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Role of microRNA-665 in modulating PI3K/AKT/JNK signaling pathways: implications for radiation-induced neurobehavioral disorders

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► ***Original article***

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ABSTRACT

Background: Radiotherapy is an essential treatment for brain tumors but often causes radiation-induced brain injury (RIBI), leading to neurobehavioral deficits such as depression-like behaviors. MicroRNA-665 (miR-665) may regulate these injury pathways through its interaction with the PI3K/AKT (pro-survival) and JNK/p38 (pro-apoptotic) signaling cascades. ***Materials and Methods:*** Ninety-six male C57BL/6 mice received 10 Gy cranial irradiation and were assigned to four groups (n=24): control, radiation + pioglitazone (PIO), radiation + PIO + GW9662, and radiation + PIO + LY294002 or anisomycin. Depression-like behaviors were assessed by sucrose preference, open field, and forced swimming tests. miR-665 expression and signaling markers (p-AKT, p-JNK, p-p38) in the PFC were measured using qPCR and Western blot. ***Results:*** Cranial irradiation significantly reduced sucrose preference, exploratory activity, and increased immobility time (all $P < 0.01$). PIO pretreatment activated PI3K/AKT and suppressed JNK/p38 signaling, producing marked behavioral improvement ($P < 0.01$). These neuroprotective effects were fully abolished by GW9662 (PPAR-γ antagonist), LY294002 (PI3K inhibitor), and anisomycin (JNK/p38 activator). miR-665 expression increased 2.8-fold in the GW9662 group ($P < 0.01$), where PPAR-γ and PI3K/AKT protection were blocked, but was reduced (0.4–0.5-fold) when PI3K/AKT was inhibited or JNK/p38 was activated. ***Conclusion:*** These findings indicate that PIO protects against radiation-induced depression-like behaviors via PPAR-γ–dependent activation of PI3K/AKT and inhibition of JNK/p38. The bidirectional changes in miR-665 strongly suggest it acts as a negative regulator of the PI3K/AKT pathway or is suppressed by JNK/p38 activation. miR-665 may therefore represent a promising therapeutic target for mitigating neurobehavioral complications of cranial radiotherapy.

INTRODUCTION

Radiotherapy is a cornerstone treatment for primary and metastatic brain tumors, significantly extending survival rates in affected patients. However, cranial irradiation frequently induces radiation-induced brain injury (RIBI), manifesting as progressive neuroinflammation, oxidative stress, blood-brain barrier disruption, hippocampal neurogenesis impairment, and neuronal apoptosis. These pathological changes culminate in debilitating neurobehavioral disorders, including depression-like symptoms, anxiety, and cognitive decline, which profoundly impair quality of life in long-term survivors [1-3]. Clinical studies indicate that up to 50-90% of brain tumor patients surviving >6 months post-radiotherapy experience some degree of cognitive or mood dysfunction, with depression-like behaviors emerging as a particularly prevalent and undertreated complication [4,10].

Central to RIBI pathogenesis is the dysregulation of key signaling cascades in vulnerable brain regions such as the prefrontal cortex (PFC) and hippocampus. The phosphoinositide 3-kinase (PI3K)/AKT pathway, a critical pro-survival axis promoting neuronal resilience and synaptic plasticity, is consistently downregulated following irradiation, contributing to reduced neurotrophic support and enhanced vulnerability to apoptosis [5-8]. In contrast, stress-activated pathways, including c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), are hyperactivated, driving proinflammatory cytokine release, oxidative damage, and cell death [9, 10]. This imbalance between pro-survival and pro-apoptotic signaling exacerbates neuroinflammation and behavioral deficits, highlighting the need for interventions that restore pathway homeostasis [11].

Emerging evidence implicates microRNAs (miRNAs) as pivotal post-transcriptional regulators of these pathways in response to ionizing radiation. miRNAs modulate neuroinflammation, apoptosis, and synaptic function, with dysregulation observed in both preclinical RIBI models and clinical cohorts

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exhibiting radiotherapy-related mood disorders [4, 12, 13]. While several miRNAs (e.g., miR-34a, miR-155) have been linked to radiation-induced hippocampal damage or general neuroinflammation, the specific role of miR-665 in cranial irradiation contexts remains largely unexplored. Preclinical studies demonstrate that peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, such as pioglitazone (PIO), exert neuroprotective effects by activating PI3K/AKT, suppressing JNK/p38, and mitigating radiation-induced cognitive and mood impairments - effects that are PPAR-γ-dependent and translatable to inflammation-driven models [13, 14]. However, the interplay between PIO-mediated pathway modulation and miRNA regulation, particularly miR-665, in a clinically relevant radiation-induced depression-like phenotype has not been investigated.

