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Selective Inhibition of PI3K Isoforms in Brain Tumors Suppresses Tumor Growth by Increasing Radiosensitivity

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Purpose: Glioblastoma (GBM) is a malignant brain tumor with poor prognosis. Radioresistance is a major challenge in the treatment of brain tumors. The development of several types of tumors, including GBM, involves the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway. Upon activation, this pathway induces radioresistance. In this study, we investigated whether additional use of selective inhibitors of PI3K isoforms would enhance radiosensitivity in GBM.
Materials and Methods: We evaluated whether radiation combined with PI3K isoform selective inhibitors can suppress radioresistance in GBM. Glioma 261 expressing luciferase (GL261-*luc*) and LN229 were used to confirm the effect of combination of radiation and PI3K isoform inhibitors in vitro. Cell viability was confirmed by clonogenic assay, and inhibition of PI3K/AKT signaling activation was observed by Western blot. To confirm radiosensitivity, the expression of phospho-γ-H2AX was observed by immunofluorescence. In addition, to identify the effect of a combination of radiation and PI3K-α isoform inhibitor in vivo, an intracranial mouse model was established by implanting GL261-*luc*. Tumor growth was observed by IVIS imaging, and survival was analyzed using Kaplan–Meier survival curves.
Results: Suppression of the PI3K/AKT signaling pathway increased radiosensitivity, and PI3K-α inhibition had similar effects on PI3K-pan inhibition in vitro. The combination of radiotherapy and PI3K-α isoform inhibitor suppressed tumor growth and extended survival in vivo.
Conclusion: This study verified that PI3K-α isoform inhibition improves radiosensitivity, resulting in tumor growth suppression and extended survival in GBM mice.

Key Words: Glioblastoma, radiosensitivity, radiation, PI3K-isoform, radioresistance

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INTRODUCTION

Glioblastoma (GBM) is a malignant grade four tumor, and the most common type of malignant brain tumor in adults.[1] The treatment of GBM consists of safe surgical resection, radiation therapy, and temozolomide chemotherapy.[2] Despite these aggressive treatments, however, the overall survival rate of patients with GBM is very poor, with an average of only 14–16 months.[3,4] Numerus strategies have been investigated to improve the health outcomes of patients, but have failed to achieve survival

benefits in large clinical trials.[5-7] Innate radioresistance and increased radioresistance, especially after recurrence, of GBM tumors are considered the main causes of treatment failure.[8,9]

Over the years, several studies have investigated the mechanisms of GBM radioresistance.[10-12] Several key factors, including tumor microenvironment, hypoxia, glioma stem cells, metabolic alterations, and DNA damage and repair, have been suggested as being involved in GBM radioresistance.[10] Hyperactivation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway is one of the crucial pathways involved in tumor growth, survival, and proliferation in a variety of cancers, including GBM.[3] Furthermore, this pathway has been shown to be correlated with mechanisms of tumor-specific radioresistance.[12,13] Various PI3K isoforms play different tumor type-specific roles.[14-16]

The inhibition of the PI3K/AKT signaling pathway improves radiosensitivity.[13,17,18] However, first-generation pan-PI3K inhibitors, which inhibit all PI3K isoforms, can cause side effects, such as vomiting, diarrhea, neutropenia, and hyperglycemia.[19-21] PI3K isoform-selective inhibitors (p110α, p110β, p110γ, and p110δ) can be effective alternatives for radioresistance regulation. The combination of a PI3K isoform-selective inhibitor with radiotherapy is expected to reduce the risk of side effects while suppressing radioresistance. Recently, the effectiveness of PI3K isoform-selective inhibitors in reducing radioresistance has been studied in clinical trials.[13,22] Our previous study demonstrated that the selective inhibition of PI3K isoforms enhances radiosensitivity in non-small cell lung cancer.[23]

In this study, we hypothesized that a combination of radiotherapy and PI3K isoform-selective inhibitors would improve radiotherapeutic response rates among GBM tumors by enhancing radiosensitivity. To develop an optimal treatment strategy, we compared and evaluated the tumor growth inhibition rates of several PI3K isoform-selective inhibitors in vitro and in vivo.

MATERIALS AND METHODS

Cell lines and cell culture

The mouse-derived cell line GL261-*luc* (purchased from PerkinElmer, Waltham, MA, USA) and human-derived cell line LN229 (purchased from ATCC, Manassas, VA, USA) were used in the present study. GL261-*luc* and LN229 cell lines were cultured in Dulbecco's modified Eagle's Medium (DMEM, HyClone, Darmstadt, Germany) with 10% fetal bovine serum (FBS, HyClone) and 1% penicillin-streptomycin (PS, HyClone), and then incubated at a controlled temperature of 37°C in a 5% CO_2 humidified atmosphere.

Irradiation

Irradiation was performed on the GL261-*luc* and LN229 cell lines using a X-rad 320 (Precision X-Ray, North Branford, CT, USA) system with a single dose of 1 Gy for the clonogenic assay. Irradiations of 2, 3, 4, and 6 Gy were applied with two, three, four, and six repeated doses of 1 Gy, respectively. The radiation fraction was emitted at 320 kVp and 12.5 mA with 2.0 mm Al filtration at a dose rate of 4.76 cGy/sec.

Phosphoinositide 3-kinases (PI3K) isoform-selective inhibitors

Pictilisib (GDC0941) is a potent PI3K class I pan-inhibitor with modest selectivity against the isoform p110 ranging from 3 nM (p110α) to 75 nM (p110γ) in vitro. Taselisib (GDC0032) is a potent PI3K-α inhibitor. Duvelisib (IPI145) is a potent PI3K-δ/γ-specific inhibitor. Idelalisib (CAL101) is a p110δ-specific inhibitor. We used the same doses of these inhibitors as used in our previous study.[23] All chemicals were purchased from Selleckchem (Houston, TX, USA).

MTT assays

The effect of the PI3K isoform-selective inhibitors on cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (MTT; Sigma-Aldrich, St. Louis, MO, USA). Cells were plated in 96-well tissue culture plates (2000 cells per well) and treated with PI3K-isoform inhibitors. Following 24 h of treatment, cells were incubated for 4 h at 37°C in a CO_2 incubator with 10% of MTT solution in complete medium (DMEM+10% FBS+1% PS). Subsequently, formazan crystals were dissolved in 100 μL of Dimethyl sulfoxide, and absorbance was confirmed at 590 nm using a microplate reader (VERSA max, Molecular Devices, San Jose, CA, USA).

