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Radiotherapy-associated changes in the miR-124/SP1 axis in a rat glioma model

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ABSTRACT

Objective(s): Glioma is an aggressive brain tumor that frequently involves microRNA dysregulation. Rno-miR-124-3p is a brain-enriched miRNA involved in gene regulation, with Specificity Protein 1 (SP1) as its validated target. Radiotherapy remains a cornerstone of glioma treatment; however, its molecular effects on miRNA-transcription factor axes are not fully understood. We hypothesized that radiotherapy up-regulates Rno-miR-124-3p, thereby down-regulating SP1. Accordingly, this study evaluated their expression following radiotherapy in a rat glioma model.

Materials and Methods: C6 glioma cells were cultured, and an effective radiation dose was determined using an MTT assay. Sixty male Wistar rats were randomly assigned to six experimental groups: control, sham, glioma, and their corresponding radiotherapy-treated groups. Glioma was induced by stereotactic injection of C6 cells into the striatum. After radiotherapy, brain tissue was collected for histological and molecular analyses. Tumor expression of Rno-miR-124-3p and SP1 was quantified by real-time PCR.

Results: In glioma-bearing rats, Rno-miR-124-3p expression was significantly down-regulated, whereas SP1 was significantly up-regulated compared with control groups ($P \leq 0.05$ and $P \leq 0.01$, respectively). After radiotherapy, an inverse pattern was observed, with increased Rno-miR-124-3p and decreased SP1 in glioma tissues ($P \leq 0.05$ and $P \leq 0.01$). Histological evaluation suggested structural differences between the glioma and radiotherapy-treated glioma groups.

Conclusion: Glioma development alters the Rno-miR-124-3p/SP1 regulatory axis. Radiotherapy modulates this molecular pattern, suggesting a potential role for Rno-miR-124-3p in glioma-related pathways. Further studies are needed to clarify its functional and clinical relevance.

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Introduction

Glioblastoma (GBM) is the most aggressive primary brain tumor (1). Despite standard therapies, including surgery followed by chemoradiotherapy, patient prognosis remains exceptionally poor, underscoring the urgent need for novel radiosensitizing strategies (1-3).

Radiotherapy is a central component of standard GBM treatment, commonly administered alongside surgical resection and temozolomide chemotherapy (4). Ionizing radiation primarily induces cytotoxicity by generating DNA double-strand breaks and oxidative stress, ultimately leading to tumor cell death (5, 6). However, the therapeutic efficacy of RT is often constrained by the activation of cellular repair mechanisms and adaptive responses, which limit its effectiveness (4). These challenges highlight the importance of identifying molecular pathways that are altered following irradiation in glioma tissue. Furthermore, ionizing radiation not only induces direct cellular damage but also triggers complex stress-responsive gene networks (7). In response to radiation-induced DNA breaks and oxidative stress, cells dynamically alter the expression of crucial regulatory molecules, particularly miRNAs. These radiation-modulated miRNAs act as rapid responders to genotoxic stress, modulating downstream transcription factors and reshaping gene expression profiles to either facilitate damage repair or induce apoptosis (7). Understanding this radiation-miRNA-transcription factor axis is essential for overcoming tumor radioresistance.

MicroRNAs (miRNAs) are small non-coding RNAs, approximately 19–25 nucleotides in length, that regulate gene expression post-transcriptionally. Aberrant miRNA expression has been extensively linked to cancer initiation, progression, and treatment response (8, 9). Among brain-enriched miRNAs, Rno-miR-124-3p is among the most

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abundantly expressed and plays a critical role in neuronal differentiation and homeostasis. Previous studies have reported marked down-regulation of Rno-miR-124-3p in GBM, and altered expression has been associated with increased tumor cell proliferation and invasion, as well as the maintenance of malignant phenotypes (10-13).