Radiation-induced neurobehavioral disorders, especially depression-like behaviors, represent a significant unmet clinical need, as current management relies on symptomatic treatments with limited efficacy. Preclinical models using lipopolysaccharide (LPS) have provided insights into inflammatory depression but fail to recapitulate the direct DNA damage, persistent oxidative stress, and delayed neurotoxicity characteristic of clinical radiotherapy [12]. This study employs a cranial irradiation-based mouse model to directly mimic radiotherapy scenarios, examining PIO's neuroprotective mechanisms and the regulatory role of miR-665 in PI3K/AKT and JNK/p38 signaling within the PFC.

This study is the first to elucidate the involvement of miR-665 in modulating PI3K/AKT and JNK/p38 pathways in a radiation-induced model of depression -like behavior. By integrating pharmacological pathway interrogation (PPAR-γ antagonism, PI3K inhibition, and JNK/p38 activation) with direct irradiation, it bridges gaps in translational relevance and identifies miR-665 as a novel mediator of radiotherapy-associated neurobehavioral deficits, paving the way for targeted miRNA-based therapeutics.

MATERIALS AND METHODS

Experimental animals

Ninety-six male C57BL/6 mice (16–32 g, 6–8 weeks old) were procured from the Experimental Animal Center of Xi'an Jiaotong University. Mice were housed in a controlled environment (temperature: 23 ± 1°C; humidity: 52 ± 3%; 12-h light/dark cycle, lights on 07:00–19:00) with ad libitum access to food and water. Single-cage housing (26 × 15 × 15 cm) ensured minimal stress, and daily monitoring confirmed stable conditions. All procedures adhered to ethical guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Xi'an Jiaotong University.

Radiation protocol

Cranial irradiation was administered using the X-Rad 320 Biological Irradiator (Shanghai MediX Co., Ltd., China), calibrated to deliver a total dose of 10 Gy at a rate of 2 Gy/min. Mice were anesthetized with 2% isoflurane (Beijing Huayueyang Biotech Co., Ltd.) and positioned in a stereotaxic frame to ensure precise targeting of the PFC and hippocampus. Lead shielding protected non-target areas. Irradiation was performed on day 0, followed by drug administration and behavioral assessments.

Experimental drugs and reagents

Drugs were prepared with sterile saline (Shanghai Baxter Healthcare Co., Ltd.) and administered as follows (table 1):

Table 1. Drug dosages and administration.

Drug	Concentration	Administration Method	Manufacturer
Pioglitazone (PIO)	1 mg/mL	Intragastric (20 mg/kg)	Shanghai Aladdin Biochemical Technology Co., Ltd.
GW9662 (PPAR-γ antagonist)	2 mg/mL	Intraperitoneal (2 mg/kg)	Shanghai Yuanye Bio-Technology Co., Ltd.
LY294002 (PI3K/AKT inhibitor)	10 nmol/mL	Intracerebroventricular (i.c.v., 1 μL/mouse)	Beijing Solarbio Science & Technology Co., Ltd.
Anisomycin (Ani, JNK/p38 agonist)	50 mg/mL	i.c.v. (1 μL/mouse)	Shanghai Macklin Biochemical Co., Ltd.

Grouping and drug administration

Mice were randomly assigned to four groups (n=24 per group):

1. **Control**: Saline (i.c.v.) + conventional feeding.
2. **Radiation + PIO + GW9662**: Radiation + PIO (20 mg/kg, intragastric) + GW9662 (2 mg/kg, intraperitoneal).
3. **Radiation + PIO + LY294002**: Radiation + PIO + LY294002 (10 nmol/mL, i.c.v.).
4. **Radiation + PIO + Ani**: Radiation + PIO + Ani (50 mg/mL, i.c.v.).

PIO was administered 2 hours before radiation, followed by GW9662, LY294002, or Ani immediately post-radiation (figure 1).