Western blot

Protein expression was confirmed using Western blotting. Cells were lysed using Cell Extraction Buffer (Thermo Fisher, Waltham, MA, USA) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher). Concentrations of whole-cell lysate were measured using a BCA protein assay kit (Thermo Fisher). Whole cell lysates were electrophoresed on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). The membrane was blocked treating with 3% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST) for 2 h at 25°C. The membranes were allowed to react overnight at 4°C with primary antibodies and rinsed three times for 15 min each with TBST. Subsequently, they were reacted with horseradish peroxidase-conjugated secondary antibody for 1 h at 25°C and rinsed with TBST for times for 15 min each. Protein expression was activated using ECL solution (WESTSAVE ECL Solution, AbFrontier, Seoul, Korea) and exposed to X-ray film (AGFA, Mortsel, Belgium). Information on the antibodies used is summarized in Table 1.

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Table 1. Western Blot Antibodies

Antibody	Cat. No.	Brand	Dilution ratio
AKT	9272	Cell signaling, Massachusetts, USA	1:1000
Phospho-AKT	9271	Cell signaling, Massachusetts, USA	1:1000
Phospho-γ-H2AX	056361	Millipore, Massachusetts, USA	1:1000
β-actin	sc-47778	Santa cruz, Dallas, Texas, USA	1:10000

Immunofluorescence

After seeding 2000 cells on a 4-well slide plate, the cells were irradiated and treated with PI3K isoform-selective inhibitors. After 3 h of irradiation, the cells were fixed with 100% chilled methanol. To permeabilize the cells, they were incubated for 10 min with phosphate-buffered saline (PBS) containing 0.25% Triton X-100. After washing with PBS three times for 5 min each, the samples were then blocked with 1% BSA in PBS containing 0.1% Tween 20 (PBST) for 1 h. The cells were allowed to react overnight at 4°C at 1:100 in anti-phospho-γ-H2AX antibody (Millipore, Burlington, MA, USA) and washed three times for 5 min with PBST. The cells were then treated with an FITC-labeled secondary antibody (Thermo Fisher) for 1 h at RT in the dark at 1:300. Finally, the cells were mounted using VECTASHIELD solution (Vector Laboratories, Burlingame, CA, USA). Phosphor-γ-H2AX foci fluorescence images were acquired with a Zeiss LSM 700 confocal fluorescence microscope (Carl Zeiss A. G., Baden-Württemberg, Germany).

Clonogenic assay

The clonogenic assay is a radiosensitivity test. After irradiation, cells were seeded in a 6-well plate in triplicate. After seeding, the cells were treated with PI3K isoform inhibitors and incubated for 9–14 days at 37°C in 5% CO_2 humidified atmosphere for colony formation. The colonies were stained with a 0.5% crystal violet solution.

The survival fractions after irradiation were calculated in accordance with the report of Chadwick and Leenhouts[24] and drawn with GraphPad Prism 5.0 (GraphPad Software Inc., Boston, MA, USA) using a linear quadratic model with the formula $Y=\exp[-1* (A*X+B*X^2)]$, where X is the radiation dose and Y is the survival fraction. An estimated survival fraction of 0.5 and the sensitizer enhancement ratio of 50% inhibition (SER50) of each PI3K isoform-selective inhibitor were calculated (Supplementary Table 1, only online).

In vivo combination therapy of intracranial mice model

An intracranial mouse model was established using BALB/c-nu mice purchased from Orient Co. (Seongnam, Korea). After placing a mouse on the ear bar of a stereotaxic frame (RWD Life Science, San Diego, CA, USA), a burr hole was drilled 1.5 mm anterior and 2.5 mm lateral from the bregma using a drill (SAESHIN PRECISION, Daegu, Korea). GL261-*luc* cells ($3×10^5$ cells in 5 μL of Dulbecco's PBS) were directly implanted into the brain 2.0 mm deep from the skull surface. Subsequently, the mice were randomly assigned to four groups: vehicle control, radiation, PI3K-α isoform inhibitor (GDC0032) treated, and PI3K-α isoform inhibitor (GDC0032)+radiation. Radiotherapy was performed specifically on the brain at 2 Gy for 5 days, with or without the PI3K-α isoform inhibitor (GDC0032). The PI3K-α isoform inhibitor (GDC0032; 5 mg/kg) was injected intraperitoneally.

Statistical analysis

Student's t-test was used to assess significant differences between two groups. We analyzed the survival periods of the treatment groups using Kaplan–Meier survival curves. Survival differences between groups were compared using the log-rank test. SPSS software (version 25.0; IBM Corp., Armonk, NY, USA) was used to conduct all statistical analyses. GraphPad Prism 5.0 (GraphPad Software Inc.) was used to create graphical representations. A two-sided p-value of less than 0.05 was considered statistically significant.

RESULTS

Effects of irradiation and PI3K isoform-selective inhibitors on human glioma cells

We performed a clonogenic assay to confirm the radiosensitivity of the human-derived glioma LN229 cells (Fig. 1A). After irradiating LN229 cells with 2, 4, and 6 Gy, they were cultured for 9–12 days in growth medium. The viability of irradiated LN229 cells was 73.6%, 39.4%, and 11.1% at 2, 4, and 6 Gy, respectively. To evaluate the ability to repair DNA double-strand breaks (DSB), we observed the expression of phospho-γ-H2AX using immunofluorescence 3 h after irradiation (Fig. 1A). The expression of phospho-γ-H2AX was upregulated with increasing irradiation doses.

We performed an MTT assay to confirm the viability of LN229 cells treated with PI3K isoform-selective inhibitors: PI3K pan-inhibitor (GDC0941), PI3K α-isoform inhibitor (GDC0032), PI3K δ-isoform inhibitor (CAL101), and PI3K γ/δ-isoform inhibitor (IPI145) (Fig. 1B). LN229 cells were treated with doses of 0.1, 1, and 10 μM for 24 h. PI3K isoform-selective inhibitors decreased the viability of LN229 cells significantly at its higher doses (all $p<0.001$). The average viabilities of LN229 cells treated with PI3K isoform-selective inhibitors were as follows: 90.21%, 94.85%, 96.92%, and 93.39% at 0.1 μM; 83.07%, 87.37%, 96.55%, and 90.86% at 1 μM; and 65.88%, 75.69%, 86.19%, and 85.83% at 10 μM.