Specificity protein 1 (SP1) is a ubiquitously expressed transcription factor belonging to the Krüppel-like factor family and regulates genes involved in cell cycle control, angiogenesis, and cell survival (14). Overexpression of SP1 has been reported in glioma and correlated with tumor progression and unfavorable clinical outcomes (15). Notably, SP1 has been identified as a direct downstream target of Rno-miR-124-3p, suggesting that disruption of the Rno-miR-124/SP1 regulatory axis may contribute to glioma-associated molecular dysregulation (9).

In the context of radiobiology, the Rno-miR-124-3p/Sp1 axis presents a highly relevant target. Sp1 is a critical transcription factor known to promote cancer cell survival and radioresistance by up-regulating anti-apoptotic proteins and facilitating DNA repair mechanisms (16). Conversely, Rno-miR-124-3p functions as a potent tumor suppressor, modulating oxidative stress (ROS) responses and inducing apoptosis (17). Down-regulation of Rno-miR-124-3p in gliomas frequently leads to the uninhibited overexpression of its downstream target, Sp1, thereby conferring a radioresistant phenotype (18). Therefore, investigating this specific axis provides a strong biological rationale: restoring Rno-miR-124-3p could potentially inhibit Sp1-mediated survival pathways, thereby sensitizing glioma cells to radiotherapy.

While the tumor-suppressive role of Rno-miR-124-3p and its targeting of SP1 have been documented in various *in vitro* models, *in vivo* investigations remain notably scarce (19, 20). The few existing animal studies have predominantly focused on chemotherapy sensitization or on the use of exogenous viral vectors for overexpression (21). Critically, the endogenous dynamic response of the Rno-miR-124-3p/Sp1 axis specifically following ionizing radiation in a whole-organism tumor microenvironment has not been adequately explored. This gap leaves it unclear whether radiotherapy alone can favorably modulate this axis *in vivo*. Therefore, the present study aimed to address this limitation by investigating the expression patterns of Rno-miR-124-3p and SP1 in a rat glioma model before and after X-ray radiotherapy.

Specifically, we hypothesized that radiotherapy induces up-regulation of Rno-miR-124-3p, which in turn leads to down-regulation of SP1. Characterizing radiotherapy-associated alterations in this regulatory axis may provide further insight into the molecular mechanisms and the translational potential of targeting this pathway in irradiated glioma tissue.

Materials and Methods

Cell culture

The C6 rat glioma cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 15% horse serum (HS) and 2.5% fetal bovine serum (FBS), in strict accordance with the American Type Culture Collection (ATCC) standard protocol for this specific cell line (ATCC® CCL-107™) to maintain optimal glial characteristics. Cells were maintained in a humidified incubator at 37 °C with 5% CO_2. For subculturing, cells were detached using a 0.25% Trypsin-EDTA solution and incubated for 3-5 min at 37 °C until complete detachment was observed.

Cell X-ray irradiation protocol

C6 glioma cells were irradiated using 6 MV X-rays delivered by a linear accelerator (Elekta Versa HD, Sweden) to deliver various absorbed doses (e.g., 2, 4, 6, 8, and 10 Gy) for dose-response evaluation. Cells were placed centrally within a solid water phantom. Specifically, 1.5 cm of buildup material was placed above the culture plates to ensure adequate dose buildup for the 6 MV beam, and a 5 cm solid water block was placed beneath the plates to provide adequate backscatter. Radiation was delivered at a source–axis distance (SAD) of 100 cm with a constant dose rate of 400 MU/min. Dosimetric validation of the prescribed dose was performed using a pinpoint ionization chamber (PTW, Freiburg, Germany; sensitive volume 0.015 cm^3). All procedures were conducted at the Radiotherapy Department, Khansari Hospital, Arak, Iran.

