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The Role of Insulin Receptor Substrate 4 (IRS4) Protein in the Radiotherapy Response of Glioblastoma Multiforme (GBM) Cells

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ABSTRACT

Objective: To explain the fundamental role of Insulin Receptor Substrate 4 (IRS4) protein in the response of Glioblastoma Multiforme (GBM) cells to radiotherapy.

Methods: LN229 cells were transfected with IRS4 expression vector using lipofectamine, and the ectopic IRS4 expression was confirmed by western blot. After irradiating LN229 cells with 5, 8, and 10 Gy doses of radiotherapy, the functional effect of IRS4 on radiotherapy was determined using MTT and colony formation assays.

Results: It was determined that increased IRS4 expression led to enhanced radiosensitivity in GBM cells. Increased IRS4 expression in the GBM cell line was found to cause a decrease in cell survival rates and colony formation rates.

Conclusion: IRS4 has been identified to potentially play an active role in the radiotherapy response of GBM cells.

Keywords: IRS4, GBM, Radiotherapy

INTRODUCTION

Glioblastoma Multiforme (GBM) is the most common and aggressive brain tumor found in adults.[1] According to the classification by the World Health Organization (WHO), GBM is considered a grade IV tumor with astrocytic differentiation. Its initial symptoms can include various mental changes such as headaches, memory loss, unexplained personality changes, and difficulty in forming sentences.[2] One of the main reasons GBM is among the most lethal cancers is the high level of heterogeneity at the cellular and genetic levels, which limits treatment options.[3] In this context, the average survival time for patients diagnosed with GBM is two years or less.[4] The standard treatment protocol involves the surgical removal of the tumor, followed by chemotherapy combined with radiotherapy (RT), which is considered reasonably safe.[5]

The underlying mechanism of many anti-cancer treatments, including RT or ionizing radiation (IR), is the induction of DNA double-strand breaks (DSBs), which lead to cell death.[6] RT-induced DNA damage causes cell death by inducing multiple death mechanisms, such as apoptosis, necrosis, and senescence.[7] The dose of radiotherapy administered to cancer cells is determined by the genomic structure of the cancer cell and the sensitivity of the surrounding microenvironment to RT.[8]

Insulin has important functions in the central nervous system, and impaired insulin response plays a critical role in the development of neurodegenerative diseases. The insulin receptor (IR), a member of the receptor tyrosine kinase (RTK) family, and the insulin-like growth factor receptor (IGF-IR) are widely expressed in various types of cancer and are generally associated with poor prognosis.[9] Insulin receptor substrate (IRS) proteins are adaptor molecules that regulate multiple cellular processes by transmitting extracellular signals to the intracellular space through transmembrane receptors. intracellular signaling in response to insulin and IGF1 stimulation.[10]

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Among the members of the IRS protein family, the expression of IRS4 in tissues is more limited compared to IRS1 and IRS2. Increased IRS4 expression has been associated with various types of cancer, such as breast, lung, colorectal, and hepatocellular cancers. In addition to its expression, genomic studies have shown frequent deletions in the cis-regulatory regions of the IRS4 gene, identifying IRS4 as an oncogenic driver.[11] It has been demonstrated that IRS4, when ectopically expressed via retroviral means, can induce the PI3K signaling pathway even in the absence of growth factors, and despite high levels of IRS1 and IRS2 expression, PI3K has a high affinity for IRS4. This suggests that IRS4 may play a significant role in cancer development.[12]

Numerous studies have provided information on the development of cancer and the response to cancer treatment due to the widespread expression of IRS1 and IRS2, members of the IRS protein family. However, information regarding IRS4 is quite limited. The aim of this study is to explain the fundamental role of the IRS4 protein in the response of GBM cells to radiotherapy.

METHODS

Cell culture

LN229 cell line is p53 mutant, PTEN wild type and p16 and p14ARF deleted was used as the Glioblastoma Multiforme cell model in this study. All cells were cultured as monolayers in DMEM (Dulbecco's Modified Eagle Medium) (Biowest, France) containing 1% L-glutamine (PanBiotech, Germany), 10% Fetal Bovine Serum (Gibco, USA), and 1% PSA (Penicillin-Streptomycin-Amphotericin) (Gibco, USA). The cultures were incubated in a 5% CO_2 atmosphere, 95% humidity, at 37°C.