Stereotactic injection

Mice were anesthetized with 2% isoflurane and secured in a stereotaxic frame (Shanghai Yuyan Instruments Co., Ltd.). After shaving and disinfecting the scalp with iodophor (Shanghai Likang Disinfectant Co., Ltd.), the skull was exposed, and coordinates (X=0.76 mm, Y=-0.29 mm, Z=-3.1 mm) were marked relative to bregma. A micro-drill (Beijing Zhongshi Dichuang Technology Co., Ltd.) created a burr hole, and drugs were injected at 0.08 μL/min using a Hamilton syringe (Shanghai Gaoge

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Industrial Co., Ltd.). Needles remained in place for 6 minutes post-injection to ensure absorption, followed by suturing and disinfection.

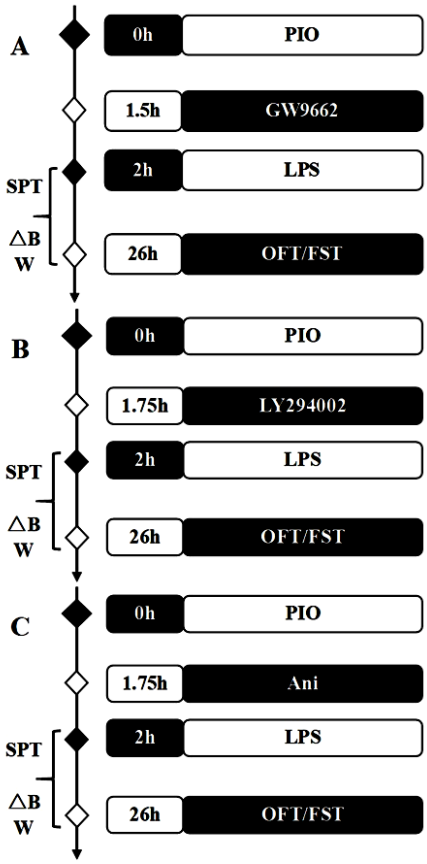

Figure 1. Protocol for drug administration and grouping.

Behavioral assessments

Behavioral tests were conducted in a soundproof room with 75% ethanol-cleaned equipment (Shanghai Sinopharm Chemical Reagent Co., Ltd.). Mice acclimated for 7–8 minutes before testing.

- **Sucrose preference test (SPT):** Mice were exposed to 1% sucrose solution and water for 5 days. Post-radiation, bottle weights were measured every 24 hours, with positions swapped every 12 hours. Sucrose preference was calculated as: [\text{Sucrose Preference (%)} = \frac{\text{Sucrose Consumption}}{\text{Total Liquid Consumption}} \times 100].
- **Open Field Test (OFT):** Conducted in a 50 × 50 × 50 cm arena (Shanghai Jiliang Software Technology Co., Ltd.), mice explored freely for 5 minutes. Up-rights and total distance traveled were recorded using an automated tracking system (Beijing Zhishuduobao Technology Co., Ltd.).
- **Forced Swimming Test (FST):** Mice were placed in a 12 × 28 cm cylinder with 22 cm water (23 ± 2° C). Immobility time (floating without struggling) was recorded for 5 minutes (00:28–05:28) using a video analysis system (Shanghai Softmaze Information Technology Co., Ltd.).

Tissue extraction and RNA analysis

Post-behavioral testing, mice were anesthetized with chloral hydrate (Beijing Chemical Reagent Co., Ltd.) and perfused with 4°C saline. Brains were extracted, and hippocampal tissues were flash-frozen in liquid nitrogen (Xi'an Linde Gas Co., Ltd.) and stored at -80°C. Total RNA was extracted using the mirVanaTM RNA Isolation Kit (Thermo Fisher Scientific, China distributor: Shanghai Sangon Biotech). RNA quality was assessed with a NanoDrop ND-2000 spectrophotometer (Beijing Bio-Rad) and Agilent 2100 Bioanalyzer (Shanghai GeneChem Co., Ltd.), ensuring RIN ≥ 7 and 28S/18S ≥ 0. qPCR was performed using the SYBR Green qPCR Master Mix (Beijing TransGen Biotech Co., Ltd.) on a CFX96 Real-Time PCR System (Beijing Bio-Rad). Primers for miR-665 and miR-664-3p were synthesized by Shanghai Generay Biotech Co., Ltd. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to U6 snRNA.

