Linc-RA1 inhibits autophagy and promotes radioresistance by preventing H2Bub1/USP44 combination in glioma cells

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Abstract
Radiotherapy is one of the standard treatments for glioma patients; however, its clinical efficacy is limited by radioresistance. We identified a mechanism of such resistance mediated by linc-RA1 (radioresistance-associated long intergenic noncoding RNA 1). Linc-RA1 was upregulated in radioresistant glioma cells and glioma tissue samples, compared with radiosensitive cells and nontumor tissues. Linc-RA1 was associated with inferior overall survival and advanced clinical stage of glioma. Linc-RA1 promoted glioma radioresistance in vitro and in vivo. Mechanistically, linc-RA1 stabilized the level of H2B K120 monoubiquitination (H2Bub1) by combining with H2B and inhibiting the interaction between H2Bub1 and ubiquitin-specific protease 44 (USP44), which inhibited autophagy, thus contributing to glioma radioresistance. These results reveal that linc-RA1-mediated autophagy is a key mechanism of radioresistance and is an actionable target for improving radiotherapy efficacy in patients with glioma.

Introduction
Gliomas are the most common primary brain tumors and are classified as grades I–IV under the World Health Organization (WHO) grading system1. High-grade glioma (HGG), including WHO grades III and IV, is the most fatal brain tumor in adults, and its treatment has been largely unsatisfactory2. Radiotherapy is one of the limited treatment options with verified clinical efficacy for patients with HGG3. Unfortunately, the efficacy of radiotherapy for HGG patients is at best modest, due to radioresistance of the tumor, the underlying mechanisms of which remain poorly characterized4. Long noncoding RNAs (IncRNAs) are noncoding transcripts containing more than 200 nucleotides that can control the expression of a gene at the transcriptional, post-transcriptional, or epigenetic levels5. Increasing evidence has shown that specific IncRNAs are implicated in the onset and progression of various cancers6. For example, dysregulated IncRNAs, including IncRNA HULC and CAMTA1, can be closely linked to key aspects of pathology, progression, and outcomes in liver cancer7,8. Specific IncRNAs, including IncRNA PCAT-1 and MALAT1, are critically involved in the development and drug resistance in gastric cancer9,10. Importantly, IncRNAs are considered as critical players in the tumorigenesis and progression of gliomas11. For example, IncRNA HOTAIR, with a higher expression level in glioma tissues than in nontumor tissues, is essential for glioma proliferation12. LncRNA CRNDE can promote the growth and invasion of glioma cells through the mammalian target of rapamycin (mTOR) signaling pathway13. However, the roles of IncRNAs in radioresistance of glioma remain largely unknown14. In our previous study, we used...
two human glioma cell lines M059J and M059K, which were derived from the same patient, with M059K cells being more resistant to irradiation (IR) than M059J cells\(^\text{15}\). The differential expression profile of IncRNAs between M059J and M059K cells was analyzed using an IncRNA microarray. We proved that IncRNA SNHG18 promoted radioresistance of glioma cells by suppressing semaphorin5A\(^\text{16}\).

In the present study, we continued to explore the other differentially expressed IncRNAs, which are considered to be involved with the radioresistance to glioma. We identified an IncRNA and named it radioresistance-associated long intergenic noncoding RNA 1 (linc-RA1). The results demonstrated that linc-RA1 is highly expressed in radioresistant glioma cells and glioma tissues, compared with radiosensitive and nontumor tissues, and promotes radioresistance of glioma. Mechanistically, linc-RA1 could stabilize the level of H2B K120 monoubiquitination (H2Bub1), thereby inhibiting the activation of autophagy and contributing to the radioresistance of glioma cells.