Furthermore, each PI3K isoform-selective inhibitor suppressed the activity of AKT in LN229 cells (Fig. 1C, Supplementary Fig. 1A, only online). The PI3K α-isoform inhibitor (GDC0032) was the most effective among the isoform-selective PI3K inhibitors. Based on these results, the dose for testing the combination therapeutic effect was determined as the

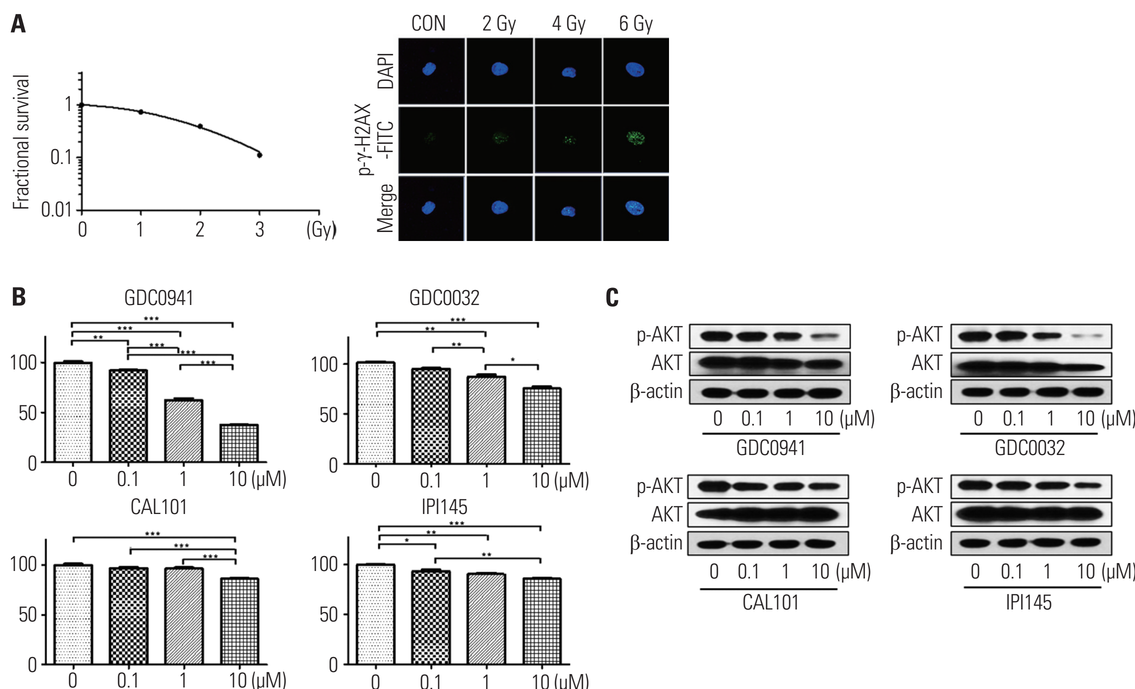

Fig. 1. Effect of irradiation and PI3K isoform inhibitors in the LN229 human glioma cell line. (A) Dose versus clonogenic survival curves and immunofluorescence images of phosphor-γ-H2AX. LN229 cells were treated with PI3K isoform-selective inhibitors for 24 h (doses ranging from 0.1 to 10 μM). Data are expressed as the percentage of (B) MTT cell proliferation assay. (C) Protein expression of AKT activation in LN229 cells. Statistical significance was set at $p<0.05$. *$p<0.05$; **$p<0.01$; ***$p<0.001$. PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

dose that minimized the cytotoxicity and activation of AKT.

PI3K isoform-selective inhibitors increase radiosensitivity in human glioma cell lines

To confirm radiosensitivity using irradiation in combination with PI3K isoform-selective inhibitors, we performed a clonogenic assay. After combination therapy (treatment with irradiation and isoform-selective inhibitors), LN229 cells were colonized for 12–14 days. The combination therapy inhibited the colonization of LN229 cells (Fig. 2A). The combination therapy using different inhibitors decreased the survival rate of LN229 cells to approximately 29% (PI3K-pan), 27% (PI3K-α), 37% (PI3K-δ), and 45% (PI3K-γ/δ) ($p<0.001$). In particular, the PI3K α-isoform inhibitor GDC0032 inhibited colony formation similar to the PI3K-pan inhibitor (GDC0941). PI3K-δ isoform inhibitor (CAL101) and PI3K-γ/δ inhibitor (IPI145) also decreased cell viability. In addition, the expression of phosphorylated AKT was suppressed by PI3K isoform-selective inhibitors (Fig. 2A, Supplementary Fig. 1B, only online). After combination therapy, PI3K pan-inhibitor (GDC0941) and PI3K α-isoform inhibitor (GDC0032) inhibited the expression of phosphorylated AKT more strongly than the PI3K-δ isoform inhibitor (CAL101) and PI3K-γ/δ inhibitor (IPI145). To confirm the inhibition of DNA DSB repair through the combination therapy, we recorded the expression of phospho-γ-H2AX using immunofluorescence and Western blotting at 3 h after irradiation (Fig. 2B). The expression of phospho-γ-H2AX was increased by the combination therapy including isoform-selective inhibitors, compared to that by irradiation alone. Particularly, the repair of DNA DSB was suppressed while using a PI3K-α isoform inhibitor, as observed in the case of using PI3K-pan inhibitor.

Effects of irradiation and PI3K isoform-selective inhibitors on mouse glioma cells

Clonogenic assay confirmed the radiosensitivity of mouse-derived glioma GL261-*luc* cells (Fig. 3A). Irradiated GL261-*luc* cells were found to be 76.38% and 38.86% at 2 Gy and 9.65% viable at 1, 2, and 3 Gy, respectively. In addition, we evaluated the ability to repair DNA DSBs with expression profiling of phospho-γ-H2AX at 3 h after irradiation using immunofluorescence (Fig. 3A). The expression of phospho-γ-H2AX increased with increasing irradiation doses.