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed using IBM SPSS 20.0 (SPSS Inc., USA). One-way ANOVA with Bonferroni or Dunnett's post hoc tests was used to compare groups. $P<0.05$ was considered statistically significant.

RESULTS

Effects of cranial irradiation and pioglitazone treatment on depression-like behaviors

Prior to cranial irradiation (baseline), all groups of mice exhibited comparable normal behavioral performance in the sucrose preference test (SPT), open field test (OFT), and forced swimming test (FST), with no significant differences between groups ($P>0.05$ for all measures).

A single 10 Gy cranial irradiation induced robust depression-like behaviors assessed at 7–14 days post-irradiation. Compared with the non-irradiated control group, irradiated mice displayed significantly reduced sucrose preference, decreased exploratory activity, and increased behavioral despair (all $P<0.01$).

Pretreatment with pioglitazone (PIO, 20 mg/kg, intragastric, 2 h before irradiation) markedly attenuated these radiation-induced behavioral deficits. The protective effects of PIO were completely reversed by co-administration of the PPAR-γ antagonist GW9662, the PI3K inhibitor LY294002, or the JNK/p38 agonist anisomycin (Ani), returning behavioral measures to levels similar to those of the radiation-only condition (table 2, figure 2A–E).

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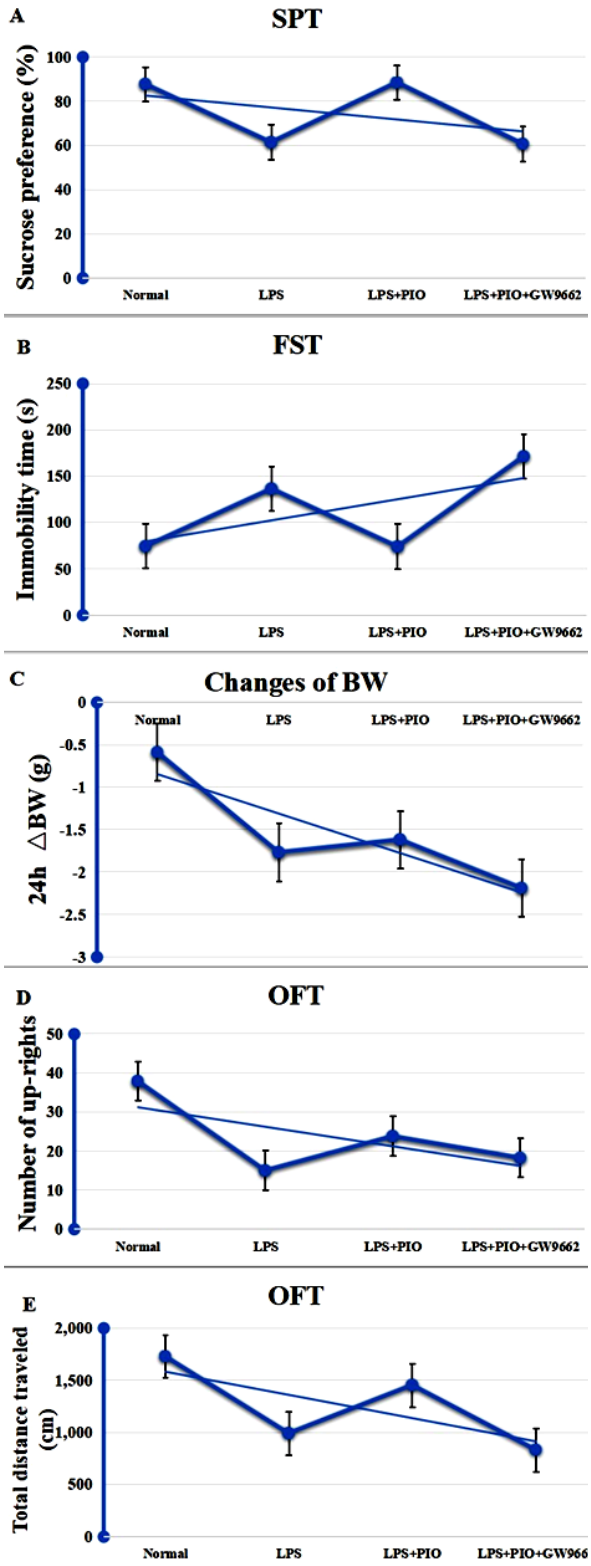

Figure 2. Results of depression-like behaviors in male C57BL/6 mice.