**Results**

**Linc-RA1 is upregulated in glioma radioresistant cell lines and correlates with advanced glioma grades and poor prognosis**

To identify IncRNAs involved in radioresistance of glioma, the expression profile of IncRNAs between the M059J and M059K cells was assessed using microarray analysis in our previous study\(^\text{16}\). M059J and M059K cell lines are established from different areas of the same tumor\(^\text{15}\), with M059K cells that were much more resistant to IR than M059J cells (Supplementary Fig. 1a). Seventy-seven IncRNAs were expressed differently (fold change > 10.0, \(p\) value < 0.05), including 30 upregulated and 47 downregulated IncRNAs in M059K cells compared with that in M059J cells. Among them, IncRNA TCONS_00009108 (GenBank Accession no. XR_949976.1), which was one of the top-scoring highly overexpressed IncRNAs in radioresistant cells, caught our attention and was named as radioresistance-associated long intergenic noncoding RNA 1 (linc-RA1). The full-length cDNA of linc-RA1 was obtained using RACE (Supplementary Table 1). Subsequent analysis showed that linc-RA1 was highly upregulated in radioresistant glioma cell lines (M059K and U87) compared with that in radiosensitive glioma cell lines (M059J and U251) (Fig. 1a). Consistently, high expression of linc-RA1 was observed in M059K cells compared with that in M059J cells using ISH (Fig. 1b). Thus, the expression of linc-RA1 in radioresistant glioma cells was significantly higher than that in radiosensitive glioma cells.

Furthermore, analysis of the expression of linc-RA1 in 120 glioma tissues and 78 nontumor brain tissues revealed significantly higher expression in glioma tissues (Fig. 1c). Moreover, linc-RA1 expression was upregulated in high-grade glioma tissues (WHO III–IV, \(n = 82\)) compared with that in low-grade tissues (WHO I–II, \(n = 38\)), indicating a positive correlation between linc-RA1 expression and the malignancy degree of glioma (Fig. 1d). However, linc-RA1 expression did not correlate with gender, age, or tumor size (Table 1). Consistently, the higher expression of linc-RA1 in glioma tissues (52.50%, 63 of 120) compared with that in nontumor brain tissue samples (29.49%, 23 of 78, Fig. 1e, f and Table 2) was also confirmed using ISH. More importantly, Kaplan–Meier analysis revealed that patients with glioma with higher linc-RA1 expression had significantly shorter progression-free and overall survival than those with lower expression (Fig. 1g, h). Thus, linc-RA1 correlated with advanced glioma grades and poor prognosis of patients with glioma.

**Linc-RA1 enhances radioresistance of glioma cells in vitro and in vivo**

To evaluate the roles of linc-RA1 in radioresistance of glioma cells, it was overexpressed in relatively radiosensitive M059J and U251 cells, and suppressed in relatively radioresistant M059K and U87 cells (Supplementary Fig. 1b, c). Overexpression or knockdown of linc-RA1 had no significant influence on the viability of the glioma cells (Supplementary Fig. 1d, e). Interestingly, linc-RA1 overexpression increased the surviving fractions of M059J and U251 cells (Fig. 2a, c), whereas suppression of linc-RA1 decreased the surviving fraction of M059K and U87 cells (Fig. 2b, d). To measure DNA damage, we detected DNA breaks using comet assays, which showed that the tail DNA% (indicating DNA damage) was significantly lower in M059J cells overexpressing linc-RA1 (Fig. 2e, f), and higher in M059K cells with downregulated linc-RA1 expression (Fig. 2g, h), compared with control cells.

Radiation damage can trigger IR-induced cell death by different pathways, such as mitotic catastrophe, necrosis, and apoptosis\(^\text{17}\). Our results showed that the combination of linc-RA1 overexpression with IR significantly decreased the percentage of IR-induced dead cell (Fig. 3a, b). Similarly, the percentage of IR-induced dead cell was significantly higher when M059K and U87 cells with downregulated linc-RA1 received IR (Fig. 3c–f).