MTT assay confirmed the viability of GL261-*luc* cells treated with the isoform-selective inhibitors (Fig. 3B). GL261-*luc* cells treated with increasing doses of inhibitor (0.1, 1, and 10 μM) for 24 h revealed that the viability of GL261-*luc* cells decreased significantly with higher doses of PI3K isoform-selective inhibitors ($p<0.001$). The average viability of GL261-*luc* cells with each PI3K isoform-selective inhibitor were as follows: 92.21%, 85.50%, 98.57%, and 94.95% at 0.1 μM; 62.42%, 67.37%, 96.68%, and 90.00% at 1 μM; and 37.55%, 43.94%, 82.11%, and 66.12% at 10 μM.

In addition, each isoform-selective PI3K inhibitor suppressed the activity of AKT in GL261-*luc* cells (Fig. 3C, Supplementary Fig. 1A, only online). The PI3K α-isoform inhibitor (GDC0032) was the most effective among the isoform-selective PI3K in-

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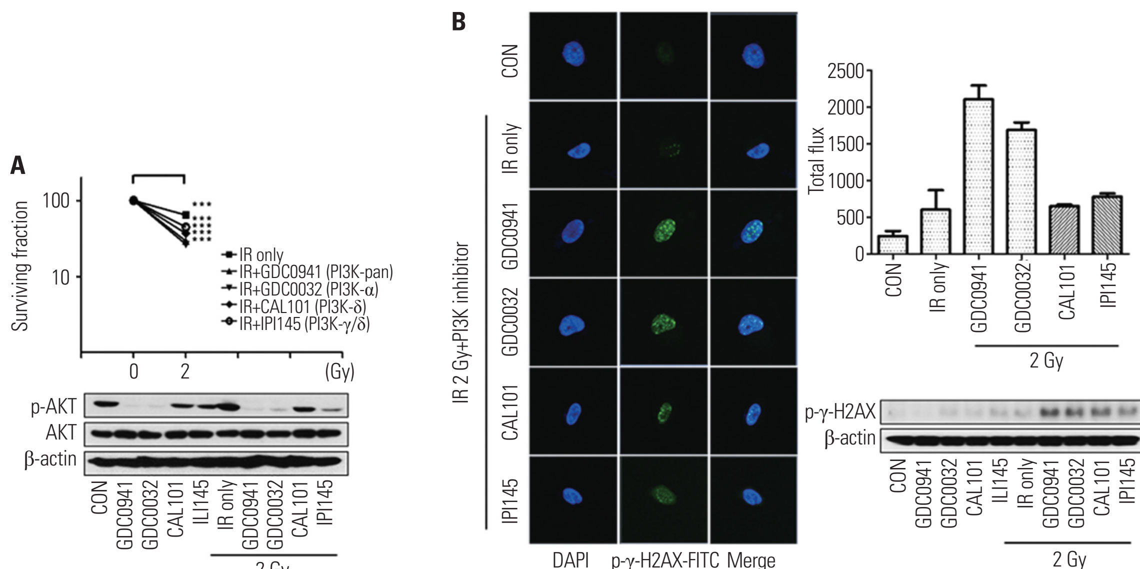

Fig. 2. Combination effect of irradiation and PI3K isoform inhibitors in the LN229 human glioma cell line. (A) Clonogenic survival curves: LN229 were treated with PI3K isoform-selective inhibitor at 1 h after 2 Gy irradiation. (B) Expression of phosphor-γ-H2AX in GL261 cells after a combination of radiation and PI3K isoform inhibitors (*p*: CON vs. IR+GDC0941; ***, CON vs. IR+GDC0032; ***). Treatment doses were GDC0941 0.1 μM, GDC0032 0.1 μM, CAL101 1 μM, and IPI145 1 μMs. Statistical significance was set at $p<0.05$. ***$p<0.001$. PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

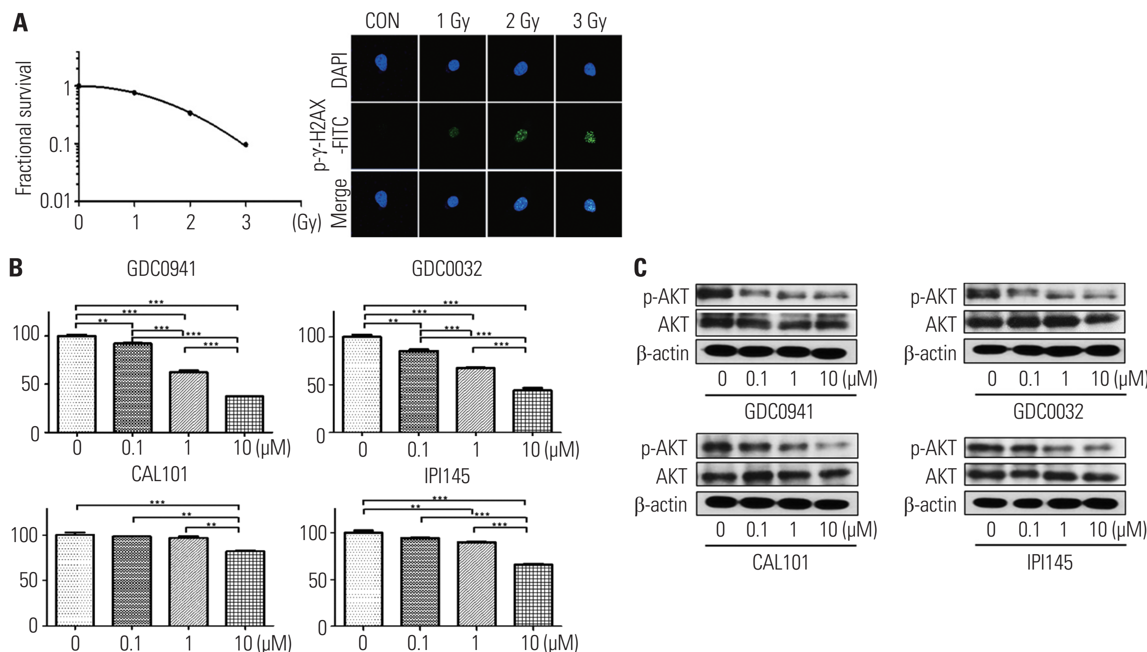

Fig. 3. Effect of irradiation and PI3K isoform inhibitors in the GL261 mouse glioma cell line. (A) Dose versus clonogenic survival curves and immunofluorescence images of phosphor-γ-H2AX. GL261-*luc* cells were treated with PI3K isoform-selective inhibitors for 24 h (doses ranging from 0.1 to 10 μM). Data are expressed as the percentage of (B) MTT cell proliferation assay. (C) Protein expression of AKT activation in GL261-*luc*. Statistical significance was set at $p<0.05$. **$p<0.01$; ***$p<0.001$. PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

hibitors. Based on these results, the dose for testing the combination therapeutic effect was determined as the dose that minimized the cytotoxicity and activation of AKT.