Radiation dose optimization via MTT assay

To determine the effective radiation dose, C6 cells (3,000 cells/well) were seeded in 96-well plates and allowed to adhere for 24 hr before irradiation with X-ray doses ranging from 2 to 10 Gy (in 2 Gy increments). Cell viability was measured at 24, 48, and 72 hr post-irradiation using an MTT assay (22). Briefly, 10 µl of MTT reagent (5 mg/mL in PBS) was added to each well, followed by 6 hr incubation at 37 °C. After removal of the medium, formazan crystals were dissolved in 100 µl DMSO, and absorbance was measured at 570 nm (reference 630 nm) using a microplate reader. All experiments were repeated three times in triplicate.

Animal model and experimental groups

Sixty adult male Wistar rats (Rattus norvegicus, Wistar) (200–220 g) were purchased from the Pasteur Institute of Iran (Tehran, Iran). All animals were housed under standard laboratory conditions (12-hr light/dark cycle, temperature of 22±2 °C, and 50±10% relative humidity) with ad libitum access to a standard rodent diet and water. They were allowed to acclimatize for one week prior to any experimental procedures. The rats were then randomly assigned using a simple randomization method (drawing lots) to six groups (n=10): (1) Intact (no intervention); (2) Sham (DMEM injection into the brain striatum); (3) Glioma (1×10^6 C6 cells in 3 µl DMEM were injected) (23); (4) Intact + radiotherapy (8 Gy X-ray), (5) Sham + radiotherapy (DMEM injection + 8 Gy X-ray); (6) Glioma + radiotherapy (C6 injection + 8 Gy X-ray). Intracerebral injections were performed stereotactically using a rat stereotaxic apparatus (Stoelting, USA) according to the rat brain atlas (24), targeting the striatum (AP −0.8 mm, ML ±3.0 mm, DV −5.0 mm relative to bregma) under surgical anesthesia induced by an intraperitoneal (IP) injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Animals were monitored for 21 days post-injection by daily neurological assessments and weekly body weight measurements. To verify successful tumor engraftment prior to treatment, a separate subset of pilot rats (n=3) was sacrificed on day 20 post-implantation, and tumor formation was confirmed histopathologically using Nissl staining. At the end of the main observation period, euthanasia was performed by administering a lethal overdose of the same anesthetic agents (ketamine, 200 mg/kg, and xylazine, 30 mg/kg, IP).

Whole-brain radiotherapy protocol

On day 21 post-implantation, rats in the radiotherapy groups received a single fraction of whole-brain X-ray irradiation (8 Gy) delivered with a 6 MV linear accelerator (Elekta Versa HD). This dose was selected based on *in vitro* dose–response experiments and was intended to assess early molecular responses to irradiation rather than long-term tumor regression. Animals were anesthetized (ketamine/xylazine, 80/10 mg/kg, IP). Given the small size of the rat brain, focal irradiation of the striatum was not technically feasible. Therefore, rats were positioned with their bodies completely shielded from the neck down with lead blocks, ensuring uniform cranial (whole-brain) exposure to the radiation field. Seventy-two hours after irradiation, rats were euthanized by intracardiac perfusion under deep anesthesia, and brain tissues were collected for subsequent histological and molecular analyses.

Histological analysis

Brain tissues were fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned for Nissl staining. Histological evaluation was performed to confirm tumor presence and assess overall tissue architecture. Although Nissl (cresyl violet) is traditionally used to visualize neuronal cell bodies, its strong affinity for nucleic acids makes it highly effective for identifying the C6 glioma mass. Rapidly dividing tumor cells exhibit dense, highly basophilic nuclei, providing excellent visual contrast between the hypercellular tumor core and the adjacent normal brain parenchyma. This analysis was qualitative and not intended for quantitative assessment of tumor size, density, or radiation-induced necrosis. Representative images were acquired using a BX51 light microscope equipped with a DP11 digital camera (Olympus, Japan).