To further verify these results, an in vivo tumor model was employed. U87 cells stably transduced with a scrambled shRNA or an shRNA targeting linc-RA1 were injected subcutaneously into nude mice. When the tumor size reached approximately 150 mm\(^3\), xenograft tumors of the IR groups received local tumor IR with a fractionated dose of 2 Gy every other day, five times (Fig. 3g). The tumor size was measured until the tumors in all groups grew to 300 mm\(^3\) (Fig. 3h, i). In the control and linc-RA1-knockdown groups, the tumors reached 300 mm\(^3\) on days 21 and 22, respectively. Importantly, in the group treated with IR alone, the time to reach 300 mm\(^3\) was 27 days, and which increased to 36 days when IR was combined with knockdown of linc-RA1.
The results showed that knockdown of \textit{linc-RA1} alone had no significant influence on tumor growth, but did inhibit tumor growth after IR. Taken together, these results indicated that \textit{linc-RA1} enhances the radioresistance of glioma cells in vitro and in vivo.

\textbf{Linc-RA1 promotes the H2Bub1 modification after exposure to IR}

The localization of lncRNAs can have important implications on their molecular functions and mechanisms. Thus, the...
**Fig. 2 Linc-RA1 enhances radioresistance of glioma cells in vitro.**

(a) Clonogenic survival assays of M059J and U251 cells transduced with vector or lentiviruses encoding human linc-RA1 sequence.  
(b) Clonogenic survival assays of M059K and U87 cells transfected with scrambled shRNA or linc-RA1 shRNA.  
(c) Representative images of clonogenic survival assays from M059J and U251 cells transduced with vector or linc-RA1.  
(d) Representative images of clonogenic survival assays from M059K and U87 cells transfected with scrambled shRNA or linc-RA1 shRNA.  
(e, f) Representative images and quantification of comet assays of M059J cells transduced with vector or linc-RA1, at the indicated time points after 6-Gy IR.  
(g, h) Representative images and quantification of comet assays of M059K cells transfected with scrambled shRNA or linc-RA1 shRNA, at the indicated time points after 6-Gy IR. Data are presented as means ± SD, n = 3, *P < 0.05, **P < 0.01, ***P < 0.001.
The distribution of linc-RA1 was examined using FISH in M059K cells. We found that linc-RA1 was localized predominantly in the nuclei, with some expression in the cytoplasm, indicating that linc-RA1 might play a major role in the nuclei (Fig. 4a).

Studies have shown that lncRNAs can function by interacting with proteins18. Thus, we identified nuclear proteins that could bind to linc-RA1 using RNA pulldown, followed by mass spectrometry analysis. The results showed that linc-RA1...
Fig. 4 (See legend on next page.)
could bind several proteins, including H2B, XPF, NUMB, and PCBP1. Among the proteins identified by mass spectrometry of the specific protein band for linc-RA1, H2B was also detected by western blotting (Fig. 4b, c). The RNA immunoprecipitation (RIP) experiment using anti-H2B antibodies in extracts from M059K cells showed enrichment of linc-RA1 (but not GAPDH mRNA) versus a nonspecific IgG control (Fig. 4d, e). Histone H2B is distributed in the nucleus and is involved with the DNA-damage response and other important pathways; therefore, it was chosen for further mechanistic research.

To explore whether linc-RA1 functions via binding to H2B to contribute to the radioresistance of glioma cells, we investigated whether the level of H2B was affected by linc-RA1 after irradiation. However, the results showed an unchanged level of H2B (Fig. 4f, g). Also, we detected the expression of XPF, NUMB, and PCBP1 with linc-RA1 overexpression in M059J cells or linc-RA1 knockdown in M059K cells. There was no change on their expression (Supplementary Fig. 2a–f). Interestingly, previous studies confirmed that some histone modifications of H2B are involved in the DNA-damage response. Thus, we hypothesized that linc-RA1 might be involved in the histone modification of H2B, which might be associated with the radioresistance of glioma cells. Among the histone modifications of H2B, H2B K120 monoubiquitination (H2Bub1) can promote DNA-damage response. Thus, we suspected that linc-RA1 might promote glioma radioresistance by regulating H2Bub1. Consistently, our results showed that linc-RA1 altered the level of H2Bub1 after IR at a dose of 6 Gy. Overexpression of linc-RA1 increased the level of the H2Bub1 modification in M059J cells after IR (Fig. 4f), while knockdown of linc-RA1 decreased the H2Bub1 modification level in M059K cells (Fig. 4g).