PI3K isoform-selective inhibitors increase radiosensitivity in mouse glioma cell lines

The radiosensitivity of cells was confirmed with the combination therapy followed by a clonogenic assay. GL261-*luc* cells colonized for 13–14 days after combination therapy involving irradiation and PI3K isoform-selective inhibitors revealed distinct inhibitory effect of the isoform-selective inhibitors on cell colonization (Fig. 4A). The combination therapy reduced the survival rate in GL261-*luc* cells to approximately 17% (PI3K-pan, $p<0.001$), 19% (PI3K-α, $p<0.001$), 28% (PI3K-δ, $p<0.001$),

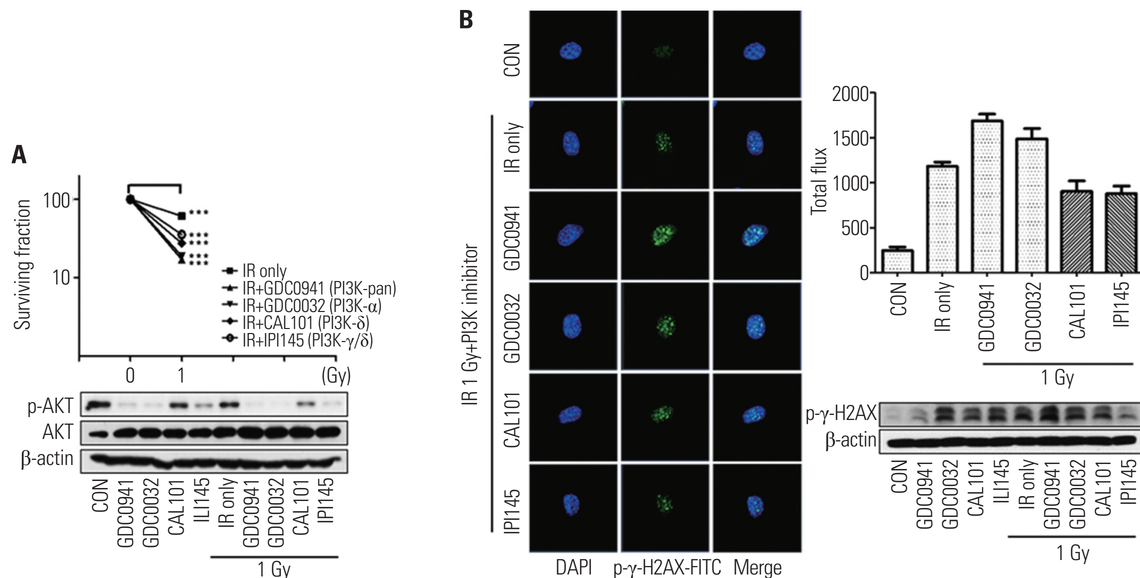

Fig. 4. Combination effect of irradiation and PI3K isoform inhibitors in the GL261 mouse glioma cell line. (A) Clonogenic survival curves: GL261-*luc* were treated with PI3K isoform-selective inhibitor at 1 h after 1 Gy irradiation. (B) Expression of phosphor-γ-H2AX in GL261 cells after combination of radiation and PI3K isoform inhibitors (CON vs. IR only; ***, CON vs. IR+GDC0941; ***, CON vs. IR+GDC0032; ***, CON vs. IR+CAL101; ***, CON vs. IR+IPI145; **). Treatment doses were GDC0941 0.1 μM, GDC0032 0.1 μM, CAL101 1 μM, and IPI145 0.1 μM. Statistical significance was set at $p<0.05$. ** $p<0.01$; *** $p<0.001$. PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

and 34% (PI3K-γ/δ, $p<0.001$). Specifically, the inhibitory effects of the PI3K α-isoform inhibitor (GDC0032) and PI3K-pan inhibitor (GDC0941) were comparable. PI3K-δ isoform inhibitor (CAL101) and PI3K-γ/δ inhibitor (IPI145) also decreased cell viability. In addition, the expression of phosphorylated AKT was suppressed by the isoform-selective inhibitors (Fig. 4A, Supplementary Fig. 1B, only online). In combination therapy, PI3K-pan inhibitor (GDC0941) and PI3K γ-isoform inhibitor (GDC0032) inhibited the expression of phosphorylated AKT more strongly than that by PI3K-α isoform inhibitor (CAL101) and PI3K-γ/δ inhibitor (IPI145). To confirm this, we observed phospho-γ-H2AX expression through immunofluorescence and Western blotting at 3 h after irradiation. The expression of phospho-γ-H2AX, indicating inhibition of DNA DSB repair was increased to a greater extent with combination therapy involving isoform-selective inhibitors, compared to that induced by irradiation alone (Fig. 4B). In particular, DNA DSB repair suppressed using the PI3K-α isoform inhibitor was similar to that by the PI3K-pan inhibitor.

PI3K-α isoform modulates radiosensitivity in glioma tumor in vivo

To confirm the resultant radiosensitivity when inhibiting the PI3K-α isoform in brain tumors, we performed combination radiotherapy in a GL261-*luc* intracranial mice model. The tumor-implanted brain was irradiated for 5 days with 2 Gy combined with or without 5 mg/kg PI3K-α isoform inhibitor (GDC0032). Inhibition of the PI3K-α isoform increased the radiosensitivity of brain tumors and markedly suppressed tumor growth, compared to that achieved without the PI3K-α isoform inhibitor in vivo ($p<0.05$) (Fig. 5A). We also evaluated the correlation between inhibition of the PI3K-α isoform and time required for biochemical progression using Kaplan–Meier analysis (Fig. 5B). When PI3K-α isoform inhibitor was administered, no significant weight change or illness was found (Supplementary Fig. 2, only online). Our data showed that combination therapy including radiation and PI3K inhibition resulted in slower biochemical progression than that in the control group. Moreover, these data indicated that the PI3K-α isoform is strongly associated with improved radiosensitivity.