Radiotherapy treatment

Before radiotherapy treatment, a simulation study was performed for plaque dose planning. The simulation material was covered with tissue-equivalent bolus material with a physical density of 1 g/cm³ and dimensions of 12x8.5x1 cm³ to ensure homogeneous dose distribution in all wells and to represent in-vivo conditions. Axial tomography images with a 2.5 mm slice thickness were obtained from these simulation plaques using the Siemens Sensation 4 model CT-Simulator system. The obtained cross-sectional images were defined in the CMS XIO 3D radiotherapy treatment planning system, and dose planning was conducted using the Theratron 1000E model cobalt-60 teletherapy device. The dose planning was completed for each well using Co-60 photon beams with an average energy of 1.25 MeV, with the cells being exposed to 5, 8, and 10 Gy doses of radiation in a single fraction. The data obtained from the treatment planning system, including field size, SSD (Source Skin Distance), irradiation angles, and irradiation durations, were used for radiotherapy application.

Transfection

LN229 cells were transfected with pcDNA3.1 Flag Tagged human IRS4 or empty pcDNA3.1 plasmids using the Qiagen Attractene Transfection reagent (Qiagen, USA) according to the protocol provided by the manufacturer.

Survival analysis (MTT)

LN229 cells, with increased human IRS4 expression or transfected with control plasmids, were seeded at 5000 cells/well in 96-well plates, with six replicates per condition. After overnight incubation, the cells were treated with 5, 8, and 10 Gray doses of radiotherapy. Following radiotherapy, the cells were incubated for 24, 48, and 72 hours under cell culture conditions. At the end of each incubation period, MTT (Sigma, USA) solution at a concentration of 5 mg/ml was added to each well, and the plates were incubated again for 4 hours under a 5% CO_2 atmosphere at 37°C with 95% humidity. After incubation, the medium was removed, and the resulting formazan crystals were dissolved in 100 µl DMSO. The amount of MTT formazan product was determined by measuring absorbance at 540 nm, with 690 nm as the reference wavelength.

Western blot

Protein lysates from LN229 cells transfected with control and pcDNA3.1 Flag Tagged human IRS4 plasmids were prepared using Triton X-100 buffer containing 50 mM HEPES pH 7.0, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 0.15 units/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A. Protein concentrations were determined using the Bradford method, and 100 µg of protein lysates were fractionated by SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked in 5% non-fat dry milk in 1X PBS-Tween 20. Membranes were probed with primary antibodies against IRS4 (Santa-Cruz,USA) and gamma H2AX at a concentration of 1:1000, and Beta-actin (Santa-Cruz,USA) at 1:1500 as a loading control. HRP-conjugated secondary antibodies were used at a concentration of 1:2000, and protein bands were detected using the Image J software. Normalization was performed relative to the beta-actin band.

Colony formation assay

LN229 cells with increased IRS4 expression or transfected with control plasmids were seeded at 500,000 cells/well in 6-well plates. After overnight incubation, one of the plates was treated with 10 Gray radiotherapy, while the other plate was not treated and used as a control. Following radiotherapy treatment, the cells were incubated under cell culture conditions for 9 days, fixed with a methanol acid solution, and stained with a methanol violet solution at a concentration of 0.01% (w/v) for 15 minutes. Colonies were counted using the Image J software.

Statistical analysis

All data from the cell survival and colony formation assays were presented as mean ± standard deviation based on three independent experiments. Multiple comparison analyses were performed using two-way ANOVA with the GraphPad Prism 9 software, and $p<0.05$ was considered statistically significant.

RESULTS

Effective radiotherapy dose and incubation time for LN229 cells

To determine the effective radiotherapy dose and incubation time, LN229 cells were treated with 5 and 10 Gy doses of radiotherapy and incubated for 48 and 72 hours. Survival analysis results showed a 10% decrease in survival after 48 hours and a 16% decrease after 72 hours in the 5 Gy-treated group compared to the control group. In the 10 Gy-treated group, survival decreased by 16% after 48 hours and 20% after 72 hours compared to the control group (Figure 1).