Furthermore, the protein levels of related enzymes, including ubiquitin ligases of H2Bub1, RNF20, and RNF40, and the deubiquitinating enzyme of H2Bub1, USP44, were detected to investigate the molecular mechanisms of linc-RA1. The RNF20–RNF40 E3 ubiquitin ligase complex can monoubiquitylate histone H2B to produce H2Bub1, while the deubiquitinase USP44 can remove this modification. The results showed that the levels of RNF20, RNF40, and USP44 were unchanged with linc-RA1 upregulation or downregulation, indicating that linc-RA1 regulates the monoubiquitination of histone H2B, independent of the expression level of ubiquitin ligases RNF20/40 or deubiquitinating enzyme USP44 (Supplementary Fig. 3a, b). Importantly, lncRNAs can also directly bind proteins that are essential for a signaling pathway, thus regulating protein–protein interactions and modulating their functions. Thus, we speculated that linc-RA1 might affect the interactions between H2Bub1 and RNF20, RNF40, or USP44. Interestingly, overexpression of linc-RA1 decreased the interaction between H2Bub1 and USP44 in M059J cells after IR (Fig. 4h), while knockdown of linc-RA1 increased the interaction between H2Bub1 and USP44 in M059K cells after IR (Fig. 4i). These results indicated that linc-RA1 could stabilize the level of H2Bub1 by inhibiting the interaction between H2Bub1 and USP44.

Linc-RA1 regulates radioresistance through H2Bub1-mediated autophagy

Several studies have indicated that the decrease in H2Bub1 levels induced by USP44 results in autophagy activation. Importantly, autophagy is involved with DNA-damage response. The induction of autophagy can enhance radiosensitivity. Therefore, we hypothesized that linc-RA1 could affect H2Bub1-mediated autophagy pathway, thereby promoting radioresistance of glioma cells. Linc-RA1 overexpression in M059J cells at 6 h after exposure to 6 Gy led to an increase in H2Bub1 levels, accompanied simultaneously with a decrease in the microtubule-associated protein 1 light-chain 3 beta (LC3B)-II/I ratio and an increase in p62 (Sequestosome 1) levels. In contrast, suppression of linc-RA1 in M059K cells at 6 h after exposure to 6 Gy reduced the level of H2Bub1 and p62, and increased the LC3B-II/I ratio (Fig. 5a, b). Linc-RA1 overexpression decreased the level of γ-H2AX, which reflects the number of DNA double-strand breaks (DSBs), in M059J cells, while linc-RA1 knockdown increased the level of γ-H2AX in M059K cells after IR.
Fig. 5 (See legend on next page.)
(Fig. 5a, b). These results indicated that linc-RA1 inhibited autophagy after IR in glioma cells.

We sought to determine whether linc-RA1 promotes glioma radioresistance through H2Bub1. We found that H2Bub1 inhibition by RNF20 knockdown could partly restore the inhibition of autophagy caused by the overexpression of linc-RA1 in M059J cells (Fig. 5c). H2Bub1 inhibition by RNF20 knockdown could partly restore the decrease of γ-H2AX level caused by the overexpression of linc-RA1 in M059J cells (Fig. 5d). A clonogenic survival assay revealed that H2Bub1 inhibition by RNF20 knockdown could partly restore the enhanced radioresistance induced by linc-RA1 overexpression in M059J cells (Fig. 5e). Consistently, H2Bub1 inhibition by USP44 overexpression also produced similar effects (Fig. 5f–h).