DISCUSSION

In this study, we confirmed that PI3K signaling activation is associated with the acquisition of radioresistance in GBM. PI3K isoform-selective inhibitors appear to be involved in the regulation of the radiosensitivity of GBMs cells. Among these inhibitors, the PI3K-α-isoform inhibitor most effectively suppressed cell proliferation and radioresistance in this study. In particular, combination radiotherapy with a PI3K-α isoform inhibitor suppressed tumor growth in GBM more effectively compared to radiotherapy alone. We suggest that combination radiotherapy with PI3K-α isoform inhibitors may help improve radiosensitivity in patients with GBM.

Radioresistance is one of the central causes of failure of GBM treatment.[11] Various mechanisms have been proposed to explain radioresistance.[25] Mechanisms of radioresistance in GBM involve several key factors, including the tumor microenvironment, hypoxia, glioma stem cells, tumor heterogeneity, and DNA damage and repair.[10] DSBs are considered a major cause of radiation-induced cell death.[26] DSBs are mainly produced by

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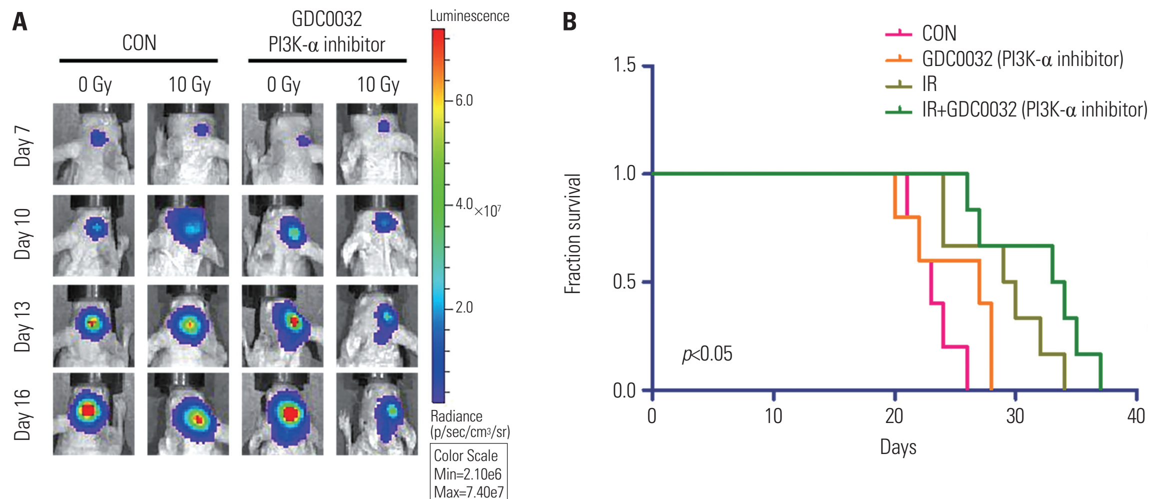

Fig. 5. Inhibition of the PI3K-α isoform increases radiosensitivity in brain tumor in vivo. After intracranial implant of GL261-*luc* cells, 10 Gy irradiation with or without GDC0032 (5 mg/kg). (A) IVIS imaging of mouse brain tumors from within 7–16 days post therapy. (B) Kaplan–Meier survival analysis (p: CON vs. IR only; *, CON vs. IR+GDC0032; **). Statistical significance was set at $p<0.05$. *$p<0.05$; **$p<0.01$. PI3K, phosphoinositide 3-kinase; AKT, protein kinase B.

exogenous agents, such as irradiation.[27] The yield of DSB starts with low doses of irradiation and increases with the dose. DNA repair is mediated by genes, such as BRCA1, which is induced by AKT.[28] In several papers on GBMs, researchers have demonstrated that inhibition of AKT activity suppresses repairs of radiation-induced DSB and increases radiation sensitivity and survival.[9,18] In the present study, we confirmed that the inhibition of AKT activity by PI3K inhibitors increases the level of phospho-γ-H2AX (a biomarker of irradiation-induced DNA DSBs). These results are consistent with those of other studies showing that inhibition of AKT activation could increase the effect of radiotherapy in GBM.[12,18]

The PI3K signaling pathway has been demonstrated to be involved in progression and radioresistance in several carcinomas.[22,29] AKT, a key molecule in the PI3K signaling pathway, is correlated with radioresistance in malignancies.[30] Another major factor is that the activation of AKT inhibits apoptosis, increasing the survival rate of cancer cells after radiotherapy.[31] Therefore, the suppression of AKT can potentially overcome radioresistance. We selected a concentration that inhibited about 70% proliferation rate for the PI3K-isoform inhibitor to minimize side effects. Additionally, several papers have revealed that the recovery of radiation-induced DSB and radioresistance is partially dependent on AKT activation in vitro and in vivo.[12,32,33]

Considering our results, inhibition of PI3K isoforms could be a potential strategy for overcoming radioresistance. In related clinical trials, inhibition of all PI3K isoforms, using first-generation inhibitors, had limited efficacy and relatively severe side effects.[34,35] The four p110 class I PI3K isoforms, including p110α, p110β, p110δ, and p110γ, have particular roles in several carcinomas.[16] The p110δ and p110γ isoforms, expressed most frequently in carcinogenic solid tissues, play a role in stimulating cell proliferation and invasive cell growth.[16,36] The p110δ and p110γ isoforms, expressed most frequently in hematopoietic lineage cells, are involved in the activation of AKT and drug resistance in myeloid leukemia cells.[15,16,36] However, the effect of PI3K selective isoforms on radioresistance has not been precisely elucidated in GBMs. We demonstrated that inhibition of individual PI3K isoforms in GBM impairs DNA DSBs repair mechanisms and modulates radiosensitivity in vitro. In particular, inhibition of the PI3K-α isoform improved radiosensitivity over other isoforms, suggesting that the PI3K-α isoform plays a major role in regulating radioresistance, compared to the other isoforms in GBMs. Epithelial–mesenchymal transition (EMT) is known to be increased in tumors that have acquired radioresistance.[23] Therefore, we confirmed that inhibition of the PI3K isoform inhibits GBM migration ability and regulates EMT-related gene expression in vitro (Supplementary Fig. 3, only online). These results suggest that PI3K isoforms-selective inhibition is another factor that suppresses tumor development. Additionally, we selected PI3K-α inhibitors that increased radiosensitivity the most in vitro and performed in vivo experiments in BALB/c nude mice for tumor growth inhibition and survival analysis due to radiosensitivity enhancement. Inhibition of the PI3K-α isoform increased the radiosensitivity of brain tumors, suppressed tumor growth, and extended the survival period in vivo. Our results are consistent with previous findings on the effect of the PI3K-α isoform in other types of cancers.[17,37,38] In our previous study, we confirmed that the inhibition of the PI3K-α isoform in NSCLC improves radiosensitivity, thereby increasing the radio-therapeutic response of tumors and suppressing EMT.[23] This suggests that PI3K-α inhibitors have promising safety and may be effective agents to increase the response rate to radiotherapy.