RNA extraction and real-time PCR

Total RNA was extracted from brain tissue using RNAX-Plus (Yekta Tajhiz, Iran). RNA concentration and purity were assessed with a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). For miRNA analysis, cDNA was synthesized using miRNA-specific stem-loop RT primers, while for mRNA (SP1), standard oligo (dT) primers with M-MuLV Reverse Transcriptase were used. Quantitative PCR was performed using SYBR Green qPCR Master Mix (Applied Biosystems). SNORD47 and GAPDH were used as internal controls for Rno-miR-124-3p and SP1, respectively. Relative gene expression was calculated using the REST 2009 software (Qiagen) (25). Primers for Rno-miR-124-3p, SP1, GAPDH, and SNORD47 were designed previously by our group (26) and used in the present study. A double-blind assessment was performed for histological and molecular analyses.

Statistical analysis

Gene expression analyses of Rno-miR-124-3p and SP1 were conducted using REST 2009, which implements a pairwise fixed reallocation randomization test. MTT assay data were analyzed separately and are presented as mean±SD (n=3 independent experiments). Statistical comparisons of cell viability were performed using two-way ANOVA with Tukey's post hoc test. Correlations between Rno-miR-124-3p and SP1 expression levels were evaluated using Spearman's rank correlation coefficient (GraphPad Prism v10.5). A *P*-value≤ 0.05 was considered statistically significant.

Results

Determination of effective radiation dose by MTT assay

MTT assay results showed that cell viability decreased in a dose- and time-dependent manner after irradiation. A two-way ANOVA with Bonferroni post hoc testing was used to evaluate these effects. Specifically, the post hoc analysis confirmed that cell viability in the 8 Gy irradiated group at 72 h (Mean ± SD = 29±2%, n=3) was significantly lower than in the non-irradiated control group (Mean ± SD = 100 ± 0%, n=3, *P*≤0.0001). This combination was selected as the optimal condition for subsequent experiments because it significantly reduced cell viability without causing immediate and complete cell death, thereby providing an ideal therapeutic window to observe downstream molecular responses (Figure 1).

Histopathological evaluation

Nissl staining provided qualitative confirmation of tumor formation and the therapeutic impact of radiotherapy. As illustrated in Figure 2, the untreated glioma group exhibited a distinct, darkly stained tumor mass characterized by severe hypercellularity and disruption of the normal cerebral cytoarchitecture. The margins of this hypercellular mass were irregular, indicating infiltrative growth into adjacent healthy tissue. In contrast, in the glioma + radiotherapy group, the tumor mass appeared more fragmented with

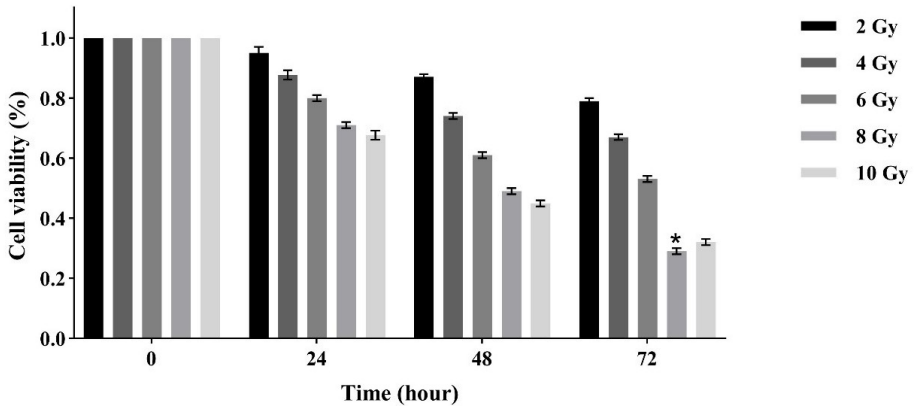

Figure1. Effects of ionizing radiation on the viability of rat C6 glioma cells
Cell viability was assessed by the MTT assay at 24, 48, and 72 hr after irradiation at different doses (2–10 Gy). A dose- and time-dependent reduction in cell viability was observed, with the most pronounced decrease at 8 Gy after 72 hr. Data are presented as mean±SD (n= 3). Statistical analysis was performed using two-way ANOVA followed by Bonferroni *post hoc* test; *P*<0.05 was considered statistically significant.