Furthermore, we also found that the knockdown of autophagy-related protein 7 (ATG7) partly restored the increased γ-H2AX level caused by knockdown of linc-RA1 in M059K cells (Fig. 5i, j). A clonogenic survival assay revealed that ATG7 knockdown partly restored the enhanced radiosensitivity induced by linc-RA1 knockdown in M059K cells (Fig. 5k). The treatment with autophagic inhibitor Spautin-1 also produced similar effects (Supplementary Fig. 4a–c).

Moreover, immunofluorescence assays demonstrated that linc-RA1 overexpression could decrease the foci number of γ-H2AX in M059 cells (Fig. 6a, c), while linc-RA1 knockdown could increase the foci number of γ-H2AX in M059K cells (Fig. 6b, d). Moreover, H2Bub1 inhibition by USP44 overexpression could partly restore the decreased γ-H2AX foci number caused by linc-RA1 overexpression in M059J cells (Fig. 6a, c). ATG7 knockdown could partly restore the increased γ-H2AX level caused by linc-RA1 knockdown in M059K cells (Fig. 6b, d). Altogether, these results suggested that linc-RA1 promoted radioresistance at least partly through the alteration of H2Bub1 level and the regulation of autophagy in glioma cells.

**Discussion**

In the present study, lncRNA linc-RA1 was identified as upregulated in radioresistant glioma cells and glioma tissues compared with radiosensitive cells and nontumor tissues. Linc-RA1 promotes the radioresistance of glioma cells. Mechanistically, linc-RA1 stabilizes H2Bub1 levels by inhibiting its binding with USP44, thereby inhibiting autophagy activation and contributing to glioma cell radioresistance (Fig. 7). These findings indicated that linc-RA1 plays an important role in regulating the radiosensitivity of glioma cells.

Recently, several studies have indicated that IncRNAs are involved in cancer radioresistance. In cervical cancer, lncRNA LINC00958 regulates RRM2 by competing for miR-5095, thereby regulating radiotherapy resistance. In prostate cancer, lncRNA UCA1 enhances tumor cell radioresistance by inhibiting cell-cycle progression. However, the study is about the roles of IncRNAs in glioma radioresistance. In our previous study, we showed that lncRNA SNHG18 promoted glioma radioresistance by inhibiting semaphorin5A. Here, we further analyzed glioma radioresistance-related IncRNAs. We demonstrated that ectopic expression of linc-RA1 in M059J and U251 cells significantly enhanced radioresistance, whereas linc-RA1 knockdown in M059K and U87 cells increased radiosensitivity. This is the first study to investigate the effect of linc-RA1 on the radiation response of glioma cells. Tumor cell sensitivity to radiotherapy is one of the major influencing factors that determine the prognosis of patients with HGGs. Thus, targeting the linc-RA1 might be an effective method to enhance glioma radiosensitivity.

LncRNAs display characteristic tissue-specific and cell-type-specific expression patterns, which could be used as biomarkers to classify and prognose tumors. Many researches indicate that the aberrant expression patterns of IncRNAs in clinical samples correlate with malignancy grade and histopathological differentiation, which are clinically important in the diagnosis and prognosis of glioma. For example, previous studies demonstrated that upregulated IncRNA CRNDE expression correlates with larger tumor size, higher WHO grade, and worse overall survival of patients with glioma. In addition, the differential expression of HOXA11-AS in different subtypes of glioma suggested it as a biomarker to identify glioma
molecular subtypes. Similarly, we found that high expression of linc-RA1 correlated with higher histological grade and poor prognosis of glioma. Thus, high expression of linc-RA1 might be a potential biomarker to classify and prognose glioma. The identification of novel glioma biomarkers is important to study molecular mechanisms and improve prognosis.