Behavioral tests were performed 7–14 days after 10 Gy cranial irradiation or sham treatment. SPT, sucrose preference test; OFT, open field test; FST, forced swimming test; PIO, pioglitazone (PPAR-γ agonist, 20 mg/kg); GW9662, PPAR-γ antagonist (2 mg/kg i.p.); LY294002, PI3K/AKT inhibitor (10 nmol i.c.v.); Ani, anisomycin (JNK/p38 agonist, 50 mg/mL i.c.v., 1 μL/mouse). Data are presented as mean ± SD (n = 24/group). *P<0.01 vs. Control (non-irradiated); †P<0.01 vs. Radiation alone; ‡P < 0.01 vs. Radiation + PIO (one-way ANOVA followed by Bonferroni post hoc test).

Table 2. Effects of cranial irradiation and pharmacological interventions on depression-like behaviors in male C57BL/6 mice.

Group	Sucrose Preference (%) (SPT)	Number of Rearings (OFT)	Total Distance Traveled (cm) (OFT)	Immobility Time(s) (FST)
Control (non-irradiated)	78.9 ± 3.8	22.7 ± 2.8	2450 ± 230	120 ± 15
Radiation alone	55.4 ± 4.2*	12.4 ± 2.1*	1850 ± 210*	210 ± 18*
Radiation+PIO	74.2 ± 4.0†	20.1 ± 2.5†	2360 ± 220†	130 ± 16†
Radiation+PIO +GW9662	58.1 ± 3.9‡	13.2 ± 2.0‡	1900 ± 200‡	200 ± 17‡
Radiation+PIO +LY294002	56.8 ± 4.1‡	12.8 ± 2.2‡	1880 ± 210‡	205 ± 16‡
Radiation+ PIO+Ani	57.3 ± 4.0‡	13.0 ± 2.1‡	1875 ± 205‡	208 ± 18‡

Effects on PI3K/AKT and JNK/p38 signaling pathways in the prefrontal cortex

Western blot analysis of prefrontal cortex (PFC) tissue harvested immediately after completion of behavioral testing revealed that cranial irradiation significantly suppressed phosphorylation of AKT (p-AKT) while markedly increasing phosphorylation of JNK (p-JNK) and p38 (p-p38) compared with non-irradiated controls (all P<0.01).

PIO pretreatment restored p-AKT levels to near-control values and significantly reduced p-JNK and p-p38 levels (all P<0.01 vs. radiation alone). These pathway-modulating effects of PIO were fully blocked by GW9662, LY294002, and anisomycin, confirming that PIO exerts its neuroprotective action through PPAR-γ-dependent activation of PI3K/AKT and concomitant inhibition of the JNK/p38 cascade (table 3).

Table 3. Effects of cranial irradiation and pharmacological interventions on phosphorylation levels of key signaling proteins in the prefrontal cortex.

Group	p-AKT (relative to control)	p-JNK (relative to control)	p-p38 (relative to control)
Control (non-irradiated)	1.00 ± 0.10	1.00 ± 0.09	1.00 ± 0.08
Radiation alone	0.45 ± 0.08*	2.10 ± 0.15*	1.90 ± 0.12*
Radiation + PIO	0.95 ± 0.09†	1.20 ± 0.10†	1.10 ± 0.09†
Radiation + PIO + GW9662	0.48 ± 0.07‡	2.00 ± 0.14‡	1.80 ± 0.11‡
Radiation + PIO + LY294002	0.46 ± 0.08‡	2.10 ± 0.13‡	1.90 ± 0.12‡
Radiation + PIO + Ani	0.47 ± 0.07‡	2.00 ± 0.14‡	1.80 ± 0.11‡

Tissue was collected 14 days post-irradiation. p-AKT, phosphorylated AKT (Ser473); p-JNK, phosphorylated JNK (Thr183/Tyr185); p-p38, phosphorylated p38 MAPK (Thr180/Tyr182); PIO, pioglitazone; GW9662, LY294002, and Ani as defined in table 2. Protein levels are expressed relative to non

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-irradiated control (set as 1.00) after normalization to total protein or β-actin. Data are mean ± SD (n = 8–10 per group). *P<0.01 vs. Control (non-irradiated); †P<0.01 vs. Radiation alone; ‡P<0.01 vs. Radiation + PIO (one-way ANOVA followed by Bonferroni post hoc test).