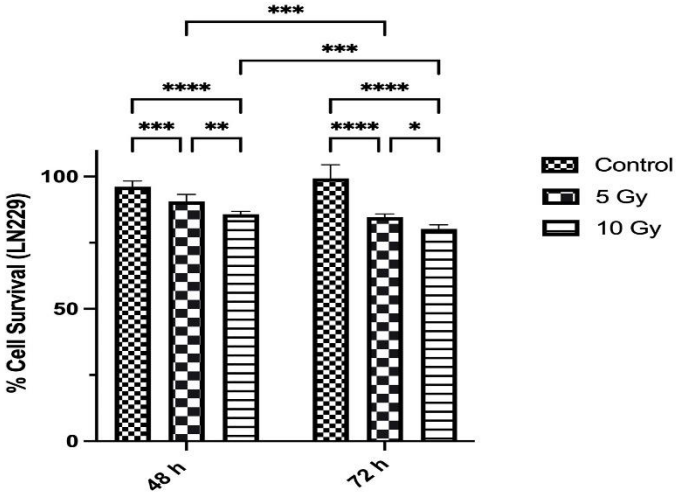

Figure 1. Survival graph of LN229 cells depending on different RT doses and incubation times (*<0.05, **<0.01, ***<0.001, ****<0.0001)

Dose-Dependent γH2AX expression changes in LN229 cells

LN229 cells were treated with 5, 8, and 10 Gy doses of radiotherapy to assess dose-dependent changes in γH2AX expression. A significant increase in γH2AX expression was observed in the 8 and 10 Gy-treated groups compared to the control group, with a 1.5-fold increase in the 10 Gy-treated group correlating with survival analysis results (Figure 2).

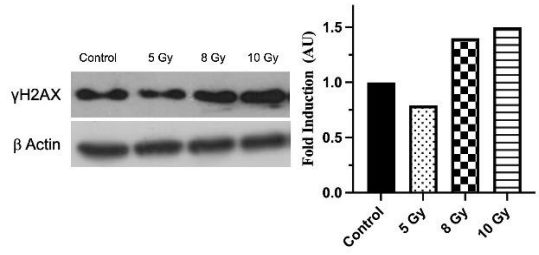

Figure 2. γH2AX expression changes depending on different RT doses in LN229 cells

Effect of IRS4 expression on survival of LN229 cells after radiotherapy

LN229 cells with increased IRS4 expression were treated with a 10 Gy dose of radiotherapy and incubated for 72 hours. No change in survival was observed between the control and pcDNA3.1-transfected groups. However, a 32% decrease in survival was observed in IRS4-expressing LN229 cells without radiotherapy (Figure 3).

In the 10 Gy-treated group, a 25% decrease in survival was observed in IRS4-expressing cells compared to radiotherapy-treated controls, and a 19% decrease compared to non-radiotherapy controls (Figure 3).

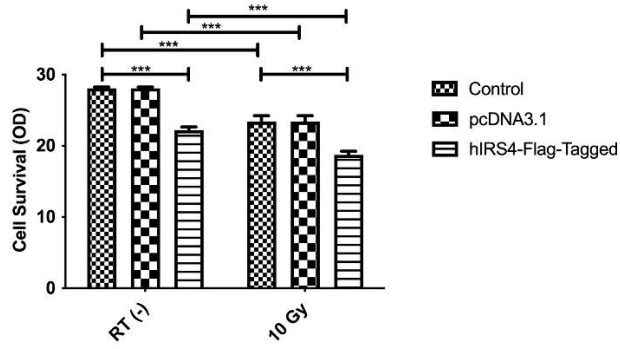

Figure 3. Survival level of IRS4 overexpressing LN229 cells after 10 Gy radiotherapy treatment (***<0.001)

Radiation-Induced γH2AX expression in LN229 cells with increased IRS4 expression

IRS4-transfected LN229 cells were treated with 10 Gy of radiotherapy, and γH2AX expression levels were assessed. A 2-fold increase in phosphorylated γH2AX expression was observed in IRS4-expressing cells compared to control

cells in both radiotherapy-treated groups (Figure 4).