Histones are small nuclear proteins that play key roles in DNA compaction. The histone tails of nucleosomes are the substrates for many post-translational modifications (PTMs), including acetylation, methylation, and ubiquitination. Histones carrying these PTMs modulate the accessibility and compaction of chromatin, which regulates transcription, DNA-damage repair, and chromosome compaction. Accordingly, histone PTM dysregulation contributes to oncogenesis, and the proteins essential for the addition and removal of certain PTMs are frequently altered in cancers. The monoubiquitin moiety from lysine 120 of H2B (H2Bub1) is an important PTM of this core histone, and is involved in transcription, the DNA-damage response, and autophagy. The H2Bub1 enzymatic cascade involves E3 RING finger ubiquitin ligases, generally...
enzymes and their targets. In this study, we demonstrated that the binding of lncRNAs could affect the availability of PTM sites or the binding between the PTM enzymes and their targets. It has been suggested that the binding of lncRNAs could affect the availability of PTM sites or the binding between the PTM enzymes and their targets. In this study, we demonstrated that linc-RA1 increased H2Bub1 levels by inhibiting its binding with USP44, thereby inhibiting autophagy activation and contributing to glioma cell radioresistance.

Radioresistance is caused by various factors, including the intrinsic biology of tumor cells (with genetic and epigenetic alterations), and the extensive heterogeneity and tumor microenvironment of gliomas. Autophagy is recognized as a double-edged sword in radioresistance, which seems to depend on tumor type, stage, genetic context, and the tumor microenvironment. On the one hand, autophagy has cytoprotective effects in cancer cells. On the other hand, autophagy-induced autophagy could represent a radioprotective mechanism in cancer cells. Some researchers believe that radiation-induced autophagy could represent a radioprotective mechanism in cancer cells. On the other hand, considering evidence shows that radiation alone, or in combination with different chemical agents, can activate autophagy, leading to increased cell death. Moreover, many researchers consider that activating autophagy could lead to cell death, called autophagic cell death, contributing to radiosensitization in gliomas. For example, GDC-0941, an autophagy-inducing agent, drastically increased the sensitivity of glioblastoma cells to the dual treatment (TMZ + IR). In our study, autophagy inhibition by knockdown of ATG7 or treatment with Spautin-1 rescued clonogenic capability of irradiated M059K cells with linc-RA1 knockdown, suggesting a pro-death autophagy contribution. Taken together, our results suggested that linc-RA1 knockdown could enhance radiosensitivity by activating autophagy in glioma cells. Our study demonstrated a new working pattern of histone modifications that promoted glioma radioresistance.

Radiation damage can trigger IR-induced cell death by various processes, such as mitotic catastrophe, necrosis, and apoptosis. Our results mainly showed that linc-RA1 promoted radioresistance of glioma cells through autophagy. Our FACS data showed that the sum of Annexin-positive, PI-negative cell population (indicative of early apoptosis) and Annexin-positive, PI-positive cell population (indicative of late apoptosis) was significantly different, which included many ways of cell death, such as apoptosis and necrosis. We supposed that the sum represented the amount of IR-induced cell death, and its differences were generated by the expression of linc-RA1. Further research is needed to understand the deeper mechanisms.

In conclusion, linc-RA1 was upregulated in radio-resistant glioma cells, and its expression correlated with high histopathological grade and poor prognosis of glioma. Mechanistically, linc-RA1 stabilizes H2Bub1 levels, thereby inhibiting autophagy activation, which contributes to glioma cell radioresistance. This study provides key insights into the roles and mechanisms of lncRNAs in glioma radioresistance, implicating linc-RA1 as a biomarker and potential therapeutic target in glioma radioresistance.

Materials and methods

Patients and tumor samples

Tumor tissues were collected from 120 patients with glioma at Nanfang Hospital of Southern Medical University (Guangzhou, 510515, China) from January 2007 and January 2012. The study was approved by the hospital ethics committee, and all specimens were collected following written consent by the patients. A diagnosis of glioma was confirmed histopathologically.