Based on preclinical results, clinical studies have been conducted and published, and a few clinical trials are ongoing for GBM. Paxalisib (GDC0084) is a potent selective, oral, brain-penetrating, small-molecule inhibitor of class I PI3K that has

been studied in preclinical and clinical trials.[39] The safety of GDC0084 in radiotherapy in patients with brain metastases or leptomeningeal metastases with PI3K pathway mutations is being evaluated in a phase 1 study (NCT04192981). An ongoing phase I study is addressing the safety, tolerability, and pharmacokinetics of GDC0084 treatment applied in pediatric patients with newly diagnosed diffuse midline glioma after radiotherapy (NCT03696355). An ongoing phase II trial is trying to determine whether the combination of GDC0084 with other drugs and radiation therapy would be effective in treating glioma (NCT05009992). However, clinical studies on the combination of PI3K isoform-selective inhibitors and radiotherapy for GBM are still lacking.

We have identified several additional points that demand further research. We could not confirm the effect of the inhibition of other PI3K isoforms on GBM tumor in vivo. Also, this study did not demonstrate the effect of PI3K-α inhibition on multiple cellular processes other than the improvement of tumor radiosensitivity. In addition, we could not demonstrate the molecular mechanism of how the inhibition of the PI3K-α-isoform improves radiosensitivity in GBM cells. Since our study focused on inhibiting tumor growth by improving the radiosensitivity of the tumor, we selected the LN229 cell line with a fast proliferation rate and low migration capacity.[40] However, since the results could differ when used in patients, ultimately, it will have to be proven in an experiment using patient-derived cells. Finally, we did not elucidate how the inhibition of the PI3K-α-isoform affected the tumor environment in addition to radiosensitivity. Further studies are needed to elucidate downstream factors and validate our findings.

In conclusion, in this study, we demonstrated that activation of PI3K signaling is associated with the development of radioresistance in GBM cells. Isoform-selective PI3K inhibitors are involved in regulating the radiosensitivity of these cells. Among them, inhibition of the PI3K-α-isoform was the most effective for inhibiting cell proliferation and increasing radiosensitivity. We believe that a combination of a PI3K-α isoform inhibitor and radiotherapy could be a potential strategy to overcome radioresistance in patients with GBM.

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AUTHOR CONTRIBUTIONS

Conceptualization: Hong In Yoon. Data curation: Mi Youn Seol. Formal analysis: Mi Youn Seol. Funding acquisition: Hong In Yoon. Investigation: Hong In Yoon. Methodology: Mi Youn Seol. Project administration: Hong In Yoon. Resources: Hong In Yoon. Software: Mi Youn Seol. Supervision: Hong In Yoon. Validation: Hong In Yoon and Mi Youn Seol. Visualization: Mi Youn Seol. Writing—original draft: Mi Youn Seol. Writing—review & editing: Mi Youn Seol, Seo Hee Choi, Ik Jae Lee, Hyung Soon Park, Hye Ryun Kim, and Sang Kyum Kim. Approval of final manuscript: all authors.

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REFERENCES

1. Gurney JG, Kadan-Lottick N. Brain and other central nervous system tumors: rates, trends, and epidemiology. Curr Opin Oncol 2001;13:160-6.
2. Davis ME. Glioblastoma: overview of disease and treatment. Clin J Oncol Nurs 2016;20(5 Suppl):S2-8.
3. Zhao HF, Wang J, Shao W, Wu CP, Chen ZP, To ST, et al. Recent advances in the use of PI3K inhibitors for glioblastoma multiforme: current preclinical and clinical development. Mol Cancer 2017;16:100.
4. Buckner JC. Factors influencing survival in high-grade gliomas. Semin Oncol 2003;30(6 Suppl 19):10-4.
5. Chinot OL, Wick W, Mason W, Henriksson R, Saran F, Nishikawa R, et al. Bevacizumab plus radiotherapy-temozolomide for newly diagnosed glioblastoma. N Engl J Med 2014;370:709-22.
6. Westphal M, Heese O, Steinbach JP, Schnell O, Schackert G, Mehdorn M, et al. A randomised, open label phase III trial with nimotuzumab, an anti-epidermal growth factor receptor monoclonal antibody in the treatment of newly diagnosed adult glioblastoma. Eur J Cancer 2015;51:522-32.
7. Stupp R, Hegi ME, Gorlia T, Erridge SC, Perry J, Hong YK, et al. Cilengitide combined with standard treatment for patients with newly diagnosed glioblastoma with methylated MGMT promoter (CENTRIC EORTC 26071-22072 study): a multicentre, randomised, open-label, phase 3 trial. Lancet Oncol 2014;15:1100-8.
8. Kuwahara Y, Roudkenar MH, Urushihara Y, Saito Y, Tomita K, Roushandeh AM, et al. Clinically relevant radioresistant cell line: a simple model to understand cancer radioresistance. Med Mol Morphol 2017;50:195-204.
9. Mukherjee B, McEllin B, Camacho CV, Tomimatsu N, Sirasanagandala S, Nannepaga S, et al. EGFRvIII and DNA double-strand break repair: a molecular mechanism for radioresistance in glioblastoma. Cancer Res 2009;69:4252-9.
10. Jamal M, Rath BH, Tsang PS, Camphausen K, Tofilon PJ. The brain microenvironment preferentially enhances the radioresistance of CD133(+) glioblastoma stem-like cells. Neoplasia 2012;14:150-8.
11. Bao S, Wu Q, McLendon RE, Hao Y, Shi Q, Hjelmeland AB, et al. Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 2006;444:756-60.
12. Li HF, Kim JS, Waldman T. Radiation-induced Akt activation modulates radioresistance in human glioblastoma cells. Radiat Oncol 2009;4:43.
13. Chen Y, Wang BC, Xiao Y. PI3K: a potential therapeutic target for cancer. J Cell Physiol 2012;227:2818-21.
14. Guerreiro AS, Fattet S, Fischer B, Shalaby T, Jackson SP, Schoenwaelder SM, et al. Targeting the PI3K p110α isoform inhibits medulloblastoma proliferation, chemoresistance, and migration. Clin