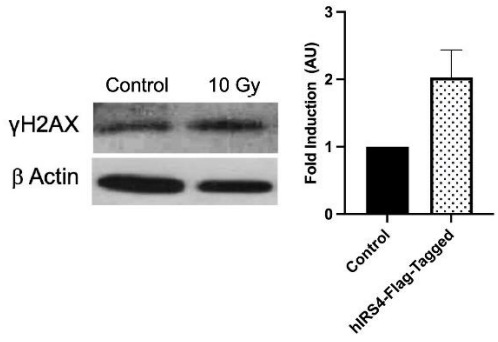

Figure 4. Expression levels of γH2AX after 10 Gy radiotherapy treatment in IRS4 overexpressing LN229 cells

Effect of increased IRS4 expression on colony formation in LN229 cells after radiotherapy

A colony formation assay was performed to compare the colony-forming ability of cells with increased IRS4 expression combined with radiotherapy treatment. A 19% decrease in colony formation was observed in IRS4-expressing LN229 cells compared to controls. In the 10 Gy-treated group, an 88% decrease in colony formation was observed in IRS4-transfected cells compared to controls, and a 98% decrease was observed compared to non-radiotherapy IRS4-expressing cells (Figure 5).

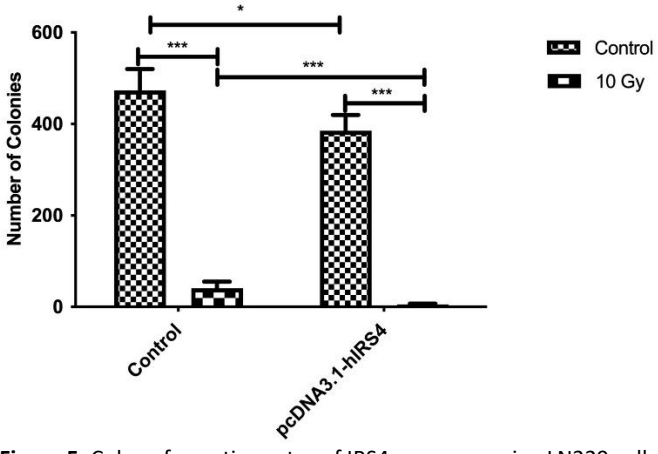

Figure 5. Colony formation rates of IRS4 overexpressing LN229 cells after 10 Gy radiotherapy treatment (*<0.05, ***<0.001)

DISCUSSION

The insulin-like growth factor receptor (IGFR) and insulin receptor (IR) are known to play a role in cancer development and progression. Activation of these signaling pathways by insulin and IGF is common in cancer cells and represents a significant resistance factor to various anticancer therapies.[13]

The tyrosine kinase domains of the IR and IGF-IR β subunits phosphorylate specific substrates such as IRS family members (IRS1-IRS4), Gab-1, Cbl, and Shc. This phosphorylation induces PI3K/AKT/mTOR and ERK/MAPK pathways, mediating cellular metabolic and mitogenic mechanisms.[14]

Among the IRS family members, IRS4 has limited expression and lacks a protein phosphatase-binding domain, leading to ongoing research into its functional effects.[15] In this study, a 25% decrease in survival was observed in cells treated with a 10 Gy dose of radiotherapy after increased IRS4 expression. The combination of IRS4 transfection and radiotherapy increased the cells' sensitivity to RT. Studies have shown that IRS1 directly binds to Rad51, enhancing DNA repair activity.[16,17] Based on these findings, IRS4, unlike IRS1, may not interact with Rad51, thus not contributing to DNA repair mechanisms. Additionally, studies have shown that IRS4 may inhibit IRS1 function when both proteins are expressed in the same cell.[18] Therefore, increased IRS4 expression in GBM cells may inhibit IRS1 function and reduced DNA repair efficiency.

In subsequent experiments, increased IRS4 expression in cells treated with 10 Gy of radiotherapy was associated with increased γH2AX expression levels compared to controls. This suggests that increased IRS4 expression in cells treated with 10 Gy of radiotherapy may induce cell cycle arrest, senescence, and apoptosis by causing unrepaired DNA damage. γH2AX expression in the control group of IRS4-transfected cells was higher than in the non-transfected control group, suggesting that IRS4 expression may promote DNA damage and cell cycle arrest independent of RT. As a result, increased IRS4 expression alone is sufficient to induce DNA damage and apoptosis in GBM cells, which may be due to reduced IRS1 function and inefficient DNA repair.

This study demonstrated that increased IRS4 expression in GBM cells increases radio sensitivity by reducing DNA repair efficiency. IRS4, which reduces cell survival even without radiotherapy, may be a potential new therapeutic target in GBM treatment. Further experiments using RNA interference methods will contribute to the development of specific treatments targeting IRS4.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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