimulating the immune system to eradicate any lingering cancer cells subsequent to radiotherapy, thereby potentially augmenting the therapeutic efficacy.[15]

In this study, we firstly identified DEGs between radiosensitive and radioresistant LUAD patients through analysis of TCGA-LUAD dataset and found that RGS20 was significantly upregulated in radioresistant LUAD patients and correlated with poor prognosis. Cellular pyroptosis is a common consequence of radiation. Knocking down RGS20 gene enhanced radiation-induced pyroptosis, which could be reversed by LDC7559, a GSDMD inhibitor. RGS20 appeared to suppress cellular pyroptosis by downregulating the expression of GSDMD-NT, but further exploration is still required to understand its underlying mechanisms.

Taken together, RGS20 contributed to radioresistance of NSCLC cells via suppressing pyroptosis. Our study highlighted the significance of RGS20 in tumor radioresistance mediated by pyroptosis and provided a basis for developing novel therapeutic approaches to enhance clinical radiosensitivity.

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Declaration of competing interest

None.

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