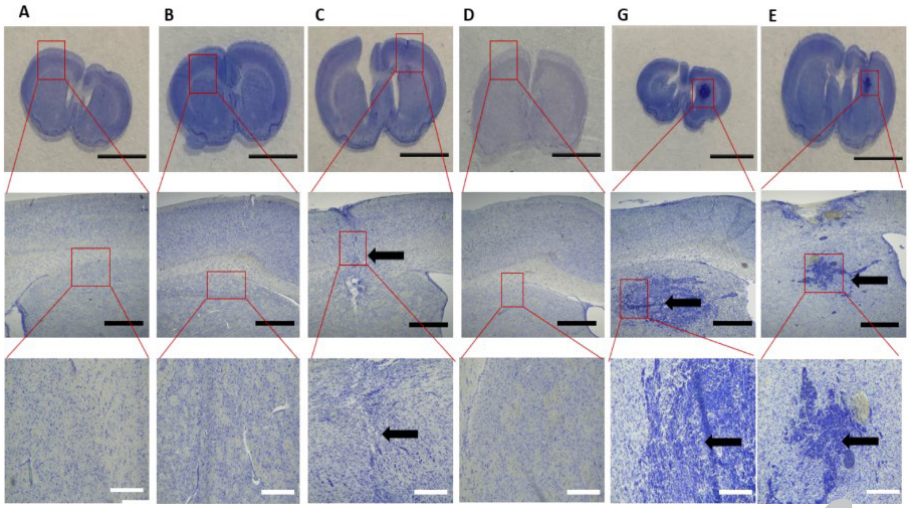

Figure 2. Rat Nissl-stained brain sections
((A) Control, (B) Control+Radiotherapy, (C) Sham, (D) Sham+Radiotherapy, (E) Glioma, (E) Glioma+Radiotherapy. Top: Whole brain (1X). Middle: Tumor regions (10X, black arrows). Bottom: 40X magnification. Glioma (G) shows dense tumor cell proliferation and disrupted tissue architecture. Radiotherapy (E) reduces tumor cell density and partially restores tissue architecture. Control and sham groups (A-D) show normal morphology, with no significant changes after radiotherapy. Scale bars: grey=2 mm, black=500 µm, white = 100 µm.

a noticeable reduction in staining intensity and cellular density. Additionally, signs of cellular degeneration and apoptosis, including pyknotic nuclei, were evident, collectively suggesting radiation-induced suppression of tumor growth. No hypercellular masses or overt structural abnormalities were observed in the intact control, sham, or irradiated non-glioma groups. It should be noted that this histological assessment was performed qualitatively to confirm tumor presence and evaluate gross morphological changes rather than to quantitatively measure tumor volume or specific cellular necrosis (Figure 2).

Gene expression analysis of Rno-miR-124-3p and SP1

Quantitative real-time PCR revealed that glioma induction significantly down-regulated relative Rno-miR-124-3p expression to approximately 0.3-fold ($P \leq 0.01$) and up-regulated relative SP1 expression to roughly 2.5-fold ($P \leq 0.01$) compared with the intact control group. Radiotherapy markedly reversed these alterations in glioma-bearing rats, increasing relative Rno-miR-124-3p expression to 1.5-fold and reducing relative SP1 levels to approximately 1.2-fold ($P \leq 0.05$ vs. glioma). Irradiation alone did not significantly affect Rno-miR-124-3p or SP1 expression in non-glioma groups (Intact+Radiotherapy and Sham+Radiotherapy). These quantitative findings indicate that radiotherapy is strongly associated with modulation of Rno-miR-124-3p and SP1 expression in glioma-bearing rats (Figure 3).