Cell culture

Human glioma cells M059J and M059K were obtained from the ATCC (Manassas, VA, USA) and cultured under conditions following the manufacturer’s instructions. U251 and U87 cells were from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured following the manufacturer’s instructions. All the cells were cultured at 37°C in a humidified incubator containing 5% CO₂.

5′ and 3′ rapid amplification of cDNA ends (RACE)

Total RNA was isolated from M059K cells as described above. 5′-RACE was performed using a 5′-Full RACE Kit.
with TAP (Takara); 3′-RACE was performed using a 3′-Full RACE Core Set with PrimeScript RTase Kit (Takara) following the manufacturer’s instructions. The full-length sequence of *linc-RA1* is listed in Supplementary Table 1.

**Quantitative real-time reverse transcription PCR (qRT-PCR)**

Total RNA was extracted using the TRIzol Reagent (Invitrogen, Waltham, MA, USA) and processed using DNase I (Takara, Dalian, China) according to the manufacturer’s instructions. After conversion to cDNA, quantitative polymerase chain reaction (qPCR) was carried out using a SYBR Green PCR kit (Takara). The data were normalized to the expression of *GAPDH* (encoding glyceraldehyde-3-phosphate dehydrogenase) as a reference gene. Primer sequences are listed in Supplementary Table 2.

**Western blotting**

Cell were lysed using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) with proteinase inhibitors (Beyotime) and phosphatase inhibitors (Beyotime). Non-specific binding was blocked by incubating the membrane with 5% nonfat dry milk for 2 h. Membranes are incubated with primary antibodies overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies. Finally, the immunoreactive proteins on the membranes were visualized using an ECL detection kit (Millipore, Billerica, MA, USA). Primary antibodies are listed in Supplementary Table 3.

**Lentiviral construction and transduction**

The lentiviral vector expressing full-length human *linc-RA1* was constructed by Genechem (Shanghai, China) and used to transfect M059J and U251 cells to generate stable cells overexpressing *linc-RA1* that could be selected using puromycin. The hU6-sh-*linc-RA1*-Ubiquitin-EGFP-IRES-puromycin lentivector (Genechem) expressing a short-hairpin RNA (shRNA) and that could be selected using puromycin lentiviral vector (Genechem) expressing a *hU6-sh-linc-RA1*-Ubiquitin-EGFP-IRES-puromycin lentivector (Genechem) expressing a short-hairpin RNS (shRNA) and that could be selected using puromycin, was used to knockdown *linc-RA1* expression in M059K and U87 cells.

**siRNA transfection**

A small-interfering RNA (siRNA) targeting RNF20 (encoding ring finger protein 20) was synthesized by Ribobio (Guangzhou, China). Transfection was accomplished using the Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s protocol.

**Clonogenic survival assay**

Equal quantities of cells were seeded in plates in triplicate. The cells were then exposed to IR at the indicated doses (Varian2300EX, Varian, Palo Alto, CA). After incubation for 10–14 days, the cells were fixed and stained with 4% paraformaldehyde and 1% crystal violet, respectively. Colonies with more than 50 cells were counted using microscopy. Survival curves were generated using the multitarget single-hit model.

**Immunofluorescence assay**

Cells were seeded and irradiated with a dose of 6 Gy after adhering. After 0.5, 2, or 6 h of IR, the cells were fixed and permeabilized using 4% paraformaldehyde and 0.1% Triton X-100 (Sigma, St. Louis, MO, USA). The cells were blocked with 1% goat serum and incubated with primary antibodies. The cells were then incubated with fluorochrome-conjugated secondary antibodies. Finally, the secondary antibodies on the membrane were incubated with 5% nonfat dry milk for 2 h. Membranes are incubated with primary antibodies overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies. Finally, the immunoreactive proteins on the membranes were visualized using an ECL detection kit (Millipore, Billerica, MA, USA). Primary antibodies are listed in Supplementary Table 3.