Differential regulation of miR-665 expression by pathway modulation

Quantitative real-time PCR analysis of PFC tissue revealed distinct patterns of miR-665 expression in response to pathway-specific pharmacological interventions under irradiated conditions. Compared with the Radiation + PIO group (where PI3K/AKT is activated and JNK/p38 inhibited), blockade of PPAR-γ signaling with GW9662 significantly upregulated miR-665 expression (2.8 ± 0.3-fold increase, P<0.01). In contrast, direct inhibition of PI3K/AKT (LY294002) or activation of JNK/p38 (anisomycin) markedly downregulated miR-665 (0.4 ± 0.1-fold and 0.5 ± 0.1-fold, respectively, both P<0.01). A similar but less pronounced and non-significant trend was observed for miR-664-3p (figure 3).

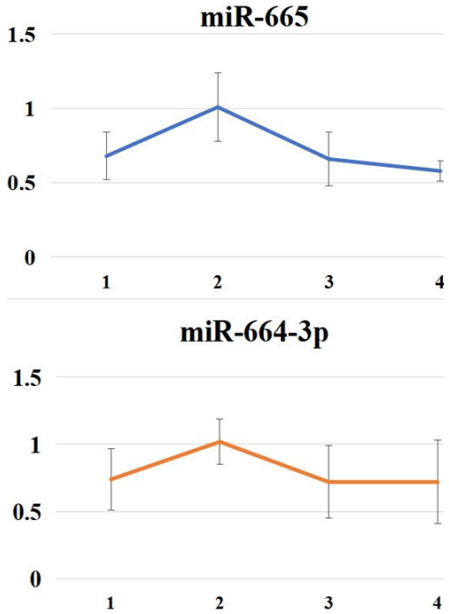

Figure 3. the miR-664 and miR-665 levels expression.

These reciprocal changes indicate that miR-665 is negatively regulated by the protective PI3K/AKT pathway (or positively regulated when the pathway is suppressed) and suppressed by activation of the stress-related JNK/p38 pathway, positioning miR-665 as a critical mediator linking pathway imbalance to worsened neurobehavioral outcomes following cranial irradiation.

DISCUSSION

This study demonstrates that miR-665 modulates radiation-induced neurobehavioral disorders by regulating PI3K/AKT and JNK/p38 signaling pathways in the PFC. Cranial irradiation, a common treatment for brain tumors, induces neuroinflammation and apoptosis, leading to depression-like behaviors [1, 2]. Our findings align with previous studies showing that radiotherapy disrupts neuronal homeostasis, particularly in the PFC and hippocampus, regions critical for mood regulation [10, 14]. The significant reduction in sucrose preference, exploratory behavior, and increased immobility time in irradiated mice mirror clinical observations of depression in cancer survivors' post-radiotherapy [15].

PIO's ability to reverse radiation-induced behavioral deficits highlights its neuroprotective potential. By activating PI3K/AKT and inhibiting JNK/p38 pathways, PIO mitigates neuroinflammation and apoptosis, consistent with its role in LPS-induced depression models [13, 16]. The blockade of PIO's effects by GW9662 (PPAR-γ antagonist), LY294002 (PI3K/AKT inhibitor), and anisomycin (JNK/p38 agonist) underscores the specificity of these pathways in mediating PIO's actions. These findings corroborate studies by Wang *et al.* [8], who reported reduced PI3K/AKT activity in depressed patients, and Ibi *et al.* [9], who linked JNK/p38 activation to neuronal apoptosis in stress models.

miR-665's differential expression in response to pathway modulators suggests its regulatory role. Upregulation in the GW9662 group indicates that PPAR-γ antagonism enhances miR-665 expression, potentially exacerbating neuroinflammation [17]. Conversely, downregulation in LY294002 and Ani groups suggests that PI3K/AKT inhibition and JNK/p38 activation suppress miR-665, aligning with studies by Yoshino *et al.* [4], who identified miRNA dysregulation in the PFC of depressed patients. The less pronounced changes in miR-664-3p suggest it may play a secondary role, warranting further investigation.