Correlation between Rno-miR-124-3p and SP1 expression

In the glioma group before radiotherapy, a significant inverse correlation was observed between Rno-miR-124-3p and SP1 expression levels (P=–0.65, P=0.045, n=6), indicating that higher Rno-miR-124-3p levels were associated with lower SP1 expression. Following radiotherapy, this inverse correlation was further strengthened (P=–0.72, P=0.02, n=6) (Figure 4). While correlation alone does not establish a direct functional effect, this notably strengthened inverse relationship suggests that radiation exposure may actively enhance the regulatory axis between these two molecules. This tighter regulatory loop

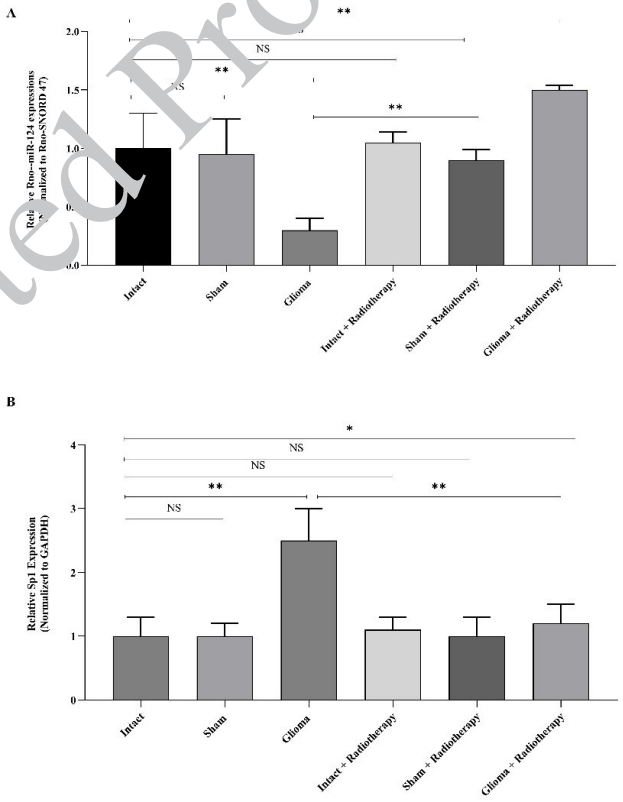

Figure 3. Relative expression of:
(A) Rno-miR-124-3p (normalized to SNORD47) and (B) Specific Protein 1 (SP1) (normalized to GAPDH) in rat control, sham, glioma, and radiotherapy-treated groups. Gene expression was assessed by quantitative real-time PCR and is presented as mean±SEM (n=6 per group). Statistical significance was evaluated using REST 2009 for qPCR data, with *$P \leq 0.05$, **$P \leq 0.01$, and NS=not significant. Observed differences reflect relative changes in transcript levels among groups and do not imply direct functional effects.

implies that radiotherapy-induced stress might sensitize the tumor cells, prompting a more robust up-regulation of Rno-miR-124-3p, which in turn more stringently suppresses SP1 expression to counteract tumor survival mechanisms.