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**Tumor radiosensitivity assay**

Animal experiments were performed strictly according to the principles approved by the Committee on the Ethics of Animal Experiments of Guangzhou Medical University (Guangzhou, China). For the in vivo experiments, suspensions of 1 × 10⁷/0.2 ml *linc-RA1*-silenced or control U87 cells were subcutaneously inoculated into the right hind limb of 4-week-old female nude mice (n = 12 mice per group). When the tumor size reached about 150 mm³ (usually the 10th day), xenograft tumors of the IR groups received local tumor IR with a fractionated dose of 2 Gy every other day for 10 days. The mice were then sacrificed when the tumor volume in the control group (NC) reached approximately 300 mm³, which was usually the 21st day after inoculation (n = 6 mice per group). Tumor growth was measured until the tumor volume reached at least 300 mm³ on the 42nd day (n = 6 mice per group).

**In situ hybridization (ISH)**

The expression of *linc-RA1* in clinical glioma specimens was detected using ISH, performed as previously described. The sections were deparaffinized with xylene, rehydrated in serial dilutions of ethanol, and treated with 0.2 N HCl. After washing for 3 times, the sections were incubated in proteinase K (40 µg/mL, Promega) for
20 min and fixed with 4% paraformaldehyde for 10 min. The sections were reconstituted using hybridization solution and incubated at 56 °C overnight in a digoxigenin-labeled linc-RA1 probe (Exiqon, Vedbaek, Denmark). After washing, the sections were blocked with 5% normal goat serum for 1 h at room temperature followed by incubation in an anti-digoxigenin alkaline phosphatase conjugate (Roche, Stockholm, Sweden) overnight at 4 °C. Colorimetric signals were obtained by incubating the sections in 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro-blue tetrazolium chloride (NBT) buffer in the dark for 4 h at room temperature. Nuclear fast red was used as the counterstain.

RNA pulldown
RNA pulldown was performed as previously described. Briefly, biotinylated linc-RA1 was in vitro transcribed with the Biotin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN, USA) and T7 RNA polymerase (Roche Diagnostics), treated with RNase-free DNase I (Roche Diagnostics), and purified with the RNeasy Mini Kit (Qiagen). Nuclear protein from M059K cells was then mixed with biotinylated RNA and incubated with streptavidin agarose beads (Invitrogen) at room temperature. The associated protein was detected by western blotting. Specific bands were excised and analyzed by mass spectrometry.

RNA immunoprecipitation
RNA immunoprecipitation was performed as previously described. Briefly, cells were lysed with RIPA buffer (Beyotime) containing proteinase inhibitors (Beyotime) and phosphatase inhibitors (Beyotime). Magnetic beads (Invitrogen) were preincubated with primary antibodies or anti-rabbit IgG (Cell Signaling Technology, Beverly, MA, USA) for 30 min. Then the lysates were immunoprecipitated with beads and rotated overnight at 4 °C. RNA was purified from RNA–protein complexes bound to the beads and then was analyzed by qRT-PCR.

Comet assay
The Comet assay ( Trevigen, Gaithersburg, MD, USA) was performed on transfected cells at 0, 2, 4, and 8 h after IR (6 Gy), according to the manufacturer’s instructions.

Immunoprecipitation
For the immunoprecipitation assay, we incubated cell lysates with anti-USP44 or normal rabbit IgG overnight at 4 °C. The mixture was then incubated with protein A/G magnetic beads (Bimake, Houston, USA) and rotated for 2 h at 4 °C. After four washes with lysis buffer, the proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Statistical analysis
The data are presented as the mean ± SD from at least three independent experiments. Student's t test and one-way analysis of variance were used to compare continuous variables, and the χ² or Fisher's exact tests were used to compare categorical variables. Survival curves were plotted using the Kaplan–Meier method and compared using the log-rank test. All statistical analyses were performed using SPSS 25 software (IBM Corp., Armonk, NY, USA). A P value < 0.05 was considered statistically significant.
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