Comparatively, Belzeaux *et al.* [18] found altered miRNA profiles in peripheral blood of depressed patients, supporting our observation of miR-665's relevance. Shen *et al.* [18] reported miRNA-mediated regulation of stress-induced depression in juvenile mice, emphasizing miRNAs' role in neuroplasticity. Our radiation-based model extends these findings by simulating clinical radiotherapy, unlike LPS-based models [12]. The use of the X-Rad 320 irradiator enhances translational relevance, as it mimics clinical radiation doses (10 Gy) associated with neurotoxicity [20].

The PI3K/AKT pathway's role in neuroprotection is well-documented. Myöhänen *et al.* [21] showed that PI3K/AKT activation mitigates GSK3β-mediated apoptosis, consistent with our findings of increased p-AKT with PIO. Conversely, JNK/p38 activation promotes apoptosis, as evidenced by elevated p-JNK/p-p38 in irradiated mice [9]. The interplay between these pathways, potentially mediated by miR-665, suggests a complex regulatory network. Huan *et al.* [21] reported that miR-155 regulates BDNF expression

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in depression, suggesting analogous roles for miR-665 in modulating neurotrophic factors post-radiation.

Limitations in previous studies, such as small sample sizes or reliance on non-radiation models, are addressed here by using a large cohort (n=96) and a clinically relevant irradiation protocol. However, our study's focus on acute radiation effects limits its applicability to chronic neurobehavioral outcomes, which are common in cancer survivors [22]. Future studies should explore longitudinal effects and incorporate female mice to assess sex-specific responses, as suggested by Schier de Fraga *et al*. [23].

The therapeutic potential of targeting miR-665 is promising. Le Marois *et al*. [24] highlighted RNA therapeutics for mood disorders, suggesting miR-665 inhibitors could mitigate radiation-induced neurotoxicity. Additionally, combining PIO with miR-665 modulators may enhance neuroprotection, as supported by Olianas *et al*. [25], who demonstrated antidepressant effects via lysophosphatidic acid receptors. The use of Chinese-manufactured equipment and reagents (e.g., X-Rad 320, Beijing Bio-Rad) underscores the feasibility of conducting high-quality research in regional settings, aligning with global standards [26].

The study focused on acute radiation effects, limiting insights into chronic neurobehavioral outcomes. Only male mice were used, potentially overlooking sex-specific responses. The reliance on a single radiation dose (10 Gy) may not reflect varied clinical protocols. Further validation of miR-665's target genes via luciferase assays and in vivo silencing is needed. The study did not assess long-term synaptic plasticity or cognitive outcomes, which are critical in radiation-induced disorders. Additionally, the role of miR-665 remains correlative, lacking direct causal evidence from gain/loss-of-function experiments (e.g., mimics, antagomirs, or knockouts). Pharmacological tools may have off-target effects and oversimplify the bidirectional interplay between PPAR-γ, PI3K/AKT, JNK/p38, and miR-665. Additionally, the acute single-dose (10 Gy) irradiation protocol in male-only mice limits translation to clinical fractionated radiotherapy and potential sex differences. Direct miR-665 manipulation and more clinically relevant models are needed to confirm its mechanistic and therapeutic roles.

CONCLUSION

Cranial irradiation induces depression-like behaviors in mice, characterized by reduced sucrose preference, impaired exploration, and increased immobility. PIO mitigates these effects by activating PI3K/AKT and inhibiting JNK/p38 pathways in the PFC, with miR-665 playing a pivotal regulatory role. These findings highlight miR-665 as a potential therapeutic target for radiation-induced neurobehavioral disorders, with implications for improving quality of life in cancer survivors undergoing radiotherapy.

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***Conflicts of interest*:** The authors declare no conflicts of interest.

***Ethical consideration*:** All procedures were approved by the IACUC of Xi'an Jiaotong University (Approval No. XJTU-2023-045) and complied with the Guide for the Care and Use of Laboratory Animals.

***Author contribution*:** J.Y., Conceptualization, methodology, data collection, writing - original draft. R.L., Formal analysis, investigation, visualization. X.Z., Supervision, project administration, writing - review & editing.

***Possible use of AI for manuscript preparation*:** This manuscript was prepared without the use of AI tools for content generation. All data analyses, writing, and interpretations were conducted by the authors. Statistical software (SPSS 20.0) was used for data analysis, and no AI-based writing or editing tools were employed.

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