https://doi.org/10.3349/ymj.2022.0414

Cancer Res 2008;14:6761-9.
15. Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, et al. Essential roles of PI(3)K-p110beta in cell growth, metabolism and tumorigenesis. Nature 2008;454:776-9.
16. Vanhaesebroeck B, Whitehead MA, Piñeiro R. Molecules in medicine mini-review: isoforms of PI3K in biology and disease. J Mol Med (Berl) 2016;94:5-11.
17. Chuang FC, Wang CC, Chen JH, Hwang TZ, Yeh SA, Su YC. PI3k inhibitors (BKM120 and BYL719) as radiosensitizers for head and neck squamous cell carcinoma during radiotherapy. PLoS One 2021;16:e0245715.
18. Narayan RS, Fedrigo CA, Stalpers LJ, Baumert BG, Sminia P. Targeting the Akt-pathway to improve radiosensitivity in glioblastoma. Curr Pharm Des 2013;19:951-7.
19. Nunnery SE, Mayer IA. Management of toxicity to isoform α-specific PI3K inhibitors. Ann Oncol 2019;30(Suppl 10):x21-6.
20. Chia S, Gandhi S, Joy AA, Edwards S, Gorr M, Hopkins S, et al. Novel agents and associated toxicities of inhibitors of the PI3K/Akt/mTOR pathway for the treatment of breast cancer. Curr Oncol 2015;22:33-48.
21. Zhang Y, Yan H, Xu Z, Yang B, Luo P, He Q. Molecular basis for class side effects associated with PI3K/AKT/mTOR pathway inhibitors. Expert Opin Drug Metab Toxicol 2019;15:767-74.
22. Massacesi C, Di Tomaso E, Urban P, Germa C, Quadt C, Trandafir L, et al. PI3K inhibitors as new cancer therapeutics: implications for clinical trial design. Onco Targets Ther 2016;9:203-10.
23. Seol MY, Choi SH, Yoon HI. Combining radiation with PI3K isoform-selective inhibitor administration increases radiosensitivity and suppresses tumor growth in non-small cell lung cancer. J Radiat Res 2022;63:591-601.
24. Chadwick KH, Leenhouts HP. Molecular theory of cell survival. Wageningen: Instituut voor Toepassing van Atoomenergie in de Landbouw; 1973.
25. Ali MY, Oliva CR, Noman ASM, Allen BG, Goswami PC, Zakharia Y, et al. Radioresistance in glioblastoma and the development of radiosensitizers. Cancers (Basel) 2020;12:2511.
26. Vignard J, Mirey G, Salles B. Ionizing-radiation induced DNA double-strand breaks: a direct and indirect lighting up. Radiother Oncol 2013;108:362-9.
27. Aparicio T, Baer R, Gautier J. DNA double-strand break repair pathway choice and cancer. DNA Repair (Amst) 2014;19:169-75.
28. Ersahin T, Tuncbag N, Cetin-Atalay R. The PI3K/AKT/mTOR interactive pathway. Mol Biosyst 2015;11:1946-54.
29. Fruman DA, Rommel C. PI3K and cancer: lessons, challenges and opportunities. Nat Rev Drug Discov 2014;13:140-56.
30. Prevo R, Deutsch E, Sampson O, Diplexcito J, Cengel K, Harper J, et al. Class I PI3 kinase inhibition by the pyridinylfuranopyrimidine inhibitor PI-103 enhances tumor radiosensitivity. Cancer Res 2008;68:5915-23.
31. Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res 2005;94:29-86.
32. Belda-Iniesta C, de Castro Carpeño J, Sereno M, González-Barón M, Perona R. Epidermal growth factor receptor and glioblastoma multiforme: molecular basis for a new approach. Clin Transl Oncol 2008;10:73-7.
33. Kao GD, Jiang Z, Fernandes AM, Gupta AK, Maity A. Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation. J Biol Chem 2007;282:21206-12.
34. Rodon J, Dienstmann R, Serra V, Tabernero J. Development of PI3K inhibitors: lessons learned from early clinical trials. Nat Rev Clin Oncol 2013;10:143-53.
35. Wymann MP, Zvelebil M, Laffargue M. Phosphoinositide 3-kinase signalling--which way to target? Trends Pharmacol Sci 2003;24:366-76.
36. Vanhaesebroeck B, Ali K, Bilancio A, Geering B, Foukas LC. Signalling by PI3K isoforms: insights from gene-targeted mice. Trends Biochem Sci 2005;30:194-204.
37. Mizrachi A, Shamay Y, Shah J, Brook S, Soong J, Rajasekhar VK, et al. Tumour-specific PI3K inhibition via nanoparticle-targeted delivery in head and neck squamous cell carcinoma. Nat Commun 2017;8:14292.
38. Elkabets M, Vora S, Juric D, Morse N, Mino-Kenudson M, Muranen T, et al. mTORC1 inhibition is required for sensitivity to PI3K p110α inhibitors in PIK3CA-mutant breast cancer. Sci Transl Med 2013;5:196ra99.
39. Wen PY, Cloughesy TF, Olivero AG, Morrissey KM, Wilson TR, Lu X, et al. First-in-human phase I study to evaluate the brain-penetrant PI3K/mTOR inhibitor GDC-0084 in patients with progressive or recurrent high-grade glioma. Clin Cancer Res 2020;26:1820-8.
40. Diao W, Tong X, Yang C, Zhang F, Bao C, Chen H, et al. Behaviors of glioblastoma cells in in vitro microenvironments. Sci Rep 2019;9:85.