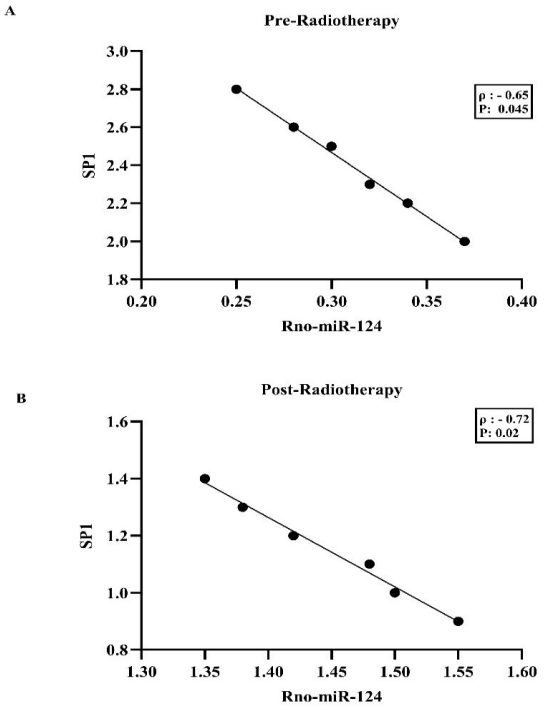

Figure 4. Spearman's rank correlation between Rno-miR-124-3p and Specific Protein 1 (SP1) expression in glioma-bearing rats
(A) Before radiotherapy, a significant inverse correlation was observed (P=–0.65, P= 0.045, n=6). (B) After radiotherapy, a significant inverse correlation was also observed (P=–0.72, P=0.02, n=6). Linear trend lines are shown for visualization purposes only; no assumption of linearity is implied. These results describe the association between transcript levels and do not imply direct functional or mechanistic effects.

Discussion

Glioma, a highly aggressive malignancy, exhibits resistance to drug and radiation therapies (27). Cancer research consistently demonstrates that altered miRNA expression, including increased oncogenic miRNAs and decreased tumor suppressor miRNAs, is implicated in tumorigenesis (28, 29). Numerous studies have shown that dysregulation of miRNA expression contributes to glioma development, progression, angiogenesis and invasion (30). The C6 glioma cell line, which mimics human GBM characteristics, is used to develop diagnostic and prognostic tools (31).

The study comprised four phases: (1) determining the effective radiotherapy dose for C6 cells using an MTT assay; (2) establishing the glioma model by stereotactic injection of C6 cells into the rat striatum, followed by radiotherapy 21 days post-injection, with subsequent histological and molecular examination of control, sham, glioma, and radiotherapy groups; (3) analyzing extracted brain tissue using Nissl staining; and (4) quantifying Rno-miR-124-3p and Sp1 expression in all groups using qRT-PCR.

Our findings in the rat glioma model demonstrate that radiotherapy significantly modulates the expression of Rno-miR-124-3p and SP1, two molecular regulators with opposing roles in tumor progression. In the baseline state of untreated glioma, the tumor suppressor Rno-miR-124-3p is significantly down-regulated, while the cell cycle and angiogenesis regulator SP1 is up-regulated, correlating with enhanced proliferation, invasion, and radioresistance (13, 32). This baseline silencing aligns with clinical observations by Fowle (2010), who noted significantly reduced miR-124-3p expression in GBM patients (33). Following radiotherapy, this oncogenic axis is effectively disrupted: Rno-miR-124-3p is significantly up-regulated, and SP1 expression concomitantly decreases. This radiation-induced reprogramming suggests a restoration of tumor-suppressive activity, supporting Deng's (2013) findings that miR-124 overexpression induces radiosensitivity (12). Similar regulatory dynamics are observed with other miRNAs in glioma, such as miR-150-3p, whose restoration also reduces SP1 expression (27). Furthermore, the suppression of SP1 is particularly relevant given its role in promoting cell metastasis and tumor progression, both of which are critically affected by radiation-based therapies (28).

As we have previously demonstrated using luciferase reporter assays (26), the inverse correlation observed in our current study can be confidently attributed to the direct regulatory action of Rno-miR-124-3p, which binds the 3'-untranslated region (3'-UTR) of the SP1 mRNA, leading to translational repression. While our underlying hypothesis involved radiation-induced stress pathways, our current data demonstrate that an 8 Gy radiotherapy dose is sufficient to hyperactivate this axis *in vivo*. The significant down-regulation of SP1 coincides with the Rno-miR-124-3p peak. This suggests that radiotherapy tightly modulates this axis, efficiently dismantling SP1-mediated cytoprotection and translating molecular suppression into the physical cellular degeneration and pyknotic nuclei we observed in the histopathological evaluation.

Radiotherapy is a primary treatment for GBM, yet many patients develop resistance, prompting research to improve radiosensitivity. In this study, C6 cells (3000, 5000, and 10000 per well) were exposed to radiotherapy (4, 8, 12 Gy) for 24, 48, and 72 hr. A dose of 8 Gy for 72 hr produced the highest lethality in 3000 cells, establishing 8 Gy as the IC50 and effective radiation dose. Rather than relying on general radiobiology principles, this specific dose selection is supported by primary studies in experimental glioma models. This finding aligns closely with Griscelli et al. (2000), who confirmed that a comparable single dose (7.5 Gy) maximizes anti-tumor effects while allowing the evaluation of molecular responses (34), as well as other primary *in vivo* glioma studies (13). The selected radiation dose of 8 Gy was based on *in vitro* cytotoxicity assays and previous literature indicating an optimal balance between tumoricidal activity and tissue preservation. Our data suggest that at this dose, radiotherapy not only induces direct cytotoxic effects but also triggers transcriptional and post-transcriptional reprogramming, favoring a less aggressive tumor phenotype.

Histopathological analysis corroborated these molecular findings, revealing that the glioma + radiotherapy group exhibited reduced tumor cell density, disrupted architecture, and evidence of necrosis compared with untreated glioma tissues. Such morphological alterations are consistent with radiation-induced tumor regression and decreased aggressiveness (35).

Taken together, these results provide *in vivo* evidence that the Rno-miR-124/SP1 axis plays a crucial role in glioma response to radiotherapy. Up-regulation of Rno-miR-124-3p and concomitant suppression of SP1 may be a key mechanism by which radiation therapy attenuates tumor growth and invasion. This mechanistic insight has potential clinical relevance: Rno-miR-124-3p could serve as a predictive biomarker for radiotherapy efficacy, while SP1 may be a viable target for combination treatments, including

miRNA-based therapeutics.

Future studies should focus on validating these findings in clinical glioma specimens, optimizing miR-124-3p delivery strategies, and evaluating the synergistic effects of radiotherapy combined with miR-124-3p mimics or SP1 inhibitors. These approaches may enhance treatment responses and improve survival outcomes for patients with high-grade gliomas.

Several limitations should be acknowledged. First, this study focused on transcriptional changes and did not include protein-level validation or functional assays of radiosensitivity. Prior work has validated SP1 protein regulation in glioma models (36), obviating the need for repetition here. Second, the C6 rat glioma model does not fully recapitulate the heterogeneity of human GBM. Finally, clinical validation will be necessary to determine the translational relevance of the observed molecular changes.

Conclusion

This *in vivo* study demonstrates an association between radiotherapy and modulation of the Rno-miR-124-3p/SP1 axis in a rat glioma model. Specifically, glioma development was characterized by decreased Rno-miR-124-3p expression and increased SP1 expression, whereas radiotherapy partially reversed this molecular pattern. Importantly, because this study primarily focused on transcriptional changes, it does not establish a causal relationship between miR-124/SP1 modulation and the functional therapeutic response. The findings suggest that the Rno-miR-124-3p/SP1 axis is responsive to irradiation at the molecular level. These findings suggest that Rno-miR-124-3p may be a candidate for future investigation as a biomarker or therapeutic target. Future studies incorporating functional knockout/knockdown assays, protein-level validation, and survival analysis are required to determine the precise biological role and ultimately translate these findings into clinical applications for glioma radiosensitivity.

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Ethics

This study was approved by the Ethics Committee of Arak University of Medical Sciences under No. IR.ARAKMU.AEC.1401.013. All animal experiments were conducted in accordance with the ARRIVE guidelines and the institutional guidelines for the care and use of laboratory animals.

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Authors' Contributions

M M designed the experiments. E G, M S, M M, B K, and F S performed the experiments and collected data. M M and M S discussed the results and strategy. M M supervised, directed, and managed the study; M M, M S, F S, E G, and B K approved the final version for publication.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors report no conflicts of interest in this work.

Declaration

We acknowledge the use of DeepSeek to improve the language and readability of the manuscript.

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